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CHARACTERIZATION AND EXPLOITMENT OF MICROBIAL STRAINS TO BE USED IN MEAT PROCESSING AND FERMENTATION

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CHAPTER 1: INTRODUCTION

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INTRODUCTION

1 MEAT PRODUCTS

1.1 Nutritional Role Of Meat In Diet

Meat has traditionally been considered an essential component of the human diet to ensure optimal growth and development. With a limited range of foods available in societies throughout history, meat was important as a concentrated source of a wide range of nutrients. Anthropological research shows that the length of the gut in primates and humans became shorter with the introduction of animal-derived food. Smaller quantities of food of high digestibility required relatively smaller guts, characterized by simple stomachs and proportionally longer small intestines, emphasizing absorption. It is perhaps due to the fact that meat has been eaten as much for enjoyment as for its nutritional qualities that consumption of meat and meat products has increased with the affluence of the consumer. The importance of meat and meat products in everyday food culture and consumer health may be questioned by the fact that populations of vegetarians living in rich countries are characterized by lower rates of cancer and cardiovascular disease. The analysis of dietary patterns, as a possible approach to examining diet–disease relations, identified two major eating patterns defined by factor analysis using dietary data collected from food frequency questionnaires. The first factor, the "prudent dietary pattern," was characterized by an high intake of vegetables, fruits, legumes, whole grains, and fish or other seafood, whereas the second factor, the "Western pattern," showed a high intake of processed meat, red meat, butter, high- fat dairy products, eggs, and refined grains. A study has been published involving Seventh-day Adventists, a well-characterized population, in which the effect of dietary intake of nutrients on biochemical parameters in blood and urine was com- pared in vegetarian and non vegetarian subjects. The dietary intake of cholesterol was higher in not vegetarian subjects (560 to 710 mg/day) compared to vegetarians (<20 mg/day) and was associated with elevated serum cholesterol levels in the not vegetarian population. These results demonstrated a correlation between dietary intake of certain food components (e.g., cholesterol) relevant for diseases (e.g., coronary heart disease) and their blood concentrations.

The meat consumption and production figures published by the U.S. Department of Agriculture and the European Union (EU) do not distinguish between fresh meat and processed or fermented meat products.

The EU production of fermented meats amounted to 689,000 tonnes of fermented sausages and approximately the same amount of raw ham in 1988. Approximately 5% of the total meat production (carcass weight) is further processed by fermentation. The major producers of fermented meat products in the EU are Germany, Italy, Spain, and France. In these countries, 20 to 40% of processed meat products can be classified as fermented meat products.

Tabel 1.1: Fermented Meat Products

FERMENTED SAUSAGES	
Cold smoked and dried but uncooked	Hungarian salami
Cold smoked and uncooked	Spreadable raw sausages (Mettwurst), very popular in Germany
Not smoked, dried and uncooked	Italian salami, Spanish chorizo
Hot smoked partially or fully cooked semi-dry sausages	Summer Sausage, Pepperoni, Salami

ITALIAN MEAT PRODUCTS	
Sausages	Salame, cotechino, soppressata, luganiga, zampone, mortadella
Whole meat cuts	Ham (prosciutto), shoulder (spalla), neck (capocollo), belly (pancetta), an aged fillet of rump (culatello), smoked flank (speck)

1.2 The Relationship Between Meat In The Diet And Disease

Numerous studies have compared the health status and mortality of vegetarians to those of omnivores. The results show a strong correlation between *per capita* consumption of meat and the incidence of colon cancer among various countries.18 In more detailed case-control and cohort studies, in which lifestyle factors were better controlled for, the consumption of red meat was associated with a high risk of colon

cancer. Results from a meta-analysis by Howe et al., including 13 of the case- control studies, indicated that total energy intake was positively associated with a higher risk of colon cancer. Surprisingly, the intakes of fat, protein, and carbohydrates were not related to cancer risk, independent of their contribution to total energy. Compared with Western vegetarians, non vegetarians have a higher mean body mass index (BMI) by about 1 kg/cm2, suggesting that higher total energy intake and meat consumption might be associated with the "Western diet pattern."

The mechanisms that increase the risk of colon cancer are not yet clear. Several *in vitro* studies suggest that DNA damage in human cell lines can be caused by food ingredients or their metabolic products. High meat consumption, for example, leads to higher levels of bile acids and *N*-nitroso compounds in the feces. Bile acid and *N*-nitroso compounds, as well as their metabolites, potentially promote colon cancer development. Animal studies show that large intestinal *N*-nitrosation does not occur in germ-free rats, but it has been shown to occur in the presence of a conventional flora. The effect of diet on the composition of the intestinal microflora was shown by Finegold et al.29 Subjects eating a Western diet were compared with subjects eating a Japanese diet. The subjects eating the Japanese diet had a lower risk of colon cancer and had significantly higher numbers of *Enterococcus faecalis, Eubacterium lentum, E. contortum, Klebsiella pneumoniae,* and various *Lactobacillus* species in their feces. The Japanese diet has been associated with low incidence of large bowel cancer. Japanese people who migrate to the United States and adopt the Western diet develop this cancer with increased frequency, approaching that of native-born Americans. The high risk group with Western dietary patterns had increased counts of species of the genera *Bacteroides, Bifidobacterium, Peptostreptococcus*, and *Clostridium* in their fecal flora. Regular consumption of meat is also associated with increased risk of death from coronary heart disease (CHD). The most compelling evidence comes from studies with Seventh-day Adventists. It was found that men and women who consumed red meat daily had around 60% greater chance of dying from CHD than those who consumed red meat less than once per week. A review of studies of the association between blood homocysteine concentrations and atherosclerotic disease showed that 16 of 21 investigations reported significantly higher homocysteine concentrations in case subjects compared with control subjects. Because red meat is a major source of methionine in the diet, and methionine is the direct metabolic precursor of homocysteine, a higher intake of red meat may be involved in cardio-

vascular disease initiation and progression.

In summary, high dietary intakes of energy, saturated fat, and red meat, all associated with the Western diet pattern, are likely to have adverse effects on chronic disease risks, particularly those of colon cancer and coronary heart disease. On the other hand, little evidence indicates that the consumption of moderate amounts of meat or meat products is harmful in regard to either cancer or cardiovascular disease.

1.3 History And Culture Of Fermented Meat

Meat is extremely susceptible to microbial spoilage. Virtually all ecological factors characterizing meat as a substrate are optimal for the growth of bacteria, which are the most efficient agents in remineralization of organic matter. For example, in meat, water activity and pH are 0.96 to 0.97 and 5.6 to 5.8, respectively, and nutrients and growth factors are abundantly available. Any storage of this nutritionally rich food and preservation of the nutrients contained therein requires the suppression of microbial growth or the elimination of microorganisms and prevention of recontamination.

The traditional methods employed for prevention of microbial spoilage are still in use, though with a different meaning in various products. These methods comprise reduction of water activity (drying, salting) and/or pH (fermentation, acidification), smoking, storage at refrigeration or freezing temperatures, and use of curing aids (nitrite and nitrate). Commonly, these methods act together in different combinations, building up hurdles against microbial growth. With regard to fermented sausages, these hurdles are low water activity (0.85 to 0.95) and pH (5.6 to 4.7), the use of nitrite (nitrate), and smoke. In addition, during fermentation and ripening, ecological factors, such as a reduced redox potential and low temperatures (10 to 12°C, at least for dry sausages), together with antagonistic compounds produced by the fermenting flora exert a selective effect against the growth of undesirable microorganisms. Basically, the same antimicrobial hurdles are effective in achieving the microbial stability of ham, except for the effect of a low pH. Since no lactic fermentation takes place, the reduced water activity is the most effective hurdle against microbial growth in ham. The understanding of these ecological factors and their control is not only a prerequisite in quality assurance, but also provides a basis for understanding to what extent these food matrices might be used to serve as probiotic foods.

The production of dried and cured meat (ham) can be traced back to prehistoric times.

It cannot be excluded that among the sausages that are mentioned in historical literature (e.g., Homer's Iliad), fermented products were included, although we do not have sufficient knowledge of their production processes to permit a conclusion that a fermentation step was part of the technology.

The origin of fermented sausages can be traced back with accuracy to ca. 1730, when salami was first mentioned in Italy. From Italy, the art of producing fermented sausages spread to other European countries and was established, for example, in Germany in 1735 and Hungary in 1835.

This very high consumption of fermented meats is an indication that such products have a long tradition of being safe. However, some specific safety aspects deserve consideration.

The high fat content commonly found in fermented sausages (usually around 50% of dry matter) has been of nutritional concern, and leaner products are now available (some as low as 5%). The sensory quality of the traditionally high-fat sausages is, however, unique and a standard for the gourmet. The body fat content of pigs has been already drastically reduced by breeding, but with respect to ham, it is left to the consumer to cut off the fat layer before consumption.

Mold-ripened varieties of both sausages and ham (e.g., Tirolean speck and Bündner Fleisch) exist. The production of such products free of mycotoxin is a concern, because of the potential of fungi for contamination. In addition, a carryover from animal feed to the meat may be a source of mycotoxin contamination. This hazard is the target of general meat inspection and control. In rare cases, mycotoxins have been detected in fermented sausages and ham. One way to overcome this hazard is the use of competitive mold strains that have a proven absence of a mycotoxigenic potential. These starter cultures usually contain strains of *Penicillium nalgiovense* or *P. chrysogenum,* and are already widely in use in Europe. For ham production, the absence of mycotoxins is still a matter of rigorous quality control.

Meat may also contain bacterial food pathogens. Because fermented meat products usually do not undergo a physical treatment to eliminate pathogenic microorganisms, the meat has to be of high quality with regard to hygiene and microbial counts. The control of pathogens is achieved by appropriate fermentation technology, including the use of starter cultures.

1.4 The Fermentation Process

The traditional aim of the fermentation process is to transform the highly perishable substrate meat into a shelf stable and safe product ensuring an optimum nutritive value and sensory quality. The factors affecting the process are the nature of the raw materials and the activity of microorganisms, as well as endogenous enzymes and process technology. For all fermented meat products, the raw material is meat with a variable amount of fat that has not been subjected to a thermal or any other germ-reducing process. Meat is the flesh (muscle tissue) of warm-blooded animals, but fermented specialties from poultry (sausages as well as cured and smoked fermented poultry) are also available. Two groups of products can be differentiated on the basis of the microbial populations involved in the fermentation process: foods from a comminuted matrix and whole meat products.

1.4.1 Fermentation of a Comminuted Meat Matrix

The comminution of muscle tissue to particles varying in size between 1 and 30 mm, together with the homogenous distribution of fermenting organisms, is the prerequisite for a fermentation process taking place throughout the matrix. Curing salt, nitrate, ascorbic acid, and, in some cases, sodium glutamate and glucono-lactone are added to the particles together with spices and above all a carbohydrate source, which is commonly glucose. These compounds exert strong effects on the growth and performance of the fermentative flora.

The fatty tissue should be as fresh as possible, as any initiated oxidative process will strongly affect the shelf life by causing early rancidity. The whole comminution process of chopping or grinding together with a mixing procedure requires temperatures below 2 to 3°C. Thereafter, the temperature is raised usually to >20°C and <28°C to initiate the fermentation process. Semidry sausages of the U.S. summer sausage type are fermented at even higher temperatures (32° to 38°C). The many types of fermented sausages are the result of a great variety of process conditions. Variables include: the particle size of the comminuted meat and fatty tissue, the selection of additives, the temperature/humidity conditions prevailing in the course of fermentation until the final ripening, the diameter of the sausages, the nature of the casings, smoking, heating after fermentation.

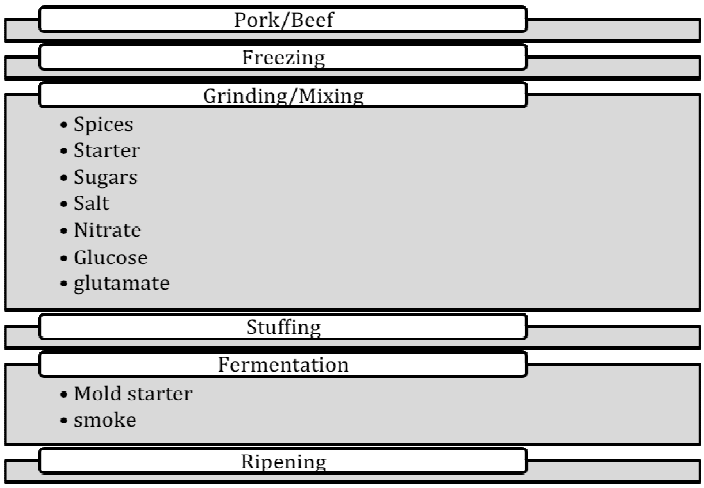


Figure 1.4.1: flow scheme of fermented meat production

The fermentation process, together with the effects of the temperature/humidity conditions, ensures that the originally highly perishable raw materials turn into a spoilage-resistant, flavor-rich product with a defined texture and stable color. Great variation exists with regard to texture, which may range from spreadable to sliceable, from soft to very hard. With regard to the microbial effects, the first days are of great importance. During that time the organisms multiply, reduce the pH to values varying between 5.4 and 4.8, exhibit enzymatic activity, and interfere with undesired microorganisms, which constitute the indigenous flora of the meat. In the course of ripening, the pH usually rises again. This rise in pH does not constitute a safety hazard, because at the same time the water activity is decreased to levels that discourage bacterial growth.

Lactobacilli and micrococci play a decisive role during the fermentation process. These organisms develop under the specific prevailing ecological conditions and were sometimes inoculated by back slopping, i.e., adding chopped fermented sausage back to a new meat mixture. Between 1950 and 1960, microorganisms were isolated and turned into preparations of starter cultures, which are now commonly used, as they exhibit numerous advantages when compared with the classical indigenous fermentation. Many of the bacteria used are lactic acid bacteria (LAB), which are of primary importance, but included in this table (tale 1.4) are also non lactic acid bacteria, which are used mainly in combination with LAB and contribute to the fermentation process as they have unique properties. For example, *Kocuria* spp. and *Staphylococcus* spp. exhibit nitrite and nitrate reductase activity, respectively, which

is important for the reddening of the sausages, i.e., the formation of the stable red color of nitrosomyoglobin. In addition, these organisms exhibit catalase activity, which counteracts the formation of hydrogen peroxide and thus helps to prevent color defects and rancidity. Yeast and fungi contribute mainly to the development of flavor and to a minor extent also to color stability.

Table 1.4: Species Employed in Meat Starter Cultures

Bacteria	*Lactic acid bacteria*	*Lb acidophilus, Lb. alimentarius, Lb. paracasei*, *Lb. rhamnosus*, *Lb. curvatus, Lb. plantarum, Lb. pentosus, Lb. sakei, Lactococcus lactis, Pediococcus acidilactici, P. pentosaceus*
	Actinobacteria	*Kocuria varians*, *Syreptomyces griseus, Bifidobacterium spp.*
	Staphylococci	*Staphylococcus xylosus, S. carnosus* ssp. *carnosus, S. carnosus* ssp. *utilis, S. equorum*
Fungi		*Penicillium nalgiovense, P. chrysogenum P. camemberti*
Yeasts		*Debaryomyces hansenii, Candida famata*

1.4.2 Fermentation Of Whole Meat Products

Immediately after slaughter, enzyme-catalyzed reactions start to act on the physical and chemical nature of muscle, turning it into meat. These reactions continue even when technological/processing measures, such as cool storage and lowering the water activity by drying or salting, are imposed. However, the reactions proceed in a predictable and controlled way. This process provides the foundation of ham production. Microorganisms do not play a role in the fermentative processes taking place in ham.

By far the majority of fermented raw ham is made from pork, but in some regions beef (Bresaola, Bündner Fleisch, Pastirma) and even meat from game, reindeer, or bear is used to produce similar products. The traditional ham in ancient Greek and Roman times as well as in China was made from the bone-containing ham of hogs. This type of ham is still considered the gold standard of quality and is produced in

many countries, e.g., Prosciutto di Parma (Italy), Jambon de Bayonne (France), Jamón Serrano (Spain), Kraskiprsut (Slovenia), Virginia ham (United States), and Yunnan ho-twe and Tshingwa ho-twe (China).

The process of ham production follows a rather simple principle.38 It consists of curing by salting (with or without the use of nitrite and/or nitrate) to achieve a water activity of <0.96, which is equivalent to 4.5% sodium chloride. At low temperatures (5°C), the salt will diffuse to the deepest part of the meat, thus overcoming the hazard of food poisoning through *Clostridium botulinum* contamination. After a phase of equilibrating the salt concentration and flavor development, the temperature is raised to 15 to 25°C to ripen the ham. This phase lasts at least 6 to 9 months and may be extended even to 18 months to achieve the optimum flavor. At the end of the ripening step, the moisture has been reduced by about 25% and a salt concentration between 4.5 and 6% results. In some countries (e.g., Germany), in addition to a nitrate cure, smoking is used to obtain a characteristic flavor and to suppress surface growth of molds. When ham is produced from only one or a few muscles, numerous methods are applied to accelerate production time and to control flavor development and water content. For example, the curing is performed in brine or by injection of curing salt and, above all, the ripening period is drastically reduced. Microorganisms may cause spoilage by growing in the inner muscle parts before the water activity is reduced to safe levels. However, microorganisms also contribute favorably to the process as they are involved in nitrate curing in brine by the formation of the reactive nitrite, which affects color by reddening, flavor, and microbial safety. The microorganisms involved are Gram-negative bacteria such as vibrios and *Halomonas* spp.

1.5 Characteristics of Fermentation

During sausage fermentation many physical, biochemical, and microbial changes happen:

- growth of LAB and concomitant acidification of the product
- reduction of nitrates to nitrites and formation of nitrosomyoglobin
- solubilization and gelification of myofibrillar and sarcoplasmic proteins
- degradation of proteins and lipids
- dehydration

1.5.1 Fermentation Microflora

The initial microbial population of sausage minces depends on the microbial load of the raw materials.

The ecological conditions of sausage minces favor the growth of Micrococcacea and lactobacilli. Lactobacilli generally grow to cell counts of 5 . 108 to 109 CFU/g, and these numbers remain stable throughout ripening. Micrococcacea (predominantly *Kocuria varians, Staphylococcus carnosus,* or *S. xylosus*) generally grow to cell counts of 10^6-10^7 CFU/g, when nitrate cure is applied. The growth of these organisms is inhibited by the application of nitrite cure as well as the decrease of pH. Higher cell counts are reached at the outer layer of the sausages, where a higher oxygen partial pressure occurs.

Because of their high salt tolerance, the predominant microorganisms found in dry cured ham fermentation belong to the classification that was formerly included in the family Micrococcacea. The species most often isolated are *Staphylococcus xylosus, S. equorum*, and *S. sciuri,* but *K. varians* is also found at appreciable cell counts. Growth of staphylococci occurs primarily at the surface of hams.

The growth of yeasts and fungi on mold-ripened products is restricted to the surface of the product, where cell counts reach 105 to 107 CFU/cm2 within four weeks of ripening. Many of the traditional fermentations involving fungal ripening rely on inoculation by the “house flora” associated with the building or equipment used for fermentation and maturation.

1.6 Acidification

Acidification to the isoelectric point of meat proteins (pH 5.3 to 5.4) and the increase of the ionic strength induces gel formation of the proteins and thus confers important structural changes. The high levels of sodium chloride and lactate in fermented sausages contribute to the development of the characteristic taste of the product. Rapid acidification and subsequent drying are of paramount importance for inhibition of the growth of pathogens and their subsequent inactivation during ripening. As there exist not dried fermented sausages of a spreadable type (e.g., in Germany, known as Streichmettwurst, Teewurst, Rohpolnische), the highest hygienic standards of the raw materials and production facilities are key to product safety. In addition to low pH and water activity, specific microbial metabolites such as diacetyl or short chain fatty acids exert an inhibitory effect towards pathogens. Several meat starter cultures

produce bacteriocins — small, heat stable peptides with antimicrobial activity. The use of bacteriocinogenic starters has been shown to contribute to the elimination of *Listeria* during sausage fermentation. However, because of the resistance of Gram-negative organisms, including *Salmonella* and *Escherichia coli* O157:H7 strains, the contribution of bacteriocins to the overall hygienic safety of fermented meats is limited.

Growth and metabolism of LAB result in a fast drop of pH during the first days of sausage fermentation, followed by a slight increase during the ripening period. Lactic and acetic acids are the major fermentation products, and the molar ratio of lactate to acetate ranges between 7 and 20. The product pH depends on the buffering capacity of the meat, the metabolic activities of the fermentation micro-flora, and the addition of fermentable carbohydrates. In Northern European and in U.S. summer sausage, the pH typically ranges from 4.8 to 5.2, corresponding to a content of 200 mmol lactate/kg dry weight. In Mediterranean-type products involving longer ripening periods of up to several months, the final pH typically ranges from 5.4 to 5.8. In mold-ripened products, the sausage pH may increase to levels close to 6.0 due to lactate consumption and the formation of ammonium. The dry matter content of fermented sausages ranges from 50 to 75% or more, corresponding to water activity (aw) values ranging from 0.86 to 0.92 upon ripening.

1.7 Proteolytic and Lipolytic Degradation

Proteolytic events during fermentation of raw sausages and dry cured ham were widely discussed by Toldra and Flores, and Ordónez et al. In the course of ripening, peptides and amino acids accumulate to levels of about 1% dry matter. Peptides and amino acids themselves may contribute to the characteristic taste of dry cured products and act as flavor enhancers and synergists. Excess proteolysis may result in bitter and metallic off-flavors because of the presence of bitter peptides. Furthermore, amino acids and peptides are utilized by microorganisms for the conversion into flavor volatiles.

The hydrolysis of muscular proteins to peptides is mainly achieved by the activity of endogenous enzymes. The endopeptidases — cathepsins B, B + L and H — were shown to remain active throughout the fermentation of dry cured ham and fermented sausages, whereas tissular calpains are inactivated during fermentation and do not contribute significantly to overall proteolysis. Furthermore, muscle exopeptidases

contribute to peptide conversion to amino acids. The proteolytic system of lactobacilli consists mainly of cell wall-associated proteinases, which convert proteins to oligopeptides. Oligopeptide transport is the main route for nitrogen entry into the bacterial cells, and virtually all peptidases are located intracellularly. The proteolytic activity of starter cultures is weak compared to that of tissue enzymes. Correspondingly, the inoculation of sausage minces with starter cultures leads to only a minor increase in amino acid levels of the sausages compared to aseptic control batches. The proteolytic activity of *Kocuria varians* is inhibited by environmental conditions prevailing during sausage ripening, yet the peptidase activity of this organism may contribute to the formation of amino acids. It was recently shown that *Lb. casei* utilizes peptides released from pork muscle sarcoplasmic and myofibrillar proteins under conditions of sausage ripening. he fat content of fermented sausages typically ranges from 40 to 60% of dry matter. During fermentation, long-chain fatty acids are released from triglycerides and phospholipids. Typically, an increase in the levels of free fatty acids up to approximately 5% of the total fatty acids has been found. The fatty acid composition of fat varies considerably depending on the previous feeding regime of the animal. The specific release of polyunsaturated fatty acids is higher than that of monounsaturated or saturated fatty acids. Lysosomal muscle acid lipase and adipose tissue lipases remain active throughout several months of dry cured ham ripening. Comparisons of aseptic fermented batches of sausages with batches inoculated with starter cultures has shown that lipolysis during fermentation is attributed mainly to meat endogenous enzymes. Lactobacilli are considered to be weakly lipolytic. Strains of *K. varians* and *S. carnosus* or *S. xylosus* have been found to exhibit lipolytic activity, which is, however, inhibited at pH values below 6. In mold-ripened products, lipolytic activities of the surface mold flora contributed to the generation of long-chain fatty acids.

1.8 Generation of Flavor Volatiles

During sausage fermentation flavor volatiles are generated by lipolysis and hydrolysis of phospholipids, followed by oxidation of free fatty acids. Furthermore microorganisms produce organic acids, convert amino acids and peptides to flavor-active alcohols, aldehydes, and acids and modify products of lipid oxidation, e.g., by esterification of acyl moieties or reduction of aldehydes.

Depending on the product formula and maturation conditions, the sausage aroma is

determined by the addition of spices, smoking, or surface- ripening with yeasts or molds too.

Despite the differences in the process technology and the fermentation microflora, it may be assumed that the generation of flavor during fermentation of dry cured ham is governed by the same principles.

The most important odor compounds in French, Italian, and Spanish salami are those originating from added spices, i.e., sulfur compounds (e.g., diallylsulfide) originating from garlic, and eugenol from nutmeg. Products of lipid oxidation, fatty acids, as well as fermentation volatiles such as acetic acid, diacetyl, and phenylethanol, further contribute to overall flavor. The comparison of flavor volatiles and sensory attributes of sausages of various origins reveals that a high level of fatty acids negatively affects the sausage aroma.

Unsaturated fatty acids are prone to autoxidation in the presence of oxygen. Many of the products of lipid oxidation are highly volatile and have a low odor threshold. Hexanal, nonenal, 2(Z)octenal, 1-octen-3-ol, and 1-octen-3-on were identified as the most potent flavor volatiles resulting from autoxidation of linoleic acid, and these compounds are present in fermented sausages. The rate of lipid oxidation is greatly enhanced by heme or nonheme iron. The increase in NaCl concentration during ripening also favors lipid oxidation. Nitrite present as part of the sausage formula or formed from nitrate by microbial nitrate reductase acts as an antioxidant. The removal of peroxides by catalase, pseudocatalase, or manganese-dependent superoxide dismutase activities of the Micrococcaceae and/or lactobacilli is crucial to limit the extent of fatty acid oxidation and to prevent off-colors.

Amino acid catabolism by staphylococci and lactobacilli yields volatile products contributing to meat flavor. *Staphylococcus xylosus* and *Staphylococcus carnosus* have been shown to produce a large variety of flavor volatiles originating from amino acid degradation during growth on sausage minces. The formation of flavor volatiles is strongly affected by the growth parameters salt, nitrate, glucose, and oxygen. Degradation of leucine was also shown for strains of *Lb. curvatus, Lb. sakei,* and *Lb. plantarum.* The addition of proteolytic enzymes or the use of proteolytic starter cultures does not enhance the microbial conversion of amino acids to flavor-active derivatives.

1.9 Use of Nitrite in Meat Processing

The origin of salting meat is lost in antiquity, but is believed that the ancient Sumerian civilization was the first to practice this process. Meat curing can be defined as the addition of salt to meats for the sole purpose of preservation; that is, to inhibit or deter microbial spoilage. The preservation of meat resulted from necessity, so that products could be held for extended periods for later consumption in times of scarcity. Salting prevented bacteria growth on account of salt's direct inhibitory effect or because of the drying action it had on meat. As the use of salt as a meat preservative spread, it was found that high concentration of salt would promote the formation of an unattractive brownish-gray color within lean muscle tissue. At some point at the development of this art, more likely accident than design, it was discovered that that salt could impart or "fix" a unique pink or red color and flavor in meats (Binkerd and Kolari, 1975). By Medieval times the use of salt in production of fermented meat is without alternatives, as it affects fundamental processes in meat conditioning, on microbial performance, hygiene, shelf life, flavor and texture (Desmond, 2006). In their literature review Ruusunen and Poulanne (2005) stated that an addition of 2.5% is the lower limit for obtaining good quality fermented sausage. With regard to curing, it is of special meaning that NaCl reacts with nitrite and forms nitrosohypochloride (NOCl), which is a stronger nitrosylating agent than nitrite itself and therefore, the visible reaction (formation of nitrosomyoglobin) is accelerated (Fox et al., 1996; Møller and Skibsted, 2002).

Beside salt, the traditional curing agent is nitrate. For fermented meat such as sausages and ham it is still in use in Europe either solely as saltpetre (potassium nitrate, E 251) or sodium nitrate (E 252), or in combination with potassium nitrite (E 249). Nitrite is always used as curing salt, which contains up to 0.9% NaNO2. As nitrate is a rather inert compound, it has to be reduced to nitrite, which is the highly reactive curing principle. This reaction is a prerequisite for all curing related events in the meat matrix. As reported by Honikel (2008), some nitrate reduction has been observed also in heated cured meat products, leaving open the possibility that chemical reactions might permit nitrate reduction.

Starter cultures are in use to control nitrate reduction in the fermentation process.

Nitrite in the curing process is either obtained as the product of nitrate reduction or from the addition in the form of curing salt and affects strongly the products.

It has the following characteristics:

i. exerts antimicrobial effects that may result in reduction of the risk of food poisoning and delay of spoilage,
ii. causes reddening, i. e. formation of pink nitrosomyoglobin,
iii. forms curing flavour,
iv. acts as an antioxidative

The use of nitrate/nitrite is still a matter of discussion as the beneficial effects are opposed by health risks, which make these curing agents basically undesired compounds in food. The use of the curing agents is regulated in the EU by the following:

Directive 2006/52/EC regulates the use of Nitrate and Nitrite under the following conditions:

Nitrate (E 251, Potassium nitrate; E 252 Sodium nitrate) is permitted:

- For curing of non-heat-treated meat products (generally permitted at a concentration of 150 mg/kg, and for certain specified traditional products at 300 mg/kg, for some others at 250 mg/kg);
- To prevent late blowing in hard, semi hard and soft cheese (150 mg/kg of cheese milk);
- For sensory effects of pickled herring and sprat (500 mg/kg).

Nitrite is exclusively for curing permitted. E 249, Potassium nitrite, at 150 mg/kg is generally permitted in meat products. E 250, Sodium nitrite, at 100 mg/kg is permitted in sterilized meat products (Fo > 3.00, corresponding to heating for 3 min at 121°C) as well as in specified traditional meat products. To further specified traditional meat products it is permitted at a concentration of 180 mg/kg.

According to (EC) 780/2006 the use of Nitrate/Nitrite in organic food (meat products) is permitted under the following conditions: NaNO2 can be applied at an ingoing concentration of 80 mg/kg and the concentration has to be reduced to 50 mg/kg. The use of KNO3 is permitted at the same concentrations, which have to be expressed as $NaNO_3$. The use of nitrite is limited by the comment that it "can only be used if it has been demonstrated to the satisfaction of the competent authority that no technological alternative giving the same sanitary guarantees and/or allowing maintaining the specific features of the product, is available."

The decisive justification for the use of nitrate/nitrite in meat products rests in their inhibitory action on food pathogens, thus exerting a preservative effect (Dykhuizen et

al., 1996; Pichner et al., 2006). Again, neither nitrate nor nitrite per se is the active inhibitory principle but they have to be converted to reactive intermediate compounds (RNIs) such as NO^-, N_2O_3, ONOO, NO^+_2, RSeNO.

The effect of nitrate/nitrite on color formation is of visual prominence among the sensory changes occurring in the curing process. The target of the reddening reaction is myoglobin (MbFeII) containing haeme as a prosthetic group, which is protoporphyrin IX with iron in the ferrous state (Fe^{2+}). With iron in the ferric state (Fe^{3+}) it is named haematin, which is contained in metamyoglobin (MbFeIII). In myoglobin a proximal histidine residue is attached directly to the iron center. On the opposite position a distal histidine residue is located, which is not bonded to the iron. Oxygen and with high affinity also nitrogen monoxide (NO) bind to this second position. The complex containing NO is named nitrosomyoglobin and represents the characteristic curing red pigment. Upon heating the apoenzyme denatures but the red color is kept in that complex named nitrosohaemochrome.

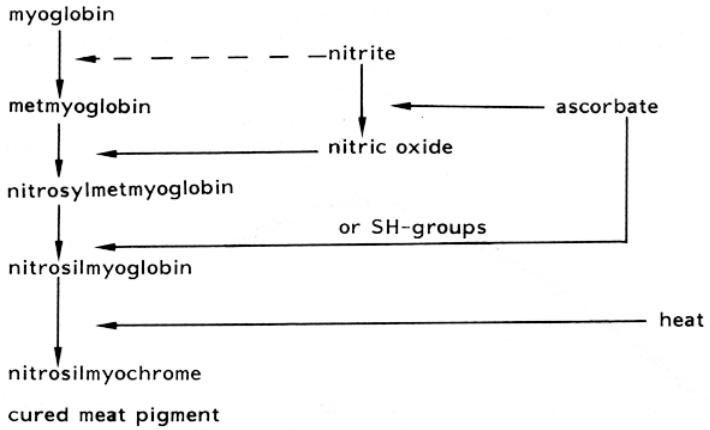


Figure 1.9. Reaction of nitrate and nitrite in fermented meat leading to the curing red pigment nitrosomyoglobin.

Under acidic conditions, created in the fermentation process by lactic acid bacteria, nitrite as the product of nitrate reduction undergoes acid catalyzed disproportionation (Hoagland, 1910, 1914). Nitrite at the oxidation state of nitrogen of +3 disproportionates to NO (oxidation state +2) and Nitrate (oxidation state +5). It is consequence of that reaction that nitrate reducing bacteria are also required for efficient nitrate reduction, even when nitrite is used as the only curing agent. Nitrite reduction and formation of NO can also occur by reaction with reductants among which ascorbate is the common agent under practical conditions.

Several other compounds in meat are reacting with the reactive nitrogen intermediates and just a fraction of 5e15% of the added nitrogen from nitrite has been recovered from myoglobin (Cassens et al., 1978, Cassens, 1990). A greater part of 20-30% has been found bound to protein and 1-15% each to SH-residues and lipids, respectively.

1.10 Use of Nitrite and Nitrate in Fermented Meat: Risks and Benefits

The major concern of nitrate/nitrite in food is related to the potential of nitrite to form cancerogenic N-nitroso compounds, which can be formed in the food matrix as well as in the human body. Clear evidences for the endogenous formation of carcinogenic N-nitrosamine acids from nitrate in the diet were provided by Tricker and Preusmann (1987). The probability of formation of stabile N-nitrosamines in meat is rather low as are also the concentrations detected in cured meat (Honikel, 2008). These compounds can, however, be formed when the products are exposed to temperatures exceeding 130°C. Epidemic studies with nearly 500,000 persons indicated that consumption of red and also processed meat increases the risk of colon cancer (Norat et al., 2005). There was not made a differentiation between meat products neither between raw or heat-treated nor fermented or otherwise preserved ones. Thus, it is not known whether or not the category of fermented meat constitutes a specific health risk.

To avoid the use of “chemical” nitrate, ingredients, which naturally contain nitrates, have been in use natural spices or natural flavorings, celery juice or celery juice concentrate (Sebranek and Bacus, 2007). The authors reported in their review that the nitrate concentration of a commercial celery juice powder was as high as 27,500 mg/kg or about 2.75%. It can be calculated that the addition of 3.6 g of this powder to 1 kg of meat batter results in a nitrate concentration of 100 mg/kg therein. On the other hand, “natural” ingredients such as sea salt, raw sugar or tubinado sugar contain nitrate at concentrations of <2 mg/kg, which is commonly to low for providing a practically usable effect. The products obtained through this type of practice are categorized under the name “clean label food”. It is obvious that the contained “natural” nitrate has to be reduced to nitrite and for this purpose starter cultures are required. Remarkably, the fermentative use of nitrate reducing starter cultures has thus been extended also to the production of cured meat products that undergo a cooking step such as emulsion type sausages. Their manufacturing process requires modification: to permit reddening the culture preparation is added when the comminution starts and thereafter the temperature is kept for 90 min at 42°C.

Smoking and cooking in the following steps are performed as usual. For production of fermented sausages the components of the formula are added together with the nitrate source at starting the comminution process. All process steps correspond to those usually employed for production of nitrate cured fermented sausages.

Increasing evidence has been obtained from human and animal studies showing that dietary nitrate contributes to the beneficial effects exerted by NO that is synthesized endogenously from arginine by nitric oxide synthetase (Gladwin et al., 2005). As nitrate as well as nitrite can be reduced and NO be formed in mammalian tissue and NO can also be oxidized to nitrite and nitrate it is evident that dietary nitrate enters a common pool (Lundberg et al., 2008, 2009). Lundberg et al. (2011) listed the following proven effects on human health of modest dietary intake of nitrate:

i. reduction of blood pressure,
ii. inhibition of platelet function,
iii. prevention of endothelial dysfunction after a mild ischaemic insult in humans,
iv. reduction of oxygen cost during exercise
v. effecting fundamental mitochondrial functions.

In addition, animal date provide evidence for prolonged blood pressure reducing effects, protection against drug and salt induced renal and cardiac injuries, enhanced post ischaemic blood flow, protection against ischaemia reperfusion injury and reversion of features of the metabolic syndrome.

1.11 Biochemical Changes During Meat Fermentation

During processing of meat, many different biochemical changes come off: proteolysis is one of the most important and relevant for final quality of the product. In fact it has an important effect on texture, taste and on aroma development (Toldrà and Flores, 1998). A good control of proteolysis is fundamental to get a final product of good quality. Muscle and microbial proteases are the main elements responsible for the proteolytical changes and thus a good knowledge of its properties and mode of action is essential for controlled proteolysis. Other changes are restricted to the beginning of the process which is the case of nucleotide breakdown reactions or the glycolysis-related enzymes and subsequent generation of lactic acid. Proteolysis and lipolysis constitute two of the most important enzymatic phenomena, responsible for the generation of compounds with direct influence on taste and aroma.

1.11.1 Glycolysis

Lactic acid is the main product resulting from carbohydrate fermentation. Once the added carbohydrates (glucose, sucrose, etc.) are transported into the cell, they are metabolized via the glycolytic or Embden-Meyerhof pathway. The ratio of the enantiomers L and D lactic acid depends on the species of lactic acid bacteria present and, more specifically, on the action of the L and D lactate dehydrogenases, respectively, and the lactate racemase. There are some key enzymes in the carbohydrate metabolism like aldolases, that generates glyceraldehyde-3-phosphate, pyruvate kinase, that generates pyruvate from phosphoethanol pyruvate and lactate dehydrogenase that generates lactic acid from pyruvate. Glucose is mainly metabolized through a homofermentative way but some other end products like acetate, formate, ethanol and acetoin, with an impact on sausage aroma, may be produced in trace amounts from alternative heterofermentative pathways. The pH drops as a consequence of lactic acid accumulation and contributes to the preservation of the sausage by preventing the growth of undesirable microorganisms. The generated lactic acid also contributes directly to acid taste and indirectly to aroma, due to the formation of metabolites, and sausage consistency due to protein coagulation as pH approaches the isoelectric point of most of the myofibrillar proteins.

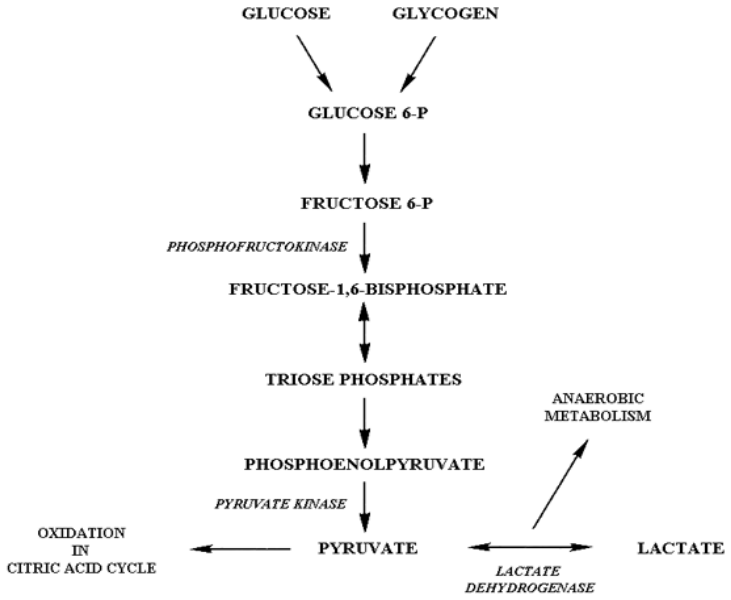


Figure1.11.1 : Embden-Meyerhof pathway

1.11.2 Proteolysis

Proteolysis consists in the progressive degradation and breakdown of major meat proteins (sarcoplasmic and myofibrillar proteins) and the subsequent generation of peptides and free amino acids. The result is a weakening of the myofibrillar network and generation of taste com- pounds but its extent depends on many factors. One of the most important is the activity of endogenous muscle enzymes, which depends on the original crossbreeds and the age of the pigs. Main muscle enzymes involved in these phenomena are cathepsins B, D and L that show a great stability in long term dry-curing processes, good activity at acid pH values and are able to act against myofibrillar proteins. Other important muscle endopeptidases like calpains exhibit poor stability and its optimal pH near 7.0 is far from that in the sausage. Muscle enzymes exert a combined action with microbial proteases although different enzymatic profiles may be found depending on the microorganisms used as starter cultures. One of the major challenges is just to establish the relative role or percentage of contribution of endogenous and microbial enzymes to proteolysis. The proteolytic system of different *Lactobacillus* has been studied and contains endopeptidases able to degrade sarcoplasmic and myofibrillar proteins as well as exopeptidases like dipeptidylpeptidase, tripeptidase , dipeptidase and aminopeptidases. However, some studies revealed that protein degradation, especially myosin and actin, is initiated by cathep- sin D, a muscle endopeptidase very active at pH values near 4,5 and able to degrade both proteins. Cathepsins B and L would be more restricted to actin and its degradation products. The latter stages of proteolysis would be pre- dominantly by bacterial peptidases and exopeptidases.

Other important factors are related with the processing technology. For instance, the temperature and time of ripening will determine the major or minor action of the enzymes, the amount of added salt, which is a known inhibitor of cathepsins and other proteases, will also regulate the enzyme action and thus the proteolysis and taste.

The generation of small peptides may be depressed by the level of salt which inhibits muscle peptidases although intense levels of non-protein-nitrogen, up to 20% of the total nitrogen content, may be reached. Some of these peptides give characteristic tastes . Final proteolysis steps by aminopeptidases, especially from microbial origin, are very important . These enzymes release free amino acids along the process and a substantial increase in the concentration of free amino acids is usually observed.

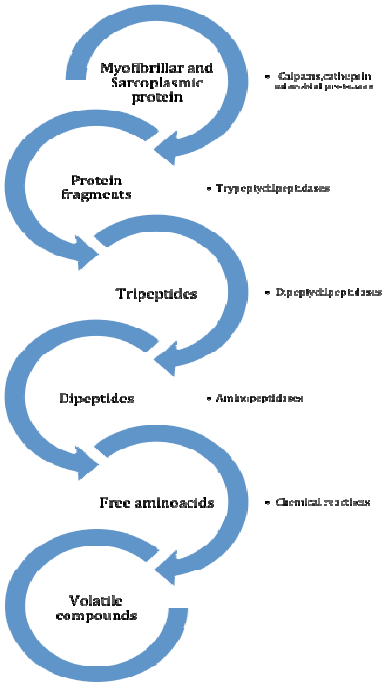


Figure 1.11.2: general scheme of proteolytic chain in dry cured meats.

1.11.2.1 Transformation of Amino Acids

The released free amino acids as a consequence of proteolyisis are then subject of a number of enzymatic and/or chemical transformations that produce different compounds that will affect the sensory characteristics of the product. So, microbial decarboxylation of amino acids may pro- duce biogenic amines. Transamination consists in the transference of the α-amino group of the first amino acid to the α carbon atom from an α-keto acid generating a keto acid from the first amino acid and a new amino acid. Dehydrogenases transform the amino acid in the corresponding keto acid and ammonia. Deamidation also generates ammonia. The microbial degradation of the amino acid side chain by liases may lead to phenol and indole formation (82). The Strecker degradation of amino acids produces branched aldehydes, like 3-methylbutanal, 2-methylbutanal and phenylacetaldehyde from leucine, isoleucine and phenylalanine, respectively, through oxidative deamination-decarboxilation reactions (Table 1.11.2).

Table 1.11.2:Volatile aldehydes from the Strecker degradation (Whitefield, 1992)

Amino acids	Aldehydes
Glycine	Formaldehyde
Alanine	Acetaldehyde
α-Aminobutyric acid	Propanal
Valine	2-Methylpropanal (Isobutyraldehyde)
Leucine	3-Methylbutanal (Isovaleraldehyde)
Isoleucine	2-Methylbutanal
Norvaline	Butyraldehyde
Norleucine	Pentanal
Serine	2-hydroxyethanal
Threonine	2-hydroxypropanal
Methionine	2-Methylthiopropanal (Methional)
Cysteine	2-Mercaptoacetaldehyde or acetaldehyde
Phenylglycine	Benzaldehyde
Phenylalanine	Phenylacetaldehyde
Tyrosine	2-(p-Hydroxyphenyl)ethanal

1.11.3 Lipolysis

Lipolysis consists on the breakdown of tri-acylglycerols by lipases and phospholipids by phospholipases resulting in the generation of free fatty acids. These fatty acids may contribute directly to taste and, indirectly to the generation of aroma compounds through further oxidative reactions. Main lipolytic enzymes, located in muscle and adipose tissue, in combination with microbial lipases, are involved in these phenomena. Although it is difficult to establish a relative role of endogenous and microbial enzymes to lipolysis, the percentage of contribution of endogenous lipolytic enzymes to total fat hydrolysis is estimated around 60 to 80% with the rest due to microbial lipases. The most important lipases located in muscle are the lysosomal acid lipase and acid phospholipase while in adipose tissue are the hormone sensitive lipase and the monoacylglycerol lipase. These enzymes show good stability through the full process . Although their activity also depends on pH, salt concentration and water activity, the conditions found in the sausages favor their action. The generation rate of free fatty acids, especially oleic, linoleic, estearic and palmitic acids, increases during the process. Most of these fatty acids proceed from phospholipids degradation although some of them generate volatile compounds through further oxidative reactions. In the case of adipose tissue, the rate of generation, especially of oleic, palmitic, linoleic, stearic, palmitoleic and myristic acids, is also high.

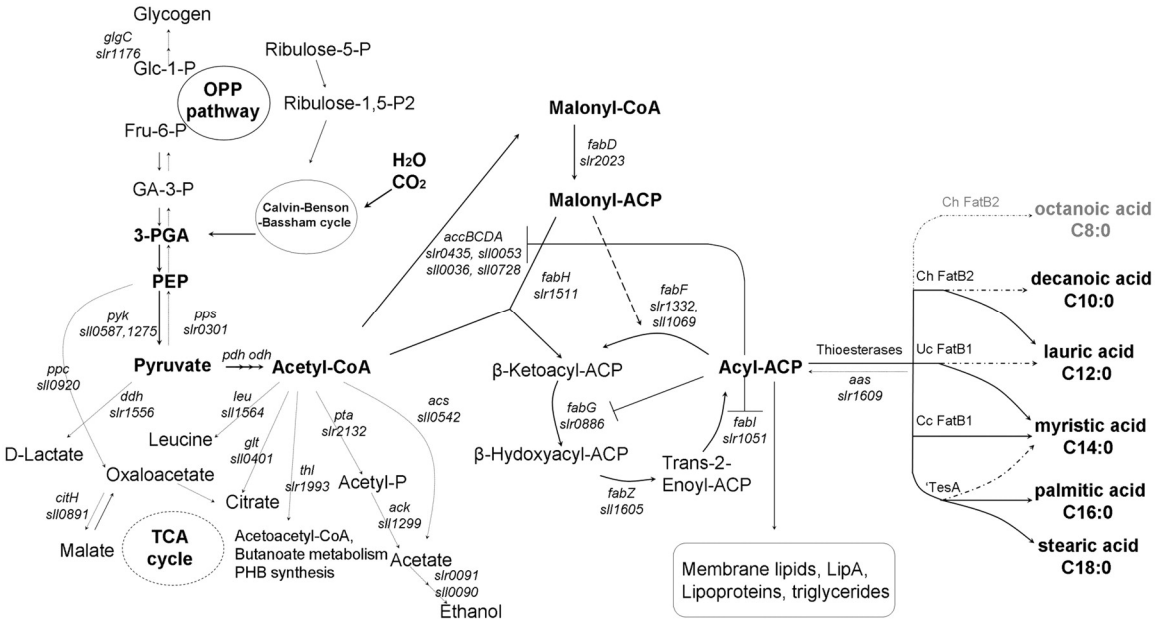


Figure 1.11.3: FFA pathway

1.11.3.1 Oxidation

The generated mono and polyunsaturated fatty acids are susceptible to further oxidative reactions to give volatile compounds. The beginning of lipid oxidation is correlated to an adequate flavor development. On the contrary, an excess of oxidation may lead to off-flavors. In fact, the generation of the characteristic aroma of dry-cured meat products is in agreement with the beginning of lipid oxidation. Free radical formation is catalyzed by muscle oxidative enzymes, like peroxydases and ciclooxygenases, external light, heating and the presence of moisture and/or metallic cations. The next step in oxidation is the formation of peroxide radicals (propagation) by reaction of free radicals with oxygen. The formed hydroxyperoxides (primary oxidation products) are flavorless but very reactive giving secondary oxidation products that contribute to flavor (92). The oxidation is finished when free radicals react each other. Main products from lipid oxidation are aliphatic hydrocarbons (poor contribution to flavor), alcohols (high odor threshold), aldehydes (low odor threshold) and ketones. Alcohols may inter- act with free carboxylic fatty acids giving esters, especially when nitrate is not used.

1.12 The Muscle Proteolytic System

Skeletal muscle contains a lot of good enzymes involved in several metabolic pathways, the most important are related to protein changes. Endopeptidases (calpains and cathepsins) are responsible for protein breakdown, tri and dipeptylpeptidases are involved in generation of small tri and dipeptides and finally, aminopeptidases and carboxypeptidases release free aminoacids (Toldrà, 1992). A general scheme of proteolytical chain was shown above (figure 1.11.2).

Most of the enzymes remain active in postmortem muscle, playing important roles in proteolysis. Some of these proteases, having optimal pH are located in organules like lysosomes whereas others are bound to membrane or free in the cytosol.

1.12.1 Endopeptidases or Proteinases

There are three important groups of endopeptidases or proteinases: cathepsins, calpains and proteasome. Cathepsins B, D, H and L are located in lysosomes. They are small in size (20-40kDa) that allow them to penetrate into the myofibrillar structure and hydrolize important proteins, like myosin and troponins that experience many important changes during dry-curing. The second group of proteinases are two calpains, also known as calcium dependent proteases. Both of them are located in the cytosol, manly in the Z-line area, and they differ in their Ca^{2+} requirement for activation. They have optimal neutral pH, around 7.5, but their activity decreases rapidly at acid pH values such as those found in dry fermented sausages. The third group of proteases is the protosome complex, a large protease with multiple catalytic sites. This enzyme is able to exhibit different activites like chymotrypsin-like activity, trypsin-like activity and peptydil-glutamyl activity. (Coux, Tanaka ad Goldberg, 1995). The 20S proteasome may have a different effect on tenderness for its ability to degrade myofibrils, but this activity is only exhibited at high pH values.

1.12.2 Exopeptidases

Trypeptidylpeptidases (TPP) and dipeptidylpeptidases (DPP) are two important groups of muscle exopeptidases**.** TPP I and II are able to hydrolyze different tripeptides, while DPP III and IV hydrolyze dipeptides and their names can vary depending on the preference for certain amino acids. Aminopeptidases are able to release a free amino acid from the ammino terminus of peptides and proteins. Arginyl, alanyl, pyroglutamyl, leucyl are the most important aminopeptidases in skeletal

muscle. All of the are active at neutral or basic pH.

Moreover there are two carboxypeptidases located in the lysosomes, with optimal activity at acid pH. Carboxypeptidase B has a wide spectrum against any amino acid but carboxypeptidase A is more specific for hydrophobic amino acid.

1.13 The Microbial Proteolytic System

The mechanism for proteolytic breakdown of proteins by microbial enzymes are similar to those described for muscle enzymes. These enzymes are also endopeptidases or proteinases and peptidases. Endopeptidases are predominantly extracellular and peptidases are located inside the cell. Extracellular proteinases may be bound either to the cell wall or to the cell membrane (Visser, 1993). In this way the mode of action may differ if using whole cells or only cell-free extracts. In the case of the whole cells, the aa and peptide transport system is necessary to supply the cells with the aa required for the growth. (Tan, Polman and Konings, 1993). The proteolytic activity associated with the cell wall is the first enzyme to degrade the proteins. The generated peptides are then transported into the cell where they are further degraded by different peptidases to small peptides and free amino acids. Many microorganisms have been used as starters for fermented meats. Some of the most important *are Lactobacillus sakei, L. curvatus, L. carnosus, L. plantarum., Kocuria varians, Staphylococcus xylosus* and the yeast *Debaryomices hansenii* (Toldrà 2004d, 2006).

LAB are widely used in food fermentation; even though the proteolytic system from dairy LAB has been well characterized, limited information is available on meat lactobacilli.

1.13.1 Endopeptidases

Lactic acid bacteria have some endopeptidases or proteinases associated with the cell envelope. These are responsible for the initial breakdown of proteins into oligopeptides. Several in vitro assays have been performed to elucidate the hydrolysis of sarcoplasmic and myofibrillar meat proteins when incubated with whole cells and cell free extract from different *L. casei, L. curvatus, L. plantarum.* The results showed that bacteria proteinases are less active than muscle proteinases in hydrolyzing myofibrillar proteins (Molly et al., 1997). However these lactobacilli can hydrolize the sarcoplasmic proteins, especially *L. plantarum* and *L. casei*, which show the

strongest degradation (Sanz et al, 1996). The substrate specificity is broad and the proteinase activity appears to e extracellurarly located, which is in agreement with the existence of a single cell wall associated proteinase in dairy lactic acid bacteria that is responsible for initial caseins hydrolysis. (Kunji et al.1996). The peptides profiles of the sarcoplasmic and myofibrillar extracts after incubation with these strains show the generations of a large number of hydrophilic peptides: this is very important because they are correlated to desirable cured meat flavors, whereas hydrophobic peptides are correlated to bitterness. (Aristoy and Toldrà, 1995).

1.13.2 Exopeptidases

There are important exopeptidases in *L.sakei*, the most prevalent species in European sausages. The first is the major or general aminopeptidase, which has an optimal neutral pH and is similar to PepL from *L. delbrueckii*. It has a broad range of activity against aminoacids especially alanine and leucine, but is unable to hydrolize basic residues. Second is the arginine aminopeptidase, activated by salt and has preference for basic residues like arginine and lysine (Sanz and Toldrà, 2002).The addition of cell free extracts from *L. sakei, L. curvatus* and *L. casei* to myofibrillar and sarcoplasmic proteins gave a net increase in free amino acids. The increase was very significant for glutamate, alanine and leucine for *L.sakei*; glutamate and alanine for *L. curvatus*; arginine and glutamate for *L. casei* (Fadda et al, 1999b).

Table 1.13.2: main purified peptidases in *L.sakei* (Toldrà,2006)

Enzyme	**Biochemical similarity**	**Activation**	**Substrates**	**Optimal T°C**	**Optimal pH**
Major aminopeptidase	PepL	Ca^{2+},Sn^{2+}, Mg^{2+}, Ba^{2+},Mn^{2+}	Leu-peptide, Ala-peptide	37	7,5
Arginine aminopeptidase	PepN	Reducing agent, salt	Arg-peptide, Lys peptide	37	5,0
X-prolyl-dipeptidylpeptidase	Pep X		X-Pro-peptides Ala-Pro-peptide	55	7,5
Dipeptidase	Pep V		Met-Ala	45	7,8
Tripeptidase	Pep T		Ala-Ala-Ala	40	7,0

1.14 Enhancement and Control of Proteolysis

Fermented sausages are common products throughout Europe. Differences in the composition, ripening and fermentation conditions exist not only in different countries, but also within the same country. Sausage fermentation is a well-known microbial process, and ecological studies during ripening date back to the 1970s (Lucke, 1974). These studies highlighted that two main populations are involved in the process. In the fermentation of sausages, the main transformations that lead to the final product involve the activity of two microbial groups: lactic acid bacteria (LAB) and micro/ staphylococci. The LAB are responsible for the acidification, while the micro/staphylococci produce lipases, eventually releasing short-chain fatty acids that are responsible for the aroma of the fermented sausage (Demeyer et al., 1974). Molecular analysis of microbial changes during fermentation showed that by three days of maturation, these two main groups of organisms were the most abundant in the sausages (Cocolin et al., 2001).

Processing conditions are important for they can increase or reduce the enzyme activity: in he hams the control can be achieved checking the relative humidity and temperature in the curing rooms, because they have an important effect on enzymatic activity (Toldrà and Flores, 1998). The pH achieved in the product during the process, near neutral in the ham and acid pH in fermented sausages, will modulate the enzymatic activity. Furthermore, proteolysis can be controlled by adding an excess of salt, that has an inhibitory effect on cathepsins and other proteases and thus reduces softening. (Toldrà, 2002). Time for ripening is important when intense flavor development is required, as most of the generated amino acids need some time to experience further reactions to aroma volatile compounds.

In summary the ability to steer the proteolysis into the meat products is very important from the economic point of view because reproducible and steerable production processes are needed to produce dry cured meat of high quality (Toldrà and Verplaetse, 1995).

1.14.1 Use of Starter Culture in Fermented Meat

Fermented sausage is prepared from seasoned, raw meat that is stuffed in casings and is allowed to ferment and mature (Campbell-Platt and Cook, 1995; Lucke, 1998). Inoculation of the sausage batter with a starter culture composed of selected lactic acid bacteria (LAB), i.e. homofermentative lactobacilli and/or pediococci, and Gram-

positive, catalase-positive cocci (GCC), i.e. nonpathogenic, coagulase-negative staphylococci and/or kocuriae, improves the quality and safety of the final product and standardizes the production process (Campbell- Platt and Cook, 1995; Lucke, 2000). Nonetheless, small manufacturers continue to use the traditional method of spontaneous fermentation without added starter culture. In the latter case, the required microorganisms originate from the meat itself or from the environment and constitute a part of the so-called house flora (Santos et al., 1998).

Such artisan fermented sausages are often of superior quality compared to controlled fermentations inoculated with industrial starters and possess distinctive qualities, partly due to the properties of the raw material and the characteristics of the technology used (Moretti et al., 2004), but also to the specific composition of the house flora. The flavour- generating, metabolic activity of GCC in artisan chorizo, for instance, has been shown to vary with the manufacturing location (Garcıa-Varona et al., 2000).

It has been suggested that commercial starter cultures in Europe, mainly produced in Northern European countries, are not always able to compete well with the house flora colonizing Southern European meat plants, so that their use often results in losses of desirable sensory characteristics (Samelis et al., 1998). The fitness of commercial meat starter cultures when applied to a particular type of salami is questionable since a culture that performs well in one type of fermented sausage is not necessarily efficient in another type. Appropriate cultures have to be selected according to the specific formulation of the batter and technology of fermentation since environmental factors will interact to select a limited number of strains that are competitive enough to dominate the process (Rebecchi et al., 1998). *Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus pentosus* and *Lactobacillus plantarum*, species sometimes found in commercial starter cultures for meats, are rarely detected in large amounts in spontaneously fermented sausages because of their inferior competitiveness compared to, for instance, *Lactobacillus sakei* or *Lactobacillus curvatus* (Doßmann et al., 1998; Coppola et al., 2000). They initiate the acidification of the meat batter well, but are not always able to prevent spontaneous outgrowth of non-starter LAB with undesirable effects on the end-product (Coventry and Hickey, 1991; Hugas and Monfort, 1997*). Lb. plantarum* may also give rise to a product with overacidity, which is not well perceived by the consumer (Garriga et al., 1996).

Even if the rapid acidification initiated by the starter culture reduces microbial risks in fermented sausages, not all concerns have been solved, mainly so in slightly fermented or ripened varieties. Whereas pseudomonads, *Enterobacteriaceae*, and aerobic sporeformers are usually not of concern (Samelis et al., 1998; Aymerich et al., 2003), the pathogens *Staphylococcus aureus, Escherichia coli, Salmonella, and Listeria monocytogenes* are posing risks to food safety.

In the last years the use of starter cultures of a new generation has been suggested, the so-called Functional starter cultures (De Vuyst, 2000; Leroy and De Vuyst, 2003, 2004) to ensure the consumer microbial safety and offer one or more organoleptic, technological, nutritional, or health advantages.

One of the main challenges is to explore the biodiversity of artisan products and to introduce qualities obtained with wild- type strains in standardized, industrial fermentations. In contrast to ill-adapted industrial starters, wild-type strains that naturally dominate traditional fermentations tend to have higher metabolic capacities which can beneficially affect product quality, for instance with regard to aroma formation or food safety. Natural selection is likely to have forced such strains to be more competitive by endowing them with ecological advantages, i.e. making them less auxotrophic (Ayad et al., 2000) and more able to produce antimicrobials (Maldonado et al., 2002).

1.14.2 Selection of Starter Culture

L. sakei, L. curvatus, L. plantarum, Lactobacillus pentosus, Lactobacillus casei, Pediococcus pentosaceus and *Pediococcus acidilactici* are the species most used as commercial meat LAB starter cultures (Hammes & Hertel, 1998; Hugas & Monfort, 1997). The first stage in designing a starter culture for a meat commodity is to characterize the LAB strains isolated from the meat product in question and then select those best suited. In meat fermentations, the main function of LAB is to obtain a rapid pH drop of the batter, which promotes

i. product safety by inactivating pathogens,
ii. product stability and shelf life by inhibiting undesirable changes caused by spoilage microorganisms or abiotic reactions,
iii. creates the biochemical conditions to attain the new sensory properties of the ripe products through modification of the raw materials (Lücke, 2000).

Currently the use of starters as functional flora is gaining importance; designed starter

cultures have properties additional to those of the more classic type, helping to optimize the sausage fermentation process, and to produce tastier, safer, and healthier products.

The production of organic acids – mainly lactic acid – from carbohydrates is the major role of LAB in sausage fermentation. This depends on several chemical, physical and microbiological reactions. While acidifying the batter, LAB participate in the coagulation of muscle proteins, resulting in the increased slice stability, firmness and cohesiveness of the final product (Hugas & Monfort, 1997; Ordóñez et al.,1999). They also enhance the spontaneous reduction of nitrites to nitric oxide, which reacts with the myoglobin to form nitrosomyoglobin, the compound responsible for the typical pink color of cured sausage (Hugas & Monfort, 1997). Moreover, they contribute to the flavor of the final product through the formation of noticeable acidic and vinegary (acetic acid) tastes. Acidic conditions are also thought to increase the activity of cathepsin D, which is responsible for muscle proteolysis (Molly et al., 1997). The production of organic acids is undoubtedly the determining factor on which the shelf life and the safety of the final product depends. The inhibition of pathogenic and spoilage flora is also dependent on a rapid and adequate formation of these organic acids. Finally, it has been reported that a rapid decrease in pH caused by amine-negative starter cultures can largely prevent biogenic amine (BA) accumulation in sausages (Maijala, Eerola, Aho, & Hirn, 1993).

The immediate and rapid formation of acid at the beginning of the fermentation process, and the production of sufficient amounts of organic acids allowing a pH below 5.1 to be reached, are therefore essential requirements of meat LAB starters. Excessive acid formation, however, is often associated with color defects (due to the inhibition of the CNC) and sometimes with gas formation – one of the most important problems in sausage fermentation (Buckenhüskes, 1993).

The ability of the starter culture to compete with the natural microbiota of the raw material and to undertake the metabolic activities expected is conditioned by its growth rate and survival in the conditions prevailing in the sausage: an anaerobic atmosphere, rather high salt concentrations, low temperatures and low pH. The salt concentration is about 2% (aw=0.94–0.98) in the batter and can reach 15% (aw = 0.85–0.86) in the final product (Lücke & Hechelmann, 1987; Montel, 1999). The manufacturing temperature ranges from 4 to 7°C when preparing the batter (Baracco, Durand, Frentz, Jacquet, & Zert, 1990), from 18 to 24°C during the fermentation

period (Montel, 1999), and from 12 to 15 °C during the drying and ripening period (Montel, 1999). The initial pH of the batter, which is generally around 6.0 decreases during fermentation and reaches values between 4.6 and 5.1. Thereafter, yeasts, mostly *Debaryomyces hansenii*, increase the pH of the product (Cook, 1995), achieving final values ranging from 5.1 to 5.5.

Thus, the growth rate at different temperatures (2–4 to 24°C), the tolerance of salt concentrations of 2–10%, and of pH in the range 4.2–6.0 are limiting factors affecting the persistence and competitiveness of the starter culture over the entire fermentation and ripening process. *L. sakei* can grow at 4 °C, in the presence of 6.5% NaCl, and at pH 4.2 (Ammor, Dufour, Zagorec, Chaillou, & Chevallier, 2005). At 15°C and in the presence of 2% NaCl, it shows growth rates which allow 0.55 generations to be produced per hour (Ammor et al., 2005). Its psychrotrophic character and salt tolerance may be due to its ability to effciently accumulate osmo- and cryoprotective solutes such as betaine and carnitine, and to its cold stress response: *L. sakei* has more putative cold-stress genes than any other lactobacilli (Chaillou et al., 2005). A combination of mechanisms, including modification of carbohydrate metabolism (down regulation of glycolysis) and stimulation of oxidative stress may also increase its resilience to cold (Marceau, Zagorec, Chaillou, Mera, & Champomier-Verges, 2004).

Heterofermentative lactobacilli are not suitable for sausage production because the formation of large amounts of carbon dioxide leads to holes of different sizes in the product (Buckenhüskes, 1993). In addition, these Lactobacilli produce concentrations of acetic acid that cause a pungent off-flavor.

Most lactobacilli are able to form hydrogen peroxide by oxidizing lactate. Hydrogen peroxide can interfere with the organoleptic properties of fermented meat products by increasing rancidity and the discoloration of the final product.

LAB participate in the formation of the typical pink color through the spontaneous reduction of nitrites to nitric oxide. Some meat LAB have also been reported to possess nitrate reductases and heme-dependent and heme-independent nitrite reductases (Hammes et al., 1990; Wolf, Arendt, Pfahler, & Hammes, 1990). These are directly involved in the mechanisms of nitrosomyoglobin formation.

Meat starter cultures are mainly mixtures of LAB and CNC, thus, to perform their expected functions, LAB starters must be able to tolerate or even show synergy with CNC starter components. Hammes et al. (1990) showed some *L. sakei and L. curvatus*

strains to inhibit others used in meat starter cultures (in addition to unwanted flora), such as Kocuria varians.

1.14.3 Use of Starter Culture for a Safer Product

The main antimicrobial effect responsible for safety is evidently the rate of acidification of the raw meat (Lucke, 2000). Nevertheless, certain antimicrobials such as bacteriocins may also play a role, in particular in slightly acidified products or to eliminate undesirable microorganisms that display acid tolerance (e.g. L. monocytogenes).

Bacteriocins produced by LAB are antibacterial peptides or proteins that kill or inhibit the growth of other Gram-positive bacteria (De Vuyst and Vandamme, 1994; Cintas et al., 2001; Cleveland et al., 2001; Diep and Nes, 2002). They often have narrow inhibitory spectra and are most active towards closely related bacteria likely to occur in the same ecological niche (Eijsink et al., 2002). LAB produce a diversity of bacteriocins that are generally active towards other LAB, contributing to the competitiveness of the producer, but also towards foodborne pathogens such as *L. monocytogenes*. The application of bacteriocin-producing LAB in the meat industry offers therefore a way of natural food preservation (Stiles and Hastings, 1991; McMullen and Stiles, 1996; Hugas and Monfort, 1997; Hugas, 1998; De Martinis et al., 2002).

Lactobacilli sausage isolates frequently produce bacteriocins or bacteriocin-like compounds, as has been shown for *L. sakei* (Sobrino et al., 1991; Tichaczek et al., 1992; Garriga et al., 1993; Samelis et al., 1994a; De Martinis and Franco, 1998; Aymerich et al., 2000b; Rosa et al., 2002; Tantillo et al., 2002), *L. curvatus* (Tichaczek et al., 1992; Sudirman et al., 1993; Mataragas et al., 2002), *L. plantarum* (Garriga et al., 1993; Rekhif et al., 1995; Enan et al., 1996; Aymerich et al., 2000b; Messi et al., 2001*), L. brevis* (Benoit et al., 1994), and *L. casei* (Vignolo et al., 1993).

The use of bacteriocin-producing *L. sakei* as starter cultures permits to decrease *Listeria* levels in fermented sausage (Schillinger et al., 1991; Hugas et al., 1995; Hugas et al., 1996; De Martinis and Franco, 1998). Antilisterial effects have also been demonstrated with bacteriocinogenic *L. curvatus* (Hugas et al., 1996; Dicks et al., 2004) and *L. plantarum* (Campanini et al., 1993; Dicks et al., 2004) sausage starter cultures.

The use of bacteriocin producers as new starter cultures may offer considerable food

safety advantages, without risk for human health due to toxicological side effects (Cleveland et al., 2001). It represents a safe way of natural food preservation that, most likely, has always occurred in fermented foods for centuries. Moreover, in situ bacteriocin production does generally not lead to organoleptic or flavour imperfections, as has been shown through taste panels with different strains (Hugas et al., 1995; Coffey et al., 1998). Still, some disadvantages have to be considered related to the fact that bacteriocin activity in situ is lower than may be expected from in vitro experiments (Schillinger et al., 1991; Campanini et al., 1993). Activity may be less effective in the sausage due to low production, genetic instability, the inability to uniformly distribute bacteriocin throughout the product, low solubility of the bacteriocin, inactivation by meat proteases, resistance of the target strain, and interference by meat components, in particular adsorption to fat and meat particles (Knorr, 1998; Ennahar et al., 1999; Cleveland et al., 2001; Aasen et al., 2003; Dicks et al., 2004). It is recommended to use strains that are well adapted to the sausage environment, preferably sausage isolates, for optimal performance and bacteriocin production (Leroy et al., 2002b). For instance, the results obtained during sausage fermentation with *L. plantarum* (Campanini et al., 1993) are less convincing than the results that are generally obtained with *L. sakei or L. curvatus*. In this context, it has been shown kinetically that the sausage isolates *L. sakei* CTC 494 (Leroy and De Vuyst, 1999a), *L. curvatus* LTH 1174 (Messens et al., 2003), and *L. curvatus* L442 (Mataragas et al., 2003) optimally produce bacteriocin under conditions of pH and temperature that prevail during European sausage fermentation. Also, appropriate cultures are to be selected according to the specific formulation and the technology used (Hugas and Monfort, 1997).

Evidently, bacteriocins are not meant to be used as the sole means of food preservation, but should be appropriately integrated in a multi hurdle preservation system, at all times respecting good manufacturing practice.

It is important during strain selection that no undesirable compounds such as toxins, biogenic amines, or D(-)-lactic acid, that could aversely affect health, are formed.

If surface mould growth is desirable, it must be checked if the mould starter culture produces mycotoxins or antibiotics (Sunesen and Stahnke, 2003). The use of moulds that are free of mycotoxin production as starter cultures could be useful in outcompeting mycotoxin-producing strains from the house flora. Moulds may also produce green or dark spots that are not acceptable to most consumers or have a

negative impact on flavour and taste (Sunesen and Stahnke, 2003).

During the ripening of fermented sausages, biogenic amines such as tyramine, histamine, tryptamine, cadaverine, putrescine, and spermidine, may be formed by the action of microbial decarboxylases on amino acids that originate from meat proteolysis (Komprda et al., 2004). Microbial decarboxylation reactions may be ascribed to both the microorganisms that were introduced via the starter culture and the ones that constitute part of the natural population of the meat. In general, starter bacteria have limited tyrosine-decarboxylating activity, but contaminant non-starter LAB, in particular enterococci, are believed to be responsible for tyramine production (Ansorena et al., 2002). The use of decarboxylase-negative starter cultures that are highly competitive and fast acidifiers prevents the growth of biogenic amine producers and leads to end-products nearly free of biogenic amines (Bover-Cid et al., 2000a, b; Suzzi and Gardini, 2003), as long as the raw material is of sufficient quality (Bover-Cid et al., 2001). Also, the introduction of starter strains that possess amine oxidase activity might be a way of further decreasing the amount of biogenic amines produced in situ (Leuschner and Hammes, 1998; Martuscelli et al., 2000; Fadda et al., 2001; Gardini et al., 2002; Suzzi and Gardini, 2003). Although it is known that the superficial inoculation with *P. camemberti* in cheeses increases the concentration of certain amines, the production of amines by moulds in fermented sausages does not appear significant but has not been fully studied yet (Bruna et al., 2003).

The nature of the lactic isomer produced by the LAB strains is of concern, since high levels of the D(-)-lactic acid isomer are not hydrolyzed by lactate dehydrogenase in humans and are thus capable of causing acidosis. Therefore, strains producing L(+)-lactic acid should be preferably selected (Holzapfel, 2002).

Antibiotic resistance is a worldwide public health problem that continues to grow. Limiting the transmission of antibiotic resistance genes to unrelated pathogenic or opportunistic bacteria is essential. The food chain has been recognized as one of the main routes for the transmission of antibiotic resistant bacteria between animal and human populations (Witte, 2000). European Authorities have recently concluded that some bacteria used for or in feed production might pose a risk to human and animal health because of harboring strains with transferable resistance genes (European Commission, 2005). Fermented meats that are not heat-treated before consumption provide a vehicle for such bacteria and can act as a direct link between the indigenous microflora of animals and the human GIT. Recently, food-associated bacteria such as

L. sakei, L. curvatus, Leuconostoc mesenteroides, and P. pentosaceus have been isolated from human feces, suggesting their ability to survive passage through the human gastrointestinal tract (GIT) (Walter et al., 2001).

Several studies have reported antibiotic resistance in LAB from meats and meat products; a few strains involved in sausage fermentation such as *L. sakei, L. curvatus and L. plantarum* have been found to show such resistance (Gevers, Danielsen, Huys, & Swings, 2003; Holley & Blaszyk, 1997; Teuber & Perreten, 2000). Although most of these resistances have been characterized as intrinsic, some genetic determinants such as chloramphenicol acetyltransferase (cat-TC), erythromycin [erm(B)] and tetracycline [tet(M), tet(S)] resistance genes have been identified, suggesting that horizontal gene transfer may have occurred (Ahn, Collins-Thompson, Duncan, & Stiles, 1992; Gevers et al., 2003; Lin, Fung, Wu, & Chung, 1996; Tannock et al., 1994) .A recent study has shown that seven out of 62 lactobacilli strains might harbor transferable resistance genes on the basis of their resistance levels to chloramphenicol, erythromycin/clindamycin, tetracycline and oxacillin (Danielsen & Wind, 2003). Therefore, before launching a starter culture or probiotic product, it is important to verify that the bacterial strains involved do not content transferable resistance genes.

1.15 Microrganisms Involved in Sausage Fermentation

The microorganisms that are primarily involved in sausage fermentation include species of LAB, GCC, moulds, and yeasts.

In spontaneously fermented European sausages, facultative homofermentative lactobacilli constitute the predominant flora throughout ripening. *L. sakei* and/or *L. curvatus* generally dominate the fermentation process (Rebecchi et al., 1998; Samelis et al., 1998; Santos et al., 1998; Andrighetto et al., 2001; Cocolin et al., 2001; Aymerich et al., 2003; Papamanoli et al., 2003; Rantsiou et al., 2004, 2005). *L. sakei* appears to be the most competitive of both strains, frequently representing half to two thirds of all LAB isolates from spontaneously fermented sausage, whereas *L. curvatus* is frequently found in amounts up to one fourth of all LAB isolates. Other lactobacilli that may be found, albeit generally at minor levels, include *L. plantarum, Lactobacillus bavaricus* (now reclassified as *L. sakei or L. curvatus*), *Lactobacillus brevis, Lactobacillus buchneri, and Lactobacillus paracasei* (Hugas et al., 1993; Rebecchi et al., 1998; Aymerich et al., 2003; Papamanoli et al., 2003). Recently, the

new species *Lactobacillus versmoldensis* has been isolated from German, quick-ripened, salami-style sausages (Krockel et al., 2003).

Pediococci are less frequently isolated from European fermented sausages but occasionally occur in small percentages (Santos et al., 1998; Papamanoli et al., 2003). They are more common in fermented sausages from the United States where they are deliberately added as starter cultures to accelerate acidification of the meat batter.

Enterococci are sometimes associated with fermented meat products, in particular artisan products from Southern Europe, where they increase during early fermentation stages and can be detected in the end-product at levels of 10^2-10^5 ufc/g (Rebecchi et al., 1998; Samelis et al., 1998; Aymerich et al., 2003; Franz et al., 2003; Papamanoli et al., 2003). They are ubiquitous in food processing establishments and their presence in the gastrointestinal tract of animals leads to a high potential for contamination of meat at the time of slaughter (Franz et al., 1999). Opinions about their significance vary, as they may enhance food flavour but also compromise safety if opportunistic pathogenic strains proliferate or antibiotic resistance is spread (Franz et al., 1999, 2001, 2003; Vancanneyt et al., 2002; Cocconcelli et al., 2003; De Vuyst et al., 2003).

Coagulase-negative staphylococci and kocuriae are GCC that participate in desirable reactions during ripening of dry fermented sausages, such as color stabilization, decomposition of peroxides, proteolysis, and lipolysis. They are poorly competitive in the presence of actively growing aciduric bacteria, often not growing more than one log cfu/g during ripening (Samelis et al., 1998). The non- pathogenic, coagulase-negative staphylococci are dominated by *Staphylococcus xylosus, Staphylococcus carnosus, and Staphylococcus saprophyticus*, but other species occur too (Papamanoli et al., 2002; Gardini et al., 2003; Blaiotta et al., 2004; Mauriello et al., 2004). In addition to staphylococci, *Kocuria varians*, formerly known *as Micrococcus varians*, or other kocuriae are sometimes isolated in small quantities from naturally *fermented sausage (*Coppola et al., 1997; Papamanoli et al., 2002; Gardini et al., 2003*).* Moulds, usually *Penicillium nalgiovense and Penicillium chrysogenum*, are used in mould-ripened sausages, particularly in Southern Europe (Lopez-Dıaz et al., 2001; Sunesen and Stahnke, 2003). A yeast population, dominated by *Debaryomyces hansenii*, may also be found on the sausage surface and originates from the house flora or is sometimes added as starter culture (Samelis et al., 1994b; Coppola et al., 2000; Encinas et al., 2000; Olesen and Stahnke, 2000).

1.15.1 The Role of Microorganisms in Meat Curing

Studies on fermented meat at the beginning of the 20th century showed that in traditional meat fermentation yeasts, moulds, lactic acid bacteria and staphylococci/micrococci are involved (Table 1.15).

Intensified studies lead to the introduction of starter cultures which enable to control the fermentation process in regard to achieving a high standard in sensory quality and hygiene as well as reduction of production time and costs. They also ensure to achieve the reduction of the added nitrate/nitrite to safe low limits. Yeasts affect flavor (Olesen and Stahnke, 2000), and may improve color through creation of anaerobic conditions, which are required for effective nitrate reduction. The species employed in starter culture are not or only weakly fermenting and also do not reduce nitrate (Nakase and Suzuki, 1985). Moulds grow on the surface, affect flavor (Sunesen and Stahnke, 2003).

Microorganisms play a crucial role in maintaining the global nitrogen cycle (Figure 1.15). The nitrate reducing reactions of the cycle take also place in meat fermentation. The most efficient nitrate reducing organisms are staphylococci and micrococci (Gøtterup et al., 2008), which affect also further important attributes of fermented meat. Lactic acid bacteria can also reduce nitrate and nitrite but their potential has not been used in commercial starter cultures. The occurrence of greyish/brownish discoloration and oxidative reactions causing off-flavour caused by accumulation of H2O2 during fermentation is prevented by their strong catalase activity of staphylococci and micrococci (Hammes and Knauf, 1994). The aroma is further positively affected by their lypolytic and free fatty acid degrading activities (Hammes and Hertel, 1998). Finally the formation of nitrite and further of RNIs contributes to hygienic safety.

Under anaerobic conditions staphylococci use nitrate as the final electron acceptor in nitrate respiration. This was shown for a set of strains of *Staphylococcus carnosus* and the closely relate *Staphylococcus piscifermentans* (Hartmann et al., 1995). It was observed that the growth yield of anaerobic cultures depended on the concentration of nitrate and was optimal at 20 mmol/L.

Some typical features need to be taken in consideration in determining the technological conditions prevailing during meat fermentation.

i. as dissimilative nitrate reduction provides bacteria with energy and is inhibited by oxygen, it is essential that anaerobic conditions prevail during

fermentation;

ii. as nitrite is temporarily accumulated and will be reduced by staphylococci to the oxidation level of ammonia after nitrate has been used up, the primary curing agent NO originates mainly from chemical reactions. These are enabled by the use of lactic acid bacteria that provide acidic conditions and/or the use of reducing additives such as ascorbate or erythorbate.

The reactions that occur are the following:

- KNO3 potassium Nitrate → KNO2 nitrite (action of bacteria)
- KNO2 → HNO2 nitrous acid (in acid medium, pH 5.2 - 5.7)
- HNO2 → NO nitric oxide
- NO → myoglobin → nitrosomyoglobin (pink color)

Potassium Nitrate worked wonderfully at 4-8°C (40-46°F) which was fine as refrigeration was not very common yet. If the temperatures dropped below 4°C (40°F) the bacteria that was needed to force Nitrate into releasing nitrite would become lethargic and the curing would stop. Potassium Nitrate was a slow working agent and the meat for sausages had to be cured for 72-96 hours.

Lactic acid bacteria fulfill numerous tasks in meat fermentation. Of superior importance is the decrease of pH that results in acid taste, preservation, hygienic safety, texture and support of reddening. They also produce aroma and may exert additional effects such as reduction of nitrate and nitrite as well as destruction of peroxides. *Lactobacillus pentosus* forms ammonia from nitrite (Wolf et al., 1990) in a process named fermentative nitrate reduction or ammonification. In this type of reaction the electrons are not used for energy conservation in a respiratory chain but to regenerate reduction equivalents and thereby gaining additional energy. Nitrate reduction was also found in strains of *L. plantarum* and *Pediococcus pentosaceus*. Strains of *L. sakei* and *L. farciminis* exhibit nitrite reductase activity which is haeme independent and results in release of NO and N_2O as intermediates of the denitrification process. *L. sakei* exhibits catalase activity in the presence of a haeme source that is abundantly available in meat.

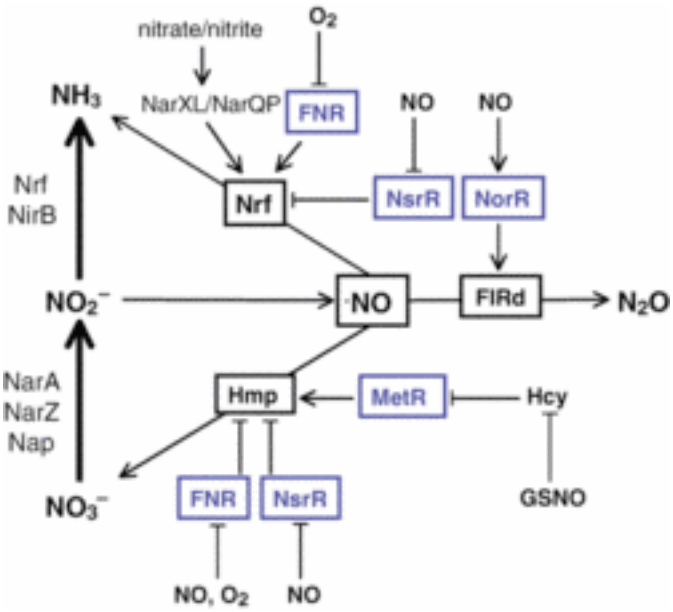


Fig. 1.15 Pathways for NO synthesis and consumption

Under anaerobic conditions, nitrate is reduced to nitrite by nitrate reductase (NarA, NarZ or Nap), and nitrite is reduced to ammonia by a respiratory or NADH-linked nitrite reductase (Nrf and NirB, respectively). Maximal rates of nitrate and nitrite reduction occur in cultures grown anaerobically in the presence of nitrate or nitrite (the regulatory mechanisms involved are not shown, but involve FNR and the two-component systems NarXL and NarPQ). Nitrite can be converted to NO by biological reduction (by nitrate and/or nitrite reductase) or by disproportionation. Under anaerobic conditions, NO is reduced to ammonia by Nrf, or to nitrous oxide by the flavorubredoxin (FlRd). In the presence of oxygen, NO is oxidized to nitrate by flavohaemoglobin (Hmp). The relevant regulators are shown along with their signals. Positive regulation is denoted by arrows, negative regulation by perpendicular lines. Hcy, homocysteine.

1.15.2 Lactic Acid Bacteria (Lab)

The primary contribution of LAB to flavor generation is ascribed to the production of large amounts of lactic acid and some acetic acid, although they also produce volatiles through fermentation of carbohydrates (Molly et al., 1996). They usually do not possess strong proteolytic or lipolytic properties, although a degree of peptidase and lipase activity has been observed for some meat strains.

Exopeptidases from meat lactobacilli contribute, in conjunction with muscle aminopeptidases, to the generation of free amino acids, contributing to flavour (Demeyer et al., 2000). LAB isolated from Greek sausages exhibited high in vitro leucine and valine aminopeptidase activities (Papamanoli et al., 2003). However, lactobacilli and pediococci display low catabolism of branched-chain amino acids, and hence do not play a major role in the formation of typical sausage aroma compounds such as 3-methyl butanal, as it is the case for staphylococci (Larrouture et al., 2000).

Little information is available about the lipolytic activity of lactobacilli during sausage fermentation, but some in vitro activity has been documented for *L. sakei, L. curvatus*, and *L. plantarum* (Hugas and Monfort, 1997; Lopes et al., 1999; Papamanoli et al., 2003). However, lipases from lactobacilli often display little or no activity under conditions found in fermented sausages (Kenneally et al., 1998a; Demeyer et al., 2000), although for some the production of lipase appears to be significant under conditions relevant for sausage ripening (Lopes et al., 1999).

In addition to lactobacilli, other LAB may be added to the sausage batter to influence flavour. The major contribution of LAB to flavour seems, however, to be limited to their carbohydrate catabolism, mainly the production of organic acids, whereas GCC appear to be more appropriate for the generation of specific aroma compounds.

Lactobacillus sakei, L. curvatus and L. plantarum are the species most widely isolated from acid-fermented meat products (Hammes et al., 1990; Hugas et al., 1993; Parente et al., 2001; Samelis et al., 1994; Schillinger and Lucke, 1987; Aymerich et al., 2003). They are responsible for lactic acid production, for the ‘‘tangy’’ flavor of sausages, and for the small amounts of acetic acid, ethanol, acetoin, carbon dioxide, and pyruvic acid that are produced during fermentation, depending on the starter applied, the carbohydrate substrate, and the sources of meat proteins and additives (Bacus, 1986; Demeyer, 1982; Thornill and Cogan, 1984).

In a study, (Rantsiou and Cocolin, 2005) natural fermentations were followed in three European countries with a long tradition in sausage production: Greece, Hungary and Italy. Differences in the LAB dynamics between the countries studied were noted and could be explained by considering the recipe used for the production as well as the fermentation conditions used in the first days. On a total number of 358 strains that were isolated, 324 were identified as *Lactobacillus spp*., representing the 90.5% of the entire population isolated and identified. Among them, three species, *L. plantarum, L.*

curvatus and L. sakei, counted for the 91.0% with a total number of isolates equal to 295. Twenty-seven strains of *L. plantarum*, 100 strains of *L. curvatus* and 168 strains of *L. sakei* were subjected to molecular characterization by RAPD-PCR and cluster analysis of the profiles. For almost all of the strains, grouping was dependent on the provenience. The majority of the clusters were formed by isolates coming from one specific country or mainly constituted by strains of only one country with a few others from one or both the other countries considered in the study. This evidence leads to the conclusion that there is a geographic distribution of LAB population among the three countries.

Another work from Bonomo et al., (2008) about the molecular characterization of the microflora present in a typical Italian sausage, showed that *L. sakei* was the predominant species (67%) followed by *Pediococcus pentosaceus* (16%), *Leuconostoc carnosum* (8%), *L. plantarum* (4%), *L. brevis* (2%) and *Leuconostoc pseudomesenteroides* (2%). The technological characterization revealed that most of the isolates had good acidifying and proteolytic properties. Moreover*, L. sakei* strains showed antimicrobial ability, while *Leuconostoc* strains the highest reduction of nitrates.

1.15.3 Micrococcaceae

Coagulase negative staphylococci (CNS) are commonly found as natural flora of fermented meat products, in relatively high number also in dry fermented sausages produced without addition of starter cultures (Miralles, Flores, & Perez-Martinez, 1996).

CNS play a major role in the development of sensory properties of fermented sausages, by reduction of nitrates to nitrite and then to nitrous oxide, by preventing rancidity through peroxide decomposition, and by proucing flavor and aroma compounds through proteolysis and lipolysis (Hammes & Hertel, 1998; Sondergaard & Stahnke, 2002).

Staphylococcus xylosus is the dominating CNS species in many Italian sausages (Cocolin, Manzano, Aggio, Cantoni, & Comi, 2001; Rossi, Tofalo, Torriani, & Suzzi, 2001) and in the Spanish sausage Chorizo (Garcıa-Varona, Santos, Jaime, & Rovira, 2000). *S. saprophyticus* and *S. carnosus* the dominating species in traditional Greek sausages (Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2002; Samelis, Metaxopoulos, Vlassi, & Pappa, 1998) and in Naples type salami (Coppola,

Mauriello, Aponte, Moschetti, & Villani, 2000). Many other CNS species (*S. haemolyticus, S. warneri, S. equorum, S. cohnii, S. epidermidis, S. hominis, S. capitis, S. intermedius*) have been reported to occur in one or more sausage types.

Staphylococcus species are commonly used as starters cultures for fermented sausages. Actually Italian law permits the use of *S. xylosus, S. carnosus and S. simulans* as starter for fermented sausage production (Repubblica Italiana, 1995) and knowledge of their technological properties is a matter of considerable interest. Nitrate reductase and catalase activities are considered to be the most important properties of staphylococci to be used as starter cultures for fermented sausages production (Weber, 1994). Mauriello et al. (2004) reported that all staphylococcal strains belonged to *S. xylosus, S. equorum and S. lentus* species were able to reduce nitrate to nitrite at 30 °C and a similar result was reported by Miralles et al. (1996) who found three strains of *S. xylosus* and one strain of *S. epidermidis* able to reduce nitrates to nitrites.

Coppola, Iorizzo, Sorrentino, Grazia (2004) studied the capability of staphylococcal strains to reduce nitrate in nitrate broth, at the temperature adopted during the ripening of the sausages: they found different strains belonging to the species of *S. xylosus*, *S. equorum* and *S. simulans* able to reduce nitrates to nitrite at 18 and 30 °C.

In relation to the proteolytic abilities, authors reported different results for CNS: it seems to be strain dependent and highly correlated to the processing conditions of every different sausage. Moreover the final proteolysis in fermented sausages is often a result of the enzymatic exogenous activity of microorganism and the endogenous activity of meat enzymes.

Staphylococci, in particular *S. xylosus* and *S. carnosus*, modulate the aroma through the conversion of amino acids (particularly the branched-chain amino acids leucine, isoleucine, and valine) and free fatty acids (Stahnke et al., 2002; Beck et al., 2004; Olesen et al., 2004; Tjener et al., 2004a,b). Aroma generation depends however on sausage technology and variety. For fast-ripened sausages, increasing inoculum levels of staphylococci may increase methyl-branched aldehyde production, whereas in slow-ripened sausages the situation is more complex (Tjener et al., 2004b). In the latter case, aroma production is particularly pronounced (Tjener et al., 2004a) and high inoculation levels favour the formation of methyl-branched acids and sulphites, whereas low levels favour diacetyl and ethyl ester production (Tjener et al., 2004b). It is thus possible to modify sausage aroma profiles by changing the inoculation level of

the Staphylococcus starter culture. In addition, additives such as nitrate, nitrite, or ascorbate, precultivation parameters, and environmental factors clearly influence the generation of aroma compounds (Olesen and Stahnke, 2003, 2004; Olesen et al., 2004).

The use of well-selected strains that generate high amounts of aroma components could permit to achieve improved sensory qualities and/or to accelerate the meat fermentation process. Selection of appropriate staphylococci in view of the application will be crucial. Strains of *S. xylosus*, for instance, predominate in Southern European salamis, which are characterized by a rounded aroma and a less acidic taste, and have been recommended when production of very aromatic sausage is intended (Samelis et al., 1998). The species has been shown to produce, amongst others, 3-methyl-1-butanol, diacetyl, 2-butanone, acetoin, benzaldehyde, acetophenone, and methyl-branched ketones (Stahnke, 1999a; Søndergaard and Stahnke, 2002).

The presence of an incomplete h-oxidation pathway in staphylococci explains the formation of methyl ketones (2- pentanone and 2-heptanone) as being derived from intermediates of this pathway (Montel et al., 1996; Stahnke, 1999a; Engelvin et al., 2000; Fadda et al., 2002a). h-Ketoacyl-CoA esters are deacylated into h-ketoacids by a thioesterase and then decarboxylated to the methyl ketone.

Besides contributing to flavour, GCC also prevent the formation of off-flavours and can be used to control the oxidation of unsaturated fatty acids, due to their nitrate reductase and antioxidant activities (Montel et al., 1998; Barrie`re et al., 2001a,b). In conclusion, selected *S. carnosus or S. xylosus* strains, with specific peptide uptake systems and branched- chain amino acid converting and fatty acid oxidising activities, could be used as functional starter cultures to obtain a tastier end-product. For instance, it has been shown that sausages with *S. carnosus 833* mature more than 2 weeks faster than control sausages (Stahnke et al., 2002). Maturity correlates significantly with higher amounts of branched-chain aldehydes and alcohols arising from the breakdown of branched-chain amino acids, and branched- and straight-chain methyl ketones in turn derived from microbial h-oxidation of fatty acids (Stahnke et al., 2002).

1.15.4 Molds and Yeasts

Moulded sausages are very common in the Mediterranean area. It has been shown that the superficial inoculation of the sausage with atoxigenic moulds, e.g. *Penicillium* or

Mucor species, contributes to sensory quality (Bruna et al., 2000, 2001, 2003; Garcıa et al., 2001). This contribution is mediated by lactate oxidation, proteolysis, degradation of amino acids, lipolysis, lipoxidation, the delay of rancidity, and reduced water loss due to slower evaporation (Sunesen and Stahnke, 2003; Benito et al., 2004; Sunesen et al., 2004). Moreover, molds contribute to the overall attractiveness of the end-product due to their characteristic white or greyish appearance, to the stabilization of color through catalase activity, oxygen consumption and protection against light, and to easy skin peeling. A characteristic popcorn odor in mold-fermented sausages has been ascribed to 2-acetyl-1-pyrroline, which may be caused by conversion of proline, often found in sausage collagen casings, by the molds (Sunesen and Stahnke, 2003). However, as with bacterial starter cultures, the selection of mold starter strains should be done carefully since the proteolytic and lipolytic capabilities, and hence the effect on the end-product, can significantly differ between strains and depend on the applied technology (Selgas et al., 1995, 1999; Sunesen and Stahnke, 2003).

The involvement of yeasts in dried fermented sausage ripening has been widely recognized over several decades (Encinas, Lopez-Diaz, Garcia-Lopez, Otero and Moreno, 2000; Flores, Toldrà 2004) Gardini et al., 2001; Geisen, Lucke, & Krockel, 1992; Hammes & Knauf 1994;). Many yeasts isolated from dry-fermented sausages are lipolytic and therefore can attack the fatty tissue and contribute to the development of taste and flavor (Geisen et al., 1992). Moreover, some authors (Papon et al., 1990) have demonstrated that dry-fermented sausages inoculated with yeasts have a greater lipolysis resulting in a stronger odor. However, the role of yeasts in sausage flavour formation is not sufficiently well characterized. In model sausage minces, Olesen and Stahnke (2000) observed that *Candida utilis* was able to produce several volatile compounds, in particular esters and alcohols, many of which were probably derived from branched-chain amino acids, whereas *Debaryomyces hansenii* had very little effect on the production of volatile compounds. In other studies, *Debaryomyces spp.* affected proteolysis (Durà, Flores and Toldra , 2004). Flores et al. (2004) showed that an appropriate inoculum level of *Debaryomyces* spp. influenced volatile production by inhibiting lipid oxidation due to its antioxidant effect and by promoting the generation of ethyl esters. However, large amounts of *Debaryomyces spp*. resulted in high quantities of acids (e.g. 2-methyl-propanoic and 2- and 3-methyl-butanoic acid) that masked the positive effect.

Studies carried out with different yeast species (mainly *Debaryomyces hansenii* and its imperfect form *Candida famata*) have shown that they can positively contribute to the stabilization of the reddening reaction (by removing oxygen) and the development of a characteristic yeast flavor, due to their ability to degrade peroxides, lipolytic and, to a lesser extent, proteolytic activities (Olesen & Stahnke, 2000). Moreover, yeasts have been reported to increase the ammonium content and reduce the amounts of lactic and acetic acids, with the concomitant diminution of the acid taste (Dura´ et al., 2004; Gehlen, Meisel, Fischer, & Hammes, 1991). The protection of sausages from the adverse effects of light is also attributed to this microbial group (Lucke & Hechelmann, 1987). However, the contribution of yeasts to fermented sausage characteristics is affected by the presence of spices and the species of starter cultures used (Flores et al., 2004). The yeast microbiota mainly found in sausages belongs to the genera *Debaryomyces, Rhodotorula, Hansenula* (synonym of *Pichia)* and *Torulopsis* (synonym of *Candida*) (Comi & Cantoni, 1980; Gardini et al., 2001). Studies on salami indicate *D. hansenii* to be the most commonly isolated yeast species. Due to its occurrence in the traditional products and its positive effects on flavour and color, *D. hansenii* is used in starter preparations and should be added to sausage mixtures at a concentration of 6.0 log cfu/g (Hammes & Knauf, 1994). However, Dura´ et al. (2004) stressed the necessity to use yeasts in combination with microorganisms having nitrate reductase activity to counteract the color defect attributable to their inhibition of the indigenous staphylococci.

1.15.4.1 The ‘Non-Conventional’’ Yeast *Yarrowia Lipolytica*

Yarrowia lipolytica is one of the most extensively studied ‘‘non-conventional’’ yeasts, being a strictly aerobic microorganism capable of producing important metabolites and having an intense secretory activity, which justifies efforts to use it in industry (as a biocatalyst), in molecular biology and in genetics studies. It is considered as nonpathogenic and several processes based on this organism were classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA).

Y. lipolytica has been considered an adequate model for dimorphism studies in yeasts, since it has an efficient system for genetic engineering transformation, and is easy to distinguish between its morphological forms, in contrast to *Saccharomyces cerevisiae,* which does not produce true filaments and exhibits pseudo-hyphae growth under

nitrogen-limited conditions. The yeast-to-mycelium transition is associated with unipolar growth, asymmetric division, large polarly located vacuoles and repression of cell separation after division. It is believed that yeast dimorphism is related to a defense mechanism to adverse conditions, such as temperature and nutritional changes. (Coelho et al, 2011).

One of the most important products secreted by this microorganism is lipase, which is an enzyme that attracts the interest of scientists and industrial researchers because it can be exploited for several applications in the detergent, food, pharmaceutical, and environmental industries. Being strictly aerobic yeast, its growth and metabolite secretion are affected by the amount of oxygen available in the culture medium. (Coelho et al, 2011).

Another ability of *Yarrowia lipolytica* strains is to grow on Olive Mill Wastewater (OMW) based medium and produce high-value compounds. In fact, this yeast has been used for bioremediation applications due to its cell wall characteristics and surfactant production. (Lanciotti *et al.*, 2005).

Y. lipolytica is unique strictly aerobic yeast with the ability to degrade efficiently hydrophobic substrates such as n- alkanes, fatty acids, fats and oils for which it has specific metabolic pathways (Fickers *et al.*, 2005) The genome sequence of the fungus has revealed that the organism is distantly related to the conventional yeast *Saccharomyces cerevisiae*.

Rodrigues and Pais have shown that *Y. lipolytica* is capable to use acetic, lactic, propionic, malic, succinic, citric and oleic acids as the sole carbon and energy source, this capacity being, in most cases, independent of the pH of the culture media. Diauxic growth was observed when the yeast was grown in glucose and citric or lactic acid suggesting that the utilization of these two acids is subjected to glucose repression. Propionic, butyric and sorbic acids also had inhibitory effects on yeast growth.

Most strains of *Y. lipolytica* grow very efficiently on acetate as sole carbon source. Concentrations up to 0.4% sodium acetate are well tolerated, higher concentrations reduce the growth rate and, concentrations above 1.0% inhibit the growth (Barth et al, 2007).

Y. lipolytica uses ethanol as carbon source at concentrations up to 3%. Higher concentrations of ethanol are toxic. Several NAD+- and NADP+-dependent alcohol dehydrogenases were observed in *Y. lipolytica*. (Barth *et al*, 2007).

The yeast *Yarrowia lipolytica*, the perfect form of *C. lipolytica*, has also frequently been isolated from fresh beef and sausages (Gardini et al., 2001). Due to its lipolytic and proteolytic activities, this species can have a high technological potential. In fact, its use as co-starter for the production of some cheese varieties has already been proposed by several authors (van den Tempel & Jakobsen, 2000). However, these authors stressed the need for an accurate strain selection based on the characteristics it is desired to impart to the products, the formulation, the production flow sheet adopted and the ripening conditions. In fact, it is well established that the microbial growth and/or the enzymatic activities can be completely inhibited by specific combinations of physico-chemical and environmental factors. Sorensen (1997) presented evidence that the lipolytic activity of several *D. hansenii* strains can be completely repressed at the pH and temperatures normally used in salami ripening. The wide intra-species variability of *D. hansenii* and *Y. lipolytica* in the lipolytic and proteolytic patterns of milk fat and proteins is well documented (Guerzoni et al., 2001; van den Tempel & Jakobsen, 2000). However, these authors presented evidence that the physico-chemical environment and composition of the system can generate more pronounced differences in the expression and activity of specific enzymes of these species than those existing among different strains of the same species.

In a recent study, Patrignani showed that the manufacture of dried fermented sausages using *D. hansenii* and *Y. lipolytica* strains did not affect the pH evolution, while positively influencing the aw decrease of the samples during ripening. Moreover the sausages made by inoculation with selected yeast strains showed more marked and earlier aw reductions; they showed at the end of ripening more pronounced proteolysis and lipolysis. In particular, the use of *D. hansenii* induced an earlier degradation of meat proteins. However, this strain showed less lipolytic potential than *Y. lipolytica* strains. The lipolytic patterns of the products were affected not only by the yeast strain but also by the degree of mincing of the meat mixture. However, important qualitative and quantitative differences in the content of FFA, that can be attributed to the yeast strain activities, were detected in the final sausages and therefore will have a high impact on the final sensory properties of the products. These results demonstrate the necessity to accurately evaluate the technological characteristics of yeast strains to be used as starters in relation to the type of sausage, the ripening time and conditions (Patrignani et al, 2006).

Martin et al investigated about the proteolytic activity of *Penicillium chrysogenum*

during ripening of meat. He showed that inoculation of meat products with *P. chrysogenum* Pg222 promoted hydrolysis of myofibrillar proteins increasing the concentrations of peptides and most amino acids. The increase in free amino acids observed in the batches inoculated with *P. chrysogenum* Pg222 may have a strong influence on the flavour of the ripened product, not only by the direct effect of the amino acids on flavour, but also through the volatile compounds that can be formed from them. This can be very important for meat products such as dry cured ham, where ripening requires several months (Cordoba et al., 1994b). Moreover this effect could contribute to shortening ripening time and improving flavor of meat products of low surface/volume ratio that require an extremely long ripening time.

Table 1.15: Species involved in meat starter cultures (Hammes et al, 2003).

Bacteria

Lactic Acid Bacteria

*Lactobacillus acidophilus,*a *Lb. alimentarius,*b *Lb. paracasei*,a *Lb. rhamnosus*, *Lb. curvatus, Lb. plantarum, Lb. pentosus, Lb. sakei, Lactococcus lactis, Pediococcus acidilactici, P. Pentosaceus*

Actinobacteria

Kocuria varians,c *Streptomyces griseus, Bifidobacterium spp*.a

Staphylococci

Staphylococcus xylosus, S. carnosus ssp. *carnosus, S. carnosus* ssp. *utilis, S. Equorum*

Halomonadaceae

*Halomonas elongata*b

Fungi

Penicillium nalgiovense, P. chrysogenum, P. camemberti

Yeasts

Debaryomyces hansenii, Candida famata

1.16 The Genus *Lactobacillus*

Lactobacilli are Gram-positive, non-spore-forming microorganisms. Considering cellular shape, they can occur as rods or coccobacilli. They are fermentative, microaerophylic and chemo-organotrophic, requiring rich media to grow. They are catalase negative, even if pseudocatalase activity can sometimes be present in some strains. Considering DNA base composition of the genome, they usually show a GC content of lower than 54 mol% (Felis and Dellaglio, 2005).

They are almost ubiquitous: they are found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), respiratory, GI and genital tracts of humans and animals, and in sewage and plant material.

According to *Taxonomic Outline of the Prokaryotes* (Release 5.0, Garrity *et al*., 2004), the genus *Lactobacillus* belongs to the phylum *Firmicutes,* class *Bacilli,* order *Lactobacillales*, family *Lactobacillaceae* and its closest relatives, being grouped within the same family, are the genera *Paralactobacillus* and *Pediococcus*.

The main discrepancy in the taxonomy of the genus *Lactobacillus* is the non-correlation between phylogenetic placement and metabolic properties. The historical subdivisions of the genus *Lactobacillus* based on the type of fermentation have been excellently reviewed by Pot *et al*. (1994), who have underlined how terms such as 'homofermentative', 'heterofermentative', 'obligately homofermentative', 'facultatively heterofermentative' and 'obligately heterofermentative' have been given different meanings by different authors and may be misleading. The accepted 'modern' definition is that given by Hammes and Vogel (1995): obligately homofermentative lactobacilli are able to ferment hexoses almost exclusively to lactic acid by the Embden–Meyerhof–Parnas (EMP) pathway while pentoses and gluconate are not fermented as they lack phosphoketolase; facultatively heterofermentative lactobacilli degrade hexoses to lactic acid by the EMP pathway and are also able to degrade pentoses and often gluconate as they possess both aldolase and phosphoketolase; finally, obligately heterofermentative degrade hexoses by the phosphogluconate pathway producing lactate, ethanol or acetic acid and carbon dioxide; moreover, pentoses are fermented by this pathway.

1.16.1 *Lactobacillus sakei*

Lactobacillus sakei is a Gram-positive anaerobic bacterium commonly found living on fresh meat and fish. This bacterium is valuable in the fermentation of meat products and exhibits properties that allow for better preservation and storage of fresh meats and fish. It is the predominant bacteria used for meat fermentation in Europe, whereas *Pediococcus pentosaceus* tends to be widely used in the United States. *Lactobacillus sakei* took its name from rice alcohol, or sake, which was the product that it was first described in. Sequencing *Lactobacillus sakei*'s genome was important in determining how this bacterium is so well adapted to meat. A team of INRA (Institut National de La Recherche Agronomique) researchers was able to determine its genome and found that its effectiveness in fermentation and food storage is indicative of its ability to sustain life even under challenging environmental conditions, its ability to produce toxics to kill other bacteria, and its capability to use nutrients in meat for self growth. The entire genome of *Lactobacillus sakei* strain 23K was determined to be a circular chromosome containing 1,884,661 base pairs. It consisted of 1,883 protein coding genes, seven rRNA gene clusters, and had a G + C content of 41.25%, (Chaillou, 2005). The presence of "one prophage remnant and 12 complete insertion sequences (IS) mostly localized in two diametrically opposed A+ T-rich regions, suggesting hotspots for genome evolution," was also found. Although raw meat provides *Lactobacillus sakei* nutrients for growth, it contains limited amounts of carbohydrates. Out of the few sugars found in meat and raw fish, *Lactobacillus sakei* can utilize only glucose and ribose (Stentz, 2001). It is no surprise that upon examination of its genome, very small transport systems are present for sugar uptake Because sugars are rapidly exhausted in meat, *Lactobacillus sakei* is also able to catabolize nucleosides such as inosine and adenosine for energy source (Chaillou, 2005). *Lactobacillus sakei* interacts with other bacteria present on meat products. Among them are the pathogenic bacteria such as *Escherichia coli* and *Listeria monocytogenes* those can be very dangerous to humans. Other bacteria include food-spoiling bacteria like *Psuedomonas fragi* and *Brochothrix thermosphacta* that do not necessarily pose danger to health, however, could damage meat products (Chaillou, 2005). *Lactobacillus sakei* has the ability to produce ribosomally-synthesized antimicrobial peptides called bacteriocins that inhibit growth of some of these bacteria. For instance, it was found in a research study that the bacteriocin-positive strain of *Lactobacillus sakei* was able to obstruct the growth of

Listeria monocytogenes in rainbow trout fillets in specific environmental conditions, whereas bacteriocin-negative strain of *Lactobacillus sakei* provided no inhibition (Husar, 2004). *Lactobacillus sakei* is able to produce bacteriocin called sakacin P that inhibits growth of several pathogenic and food-spoiling bacteria present in meat and fish products. It is no wonder that *Lactobacillus sakei* is widely used for meat fermentation. Several research studies have been performed on *Lactobacillus sakei* to further determine optimal environmental conditions, as well as its capacity to ferment meat. *Lactobacillus sakei* is able to grow under both anaerobic and aerobic conditions. With this characteristic, a research study was performed to determine the proteins and genes involved when *Lactobacillus sakei* is growing anerobically. Using two-dimensional electrophoresis, they found that the protein that is over-expressed during anaerobic growth was a peptidase and its corresponding gene is pepR. After performing several experiments on this gene, they found that pepR was indeed responsible for the *Lactobacillus sakei*'s ability to live in an anaerobic environment (Champomier-Vergès, 2002). In another study, it was found that four genes of *Lactobacillus sakei* namely ctsR, asnA2, LSA1065, and LSA1194 were involved during fermentation of raw sausage (Hufner, 2007).

1.16.2 *Lactobacillus casei*

Lactobacillus casei is one of the many species of bacteria belonging in the genus *Lactobacillus*. It is a mesophilic bacteria that is gram positive, rod shaped, nonsporing, nonmotile, anaerobic, and contains no cytochromes. *L. casei* can be found in various environments such as raw and fermented dairy products, intestinal tracts and reproductive systems of humans and animals, and fresh and fermented plant products (Holzapfel, 2001). The optimum pH for *L. casei* is 5.5. The lactic acid produced by *L. casei* through fermentation is very important since it can be used to make cheeses and yogurts, reduce cholesterol levels, enhance immune response, control diarrhea, alleviate lactose intolerance, inhibit intestinal pathogens, and serve as probiotics (Mishra, 2005). Probiotics are viable microorganisms that promote or support a beneficial balance of microbes to live in the gastrointestinal tract (Holzapfel, 2001). There are many strains/isolates of *L.casei* from different origins and geographical locations. That is why molecular typing of *L.casei* is crucial to understanding the evolutionary adaptation of this species to different ecological niches (Cai et al, 2001). Another reason for having *L. casei's* genome sequenced is to

determine the phylogenetic relationships between various groups of bacteria in *Lactobacillus*. *L. casei, L. paracasei, L. rhamnosus*, and *L. zeae* form a closely related taxonomic group within *Lactobacillus*. Sometimes the classification *L. casei* is cloosely applied to strains of any of these species by commercial companies. By having the genome sequenced, species boundaries could be drawn and names can be attached to those species (Desai, 2006). The genome of *Lactobacillus casei* strain ATCC 334 is composed of one circular chromosome and one plasmid. The chromosome has 2.9 million base pairs and the plasmid has 0.029 million base pairs. The chromosome encodes for 2,751 proteins and the sequencing was completed at the US DOE Joint Genome Institute and The Lactic Acid Bacteria Genome Consortium and Fidelity Systems, Inc. Currently, the genome of the plasmid of *Lactobacillus casei* is being sequenced. One of *L. casei's* qualities is its ability to live in various diverse ecological niches. Research through comparative genomic analyses has suggested that extensive gene loss and gene acquisitions during the evolution of lactobacilli, presumably via bacteriophage or conjugation-mediated horizontal gene transfers have facilitated *L. casei's* adaptation to diverse ecological niches (Desai, 2006). The study used 40 different strains of *L. casei* showing that there is a high degree of recombination and phylogenetic diversity among the species. Another feature in the genome of *L.casei* is the *csp-A* gene. This gene codes for a cold shock protein Csp A (66 amino acid residues) which allows the bacteria to adapt to low temperatures (Sauvageot et al, 2006). It is a facultatively anaerobic organism that gets its energy through fermentation. Most *L. casei* strains can ferment galactose, glucose, fructose, mannose, mannitol, N-acetylglucosamine, and tagatose (Cai et al, 2001). The ability to ferment lactose is less common in strains isolated from plant materials than in those from cheese and human gastrointestinal tracts . The conditions of fermentation such as temperature, pH, the type of growth media, oxygen, and some neutralizers also play a role in the growth activity of *L. casei* (Ha et al, 2003). The most important compound that *L. casei* produces is lactic acid. It is obtained by fermenting glucose and lactate formation. Lactic acid is a hydroxy acid that can be produced chemically from acetaldehyde and hydrogen cyanide or by microbial fermentation. It is used for numerous industrial processes such as chemical and biological production of organic acids, the use as a flavoring in food, the manufacturing of cosmetics, and the production of biodegradable plastics. *Lactobacillus casei* has the ability to adapt to a variety of ecological niches. One of

these niches is the gastrointestinal tract. *L. casei* functions as a probiotic in the gastrointestinal tract. Probiotics are originally defined as microorganisms promoting the growth of other microorganisms (Holzapfel et al, 2001). The characteristics of a successful probiotic are acid and bile tolerance, antimicrobial activity against intestinal pathogens, and ability to adhere and colonize the intestinal tract (Mishra et al, 2005). In order for the probiotics to carry out their functions, the probiotic live cells must not be lower than $10^6/10^7$ cfu/g (Nebesny, 2007). The strains of *L. casei* that live in the intestines are sensitive to the intestinal conditions by having high bile salt concentrations and have the permeabilization and release of intracellular lactase to produce lactic acid (Holzapfel et al, 2001). *L. casei* is very important in regulating the immune system of the gastrointestinal tract. *L. casei* will bind to the luminal surface of gastrointestinal cells and stimulate gut-associated lymphoid tissue . This will strengthen the innate immune response and give local and systemic immunity to the body. To fight off the pathogens that may invade the immune system, *L. casei* can compete for nutrients or adhesion site against the pathogens. They can also inhibit the growth of pathogenic bacteria by a pH reduction through the production of organic acids such as acetic, propionic, or lactic acid, or by producing hydrogen peroxide (Millette et al, 2007). Furthermore, *L. casei* can secrete bacteriocins, antimicrobial peptides of cationic, amphiphilic molecules, to get rid of the pathogens in the body. Another interesting characteristic of *L. casei* is its ability to adapt to colder temperatures, cold shock response. Research has shown that cold shock can cause a sudden growth stop or significantly reduced growth rate by decreasing membrane fluidity, and arrest or decrease of the synthesis of most housekeeping proteins. The cold shock would turn on the *csp-A* gene to make cold shock proteins (CSP A) to help the cell adjust to its colder environment. The research has also shown that CSP is needed not only for cold shock response, but for optimal growth in normal, unstressed cells (Sauvageot, 2006). *L. casei* is generally considered nonpathogenic and safe. However, cases of sepsis, meningitis, and infections localized in organs have been reported (Salvatore, 2007). *Lactobacillus casei* produces lactic acid which is used in various applications in biotechnology since it has numerous beneficial effects such as an increase in immune system response, a decreased risk for bladder cancer, and reduced cholesterol levels. Most of the biotechnology applications are related to the food industry.

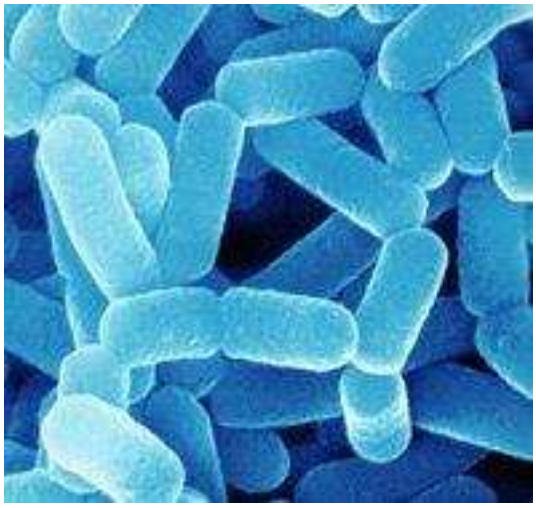


Figure 2.1.2 : *L.casei*

1.16.3 *Lactobacillus rhamnosus*

Lactobacillus rhamnosus is a bacterium that was originally considered to be a subspecies of *L. casei*, but later genetic research found it to be a species of its own. Some strains of *L. rhamnosus* are being used as probiotics.

Probiotics, as defined by the Food and Agricultural Organization of the United Nations, are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." *L. rhamnosus* was first isolated in 1983 in the intestines of a healthy human subject by scientists Barry Goldin and Sherwood Gorbach, when it was shown to have remarkable tolerance for the harsh acids normally found in the stomach and digestive tract. The "GG" in the title of the strain *L. rhamnosus* GG is derived from the last names of the two scientists. Like other probiotics, *L. rhamnosus* has properties that are beneficial to the intestinal tract. It is also believed to be of considerable assistance with the immune system, particularly in combating intestinal and urinary tract pathogens. It is also used as a natural preservative in yogurt-based products, where the bacterium attaches to the lining of the intestines, where it encourages the growth of helpful organisms that aid in digestion.

Figure 2.1.3: *Lactobacillus rhamnosus*

Lactobacillus rhamnosus is a probiotic bacterium that helps eliminate and prevent the growth of harmful bacteria in the intestines. Many consumers may be familiar with lactobacillus probiotics, which are touted today by some yogurt manufacturers as an aid in digestion and in promoting regular bowel activity. In fact, lactobacilli have been used for centuries to aid in the fermentation of dairy products. During the 20th century, researchers began evaluating these organisms and their positive effects on the human body and its ability to naturally ward off disease and infection. The *Lactobacillus rhamnosus* bacterium was first isolated by researchers in 1983, when it was shown to have remarkable tolerance for the harsh acids normally found in the stomach and digestive tract (Conway et al, 1987). The following are some of the well noted benefits of *L. rhamnosus:*

i. Helps Fight Intestinal Tract Illnesses: according to studies published by Goldin and Gorbach, *L. rhamnosus* is said to be able to survive the highly acidic conditions of the human stomach, as well as the intestinal tract. It is also believed to be bile-stable (Conway et al, 1987). This makes the probiotic highly desirable in its ability to conquer intestinal ailments.

ii. Suppresses Bacterial Infections in Renal Patients: in 2005, it was demonstrated that with patients experiencing kidney-related illnesses, *L. rhamnosus* is capable of interrupting the gastrointestinal transportation of the variety of enterococcus that is resistant to the antibiotic vancomycin.

iii. Assists in Prevention of Urinary Tract Infections: according to an article published in the November 2009 issue of Renal and Urology News, daily ingestion of *L. rhamnosus* Gr-1 may be effective in helping postmenopausal women who suffer from chronic urinary tract infections. While dosage of trimethoprim-sulfamethoxazole is considered to be a standard treatment for a

UTI, *L. rhamnosus* is a viable alternative when antibiotic resistance is a consideration (Schieszer, 2009). The probiotic seems to be capable of safeguarding the urogenital tract by its ability to excrete biosurfactants. This enables the tract to limit the adhesion of pathogens.

iv. Helps Build a Superior Immune System: while blood cells are certainly a major agent in managing the body's immune system, the gut is also a huge contributor in this area. Because of the ability of *L. rhamnosus* to survive in extremely acidic environments such as the digestive system, the probiotic can thrive in the gut. It stimulates the production of antibodies and also assists in the process of phagocytosis, a means by which the body combats dangerous invasive bacteria.

v. Decreases Duration of Diarrhea: research conducted in 2000 in several European countries indicated that the administration of *L. rhamnosus* GG to children suffering from rotavirus shortened the duration by at least one day of the pervasive diarrhea associated with the illness. (Guandalini et al., 2006) Another study showed that ingestion of *L. rhamnosus* GG was helpful in reducing the extent of diarrhea when it exists as a side effect of antibiotic use to combat *H. pylori* infections.

Lactobacillus rhamnosus is one of the most widely studied probiotics, noted and valued for its ability to survive and even thrive in the harsh conditions of the digestive and urinary tracts. Multiple clinical trials have determined the bacterium to be especially beneficial in promoting and maintaining digestive tract health. It is extremely well tolerated by men and women, and has been associated with only very rare side effects. Studies have shown that, taken regularly, *L. rhamnosus* can be an effective supplement in promoting and maintaining digestive tract health.

1.16.4 *Lactobacillus plantarum*

L. plantarum is a gram positive bacterium that is found in a variety of niches. These niches include dairy, meat, and much vegetable fermentations, it is also found in the human gastrointestinal tract. It is a facultative heterofermentative lactic acid bacterium that utilizes an extensive range of fermentatable carbon sources. Lactic acid bacteria are Gram-positive and they are non-spore forming, fermentative bacteria that grow anaerobically. The main function of these bacteria is the fermentative

conversion of sugars present in raw materials into lactic acid. *L. plantarum* also produces anti-microbial peptides and exopolysaccharides. It has the ability to maintain a pH gradient between the inside and outside of the cell in the presence of large amounts of acetate or lactate. (De Vries et al, 2006). *L. plantarum* is one of the most common microbes used as a silage inoculant. Silage is a fermented fodder that can be fed to ruminants or used as a biofuel feedstock for anaerobic digesters. (Giraud et al, 1994). *L. plantarum* has one of the largest genomes among lactic acid bacteria. In its circular chromosome it contains 3,308,274 base pairs. The genome was sequenced by using whole genome sequencing as assembly approach. The overall GC content of its chromosome is 44.5%, the plasmids tend to have a lower percent GC content. Putative biological functions have been given to 2,120 of the predicted proteins. One particular interesting region of the chromosome is the 213-kb region from 3,072,500 – 3,28,500, which encodes proteins for sugar transport, metabolism, and regulation. This region has a lower percent CG content (41.5%), leading researchers to believe that horizontal gene transfer has acquired many of these genes (Kleerebezem et al, 2007). *L. plantarum* has three plasmids, pWCFS101, pWCFS102, and pWCFS103. The plasmid sizes are as follows: pWCFS101 contains 1,917 bp, pWCFS102 contains 2,365 bp, and pWCFS103 contains 36,069 bp. Plasmid pWCFS101 is believed to contain replication proteins. Plasmid pWCFS102 is believed to contain replication proteins as well as proteins that function as copy number controls. Plasmid pWCFS103 contains genes that are predicted to be involved in arsenate and/or arsenite resistance as well as cadmium resistance; it also has genes that are believed to encode replication proteins, resolvases, DNA-damage-inducible proteins, and oxidases. *L. plantarum* contains two apparently complete prophage genomes, as well as some prophage remnants (Kranenburg et al, 2005). The *L. plantarum* chromosome reveals that this microbe has a major focus on carbon catabolism. The sequence of its chromosome also supports its extreme flexibility, versatility, and ability to adapt to different environmental conditions (Kleerebezem et al, 2007). *L. plantarum* has a rod shaped structure with rounded ends. This microbe is a gram positive bacteria meaning there is a high concentration of peptidoglycan in the cell wall, and lack an outer cellular membrane. The organism is also Auxotrophic meaning that it synthesizes few organic compounds, when it has the ability to break down sugars and pyruvate. It is also a facultative heterofermentative lactobacilli microorganism, this means that the organism takes carbon from sugars and pyruvate

and the byproduct is either alcohol or lactic acid. This process happens in an aerotolerant environment meaning that oxygen is not present. When oxygen is present it is released as H_2O_2 which can be used as a weapon that kills off other bacteria. Do to the inability to handle oxygen the organism uses a manganese dependent process. This process uses metal as a pseudo catalase and lowers oxygen concentration that is favorable to the aero tolerant environment. Sugar is a key source of energy for the microorganism to degrade (Dudley et al, 2001). During the degradation of sugar carbon is released and becomes a source of energy for the *L. plantarum*. When it is exhibiting a pyruvate metabolism it is similar to homolactic fermentation. This happens when growth occurs on glucose that is degraded to pyruvate though an EMP pathway. Once the pyruvate is formed it is converted to d and l-lactate though sterospecific lactate dehydrogenase enzymes (Dudley et al, 2001). Researchers believe that the sequence of the *L. plantarum* genome has certain features that allow this microbe to be versatile and adaptive to different environments (Kleerebezem et al, 2007). It can grow in-between 15-45 °C and can grow at pH levels of 3.2 and greater. This versatility allows *L. plantarum* isolates to be found in human saliva, fermenting dairy products, plant material, silage, and even certain waste waters. It gains its energy through the fermentative conversion of sugars to lactic acid, as long as is able to go through this process, most environments will allow the growth of this microbe. Experts believe that the high number of regulatory genes causes this microorganism to be so adaptable. The most common habitat is in a protein enriched environment such as dairy because of its primary protein-degradation which produces peptides. A study showed that there are 144 N-terminals that can be used for peptidase cleavage. Another key part to describe the adaptability of this microorganism is its ability to perform horizontal gene transfer. This process is accomplished though natural competition, bacteriophage infection and more. *L. plantarum* can perform these transformations because it can bind DNA and uptake that DNA (Kleerebezem et al, 2007). Recently *L. plantarum* has been identified as a probiotic. Probiotics are non-pathogenic microorganisms that can have a positive impact on human health when they are digested. They are becoming a very popular dietary supplement to many people, especially those who have gastrointestinal problems (Adrian et al, 2008). In this case, *L. plantarum* can be considered a human symbiotic. When the probiotics are ingested regularly it is possible that the composition of microflora in the intestinal tract can be manipulated. This

manipulation may allow an improvement of microbe balance, stabilization of digestive enzyme patterns, and immunomodulation by activating and regulating mucosa-associated and systemic immune system responses. The microflora found in the intestinal tract are thought to provide protection from pathogens. Some companies currently sell bottles containing *L. plantarum* as a probiotic to help with intestinal problems including IBS and IBD, stating that these bacteria help to "balance the intestinal ecosystem". Along with its possible use as a probiotic, there is another very important use for *L. plantarum,* in fact it is currently being explored to convert lignocellulosic biomass to biofuel and bioproducts. Current research into this idea is looking at a strain of *L. plantarum* which has certain genes inactivated to eliminate undesirable fermentation products (Liu et al, 2006). It is also able to degrade cassava raw starch. Its ability to degrade raw starch is useful because it could potentially be used as a starter in certain traditional fermentation processes. There are also potential uses for *L. plantarum* to be used in treatments of certain wastewater due to its ability to degrade phenolic compounds, such as those in olive mill wastewaters (Bronze et a, 2008). The ability of the this microbe to adapt and thrive in a range of environments, its ability and capacity to be genetically manipulated, as well as its ability to ferment and degrade different materials makes *L. plantarum* a very interesting and important bacteria to study.

1.16.5 *Lactobacillus reuteri*

Lactobacillus reuteri is a Gram-positive, rod-shaped, and anaerobic. This heterofermentatic lactic acid bacterium naturally inhabits the gut of a wide range of organisms, including humans, pigs, chickens and mice (Morita et al, 2008). It can also be isolated from human breast milk. In vitro, *Lactobacillus reuteri* grows optimally on MRS media at 37°C (Morita et al, 2008). They have also been found to grow in biofilms.

L. reuteri produces reuterin, an antimicrobial that inhibits growth of harmful bacteria, fungi, and protozoa. Due to these probiotic properties, *L. reuteri* is believed to be a promising therapy for the alleviation and reduction of certain illnesses related to gastrointestinal health, oral health, and urogenital health, including infantile colic, eczema, and H. pylori infection.

The genome of the strain JCM1112 was fully sequenced at Kitasato Institute for Life

Sciences by April 21, 2008. The genome contains one circular chromosome and does not contain any plasmids. The entire genome is 2,039,414 nucleotides long, with a GC content of 38%. The genome contains 1901 genes, 83% of which are protein coding. The chromosome contains 1,820 open reading frames (ORFs), 53 of which are phage related. *L. reuteri* is a Gram-positive rod that forms chain arrangements and does not produce endospores. Some strains have also been shown to form relatively thin biofilms (5-7µm thick.) (Jones et al, 2009). *L. reuteri* is a mutualistic host-associated microbe, living in the guts of animals. As such, it requires a host habitat and is a mesophilic, facultative anaerobe with a preference for acidic environments. It is an obligate heterofermentative microbe, producing carbon dioxide, ethanol, acetate, and lactic acid from glucose fermentation. It can also anaerobically metabolize glycerol, producing the antimicrobial reuterin (3-hydroxypropionaldehyde) (Morita et al, 2008). *L. reuteri* has also been shown to produce folate and cobalamin, also known as vitamin B12, nutrients that many animals, including humans, require.

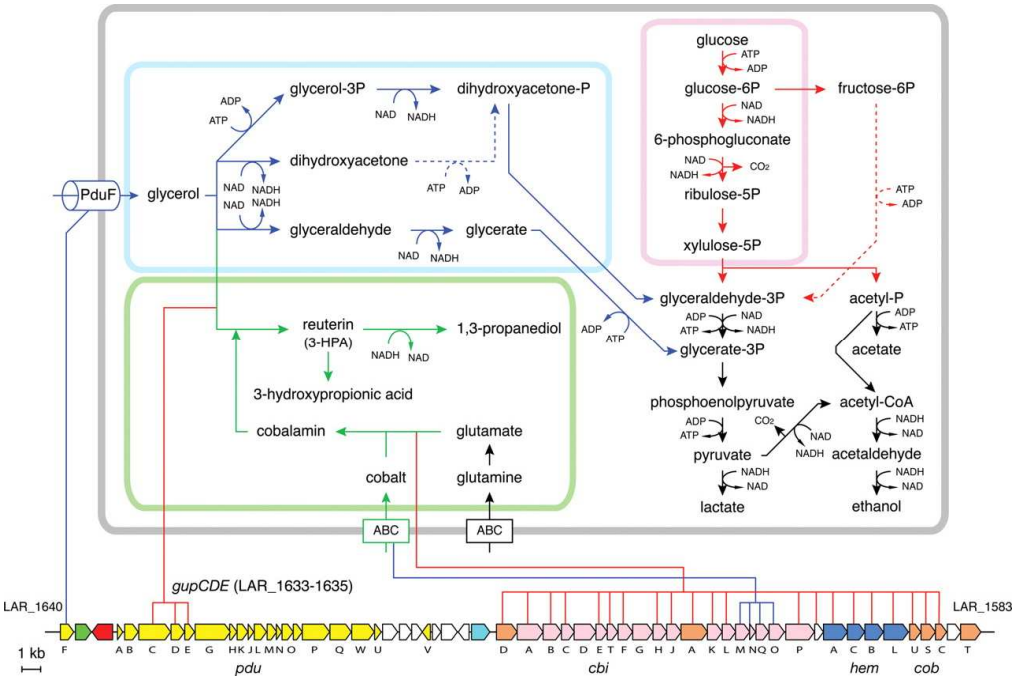


Figure 2.1.5: Proposed metabolic pathways of *Lactobacillus reuteri*

Lactobacillus reuteri is a naturally occurring component of the animal stomach and intestine microflora. It has not been known to be pathogenic; rather, it has been studied as a probiotic organism; it produces reuterin by fermenting glycerol. (Morita et al, 2008). Reuterin is a broad-range antibiotic, affecting both Gram-positive and Gram-negative species, that most pathogenic intestinal bacteria are sensitive to. Studies have shown reuterin produced by *L. reuteri* is useful against a wide array of

harmful bacteria such as pathogenic *E. coli* strains, *Clostridium difficile, Salmonella enterica, Vibrio cholerae*, and many others (Cleusix et al, 2007). While it is known how reuterin is produced, the mechanism of how reuterin inhibits growth of such a broad range of pathogenic microbes is still unclear. Its antimicrobial ability is also contributed by its colonization of the epithelial cells, preventing other bacteria from colonizing. Studies have shown that some strains may prevent *Helicobacter pylori* infection, the main cause of gastric ulcers, via colonizing competition (Mucai et al, 2001). *L. reuteri's* ability to produce the nutrients folate and vitamin B12 has also been studied as a possible nutritional supplement. Humans require daily intakes of both of these nutrients, and vitamin B12 deficiency has been linked to heart disease, anemia, cancer, and other problems. Vitamin B12 deficiency is particularly prevalent in people with strict vegetarian diets, where supplements of *L. reuteri* would be useful.

1.16.6 *Lactobacillus fermentum*

L. fermentum is a closely related species of *L. reuteri* based on a sequence analysis of the 16S ribosomal RNA gene (95% identity) (Ennahar, 2003) and on phenotypic properties, including being an obligate heterofermentative organism. Previously, both these species were classified as a single species (Ennahar, 2003), but were subsequently separated based primarily on DNA hybridization and GC content (Klein, 1998). Despite many similar phenotypic characteristics, probiotic effects have primarily been observed for *L. reuteri*, with a few studies suggesting probiotic properties for *L. fermentum* (Strompfova et al, 2006; Mikelsaar et al, 2009). The use of gut microbes as probiotics in food is aimed towards preventing and treating various health problems. Among these health problems allergies, neoplastic growth, and inflammatory bowel disease are included. Recent areas of study have focused on the influence of probiotics on metabolic functions of their host. One area has been the metabolism of cholesterol by LABs acting as probiotics. Research has shown that *Lactobacillus* species have been proven to remove cholesterol in vitro through various ways such as assimilation, binding to the surface cells, and incorporation into cellular membranes (Mikelsaar et al, 2009). Recent studies have shown that *L. fermentum* has antibiotic resistances. DNA was isolated from *Lactobacillus fermentum* and tested for antibiotic resistance against clinically important agents by using broth dilution tests.

Different strains of *Lactobacillus fermentum* demonstrated uniform resistance patterns demonstrating resistance to glycopeptide vancomycin and to tetracycline (Klein et al, 2011). This is in contrast with other studies, where it's shown that *Lactobacillus fermentum* is sensitive to some common antibiotics such as gentamicin, cefazolin, penicillin, trimethoprim/sulfamethoxazole, ampicillin, carbenicillin, erythromycin, amikacin, and cholorampehnicol (Zheng et al, 2010).

1.17 The Lab Metabolism and Its Importance in Fermented Foods.

Lactic acid bacteria (LAB) are the predominant microorganisms in a majority of food fermentations and their metabolic activity determines and maintains the quality of fermented foods. The application of starter cultures has become the state of the art in a majority of industrial food fermentation as it allows process standardization, mitigation of hygienic risks, and product diversification (Gänzle, 2009).

Strains suitable as starter cultures exhibit a combination of several desirable properties. Generally, strains must be suitable for large scale production and culture preservation. Metabolites that make an important contribution to food quality in one application may constitute a spoilage event in another (Gänzle, 2009).

1.18 Antimicrobial Activity

Food preservation by lactic fermentation generally relies on the removal of fermentable carbohydrates, the consumption of oxygen, the formation of organic acids and the concomitant decrease in pH (table 2.3.1). The preservative effect can be increased by the strain-specific formation of specific inhibitors. Diacetyl, acetaldehyde or H_2O_2 contribute to the inhibitory effect of LAB but these compounds strongly affect the sensory properties of food when present in inhibitory concentrations (De Vuyst and Vandamme, 1994; Vandenbergh, 1993). Bacteriocin application in food can be considered a mature technology and bacteriocin producing LAB are primarily used to inhibit or to eliminate *Listeria monocytogenes* from ready-to-eat meats and dairy products (Stiles, 1996; Montville et al., 2001; Drider et al., 2006).

Table 2.3.1. Metabolic activities of lactic starter cultures

UNSPECIFIC FACTORS	SPECIFIC INIHIBITORS
Substrate utilization	Diacetyl, acetaldehyde
Occupation of surfaces	Short chain fatty acids, hydroxy-fatty acids
Consumption of oxygen	Reuterin, reutericyclin
Acid formation (lactate, acetate, propionate)	Bacteriocins, antifungal peptides, and cyclic dipeptides (cyclo[Phe-Pro], cyclo[Phe-OH-Pro], cyclo[Gly-Leu])
Decrease of pH	H_2O_2
Decrease of redox potential	Benzaldehyde, (hydroxy-phenyllactate, Benzoate

Although fungi represent major spoilage organisms on fermented foods, food applications of antifungal compounds from LAB were only recently described. Antifungal metabolites from LAB include propionate, phenyllactate, hydroxyphenyllactate, several cyclic dipeptides, and 3-hydroxy fatty acids (Table). Phenyllactate and hydroxyphenyllactate are produced from phenylalanine and tyrosine, respectively (Lavermicocca et al., 2000 and 2003). Fungistatic cyclic dipeptides are produced by several strains of *Lactobacillus plantarum* (Ström et al., 2002; Schnürer and Magnusson, 2005; Broberg et al., 2007). Minimum inhibitory concentration of phenyllactate and cyclic dipeptides are in the range of 5–50 g/L but the concentrations generated in food fermentations are well below this MIC (Vermeulen et al., 2006a). These compounds are thus active only when acting in concert with other antimicrobial agents (Schwenninger et al., 2008).

1.19 Acidifying Activity

The energy yield in glucose metabolism of obligate heterofermentative lactobacilli is dependent on the availability of hydrogen acceptors for NADH regeneration (Gänzle et al., 2007). Oxygen, fructose, and oxidized glutathione are used as hydrogen acceptors and are reduced to H_2O mannitol, and thiols, respectively, with concomitant oxidation of NAD(P)H to NAD(P).

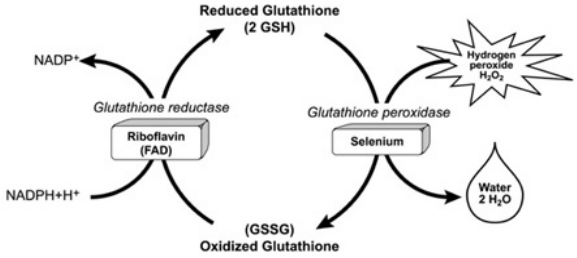


Figure 2.4: Overview on the effect of intracellular, NADH dependent glutathione reductase (GshR) on intra- or intermolecular disulfide bonds: one molecule of hydrogen peroxide is reduced to two molecules of water, while two molecules of glutathione (GSH) are oxidized in a reaction catalyzed by the selenoenzyme, glutathione peroxidase. Oxidized glutathione may be reduced by the flavin adenine dinucleotide (FAD)-dependent enzyme, glutathione reductase.

1.20 Carbohydrate Metabolism

Most obligatory heterofermentative lactobacilli but not *L. sanfranciscensis* metabolize pentoses (Hammes and Hertel, 2003). Simultaneous metabolism of maltose and pentoses was observed *for L. brevis, L. fermentum and Lactobacillus hilgardii* during growth in sourdough (Gobbetti et al., 1999). Sucrose is metabolized by fructosyltransferases in some strains of *L. sanfranciscensis* .

Lactate, ethanol and CO2 are the major products of hexose metabolism unless co-substrates are present that enable the regeneration of reduced cofactors. In the presence of electron-acceptors, acetyl-phosphate is converted to acetate with the yield of an additional molecule of ATP. Glycerol and erythritol are alternative metabolites of *L. sanfranciscensis* from glucose.

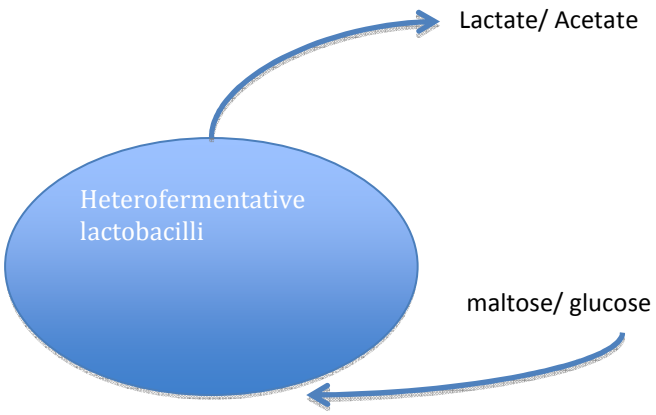


Figure 2.5.1 : the heterofermentative pathway of lactic acid bacteria

Obligate homofermentative and facultative heterofermentative lactobacilli degrade

hexoses via the Emden-Meyerhoff pathway, in these organisms, maltose and fructose utilization generally occurs only after glucose depletion. Maltose is hydrolyzed by a-glucosidase activity. Pentose metabolism by LAB is repressed by glucose (Lokman et al., 1997; Titgemeyer and Hillen, 2002) but simultaneous metabolism of maltose and arabinose was observed during growth of *L. plantarum* in sourdough (Gobbetti et al., 2000).

Hexose metabolism via the Emden-Meyerhoff pathway leads to lactate production with pyruvate as central intermediate of metabolism. Numerous alternative pathways exist in lactobacilli for alternative fates of pyruvate, and metabolites other than lactate may be the major metabolites of hexose metabolism at conditions of glucose-limitation or in the presence of oxygen as electron acceptor (Cocaign-Bousquet et al., 1996; Axelsson, 2004). However, lactate is the major end product of homofermentative hexose fermentation in many fermented foods.

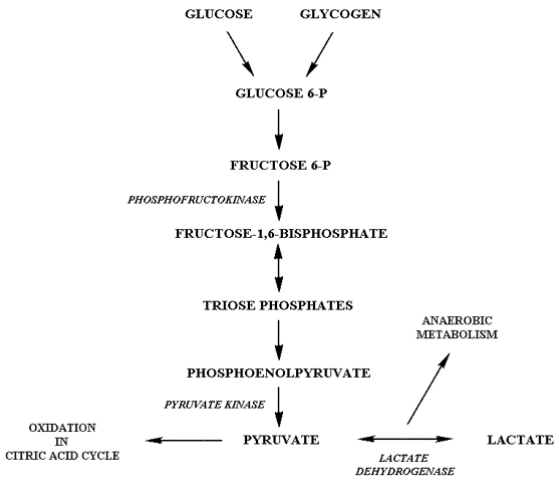


Figure 2.5.2: Emden-Meyerhoff pathway

The initial steps for citrate metabolism in LAB are transport by the citrate permease and the citrate-lyase reaction. Two alternative routes exist for oxaloacetate conversion, one pathway results in succinic acid formation, the other resulting in the decarboxylation to pyruvate (Cselovszky et al., 1992; Ferain et al., 1996). In *Lactococcus lactis*, pyruvate is partially converted to α-acetolactate when electron acceptors such as citrate are present that create a pyruvate surplus relative to the need of NADH regeneration via the lactate dehydrogenase reaction. α-acetolactate is enzymatically reduced to acetoin or converted non-enzymatically to the flavour

compound diacetyl (for review, see Hugenholtz et al., 2002). *L. sanfranciscensis* strains use the pyruvate branch to convert citrate to lactate and acetate (Gobbetti and Corsetti, 1996); the cofactor regeneration in the lactate dehydrogenase reaction enables the additional formation of acetate. Malate and fumarate are converted to lactate by *L. sanfranciscensis. L. reuteri and L. pontis* do not utilize citrate and convert malate and fumarate to succinate (Stolz et al., 1995a, b). The growth of *L. sanfranciscensis* in sourdough is limited by the pH (Ganzle et al., 1998) and lactate formation from citrate does not decrease the pH. Therefore, the use of citrate results in increased lactate and acetate levels.

1.21 Peptide and Amino Acid Metabolism

The majority of sourdough LAB does not exhibit cell-wall associated proteinase activity (Pepe et al., 2003; Vermeulen et al., 2005). Generally, a comparable extent of protein degradation is observed in wheat sourdough and in chemically acidified dough (Thiele et al., 2002, 2003, 2004; Loponen et al., 2004). However, several strains of sourdough LAB strains exhibiting proteolytic activity were characterized (Gobbetti et al., 1996; Di Cagno et al., 2002; Pepe et al., 2003) and a contribution of selected LAB to proteolysis could be demonstrated by analysis of the degradation of albumins, globulins, and gliadins in wheat sourdoughs (Di Cagno et al., 2002; Pepe et al., 2003; Zotta et al., 2006). LAB predominantly use peptides to meet their demand of complex nitrogen (Kunji et al., 1996). Comparable to *Lc. lactis and L. plantarum, L. sanfranciscensis* expresses transport systems for oligo and dipeptides (Vermeulen et al., 2005) and peptides are hydrolysed by intracellular peptidases, several of which were characterized on biochemical or genetical level (Gobbetti et al., 1996; Gallo et al., 2005; Vermeulen et al., 2005). Vermeulen et al. (2006b) compared phenylalanine turnover by *L. plantarum* and *L. sanfranciscensis* using phenylalanine and defined dipeptides as substrates. In N-limited media, both strains produced higher levels of phenyllactate from phenylalanine when the dipeptides FL, FS or PF were offered as substrate. Thus, transport limitations to the metabolism of amino acid in *L. sanfranciscensis* and *L. plantarum* are overcome by the addition of peptides as substrate. These finding are in keeping with previous observations in *Lc. lactis* and *L. helveticus* that the transamination of amino acids can be enhanced by cell lysis or permeabilization (Martinez-Cuesta et al., 2002; Valence et al., 2000).

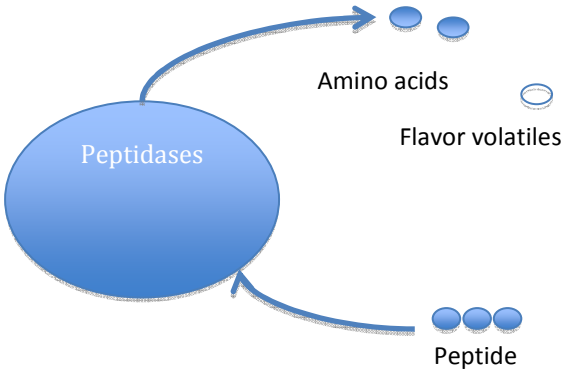


Figure 2.6: peptide hydrolysis and amino acid metabolism

Fadda and Vignolo studied the acidogenic metabolism of a strain of *L.plantarum* isolated from fermented sausage (2008). They focused on the hydrolysis of sarcoplasmic protein during fermentation in a meat model system under controlled pH and Temperature. Results showed that the hydrolysis of sarcoplasmic protein at pH 4, as during a sausage fermentation, was carried out from the synergic action of meat proteases, LAB proteolytic system and lactic acid promoted soluble-protein hydrolysis, while at pH 6 the hydrolysis was carried out exclusively from the proteolytic system of the *L. plantarum*. Moreover they observed that at acid pH (4 or lower) all the aminopeptidases are inhibited, that's a limiting factor for amino acids release.

1.22 Glutamine and Glutamate Metabolism

Proteolysis and amino acid metabolism by starter cultures are of particular relevance for food quality as amino acid metabolites are active as flavour compounds, biogenic amines, taste compounds, or antifungals. For example, the strain specific conversion of phenylalanine may yield phenylethanolamine, a biogenic amine, phenylacetate or phenylacetaldehyde, two flavour compounds which impart a "flowery" or "honey like" flavour impression, or phenyllactate, a compound with antifungal activity. The spectrum of metabolites produced by LAB from amino acids varies greatly among species and within different strains of a species (Liu et al., 2008). Recent advances in the selection of starter cultures are particularly related to the biochemical and genetic characterization of glutamine and glutamate metabolism.

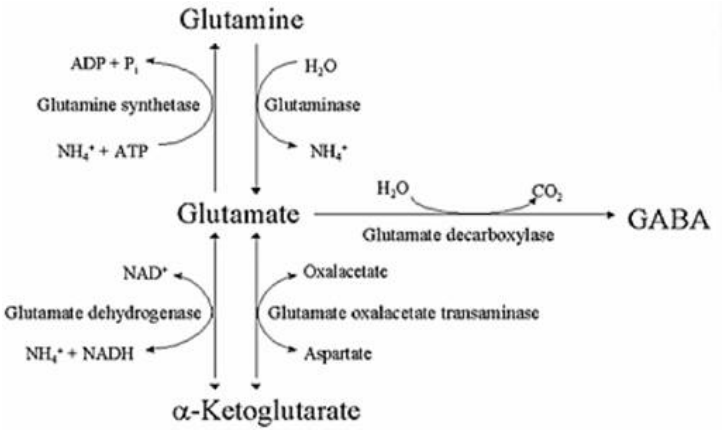


Figure 2.7: glutamine and glutamate metabolism in LAB.

An overview of the metabolism in Lactic acid bacteria is reported in figure 2.7.

- **i.** Amino acids are taken up as peptides and cleaved by an array of intracellular peptidases (Gänzle et al., 2007).
- **ii.** Glutamine is converted to glutamate by glutaminase, an enzyme activity that was reported in *L. rhamnosus* (Weingand-Ziade et al., 2003), *L. sanfranciscensis* and *L. reuteri (*Vermeulen et al., 2007b).
- **iii.** Strain specific conversion of glutamate to γ-aminobutyrate (GABA) was reported for *L. brevis, L. paracasei, L. delbrueckii, L. plantarum and L. lactis* (Yokoyama et al., 2002; Siragusa et al., 2007).
- **iv.** NADH-dependent conversion of glutamate to α-ketoglutarate (αKG) by glutamate dehydrogenase is a strain specific property of *L. plantarum, L. sanfranciscensis, L. paracase*i and other lactic acid bacteria (Tanous et al., 2002 and 2005; Vermeulen et al., 2006a).
- **v.** LAB generally exhibit transaminase activity and convert αKG to glutamate in the presence of other amino acids as amino donors.
- **vi.** The conversion of αKG to GABA was reported for *Oenococcus oenii* (Radler and Bröhl, 1984).

Glutamate contributes to the umami taste of fermented foods (Drake et al., 2007). LAB liberate glutamate from peptides, or by conversion of glutamine. Glutamine conversion is particularly relevant during growth of LAB in cereal substrates as glutamine is the predominant amino acid in cereal prolamins (Wieser, 2004). *L. rhamnosus, L. reuteri* and *L. sanfranciscensis* exhibit glutaminase activity and convert glutamine or glutamine containing peptides to glutamate (Weingand-Ziade et al., 2003; Vermeulen et al., 2007b).

GABA possesses multiple physiological functions and some studies indicate that dietary GABA has antihypertensive properties (Inoue et al., 2003). GABA is a non-proteinogenic amino acid formed by glutamate decarboxylase activity. A strain of *Lactobacillus brevis* quantitatively decarboxylated glutamate to GABA in cereal mashes (Yokoyama et al., 2002). LAB exhibit glutamate dehydrogenase activity in a strain specific manner. The enzyme catalyses the NAD(P)H-dependent recycling of glutamate to a-ketoglutarate, and consequently increases the flux through the transaminase reaction (Rijnen et al., 2000; Tanous et al., 2002). Remarkably, the cofactor-dependency of the glutamate dehydrogenase linked glutamate recycling and the metabolic flux through the transaminase reaction in *L. sanfranciscensis* DSM20451 to NAD(P)H regeneration in central carbohydrate metabolism (Vermeulen et al., 2006b). The conversion of hydrophobic and branched chain amino acids in LAB is initiated by transamination and requires a α-ketoacid as amino group acceptor. The preferred acceptor in lactococci and lactobacilli is α- ketoglutarate (αKG, Fernández and Zúñiga, 2006).

α-Ketoglutarate serves as amino acceptor in the transamination reaction of leucine, phenylalanine and other amino acids, correspondingly, the addition of α-ketoglutarate strongly increases amino acid conversion *of L. sakei, L. plantarum and Lc. lactis* (Yvon et al., 1998; Larrouture et al., 2000; Rijnen et al., 2000). Glutamate conversion to αKG occurs via transamination with oxaloacetate as amino acceptor, and simultaneous addition of citrate and glutamate increased the conversion of phenylalanine and leucine to the corresponding α- ketoacids (Tanous et al., 2005). NADH and NADPH dependent glutamate dehydrogenase (GDH) activity is an alternative pathway for αKG formation in lactococci and lactobacilli and differences in the conversion of phenylalanine and leucine by transamination were explained by the strain-dependent differences in GDH activity (Tanous et al., 2002).

1.23 Role and Importance of Glutamate in Foods.

It is merely one decade that we recognize the taste of glutamate as one of the basic tastes termed as “umami”. The recognition of glutamate as an excitatory neurotransmitter in mammalian nervous system as well as umami as the fifth basic taste has an interesting historical background. Historically, glutamate was first isolated as glutamic acid from acid hydrolysate of wheat gluten, by the German

scientist Ritthausen in 1866 and thus named it as “glutamic acid”. The history of glutamate in food is older than the history of science of nutrition. Practice of adding large seaweed (*Laminaria japonica*) to soup stocks has been in use in Japan for last 12 centuries. This seaweed markedly increases the taste of the soup. But what was unknown that it contained high amount of glutamate. It was not until 1908 that the link between the seaweed and glutamate was discovered. The brown crystals left behind after evaporation of a large amount of kombu broth, was scientifically identified as glutamate by Prof Ikeda of Tokyo University. He termed this unique flavour as “umami” (Ikeda, 1908).

Glutamate is one of the most abundant amino acids in nature. Since glutamate is a building block of protein and free glutamate exists in organs and tissues, it is found naturally in virtually all foods such as milk, vegetables, seafood, poultry, meats, traditional seasonings like fish sauce and soy sauce, and many other foods (Yoshida, 1998). It has long been used around the world to enhance the palatability of foods before the discovery of its taste. Foods rich in free glutamate, such as tomatoes, cheese and mushrooms have been used in cooking for their flavour favoring qualities. Glutamate also has been a component of traditional seasonings such as fish and soy sauces. More than 1200 years ago, in ancient Rome, fish sauce called “Garum” was used. Fish and soy sauces have been used in South Eastern Asian countries, China and Japan for more than several centuries. Mother’s milk the first food for babies, and is the only food when they are just born. It has to give them the entire nutrient they need. It was reported that glutamate is the most abundant amino acid in mother’s milk in all the species analyzed (Kare and Kawamura, 1998).

MSG, sodium salt of glutamate, is widely used as a flavor enhancer all over the world and also one of the most studied food ingredient, in history. After marketed in Japan in 1909, MSG had been used as a food ingredient in many countries for half century, into the late 1960’s. Although there was not much safety data, MSG was generally regarded as a safe substance, similar to GRAS (Generally Recognized As Safe) status in the United States (Giacometti, 1979) in part because glutamate is one of the most abundant amino acids found in nature and a component of all protein. It was also thought that intake of glutamate from added MSG was much less than that normally ingested from foods. The range oral intake varies from 0.4 g/person in Italy to 3 g/person in Taiwan (Giacometti, 1979).

Glutamate is the principal excitatory neurotransmitter in the CNS. During recent advances in the field it is realized that it is much more than a conventional neurotransmitter. It is not only the predominant excitatory neurotransmitter in the mature neurons but also it can influence immature neural cell proliferation, migration, differentiation and survival processes (Schlett K., 2006). Extracellular glutamate level has been shown to be high in embryonic CNS. Glutamate and its receptors are essential for the normal functioning of the CNS. However their excessive activation by glutamate is thought to contribute to neuronal damage in many neurological disorders ranging from hypoxic–ischemic and traumatic brain injuries to chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS (Platt SR.).

Glutamate is important and indispensable for the functioning of the CNS and important in food. The physiological control mechanisms of our body keep a check on its excitotoxic properties (Mallick, 2007).

Glutamate is a dietary essential amino acid and its level in the diet can affect the oxidation of some essential amino acids, such as leucine. The human body metabolizes added glutamate in the same manner it metabolizes glutamate found naturally in many foods. Glutamate is a multifunctional amino acid involved in taste perception, intermediary metabolism, and excitatory neurotransmission.

In Western societies, there is a general trend to an increased consumption of flavored convenience food. Theoretically, this change in behavior might lead to an increased glutamate intake, which is used in these products as flavor enhancer. However, the food industry steadily increases the number of glutamate-free products due to an enhanced reservation of the consumer against food additives. Therefore glutamate enriched protein hydrolysates are currently used in food production to replace the use of glutamate as additive and to enable the reduction of salt in processed foods.

Then the research and study of bacterial strains able to convert glutamine in glutamate, during several fermentations, become very important to ensure the consumer's health and improve the taste of processed foods.

1.24 GABA: γ-Amino Butyric Acid

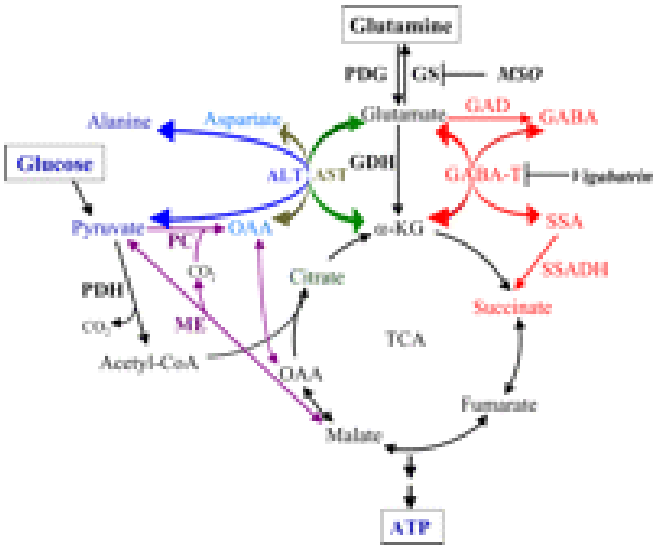


Figure 2.9: Metabolic pathway depicting the synthesis and catabolism of γ-aminobutyric acid (GABA) in the GABA shunt and its relationship to glucose metabolism and the tricarboxylic acid (TCA) cycle.

γ-amino butyric acid (GABA) is the major inhibitory neurotransmitter in mammalian brain and is found widely throughout the central and peripheral nervous systems. In the neocortex GABAergic neurons are plentiful, constituting 15–30% of all neurons. GABA serves both metabolic and trophic functions, in addition to its role as a neurotransmitter, influencing the migration of neurons and astroglia to their target locations in the cortex. During early brain development GABA elicits excitatory (depolarizing) rather than inhibitory (hyperpolarizing) postsynaptic responses. Later in development GABA influences the synaptic organization and fine-tuning of local circuits (Behar, 2009).

Due to the physiological functions of GABA, development of functional foods containing GABA at high concentration has been actively pursued (Tsushida and Murai, 1987; Saikusa et al., 1994). GABA enrichment has been achieved in anaerobic-incubated tea (gabaron tea) (Tsushida and Murai, 1987) and in rice germ soaked in water (Saikusa et al., 1994). GABA production by various microorganisms has been reported, including bacteria (Smith et al., 1992; Maras et al., 1992), fungi (Kono and Himeno, 2000), and yeasts (Hao and Schmit, 1993).

Screening various types of LAB that have GABA-producing ability is important for the food industry, because individual LAB have specific fermentation profiles, such as acid production and flavor formation ability. Because quality of fermented foods, such as taste and flavor, depends on the fermentation profiles of the LAB, such

profiles are considered an important factor in the use of LAB as starters in the production of fermented foods (Kato et al., 2001; Gran et al., 2003). Glutamate decarboxylase (GAD) is considered responsible for GABA production in GABA-producing strains of LAB. GAD has been isolated from a wide variety of sources, and its biochemical properties have been characterized (Nomura et al., 1999, 2000). Although GAD is widely distributed in LAB, GABA-producing ability varies widely among LAB. GABA-producing LAB is expected to enhance development of functional fermented foods containing GABA.

Komatsuzaki et al (2005), based on the hypothesis that GABA production during cultivation of the strains can be improved by adjusting the culture conditions suitable for GAD reaction, adjusting culture conditions, and adding a coenzyme of GAD during cultivation of screened LAB that have high GABA-producing ability, showed that *L. paracasei* isolated from a traditional fermented fish (funa sushi) produced GABA at high level (4300mM), and that cultivation conditions suitable for GABA production can be improved by utilizing the biochemical characteristics of GAD.

CHAPTER 2

OBJECTIVES

The role of microorganisms in fermented meats is well known, even if there are several models of fermentation and the different types of salami are well distinguished ecosystems. However, the role of yeasts and lactic acid bacteria in dry-cured meats (i.e. speck and ham) is often underestimated.

The ripening process of these type of products can last up to 24 months and their water activity (aw) values can decrease reaching values of 0,76. Only Staphylococci, micrococci, yeasts and moulds can colonize the superficial parts of these products thus being considered as ripening index (Munez *et al.,* 1996).

Yeasts are one of the predominant microbial groups during the ripening phase of several food products of intermediate moisture, such as Iberian dry-cured meat products. In particular *Rhodotorula rubra, Hansenula holstii* and *Hansenula sydowiorum* are the dominant microbial species during the first stage of ripening, while *Debaryomyces* spp. can be found at the end of ripening in Iberian hams (Monte *et al.,* 1986; Huerta *et al*., 1988) and "Prosciutto di Parma" (Comi *et al.*, 1990).

In particular, depending on the part of the products that is analyzed, yeasts can reach values of 10^7 cfu/g in some parts, while attain lower levels, i.e. 10^4-10^5 cfu/g, in some others at the end of ripening.

The position and particularly the presence of superficially spread fat in several positions seem to play an important role on the distribution of different biotypes of *Debaryomyces hansenii* in the product (Munez *et al.,* 1996). However, the principal aim of this study was the ecological characterization of the products rather than the assessment of the role of yeasts during ripening processes that is still not well known.

Andrade *et al*., (2009) tried to find a relationship between the molecules produced *in vitro,* i.e. in a designed model culture medium under conditions representative of dry-cured ham processing, by different biotypes of of *Debaryomyces* spp. and other dominant microbial species isolated from Iberian hams and the volatiles involved in the dry-cured flavour. According to their findings most of the biotypes produced brached aldehydes and alchools, which derive from valine, leucine and isoleucine. Such compounds are very important molecules as they contribute to the aromatic profiles of hams and all fermented and not fermented meat products. On the other hand the same metabolites are produced by lactic acid bacteria too.

The principal aim of this research project has been the evaluation of the specific role of yeasts in ripening processes of dry-cured meat products, i.e. speck and in salami produced by adding *Lactobacilli.* produced by adding *Lactobacilli* starter cultures, i.e. *L. sakei, L. casei, L. fermentum, L. rhamnosus, L.sakei* in combination with *S.xylosus*.

In particular the role of the predominant yeasts on the hydrolytic patterns of meat proteins has been studied. As yeasts are not used as starter cultures for these products, the first part of the work has been focused on the identification and characterization of the yeasts strains isolated from speck and salami during the ripening process. Also the evolution of their cell loads and changes in the composition of the microflora has been monitored. Afterwards, their contribution to the proteolytic activity of the products has been evaluated.

Therefore this research work has been developed according to the following activities:

1. Characterization of the yeasts and lactic acid bacteria in samples of speck produced by different farms and analyzed during different production and ripening stages
2. Characterization of the superficial or internal yeasts population in salami produced with or without the use of lactobacilli as starter cultures.
3. Molecular characterization of different strains of yeasts and detection of the dominant biotypes able to survive despite environmental stress factors, (such as smoke, salt i.e.)
4. Study of the proteolytic profiles of speck and salami during the ripening process and comparison with the proteolytic profiles in meat model system of a relevant number of yeasts isolated from speck and salami.
5. Study of the proteolytic profiles of *Lactobacilli* starter cultures in salami
6. Comparative statistical analysis of the proteolytic profiles to find a relationship between specific bands and peptides and specific microorganisms.
7. Evaluation of the aromatic characteristics of speck and salami, during the production process, to detect metabolic differences and find relationships among the metabolites released by starter cultures or dominant microflora.

CHAPTER 3

MATERIAL and METHODS

Table 3.1: List of the strains used

FUA Nr	Microbial species	Source
Lbcd	*Lactobacillus casei*	Milk whey
MR13	*Lactobacillus fermentum*	Cassava
C249	*Lactobacillus rhamnosus*	Milk
DSM-20531	*Lactobacillus amylovorus*	Meat
63	*Lactobacillus plantarum*	Sausage
BB12	*Lactobacillus sanfranciscensis*	Sourdoughs
	Lactobacillus sakei	Commercial starter
CLCD	*Yarrowia lipolytica*	Salami
CSLF2	*YarrowialLipolytica*	Salami
CLF1	*Yarrowia lipolytica*	Salami
CLR1	*Yarrowia lipolytica*	Salami
CsLR4	*Yarrowia lipolytica*	Salami
C1	*Yarrowia lipolytica*	Salami
C4	*Yarrowia lipolytica*	Salami
1 II YL 1a	*Yarrowia lipolytica*	Speck
1 II YL 3b	*Yarrowia lipolytica*	Speck
1 II YL 4a	*Yarrowia lipolytica*	Speck
1 II YL 6b	*Yarrowia lipolytica*	Speck
1 II YL 7a	*Yarrowia lipolytica*	Speck
1 II YL 8a	*Yarrowia lipolytica*	Speck
1 II YL 9a	*Yarrowia lipolytica*	Speck
4B	*Yarrowia lipolytica*	Irradiated poultry meat
16B	*Yarrowia lipolytica*	Irradiated poultry meat
5D	*Yarrowia lipolytica*	Irradiated poultry meat
27D	*Yarrowia lipolytica*	Irradiated poultry meat
LM2	*Yarrowia lipolytica*	Pecorino cheese
PO1	*Yarrowia lipolytica*	Superficial waters of Po river
PO11	*Yarrowia lipolytica*	Superficial waters of Po river
PO17	*Yarrowia lipolytica*	Superficial waters of Po river
PO19	*Yarrowia lipolytica*	Superficial waters of Po river
PO14	*Yarrowia lipolytica*	Superficial waters of Po river
PO23	*Yarrowia lipolytica*	Superficial waters of Po river
RO3	*Yarrowia lipolytica*	Commercial Light butter
RO9	*Yarrowia lipolytica*	Commercial Light butter
RO12	*Yarrowia lipolytica*	Commercial Light butter
RO19	*Yarrowia lipolytica*	Commercial Light butter
RO22	*Yarrowia lipolytica*	Commercial Light butter
RO25	*Yarrowia lipolytica*	Commercial Light butter
Y9	*Yarrowia lipolytica*	Chilled food
Y10	*Yarrowia lipolytica*	Chilled food
Y14	*Yarrowia lipolytica*	Chilled food
Y22	*Yarrowia lipolytica*	Chilled food
Ly56	*Lysyeria monocytogenes*	Fish
G	*Pseudomonas fulva*	Soy derived products
BT	*Pseudomonas fragi*	Soy derived products
PA22	*Leuconostoc lactis*	Soy derived products

T2	*Enterococcus faecium*	Soy derived products

Table 3.2: Composition of M17 media for *Lactococci*

Ingredients	**Quantity**
Tripton	5 g/l
Meat extract	5 g/l
Yeast extract	2.5 g/l
Soy Peptone	5 g/l
Acid ascorbic	0.5 g/l
Magnesium sulphate	0.25 g/l
Di-sodium-glycerol sulfate	19 g/l

Add 50ml/l of sterile lactose after autoclave.

Table 3.3 : Composition of MRS media for Lactobacilli

Ingredients	**Quantity**
Universal peptone	10 g/l
Lab-lemco	10 g/l
Yeast extract	5 g/l
Glucose	20 g/l
K_2HPO_4	2 g/l
sodium acetate anhydrous	5 g/l
tri-ammonium citrate	2 g/l
$MgSO_4 \cdot 7H_2O$	0.2 g/l
SO_4 (4 H_2O)	0.05 g/l
Tween 80	1 g/l

Table 3.4: Composition of YPD media for yeast

Ingredients	Quantity
Universal peptone	10 g/l
Glucose	20 g/l
Yeast extract	5 g/l

Table 3.5: Composition of PCA media for Total Bacterial Growth

Ingredients	Quantity
Universal peptone	5 g/l
Glucose	1 g/l
Yeast extract	2.5 g/l

3.1 Meat Model System Development For Proteolytic Activity Assessment

The meat model system, which was used for the *in vitro* evaluation of the proteolytic activity of both yeasts and lactic acid bacteria strains, was prepared according to the protocol described by Vignolo *et al.,* (2004).

3.1.1 Culture media

- MRS broth culture medium for lactic acid bacteria
- YPD broth culture medium for yeasts
- PCA broth culture medium for Total Bacterial Growth

3.1.2 Soluble muscle extracts

1) Weigh 10g of lean muscle in a stomacher bag
2) Add 90 ml of 20mM phosphate buffer, pH7
3) Homogenize in a stomacher 400 blender for 3 minutes
4) Centrifuge the protein solution (12000g ,4°C for 20minutes)
5) Adjust the pH to 6.5 with 1N NaOH
6) Add 1% (w/v) of glucose
7) Filter the supernatant containing the proteins through Whatman paper
8) Filter-sterilize this solution by using a 250 ml capacity filter (Millipore) with a vacuum-pump
9) Add 0.1% of Tween-80 previously sterilized

3.1.3 Sterility Control of the soluble muscle extract

1) To quantify the total aerobic organism, inoculate in duplicate Petri dishes with 0.5 ml of protein extract
2) Add 10 ml of the melted PCA and homogenize appropriately
3) After incubation at 37°C for 96 hours, check for the growth of microorganisms that must be negligible, i.e. lower than $1*10^2$ CFU/ml

3.2 Proteolytic activity of the soluble muscle extract by LAB strains

3.2.1 Culture condition of the strains

1) Inoculate *Lactobacillus* spp. strains in MRS broth media and incubate at 37°C overnight
2) Harvest exponential-phase cells and wash twice with 20mM phosphate buffer, pH7
3) Remove the supernatant and resuspend the pellet in 20mM phosphate buffer, pH7

3.2.2 Growth in the Soluble Muscle Extract

1) Inoculate 30 ml of the soluble muscle extract with 30 ml of an overnight culture to yield an initial cell number of 10^5 CFU/ml corresponding to an OD_{680} OF 0.15
2) Incubate at 30°C for 96 hours
3) Withdrawn samples every 24 hours for pH, bacterial growth, and take proteolytic activity measurements
4) Measure the pH values with a pH meter
5) Determine bacterial cell counts using 0.1% peptone water as the diluent
6) Plate 0.5 ml of each dilution in Petri dishes adding the MRS media
7) Incubate at 30°C for 48 hours

3.3 Cell Wall Extraction Of *Lactobacilli* for Meat Proteins Hydrolysis

3.3.1 Total Meat Protein Extraction

Total proteins were extracted from 2 g samples with 40 ml of 1.1 M potassium iodide, 0.1 M sodium phosphate, pH 7.4 buffer (Cordoba, 1994). The samples were homogenized for 3 minutes. The extracts were centrifuged at 8000g for 15 min at 4

°C (Beckman Coulter Avanti J-10, Fullerton, CA, USA), and the supernatants were filtered through a 0.22 μm filter.

Sarcoplasmic proteins were extracted from 2 g samples with 40 ml of 0.02 M, pH 6.5 sodium phosphate buffer (Fadda *et al*., 1999) following the steps indicated for the above extraction. The protein content of the sarcoplasmic extract was 0.80 mg/ml.

3.3.2 Lab strains growth conditions

The strains of *Lactobacillus casei* lbcd, *L. rhamnosus C249, L.sanfranciscensis BB12, L. fermentum* M13*, L amylovorus* DSM-20531 and *L. plantarum 63* isolated from different food sources (table3.1) were used for the proteolytic assay. All the strains were usually grown in MRS broth at 37°C for 24 hours and maintained at -80°C in 15% (v/v) glycerol. For enzymatic assays liquid medium was inoculated (1%, v/v) with the selected microorganism, previously sub-cultured once and incubated overnight at 37°C.

3.3.3 Preparation of Cell Suspensions and Extracts

Cells were harvested by centrifugation (10000g for 20 minutes at 4°C), washed twice in 0,085% (w/v) NaCl, containing 20 mM $CaCl_2$ and resuspended in 2% initial volume of 50 mM Tris-HCl, pH 6.5. This was designated as whole-cell suspension (WCS) (Sanz *et al*, 1999).

Cell free extracts (CFE) were obtained for each strain by the procedure of Sanz and Fadda (1999). Cells were collected as above, washed twice in 20 mM phosphate buffer (pH 7.0) and resuspended in the same buffer (10% of initial volume) supplemented with lysozyme (1mg/ml). After incubation at 30°C for 1 hour, the cell wall fraction was removed by centrifugation (15000g for 20 minutes at 4°C). The pellet was washed twice in 20 mM phosphate buffer (pH 7.0) resuspended in the same buffer and sonicated for 15 mnutes. Cell debris was removed by centrifugation (10000 g for 20 minutes at 4°C).

3.3.4 Enzymatic Mixtures

Three independent assays were carried out for the protein extracts (i.e. total protein or meat soluble muscle extract) using as enzymatic sample either whole cell suspension, CFE, or a combination of both (1:1). The reaction mixture consisted of 6ml of whole

cell suspension or CFE aseptically added at 30ml of protein extract. For the combined mixture, 3ml of whole cell suspension and 3ml of CFE were added to 30ml of protein extract. The mixtures were incubated at 37C for 96 hours (Sanz and Fadda, 1999). Bacterial counts were determined on MRS agar plate and pH values were monitored. The hydrolysis of muscle proteins was monitored by SDS-PAGE analysis, using a 4-15% polyacrylamide precast gels (BioRad).

3.4 Hydrolysis Of Meat Protein extracts by *Yarrowia lipolytica*

3.4.1 Yeasts growth conditions

Several strains of *Yarrowia lipolytica* isolated from different food sources were grown in YPD broth at 28°C for 72 hours.

Cells were separated from culture broth by centrifugation at 7000 rpm for 15 minutes. The supernatants were used for the hydrolysis with total meat proteins.

Reaction mix was:

- 12,5 ml of meat protein (1,25 g/ml)
- 2,5 ml of supernatant
- 0,1 µl of a sodium azide solution (0,002%)

The mix was incubated at 28°C for 24 and 48 hours; the enzymatic reaction was then stopped by heating at 100°C for 5 minutes.

The samples were stocked at -20°C and then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

3.5 Protein Quantification By Bradford Assay

The Bradford assay was used to determine the total protein concentration of the samples. The method is based on the proportional binding of the dye Coomassie to proteins. Coomassie absorbs at 595 nm. The protein concentration of a test sample is determined by comparison to those of a series of protein standards which are known to give rise to a reproducible linear absorbance profile. Bovine Serum Albumin (BSA) was used as standard protein.

Procedure

- Prepare a 4-fold dilution of a 2 mg/mL BSA sample by adding 50µl of 2 mg/mL BSA to 150 µl of distilled water to make 200 µl of 0.5 mg/mL BSA.
- Generate test samples for the reference cell, blank, BSA standards and the protein sample to be tested according to Table 1 in disposable cuvettes.
- Allow each sample to incubate at room temperature for 10-30 minutes.
- Measure the absorbance of each sample at 595nm using a UV-visible spectrophotometer.
- Plot the absorbance of each BSA standard as a function of its theoretical concentration.
- Determine the best fit of the data to a straight line in the form of the equation

  "y = mx + b"

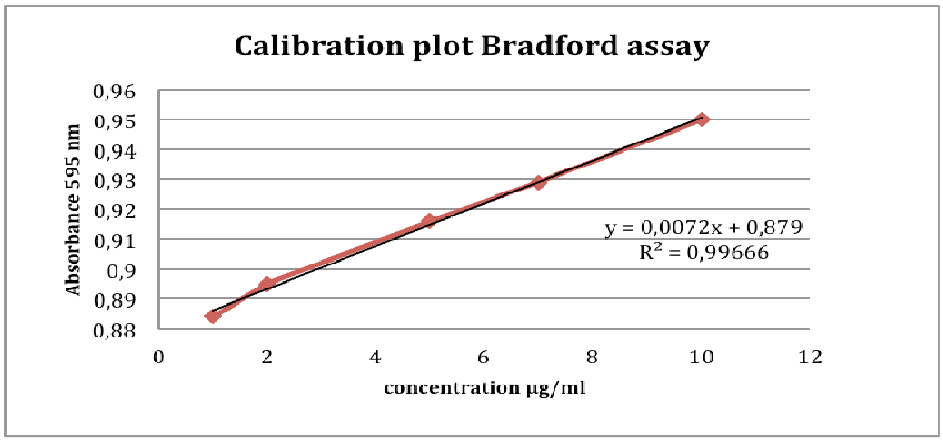
  where y = absorbance at 595 nm

  and x = protein concentration.
- Use this equation to calculate the concentration of the protein sample based on the measured absorbance.

Table 3.6: preparation of the test samples for the Bradford protein assay

Test Sample	Sample volume (µl)	Water volume (µl)	Bradford Reagent Volume (µl)
Blank	0	800	200
BSA Standard-5 µl /ml	10	790	200
BSA Standard-10 µl /ml	20	780	200
BSA Standard- 15µl ml	30	770	200
BSA Standard-20 µl /ml	40	760	200
BSA Standard-25 µl /ml	50	750	200
Protein Sample	100	700	200

Figure 3.1 : Calibration plot for Bradford assay



3.6 SDS Page Electrophoresis

The hydrolysis of muscle proteins was monitored by sodium dodecyl sulfate gel (SDS)-polyacrylamide electrophoresis (PAGE).

For the denaturation of each protein fraction, 50 µl of extract were mixed with 25 µl of Laemmli sample buffer (Bio-Rad Laboratories, Milan, Italy) containing β-mercaptoethanol. The mixture was incubated at 100 °C for 5 minutes.

The SDS-PAGE analysis was then carried out with two different methods: by using precast gels or 15% polyacrylamide gels.

As far as pre casted gels, Ready Tris-HCl Gel, 10–20% resolving gel, 4% stacking gel supplied by Bio-Rad Laboratories were used. 10 µl of a Precision Plus Protein Standard Unstained (Biorad) was used as standard.

The wells of the electrophoresis gels were loaded with 20 µl of the denatured protein samples.

Gels were run in a Mini Protean Cell System with a 10% SDS buffer, at 250 V for 30 minutes.

Staining was 1 hour in the following solution

Bromophenol blue	0.1%
Methanol	50%
Glacial acetic acid	7%

De-staining was made for 2 hours in a solution of

Methanol	50%
Glacial acetic acid	10%

Several washes were then made with distilled water, until obtaining a clear gel.

3.6.1 PREPARATION OF 15% POLYACRYLAMIDE GELS

Running gel (30 ml) 15%:

Water	6.9 ml
30% acrylamide and bis-acrylamide solution, 19:1 (BioRad)	15 ml
1.5 M Tris (pH8.8)	7.5 ml
10% SDS	0.3 ml
10% APS	0.3 ml
TEMED	0.012 ml

Stacking gel (10ml) 5%:

Water	6.8 ml
30% acrylamide and bis-acrylamide solution, 19:1 (BioRad)	1.7 ml
0.5 M Tris (pH6.8)	1.25ml
10%SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.01 ml

10 µl of a Broad Range (Bio-Rad Laboratories, Milan, Italy) were used as standard. The protein denaturation was performed according to the above described procedure. The wells of the electrophoresis gels were loaded with 25 µl of the denatured proein samples.

Gels were run in a buffer tank supplied by Bio-Rad with a 10% SDS buffer.

Voltage was at 90V for 60 minutes and 250 V for 3 hours.

The gels were then stained overnight in a staining solution made of:

Comassie Blue	0.3mM
Methanol	40%
Glacial acetic acid	7%

Destaining solution was:

Methanol	5%
Glacial acetic acid	7%

3.7 Antimicrobial Activity Of Meat Hydrolyzates.

Antimicrobial activity of meat protein extracts hydrolyzeds by *Yarrowia lipolitica* and *Lactobacillus* spp. strains was determined with the MIC method, using 96 wells sterile plates with lid (Corning Incoporated, Corning, New York). Each plate was set up as follows: column 1, 100µl of culture media plus 50µl of stock solution, i.e. meat protein extract hydrolyzate (positive control), column 2-12: 100µl of media plus 50µl of an overnight inoculum of different target microorganisms: *Lysyeria monocytogenes* Ly56*, Pseudomonas fulva* G*, Pseudomonas fragi* BT*, Leuconostoc lactis* PA22 and *Enterococcus faecium* T2. BHI was used as media for *Lysyeria monocytogenes, Pseudomonas fulva* G and *Pseudomonas fragi,* while MRS for *Leuconostoc lactis* and M17 for *Enterococcus faecium.* The inoculum of each microorganism was about 10^3 cfu/ml. 100µl of a stock solution (meat protein extract hydrolysate) were added at column 3, mixed with micropipette and transferred into the column 4. The same procedure was followed until column 12. Microtiter plates were incubated at 37°C for 24 hours and examined for microbial growth by observing the presence of turbidity. To determine the Minimum Bactericidal Concentration (MBC), 10µl of the mixture used in MIC plates was collected from the wells of each target microorganism that showed very little growth, and inoculated onto sterile nutrient agar. The plates were then be incubated at 37^{o}C for 24 hours. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria is observed on the nutrient agar plates. Therefore the concentration that showed no visible growth after incubation has been considered as MBC (Akinjogunla *et al.,* 2010; Petrus *et al.,* 2011).

3.8 Nitrate Reductase Activity

Nitrate reductase activity was determined as described by Miralles *et al.* (1996) on YTA (1.0% tryptone, 0.5% yeast extract, 1.5% agar, pH 7.0) supplemented with 1 g/l of KNO_3 (Sigma Aldrich, Milan, Italy).

The cell pellet of an overnight culture was resuspended in 10 ml of 50 mM phosphate buffer pH 7.0 and 3 spots of 10 µl loaded in YT agar plates (16g/l Bacto Tryptone, 10g/l Bacto Yeast Extract, 5g/l NaCl, adjusted at pH 7.0 with 5N NaOH). After incubation at 30°C for 24 and 72 hours, the plates were flooded with 1 ml of a 1:1 solution of NIT1 (0.8 g of sulphanilic acid in 100 ml of 5 N acetic acid) and NIT 2 (0.6 g of N-N-dimethyl-1-Naphthylamine in 100 ml of 5 N acetic acid) for the detection of nitrite. The appearance of red haloes surrounding the spots indicates the presence of nitrate reductase activity. It was considered high if the assay was positive for all the 3 spots, medium if positive for 2, low for 1 and absent if the assay was negative.

3.9 Gas-Chromatographic Analysis Of Amino Acids

This technique allows the determination of the amminoacidic fraction of the samples with EFC. The derivatization makes the sample volatile, thus allowing their detection by a gas-chromatograph couples with a mass spectrometer GC-MS).

3.9.1 Derivatization Method Of Meat Protein Extract Hydrolyzates Samples

1. Take 1000 µl of sample and add 10 µl of standard (decanoic acid 500ppm)
2. Add 200 µl of NaOH 0.1 N and mix by vortexing for 20 seconds
3. Add 169 µl of methanol and mix by vortexing for 20 seconds
4. Add 36 µl of pyridine and vortex 20 seconds
5. Add 22 µl of ECF, wait for the heat dispersion, and vortex 20 seconds
6. Repeat point 5
7. Add 400 µl of chloroform and vortex 20”
8. Add 400 µl of $NaHCO_3$ 50mM and mix by vortexing for 20 seconds
9. Separate the superior phase from the lower organic one
10. Add a spatula of Na_2SO_4 and vortex Transfer into a clean conic tube
11. Inject 1 µl of derivatized solution into the GC-MS.

12. Inject 1 µl of derivatized solution into the GC-MS.

Samples were analyzed with an Agilent Hewlett–Packard 7890 GC gas-chromatograph equipped with a mass spectrometer detector 5975 MSD (Hewlett–Packard, Geneva, Switzerland) and a 60 m ·250 µm i.d. fused silica capillary column coated with a 5% difenil dimethylpolysiloxane (Supelco, Palo Alto, Ca, USA). The conditions were as follows: injection temperature, 250 °C; detector temperature, 230°C; carrier gas (He); flow rate, 1 ml/min; splitting ratio, 1:20 (v/v). The oven temperature was programmed as follows: 80 °C for 1min; from 80°C to 240 °C, at 4°C/min then holding for 14 min.

3.10 Salami Manufactures

For the salami preparations, two mixtures were used: a basic salami mixture (20kg) and a mixture with added the commercial starter currently used by the company (20kg).

The basic salami mixture comprised (% w/w) lean pork (70%) and lard (30%), plus KNO_3 (0.015%), $NaNO_2$ (0.015%), NaCl (3.0%), sucrose, dextrose, spices and ascorbic acid. After mincing, one mixture was inoculated with *S. xylosus* e *L. sakei* at a level of 6.0 log cfu/g (mixture with starter). Four species of Lactobacilli were selected for the fermentations of the other one: *L. rhamnosus* C243, *L. casei* lbcd, *L. fermentum* MR13 and *L.sakei* (commercial). They were pre-grown twice in MRS broth (Oxoid, Basingstoke, UK) at 37 °C for 24 hours. After the second incubation period, cells were harvested by centrifugation (8500 g, 10 min, 4 °C), washed and suspended in sterile physiological solution for their utilization.

Five types of salami were produced with the basic mixture and the co-addition of the Lactobacilli species (table 3.7).

After inoculation, the mixtures were stuffed into synthetic gut (diameter of about 5.0 cm), obtaining salami of about 500g. They were kept in a fermentation chamber at about 20 °C for the first 10 days, with the temperature down to 18°C for the following 3 days, at 15°C for one day and then was kept at 13°C until the end of ripening (table 3.7).

Table 3.7: experimental plan for the microfermentations

inoculum / mixture	No inoculum	*L. rhamnosus* C243	*L. casei* lbcd	*L. fermentum* MR13	*L. sakei*	*S. xylosus* and *L.sakei*
Meat with starter	C	CLR	CLC	CLF	CLS	CS

Table 3.8 : fermentation plan for the sausages

Stage	Time (hours)	Temperature (°C)	U.R. %
1	3	18	
2	3	20	
3	3	22	
4	3	24	
5	12	22	50-60%
6	4	21	
7	20	20	55-90%
8	4	18	
9	20	20	55-90%
10	4	18	
11	30	18	55-90%
Ripening	24	13-15	
Ripening	1752	12-13	

3.10.1 Microbiological Analysis

About 10 g of sample were transferred aseptically to 90ml of sterile water and homogenized in a stomacher (BagMixer, interscience, France) for 2 min at low speed at room temperature. Serial decimal dilutions in physiological solution were prepared and 1 ml or 0.1 ml samples of appropriate dilutions were poured or spread on total count and selective agar plates. Total aerobic mesophilic flora was determined on Plate Count Agar (Oxoid), incubated at 30 °C for 72 h; Lactococcus spp. on M17 agar (Oxoid, CM0785) to which 10% v/v lactose was added, incubated at 37 °C for 48 h; Lactobacillus spp. on de Man Rogosa Sharpe (MRS) Agar (Oxoid), incubated at 30°C for 72 h under anaerobic conditions (Gas generating kit, Oxoid, BR0038); staphylococci on Baird Parker Agar (Oxoid) incubated at 37 °C for 48 h; yeasts on Sabouroud Agar (10g/l Universal peptone, 20 g/l glucose), incubated at 30°C for 48 hours.

3.10.2 Physico-Chemical And Chemical Analysis

The measurements of pH were performed by diluting 5 g of sample in 5 ml of distilled water and using a Hanna Instruments 8519 (Incofar, Modena, Italy) pH-

meter.

Water activity (aw) was measured using an Aqualab Series 3 instrument (Decagon Device, Inc., Pullman, Washington, USA).

3.10.3 Volatile Profiles

Volatile compounds were monitored at the end of ripening using a gas-chromatographic–mass spectrometry coupled with a solid phase microextraction (GC–MS-SPME) technique. For each sausage, 5 g samples (a slice about 0.5 cm thick broken in small pieces) were placed in 10 ml sterilized vials, and the vials were sealed by PTFE/silicon septa. The samples were then equilibrated for 20 min at 60 °C and volatiles adsorbed on a fused silica fibre covered by 65 lm polydimethylsiloxane-divinyl benzene (PDMS- DVB), (Supelco, Steiheim, Germany). Adsorbed molecules were desorbed in the gas-chromatograph for 5 min. For peak detection, an Agilent Hewlett–Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett–Packard, Geneva, Switzerland) and a 50 m · 0.32 i.d. fused silica capillary column coated with a 1.2 lm polyethylenglycol film (Chrompack CP-Wax 52 CB, Middelburg, The Netherlands), as stationary phase were used. The conditions were as follows: injection temperature, 250 °C; detector temperature, 220°C; carrier gas (He) flow rate, 1 ml/min; splitting ratio, 1:20 (v/v). The oven temperature was programmed as follows: 50 °C for 2min; from 50°C to 65°C, at 1°C/min; from 65°C to 220 °C, at 5 °C/min, then holding for 22 min. Volatile peak identification was carried out by computer matching of mass spectral data with those of the compounds contained in the Agilent Hewlett–Packard NIST 98 and Wiley vers. 6 mass spectral database.

3.10.4 Strains Isolation

During sausages manufacture and ripening isolations of different microorganisms were made: yeasts on YPD agar, lactobacilli on MRS agar and lactococci on M17 agar.

Colonies from countable plates were initially selected for morphologic differences.

Briefly, DNA extraction was carried out from a single colony by using an Insta Gene Matrix (Bio-Rad Laboratories, Hercules, CA).

3.10.5 Rapd PCR

Strains were analyzed with RAPD-PCR, using M13 as universal primer. Amplification was carried out with RAPD-PCR on 10 µl of genomic DNA, using the thermocycler T3000 (Biometra). The reaction mix had 25 µl of volume:

- Taq polimerasi 0,2 µl
- buffer $MgCl_2$ 1X 1,875 µl
- dNTPs 2 µl
- primer M13 1,25 µl (MWG, Milano, Italy)
- dH_2O steril 17, 675 µl
- DNA 2 µl

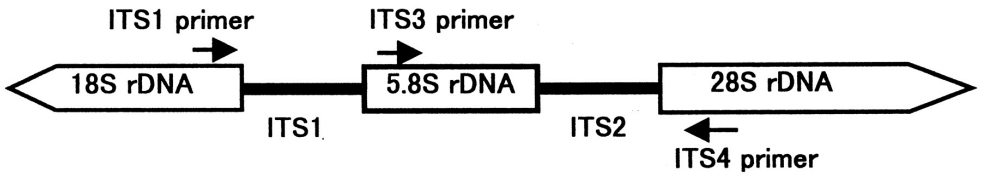
Denaturation was at 94°C for 1 minute; annealing, 45°C for 20 seconds; extension, 72°C for 2 minutes; final extension, 72°C for 5 minutes.

The PCR products were separated on 1,5% agarose gel (Sigma-Aldrich, Milano, Italy) with 0,5 mg/ml of ethidium bromide. DNA was analysed with UV trans illumination and the profiles acquired with a Gel Doc EQ System (Bio-Rad, Germany). RAPD patterns were compared using a Fingerprinting II Informatix TM Software (Bio-Rad).

3.11 Yeast identification and PCR fingerprinting

For the yeast identification, restriction analysis of rDNA region with gene 5.8 r RNA was made and the 2 not codificant regions Internal Transcribed Spacers (ITS) ITS1 and ITS2.

The primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') were used to amplify the region 5.8 rDNA and the 2 next regions ITS1 and ITS2.



Amplifications were made in a reaction volume of 30µl. 2 µl of DNA were added at the PCR mix, containing 0.5µM of every primer, 200µM of every deoxynucleotide 1.5mM $MgCl_2$ and 1X di Buffer and 1U of Ex*Taq* DNA polymerase (Takara

Biomedicals). Amplifications were made in a Personal Cycler Vers 3.26 Biotron (Biometra) at the following conditions:

- initial denaturation at 95°C for 5 min,
- 35 denaturation cycles at 94°C for 1 min,
- annealing at 55.5°C for 2 min and extension at 72°C for 2 min
- final extension at 72°C for 10min.

Amplification products were digested with endonucleases of restriction HaeIII, HinfI, CfoI and Taq I (Promega). 10 µl of every sample were added in a mix made of 2µl ultrapure dH_2O, (Eppendorf), 2µl Buffer and 1µl of enzyme. Then the samples were put for 2 hours at 37°C except for enzyme1 Taq I, where the temperature was 65°C.

For the polymorphism analysis of DNA a RAPD-PCR was made using two universal primers M13 (5' GAGGGTGGCGGTTCT 3') and RF2 (5' CGGCCCCTGT 3') (Andrighetto *et al.*, 2000).

The 2 amplification reactions were made in a 25µl volume. 2 µl of DNA were added at the PCR Mix containing 1X Buffer, 1.5mM $MgCl_2$, 200µM of deoxynucleotides, 1µM of primer and 1U of Ex*Taq* DNA polymerase (Takara Biomedicals). The conditions of amplification were as follow:

- for the primer M13: initial denaturation at 95°C for 5 min, 35 cycles at 94°C for 1 min, 45°C for 20 sec and 72°C for 2 min; final extension at 72°C for 5 min;
- for the primer RF2: initial denaturation at 95°C for 1 min 30s, 35 cycles at 94°C for 30s, 36°C for 1 min and 72°C for 1min 30s; and final extension at 72°C for 5 min;

RAPD –PCR profyles were analysed with the software Fingerprinting II (Biorad).

The calculation of similarity between the bands was based on the Pearson coefficient profiles and grouped in a dendrogram, with clustering methods using unweighted arithmetic average of the groups.

3.11.1 Electrophoresis On Agarose Gel

Amplifications products were separated by Electrophoresis on an agarose gel (1,5%), in a TAE 1X Buffer (2M Tris, 1M acetate, 100mM EDTA). The length of the bands was evaluated using a 100bp ladder (Promega).

RAPD profyles were separated on a 1,5% agarose gel and the bands compared with a 1kb ladder (Promega).

After the electrophoresis, gels were stained with an ethidium bromure solution (0,5μg/ml) (Sigma) and visualized with UV light. Imagine was acquired with the program Gene Genius Bio Imaging System (SynGene).

3.12 Proteolysis Assessment

The evolution during ripening of the proteolysis products was monitored by SDS-PAGE. Low ionic strength-soluble proteins were extracted from 2 g samples with 40 ml of 0.03 M, pH 7.4 sodium phosphate buffer (Cordoba et al., 1994). The samples were homogenized. The extracts were centrifuged at 8000g for 15 min at 4 °C (Beckman Coulter Avanti J-25, Fullerton, CA, USA), and the supernatants were filtered through a 0.45 μm filter. Total proteins were extracted from 2 g samples with 40 ml of 1.1 M potassium iodide, 0.1 M sodium phosphate, pH 7.4 buffer following the steps indicated for the above extraction. For denaturation of each protein fraction, 10 μl of extract were mixed with 20 μl of Laemmli sample buffer (Bio-Rad Laboratories, Milan, Italy) containing β-mercaptoethanol. The mixture was incubated at 100°C for 5 min. The proteins were then characterized by SDS-PAGE.

CHAPTER 4

RESULTS

CHAPTER 1

INTRODUCTION

1 MEAT PRODUCTS

1.1 Nutritional Role Of Meat In Diet

Meat has traditionally been considered an essential component of the human diet to ensure optimal growth and development. With a limited range of foods available in societies throughout history, meat was important as a concentrated source of a wide range of nutrients. Anthropological research shows that the length of the gut in primates and humans became shorter with the introduction of animal-derived food. Smaller quantities of food of high digestibility required relatively smaller guts, characterized by simple stomachs and proportionally longer small intestines, emphasizing absorption. It is perhaps due to the fact that meat has been eaten as much for enjoyment as for its nutritional qualities that consumption of meat and meat products has increased with the affluence of the consumer. The importance of meat and meat products in everyday food culture and consumer health may be questioned by the fact that populations of vegetarians living in rich countries are characterized by lower rates of cancer and cardiovascular disease. The analysis of dietary patterns, as a possible approach to examining diet–disease relations, identified two major eating patterns defined by factor analysis using dietary data collected from food frequency questionnaires. The first factor, the "prudent dietary pattern," was characterized by an high intake of vegetables, fruits, legumes, whole grains, and fish or other seafood, whereas the second factor, the "Western pattern," showed a high intake of processed meat, red meat, butter, high- fat dairy products, eggs, and refined grains. A study has been published involving Seventh-day Adventists, a well-characterized population, in which the effect of dietary intake of nutrients on biochemical parameters in blood and urine was com- pared in vegetarian and non vegetarian subjects. The dietary intake of cholesterol was higher in not vegetarian subjects (560 to 710 mg/day) compared to vegetarians (<20 mg/day) and was associated with elevated serum cholesterol levels in the not vegetarian population. These results demonstrated a correlation between dietary intake of certain food components (e.g., cholesterol) relevant for diseases (e.g., coronary heart disease) and their blood concentrations.

The meat consumption and production figures published by the U.S. Department of Agriculture and the European Union (EU) do not distinguish between fresh meat and processed or fermented meat products.

The EU production of fermented meats amounted to 689,000 tonnes of fermented sausages and approximately the same amount of raw ham in 1988. Approximately 5% of the total meat production (carcass weight) is further processed by fermentation. The major producers of fermented meat products in the EU are Germany, Italy, Spain, and France. In these countries, 20 to 40% of processed meat products can be classified as fermented meat products.

Tabel 1.1: Fermented Meat Products

FERMENTED SAUSAGES	
Cold smoked and dried but uncooked	Hungarian salami
Cold smoked and uncooked	Spreadable raw sausages (Mettwurst), very popular in Germany
Not smoked, dried and uncooked	Italian salami, Spanish chorizo
Hot smoked partially or fully cooked semi-dry sausages	Summer Sausage, Pepperoni, Salami

ITALIAN MEAT PRODUCTS	
Sausages	Salame, cotechino, soppressata, luganiga, zampone, mortadella
Whole meat cuts	Ham (prosciutto), shoulder (spalla), neck (capocollo), belly (pancetta), an aged fillet of rump (culatello), smoked flank (speck)

1.2 The Relationship Between Meat In The Diet And Disease

Numerous studies have compared the health status and mortality of vegetarians to those of omnivores. The results show a strong correlation between *per capita* consumption of meat and the incidence of colon cancer among various countries.18 In more detailed case-control and cohort studies, in which lifestyle factors were better controlled for, the consumption of red meat was associated with a high risk of colon

cancer. Results from a meta-analysis by Howe et al., including 13 of the case- control studies, indicated that total energy intake was positively associated with a higher risk of colon cancer. Surprisingly, the intakes of fat, protein, and carbohydrates were not related to cancer risk, independent of their contribution to total energy. Compared with Western vegetarians, non vegetarians have a higher mean body mass index (BMI) by about 1 kg/cm2, suggesting that higher total energy intake and meat consumption might be associated with the "Western diet pattern."

The mechanisms that increase the risk of colon cancer are not yet clear. Several *in vitro* studies suggest that DNA damage in human cell lines can be caused by food ingredients or their metabolic products. High meat consumption, for example, leads to higher levels of bile acids and *N*-nitroso compounds in the feces. Bile acid and *N*-nitroso compounds, as well as their metabolites, potentially promote colon cancer development. Animal studies show that large intestinal *N*-nitrosation does not occur in germ-free rats, but it has been shown to occur in the presence of a conventional flora. The effect of diet on the composition of the intestinal microflora was shown by Finegold et al.29 Subjects eating a Western diet were compared with subjects eating a Japanese diet. The subjects eating the Japanese diet had a lower risk of colon cancer and had significantly higher numbers of *Enterococcus faecalis, Eubacterium lentum, E. contortum, Klebsiella pneumoniae,* and various *Lactobacillus* species in their feces. The Japanese diet has been associated with low incidence of large bowel cancer. Japanese people who migrate to the United States and adopt the Western diet develop this cancer with increased frequency, approaching that of native-born Americans. The high risk group with Western dietary patterns had increased counts of species of the genera *Bacteroides, Bifidobacterium, Peptostreptococcus*, and *Clostridium* in their fecal flora. Regular consumption of meat is also associated with increased risk of death from coronary heart disease (CHD). The most compelling evidence comes from studies with Seventh-day Adventists. It was found that men and women who consumed red meat daily had around 60% greater chance of dying from CHD than those who consumed red meat less than once per week. A review of studies of the association between blood homocysteine concentrations and atherosclerotic disease showed that 16 of 21 investigations reported significantly higher homocysteine concentrations in case subjects compared with control subjects. Because red meat is a major source of methionine in the diet, and methionine is the direct metabolic precursor of homocysteine, a higher intake of red meat may be involved in cardio-

vascular disease initiation and progression.

In summary, high dietary intakes of energy, saturated fat, and red meat, all associated with the Western diet pattern, are likely to have adverse effects on chronic disease risks, particularly those of colon cancer and coronary heart disease. On the other hand, little evidence indicates that the consumption of moderate amounts of meat or meat products is harmful in regard to either cancer or cardiovascular disease.

1.3 History And Culture Of Fermented Meat

Meat is extremely susceptible to microbial spoilage. Virtually all ecological factors characterizing meat as a substrate are optimal for the growth of bacteria, which are the most efficient agents in remineralization of organic matter. For example, in meat, water activity and pH are 0.96 to 0.97 and 5.6 to 5.8, respectively, and nutrients and growth factors are abundantly available. Any storage of this nutritionally rich food and preservation of the nutrients contained therein requires the suppression of microbial growth or the elimination of microorganisms and prevention of recontamination.

The traditional methods employed for prevention of microbial spoilage are still in use, though with a different meaning in various products. These methods comprise reduction of water activity (drying, salting) and/or pH (fermentation, acidification), smoking, storage at refrigeration or freezing temperatures, and use of curing aids (nitrite and nitrate). Commonly, these methods act together in different combinations, building up hurdles against microbial growth. With regard to fermented sausages, these hurdles are low water activity (0.85 to 0.95) and pH (5.6 to 4.7), the use of nitrite (nitrate), and smoke. In addition, during fermentation and ripening, ecological factors, such as a reduced redox potential and low temperatures (10 to 12°C, at least for dry sausages), together with antagonistic compounds produced by the fermenting flora exert a selective effect against the growth of undesirable microorganisms. Basically, the same antimicrobial hurdles are effective in achieving the microbial stability of ham, except for the effect of a low pH. Since no lactic fermentation takes place, the reduced water activity is the most effective hurdle against microbial growth in ham. The understanding of these ecological factors and their control is not only a prerequisite in quality assurance, but also provides a basis for understanding to what extent these food matrices might be used to serve as probiotic foods.

The production of dried and cured meat (ham) can be traced back to prehistoric times.

It cannot be excluded that among the sausages that are mentioned in historical literature (e.g., Homer's Iliad), fermented products were included, although we do not have sufficient knowledge of their production processes to permit a conclusion that a fermentation step was part of the technology.

The origin of fermented sausages can be traced back with accuracy to ca. 1730, when salami was first mentioned in Italy. From Italy, the art of producing fermented sausages spread to other European countries and was established, for example, in Germany in 1735 and Hungary in 1835.

This very high consumption of fermented meats is an indication that such products have a long tradition of being safe. However, some specific safety aspects deserve consideration.

The high fat content commonly found in fermented sausages (usually around 50% of dry matter) has been of nutritional concern, and leaner products are now available (some as low as 5%). The sensory quality of the traditionally high-fat sausages is, however, unique and a standard for the gourmet. The body fat content of pigs has been already drastically reduced by breeding, but with respect to ham, it is left to the consumer to cut off the fat layer before consumption.

Mold-ripened varieties of both sausages and ham (e.g., Tirolean speck and Bündner Fleisch) exist. The production of such products free of mycotoxin is a concern, because of the potential of fungi for contamination. In addition, a carryover from animal feed to the meat may be a source of mycotoxin contamination. This hazard is the target of general meat inspection and control. In rare cases, mycotoxins have been detected in fermented sausages and ham. One way to overcome this hazard is the use of competitive mold strains that have a proven absence of a mycotoxigenic potential. These starter cultures usually contain strains of *Penicillium nalgiovense* or *P. chrysogenum,* and are already widely in use in Europe. For ham production, the absence of mycotoxins is still a matter of rigorous quality control.

Meat may also contain bacterial food pathogens. Because fermented meat products usually do not undergo a physical treatment to eliminate pathogenic microorganisms, the meat has to be of high quality with regard to hygiene and microbial counts. The control of pathogens is achieved by appropriate fermentation technology, including the use of starter cultures.

1.4 The Fermentation Process

The traditional aim of the fermentation process is to transform the highly perishable substrate meat into a shelf stable and safe product ensuring an optimum nutritive value and sensory quality. The factors affecting the process are the nature of the raw materials and the activity of microorganisms, as well as endogenous enzymes and process technology. For all fermented meat products, the raw material is meat with a variable amount of fat that has not been subjected to a thermal or any other germ-reducing process. Meat is the flesh (muscle tissue) of warm-blooded animals, but fermented specialties from poultry (sausages as well as cured and smoked fermented poultry) are also available. Two groups of products can be differentiated on the basis of the microbial populations involved in the fermentation process: foods from a comminuted matrix and whole meat products.

1.4.1 Fermentation of a Comminuted Meat Matrix

The comminution of muscle tissue to particles varying in size between 1 and 30 mm, together with the homogenous distribution of fermenting organisms, is the prerequisite for a fermentation process taking place throughout the matrix. Curing salt, nitrate, ascorbic acid, and, in some cases, sodium glutamate and glucono-lactone are added to the particles together with spices and above all a carbohydrate source, which is commonly glucose. These compounds exert strong effects on the growth and performance of the fermentative flora.

The fatty tissue should be as fresh as possible, as any initiated oxidative process will strongly affect the shelf life by causing early rancidity. The whole comminution process of chopping or grinding together with a mixing procedure requires temperatures below 2 to 3°C. Thereafter, the temperature is raised usually to >20°C and <28°C to initiate the fermentation process. Semidry sausages of the U.S. summer sausage type are fermented at even higher temperatures (32° to 38°C). The many types of fermented sausages are the result of a great variety of process conditions. Variables include: the particle size of the comminuted meat and fatty tissue, the selection of additives, the temperature/humidity conditions prevailing in the course of fermentation until the final ripening, the diameter of the sausages, the nature of the casings, smoking, heating after fermentation.

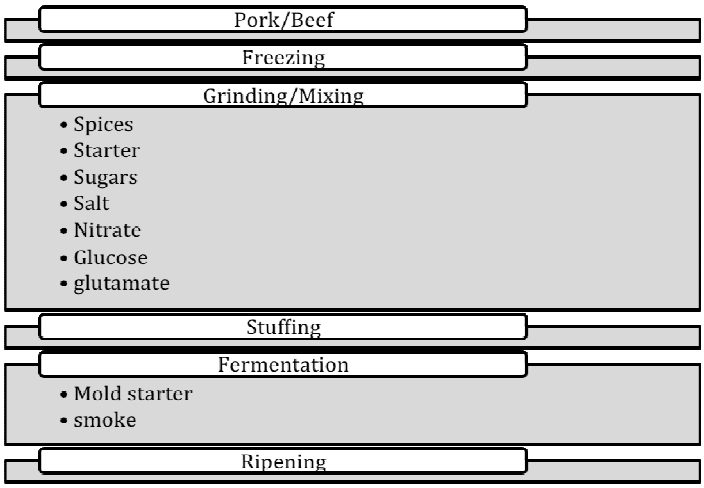


Figure 1.4.1: flow scheme of fermented meat production

The fermentation process, together with the effects of the temperature/humidity conditions, ensures that the originally highly perishable raw materials turn into a spoilage-resistant, flavor-rich product with a defined texture and stable color. Great variation exists with regard to texture, which may range from spreadable to sliceable, from soft to very hard. With regard to the microbial effects, the first days are of great importance. During that time the organisms multiply, reduce the pH to values varying between 5.4 and 4.8, exhibit enzymatic activity, and interfere with undesired microorganisms, which constitute the indigenous flora of the meat. In the course of ripening, the pH usually rises again. This rise in pH does not constitute a safety hazard, because at the same time the water activity is decreased to levels that discourage bacterial growth.

Lactobacilli and micrococci play a decisive role during the fermentation process. These organisms develop under the specific prevailing ecological conditions and were sometimes inoculated by back slopping, i.e., adding chopped fermented sausage back to a new meat mixture. Between 1950 and 1960, microorganisms were isolated and turned into preparations of starter cultures, which are now commonly used, as they exhibit numerous advantages when compared with the classical indigenous fermentation. Many of the bacteria used are lactic acid bacteria (LAB), which are of primary importance, but included in this table (tale 1.4) are also non lactic acid bacteria, which are used mainly in combination with LAB and contribute to the fermentation process as they have unique properties. For example, *Kocuria* spp. and *Staphylococcus* spp. exhibit nitrite and nitrate reductase activity, respectively, which

is important for the reddening of the sausages, i.e., the formation of the stable red color of nitrosomyoglobin. In addition, these organisms exhibit catalase activity, which counteracts the formation of hydrogen peroxide and thus helps to prevent color defects and rancidity. Yeast and fungi contribute mainly to the development of flavor and to a minor extent also to color stability.

Table 1.4: Species Employed in Meat Starter Cultures

Bacteria	*Lactic acid bacteria*	*Lb acidophilus, Lb. alimentarius, Lb. paracasei*, *Lb. rhamnosus*, *Lb. curvatus, Lb. plantarum, Lb. pentosus, Lb. sakei, Lactococcus lactis, Pediococcus acidilactici, P. pentosaceus*
	Actinobacteria	*Kocuria varians*, *Syreptomyces griseus, Bifidobacterium spp.*
	Staphylococci	*Staphylococcus xylosus, S. carnosus* ssp. *carnosus, S. carnosus* ssp. *utilis, S. equorum*
Fungi		*Penicillium nalgiovense, P. chrysogenum P. camemberti*
Yeasts		*Debaryomyces hansenii, Candida famata*

1.4.2 Fermentation Of Whole Meat Products

Immediately after slaughter, enzyme-catalyzed reactions start to act on the physical and chemical nature of muscle, turning it into meat. These reactions continue even when technological/processing measures, such as cool storage and lowering the water activity by drying or salting, are imposed. However, the reactions proceed in a predictable and controlled way. This process provides the foundation of ham production. Microorganisms do not play a role in the fermentative processes taking place in ham.

By far the majority of fermented raw ham is made from pork, but in some regions beef (Bresaola, Bündner Fleisch, Pastirma) and even meat from game, reindeer, or bear is used to produce similar products. The traditional ham in ancient Greek and Roman times as well as in China was made from the bone-containing ham of hogs. This type of ham is still considered the gold standard of quality and is produced in

many countries, e.g., Prosciutto di Parma (Italy), Jambon de Bayonne (France), Jamón Serrano (Spain), Kraskiprsut (Slovenia), Virginia ham (United States), and Yunnan ho-twe and Tshingwa ho-twe (China).

The process of ham production follows a rather simple principle.38 It consists of curing by salting (with or without the use of nitrite and/or nitrate) to achieve a water activity of <0.96, which is equivalent to 4.5% sodium chloride. At low temperatures (5°C), the salt will diffuse to the deepest part of the meat, thus overcoming the hazard of food poisoning through *Clostridium botulinum* contamination. After a phase of equilibrating the salt concentration and flavor development, the temperature is raised to 15 to 25°C to ripen the ham. This phase lasts at least 6 to 9 months and may be extended even to 18 months to achieve the optimum flavor. At the end of the ripening step, the moisture has been reduced by about 25% and a salt concentration between 4.5 and 6% results. In some countries (e.g., Germany), in addition to a nitrate cure, smoking is used to obtain a characteristic flavor and to suppress surface growth of molds. When ham is produced from only one or a few muscles, numerous methods are applied to accelerate production time and to control flavor development and water content. For example, the curing is performed in brine or by injection of curing salt and, above all, the ripening period is drastically reduced. Microorganisms may cause spoilage by growing in the inner muscle parts before the water activity is reduced to safe levels. However, microorganisms also contribute favorably to the process as they are involved in nitrate curing in brine by the formation of the reactive nitrite, which affects color by reddening, flavor, and microbial safety. The microorganisms involved are Gram-negative bacteria such as vibrios and *Halomonas* spp.

1.5 Characteristics of Fermentation

During sausage fermentation many physical, biochemical, and microbial changes happen:

- growth of LAB and concomitant acidification of the product
- reduction of nitrates to nitrites and formation of nitrosomyoglobin
- solubilization and gelification of myofibrillar and sarcoplasmic proteins
- degradation of proteins and lipids
- dehydration

1.5.1 Fermentation Microflora

The initial microbial population of sausage minces depends on the microbial load of the raw materials.

The ecological conditions of sausage minces favor the growth of Micrococcacea and lactobacilli. Lactobacilli generally grow to cell counts of 5 . 108 to 109 CFU/g, and these numbers remain stable throughout ripening. Micrococcacea (predominantly *Kocuria varians, Staphylococcus carnosus,* or *S. xylosus*) generally grow to cell counts of 10^6-10^7 CFU/g, when nitrate cure is applied. The growth of these organisms is inhibited by the application of nitrite cure as well as the decrease of pH. Higher cell counts are reached at the outer layer of the sausages, where a higher oxygen partial pressure occurs.

Because of their high salt tolerance, the predominant microorganisms found in dry cured ham fermentation belong to the classification that was formerly included in the family Micrococcacea. The species most often isolated are *Staphylococcus xylosus, S. equorum*, and *S. sciuri,* but *K. varians* is also found at appreciable cell counts. Growth of staphylococci occurs primarily at the surface of hams.

The growth of yeasts and fungi on mold-ripened products is restricted to the surface of the product, where cell counts reach 105 to 107 CFU/cm2 within four weeks of ripening. Many of the traditional fermentations involving fungal ripening rely on inoculation by the “house flora” associated with the building or equipment used for fermentation and maturation.

1.6 Acidification

Acidification to the isoelectric point of meat proteins (pH 5.3 to 5.4) and the increase of the ionic strength induces gel formation of the proteins and thus confers important structural changes. The high levels of sodium chloride and lactate in fermented sausages contribute to the development of the characteristic taste of the product. Rapid acidification and subsequent drying are of paramount importance for inhibition of the growth of pathogens and their subsequent inactivation during ripening. As there exist not dried fermented sausages of a spreadable type (e.g., in Germany, known as Streichmettwurst, Teewurst, Rohpolnische), the highest hygienic standards of the raw materials and production facilities are key to product safety. In addition to low pH and water activity, specific microbial metabolites such as diacetyl or short chain fatty acids exert an inhibitory effect towards pathogens. Several meat starter cultures

produce bacteriocins — small, heat stable peptides with antimicrobial activity. The use of bacteriocinogenic starters has been shown to contribute to the elimination of *Listeria* during sausage fermentation. However, because of the resistance of Gram-negative organisms, including *Salmonella* and *Escherichia coli* O157:H7 strains, the contribution of bacteriocins to the overall hygienic safety of fermented meats is limited.

Growth and metabolism of LAB result in a fast drop of pH during the first days of sausage fermentation, followed by a slight increase during the ripening period. Lactic and acetic acids are the major fermentation products, and the molar ratio of lactate to acetate ranges between 7 and 20. The product pH depends on the buffering capacity of the meat, the metabolic activities of the fermentation micro-flora, and the addition of fermentable carbohydrates. In Northern European and in U.S. summer sausage, the pH typically ranges from 4.8 to 5.2, corresponding to a content of 200 mmol lactate/kg dry weight. In Mediterranean-type products involving longer ripening periods of up to several months, the final pH typically ranges from 5.4 to 5.8. In mold-ripened products, the sausage pH may increase to levels close to 6.0 due to lactate consumption and the formation of ammonium. The dry matter content of fermented sausages ranges from 50 to 75% or more, corresponding to water activity (aw) values ranging from 0.86 to 0.92 upon ripening.

1.7 Proteolytic and Lipolytic Degradation

Proteolytic events during fermentation of raw sausages and dry cured ham were widely discussed by Toldra and Flores, and Ordónez et al. In the course of ripening, peptides and amino acids accumulate to levels of about 1% dry matter. Peptides and amino acids themselves may contribute to the characteristic taste of dry cured products and act as flavor enhancers and synergists. Excess proteolysis may result in bitter and metallic off-flavors because of the presence of bitter peptides. Furthermore, amino acids and peptides are utilized by microorganisms for the conversion into flavor volatiles.

The hydrolysis of muscular proteins to peptides is mainly achieved by the activity of endogenous enzymes. The endopeptidases — cathepsins B, B + L and H — were shown to remain active throughout the fermentation of dry cured ham and fermented sausages, whereas tissular calpains are inactivated during fermentation and do not contribute significantly to overall proteolysis. Furthermore, muscle exopeptidases

contribute to peptide conversion to amino acids. The proteolytic system of lactobacilli consists mainly of cell wall-associated proteinases, which convert proteins to oligopeptides. Oligopeptide transport is the main route for nitrogen entry into the bacterial cells, and virtually all peptidases are located intracellularly. The proteolytic activity of starter cultures is weak compared to that of tissue enzymes. Correspondingly, the inoculation of sausage minces with starter cultures leads to only a minor increase in amino acid levels of the sausages compared to aseptic control batches. The proteolytic activity of *Kocuria varians* is inhibited by environmental conditions prevailing during sausage ripening, yet the peptidase activity of this organism may contribute to the formation of amino acids. It was recently shown that *Lb. casei* utilizes peptides released from pork muscle sarcoplasmic and myofibrillar proteins under conditions of sausage ripening. he fat content of fermented sausages typically ranges from 40 to 60% of dry matter. During fermentation, long-chain fatty acids are released from triglycerides and phospholipids. Typically, an increase in the levels of free fatty acids up to approximately 5% of the total fatty acids has been found. The fatty acid composition of fat varies considerably depending on the previous feeding regime of the animal. The specific release of polyunsaturated fatty acids is higher than that of monounsaturated or saturated fatty acids. Lysosomal muscle acid lipase and adipose tissue lipases remain active throughout several months of dry cured ham ripening. Comparisons of aseptic fermented batches of sausages with batches inoculated with starter cultures has shown that lipolysis during fermentation is attributed mainly to meat endogenous enzymes. Lactobacilli are considered to be weakly lipolytic. Strains of *K. varians* and *S. carnosus* or *S. xylosus* have been found to exhibit lipolytic activity, which is, however, inhibited at pH values below 6. In mold-ripened products, lipolytic activities of the surface mold flora contributed to the generation of long-chain fatty acids.

1.8 Generation of Flavor Volatiles

During sausage fermentation flavor volatiles are generated by lipolysis and hydrolysis of phospholipids, followed by oxidation of free fatty acids. Furthermore microorganisms produce organic acids, convert amino acids and peptides to flavor-active alcohols, aldehydes, and acids and modify products of lipid oxidation, e.g., by esterification of acyl moieties or reduction of aldehydes.

Depending on the product formula and maturation conditions, the sausage aroma is

determined by the addition of spices, smoking, or surface- ripening with yeasts or molds too.

Despite the differences in the process technology and the fermentation microflora, it may be assumed that the generation of flavor during fermentation of dry cured ham is governed by the same principles.

The most important odor compounds in French, Italian, and Spanish salami are those originating from added spices, i.e., sulfur compounds (e.g., diallylsulfide) originating from garlic, and eugenol from nutmeg. Products of lipid oxidation, fatty acids, as well as fermentation volatiles such as acetic acid, diacetyl, and phenylethanol, further contribute to overall flavor. The comparison of flavor volatiles and sensory attributes of sausages of various origins reveals that a high level of fatty acids negatively affects the sausage aroma.

Unsaturated fatty acids are prone to autoxidation in the presence of oxygen. Many of the products of lipid oxidation are highly volatile and have a low odor threshold. Hexanal, nonenal, 2(Z)octenal, 1-octen-3-ol, and 1-octen-3-on were identified as the most potent flavor volatiles resulting from autoxidation of linoleic acid, and these compounds are present in fermented sausages. The rate of lipid oxidation is greatly enhanced by heme or nonheme iron. The increase in NaCl concentration during ripening also favors lipid oxidation. Nitrite present as part of the sausage formula or formed from nitrate by microbial nitrate reductase acts as an antioxidant. The removal of peroxides by catalase, pseudocatalase, or manganese-dependent superoxide dismutase activities of the Micrococcaceae and/or lactobacilli is crucial to limit the extent of fatty acid oxidation and to prevent off-colors.

Amino acid catabolism by staphylococci and lactobacilli yields volatile products contributing to meat flavor. *Staphylococcus xylosus* and *Staphylococcus carnosus* have been shown to produce a large variety of flavor volatiles originating from amino acid degradation during growth on sausage minces. The formation of flavor volatiles is strongly affected by the growth parameters salt, nitrate, glucose, and oxygen. Degradation of leucine was also shown for strains of *Lb. curvatus, Lb. sakei,* and *Lb. plantarum.* The addition of proteolytic enzymes or the use of proteolytic starter cultures does not enhance the microbial conversion of amino acids to flavor-active derivatives.

1.9 Use of Nitrite in Meat Processing

The origin of salting meat is lost in antiquity, but is believed that the ancient Sumerian civilization was the first to practice this process. Meat curing can be defined as the addition of salt to meats for the sole purpose of preservation; that is, to inhibit or deter microbial spoilage. The preservation of meat resulted from necessity, so that products could be held for extended periods for later consumption in times of scarcity. Salting prevented bacteria growth on account of salt's direct inhibitory effect or because of the drying action it had on meat. As the use of salt as a meat preservative spread, it was found that high concentration of salt would promote the formation of an unattractive brownish-gray color within lean muscle tissue. At some point at the development of this art, more likely accident than design, it was discovered that that salt could impart or "fix" a unique pink or red color and flavor in meats (Binkerd and Kolari, 1975). By Medieval times the use of salt in production of fermented meat is without alternatives, as it affects fundamental processes in meat conditioning, on microbial performance, hygiene, shelf life, flavor and texture (Desmond, 2006). In their literature review Ruusunen and Poulanne (2005) stated that an addition of 2.5% is the lower limit for obtaining good quality fermented sausage. With regard to curing, it is of special meaning that NaCl reacts with nitrite and forms nitrosohypochloride (NOCl), which is a stronger nitrosylating agent than nitrite itself and therefore, the visible reaction (formation of nitrosomyoglobin) is accelerated (Fox et al., 1996; Møller and Skibsted, 2002).

Beside salt, the traditional curing agent is nitrate. For fermented meat such as sausages and ham it is still in use in Europe either solely as saltpetre (potassium nitrate, E 251) or sodium nitrate (E 252), or in combination with potassium nitrite (E 249). Nitrite is always used as curing salt, which contains up to 0.9% NaNO2. As nitrate is a rather inert compound, it has to be reduced to nitrite, which is the highly reactive curing principle. This reaction is a prerequisite for all curing related events in the meat matrix. As reported by Honikel (2008), some nitrate reduction has been observed also in heated cured meat products, leaving open the possibility that chemical reactions might permit nitrate reduction.

Starter cultures are in use to control nitrate reduction in the fermentation process.

Nitrite in the curing process is either obtained as the product of nitrate reduction or from the addition in the form of curing salt and affects strongly the products.

It has the following characteristics:

v. exerts antimicrobial effects that may result in reduction of the risk of food poisoning and delay of spoilage,
vi. causes reddening, i. e. formation of pink nitrosomyoglobin,
vii. forms curing flavour,
viii. acts as an antioxidative

The use of nitrate/nitrite is still a matter of discussion as the beneficial effects are opposed by health risks, which make these curing agents basically undesired compounds in food. The use of the curing agents is regulated in the EU by the following:

Directive 2006/52/EC regulates the use of Nitrate and Nitrite under the following conditions:

Nitrate (E 251, Potassium nitrate; E 252 Sodium nitrate) is permitted:

- For curing of non-heat-treated meat products (generally permitted at a concentration of 150 mg/kg, and for certain specified traditional products at 300 mg/kg, for some others at 250 mg/kg);
- To prevent late blowing in hard, semi hard and soft cheese (150 mg/kg of cheese milk);
- For sensory effects of pickled herring and sprat (500 mg/kg).

Nitrite is exclusively for curing permitted. E 249, Potassium nitrite, at 150 mg/kg is generally permitted in meat products. E 250, Sodium nitrite, at 100 mg/kg is permitted in sterilized meat products (Fo > 3.00, corresponding to heating for 3 min at 121°C) as well as in specified traditional meat products. To further specified traditional meat products it is permitted at a concentration of 180 mg/kg.

According to (EC) 780/2006 the use of Nitrate/Nitrite in organic food (meat products) is permitted under the following conditions: NaNO2 can be applied at an ingoing concentration of 80 mg/kg and the concentration has to be reduced to 50 mg/kg. The use of KNO3 is permitted at the same concentrations, which have to be expressed as $NaNO_3$. The use of nitrite is limited by the comment that it "can only be used if it has been demonstrated to the satisfaction of the competent authority that no technological alternative giving the same sanitary guarantees and/or allowing maintaining the specific features of the product, is available."

The decisive justification for the use of nitrate/nitrite in meat products rests in their inhibitory action on food pathogens, thus exerting a preservative effect (Dykhuizen et

al., 1996; Pichner et al., 2006). Again, neither nitrate nor nitrite per se is the active inhibitory principle but they have to be converted to reactive intermediate compounds (RNIs) such as NO^-, N_2O_3, ONOO, NO^+_2, RSeNO.

The effect of nitrate/nitrite on color formation is of visual prominence among the sensory changes occurring in the curing process. The target of the reddening reaction is myoglobin (MbFeII) containing haeme as a prosthetic group, which is protoporphyrin IX with iron in the ferrous state (Fe^{2+}). With iron in the ferric state (Fe^{3+}) it is named haematin, which is contained in metamyoglobin (MbFeIII). In myoglobin a proximal histidine residue is attached directly to the iron center. On the opposite position a distal histidine residue is located, which is not bonded to the iron. Oxygen and with high affinity also nitrogen monoxide (NO) bind to this second position. The complex containing NO is named nitrosomyoglobin and represents the characteristic curing red pigment. Upon heating the apoenzyme denatures but the red color is kept in that complex named nitrosohaemochrome.

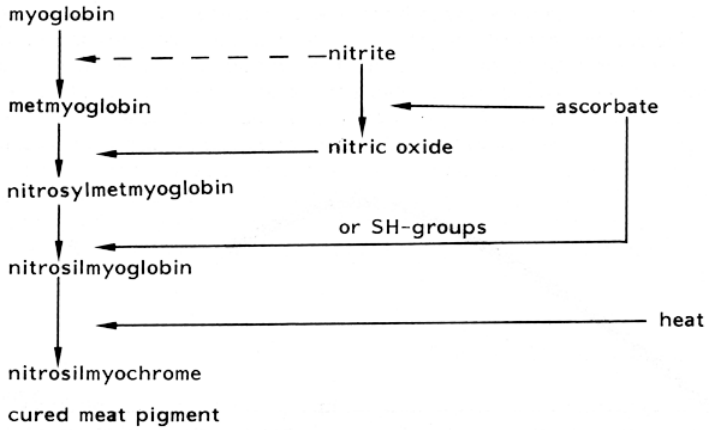


Figure 1.9. Reaction of nitrate and nitrite in fermented meat leading to the curing red pigment nitrosomyoglobin.

Under acidic conditions, created in the fermentation process by lactic acid bacteria, nitrite as the product of nitrate reduction undergoes acid catalyzed disproportionation (Hoagland, 1910, 1914). Nitrite at the oxidation state of nitrogen of +3 disproportionates to NO (oxidation state +2) and Nitrate (oxidation state +5). It is consequence of that reaction that nitrate reducing bacteria are also required for efficient nitrate reduction, even when nitrite is used as the only curing agent. Nitrite reduction and formation of NO can also occur by reaction with reductants among which ascorbate is the common agent under practical conditions.

Several other compounds in meat are reacting with the reactive nitrogen intermediates and just a fraction of 5e15% of the added nitrogen from nitrite has been recovered from myoglobin (Cassens et al., 1978, Cassens, 1990). A greater part of 20-30% has been found bound to protein and 1-15% each to SH-residues and lipids, respectively.

1.10 Use of Nitrite and Nitrate in Fermented Meat: Risks and Benefits

The major concern of nitrate/nitrite in food is related to the potential of nitrite to form cancerogenic N-nitroso compounds, which can be formed in the food matrix as well as in the human body. Clear evidences for the endogenous formation of carcinogenic N-nitrosamine acids from nitrate in the diet were provided by Tricker and Preusmann (1987). The probability of formation of stabile N-nitrosamines in meat is rather low as are also the concentrations detected in cured meat (Honikel, 2008). These compounds can, however, be formed when the products are exposed to temperatures exceeding 130°C. Epidemic studies with nearly 500,000 persons indicated that consumption of red and also processed meat increases the risk of colon cancer (Norat et al., 2005). There was not made a differentiation between meat products neither between raw or heat-treated nor fermented or otherwise preserved ones. Thus, it is not known whether or not the category of fermented meat constitutes a specific health risk.

To avoid the use of “chemical” nitrate, ingredients, which naturally contain nitrates, have been in use natural spices or natural flavorings, celery juice or celery juice concentrate (Sebranek and Bacus, 2007). The authors reported in their review that the nitrate concentration of a commercial celery juice powder was as high as 27,500 mg/kg or about 2.75%. It can be calculated that the addition of 3.6 g of this powder to 1 kg of meat batter results in a nitrate concentration of 100 mg/kg therein. On the other hand, “natural” ingredients such as sea salt, raw sugar or tubinado sugar contain nitrate at concentrations of <2 mg/kg, which is commonly to low for providing a practically usable effect. The products obtained through this type of practice are categorized under the name “clean label food”. It is obvious that the contained “natural” nitrate has to be reduced to nitrite and for this purpose starter cultures are required. Remarkably, the fermentative use of nitrate reducing starter cultures has thus been extended also to the production of cured meat products that undergo a cooking step such as emulsion type sausages. Their manufacturing process requires modification: to permit reddening the culture preparation is added when the comminution starts and thereafter the temperature is kept for 90 min at 42°C.

Smoking and cooking in the following steps are performed as usual. For production of fermented sausages the components of the formula are added together with the nitrate source at starting the comminution process. All process steps correspond to those usually employed for production of nitrate cured fermented sausages.

Increasing evidence has been obtained from human and animal studies showing that dietary nitrate contributes to the beneficial effects exerted by NO that is synthesized endogenously from arginine by nitric oxide synthetase (Gladwin et al., 2005). As nitrate as well as nitrite can be reduced and NO be formed in mammalian tissue and NO can also be oxidized to nitrite and nitrate it is evident that dietary nitrate enters a common pool (Lundberg et al., 2008, 2009). Lundberg et al. (2011) listed the following proven effects on human health of modest dietary intake of nitrate:

vi. reduction of blood pressure,
vii. inhibition of platelet function,
viii. prevention of endothelial dysfunction after a mild ischaemic insult in humans,
ix. reduction of oxygen cost during exercise
x. effecting fundamental mitochondrial functions.

In addition, animal date provide evidence for prolonged blood pressure reducing effects, protection against drug and salt induced renal and cardiac injuries, enhanced post ischaemic blood flow, protection against ischaemia reperfusion injury and reversion of features of the metabolic syndrome.

1.11 Biochemical Changes During Meat Fermentation

During processing of meat, many different biochemical changes come off: proteolysis is one of the most important and relevant for final quality of the product. In fact it has an important effect on texture, taste and on aroma development (Toldrà and Flores, 1998). A good control of proteolysis is fundamental to get a final product of good quality. Muscle and microbial proteases are the main elements responsible for the proteolytical changes and thus a good knowledge of its properties and mode of action is essential for controlled proteolysis. Other changes are restricted to the beginning of the process which is the case of nucleotide breakdown reactions or the glycolysis-related enzymes and subsequent generation of lactic acid. Proteolysis and lipolysis constitute two of the most important enzymatic phenomena, responsible for the generation of compounds with direct influence on taste and aroma.

1.11.1 Glycolysis

Lactic acid is the main product resulting from carbohydrate fermentation. Once the added carbohydrates (glucose, sucrose, etc.) are transported into the cell, they are metabolized via the glycolytic or Embden-Meyerhof pathway. The ratio of the enantiomers L and D lactic acid depends on the species of lactic acid bacteria present and, more specifically, on the action of the L and D lactate dehydrogenases, respectively, and the lactate racemase. There are some key enzymes in the carbohydrate metabolism like aldolases, that generates glyceraldehyde-3-phosphate, pyruvate kinase, that generates pyruvate from phosphoethanol pyruvate and lactate dehydrogenase that generates lactic acid from pyruvate. Glucose is mainly metabolized through a homofermentative way but some other end products like acetate, formate, ethanol and acetoin, with an impact on sausage aroma, may be produced in trace amounts from alternative heterofermentative pathways. The pH drops as a consequence of lactic acid accumulation and contributes to the preservation of the sausage by preventing the growth of undesirable microorganisms. The generated lactic acid also contributes directly to acid taste and indirectly to aroma, due to the formation of metabolites, and sausage consistency due to protein coagulation as pH approaches the isoelectric point of most of the myofibrillar proteins.

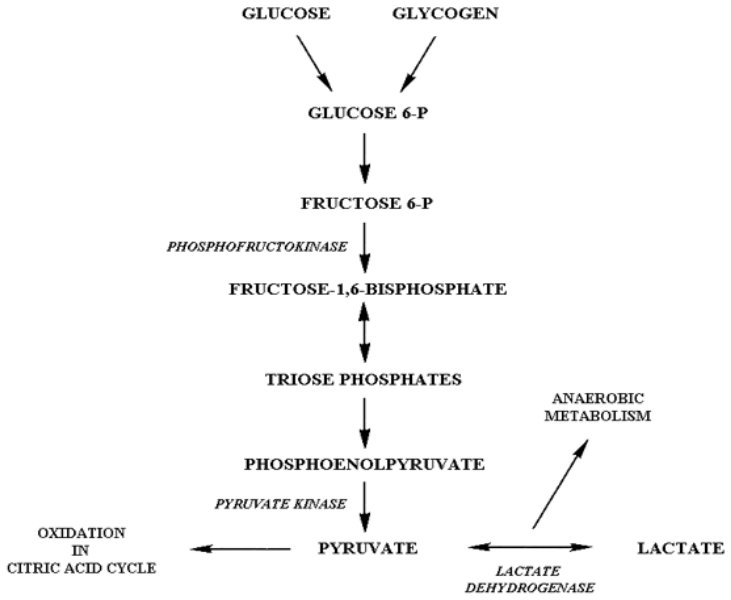


Figure1.11.1 : Embden-Meyerhof pathway

1.11.2 Proteolysis

Proteolysis consists in the progressive degradation and breakdown of major meat proteins (sarcoplasmic and myofibrillar proteins) and the subsequent generation of peptides and free amino acids. The result is a weakening of the myofibrillar network and generation of taste com- pounds but its extent depends on many factors. One of the most important is the activity of endogenous muscle enzymes, which depends on the original crossbreeds and the age of the pigs. Main muscle enzymes involved in these phenomena are cathepsins B, D and L that show a great stability in long term dry-curing processes, good activity at acid pH values and are able to act against myofibrillar proteins. Other important muscle endopeptidases like calpains exhibit poor stability and its optimal pH near 7.0 is far from that in the sausage. Muscle enzymes exert a combined action with microbial proteases although different enzymatic profiles may be found depending on the microorganisms used as starter cultures. One of the major challenges is just to establish the relative role or percentage of contribution of endogenous and microbial enzymes to proteolysis. The proteolytic system of different *Lactobacillus* has been studied and contains endopeptidases able to degrade sarcoplasmic and myofibrillar proteins as well as exopeptidases like dipeptidylpeptidase, tripeptidase , dipeptidase and aminopeptidases. However, some studies revealed that protein degradation, especially myosin and actin, is initiated by cathep- sin D, a muscle endopeptidase very active at pH values near 4,5 and able to degrade both proteins. Cathepsins B and L would be more restricted to actin and its degradation products. The latter stages of proteolysis would be pre- dominantly by bacterial peptidases and exopeptidases.

Other important factors are related with the processing technology. For instance, the temperature and time of ripening will determine the major or minor action of the enzymes, the amount of added salt, which is a known inhibitor of cathepsins and other proteases, will also regulate the enzyme action and thus the proteolysis and taste.

The generation of small peptides may be depressed by the level of salt which inhibits muscle peptidases although intense levels of non-protein-nitrogen, up to 20% of the total nitrogen content, may be reached. Some of these peptides give characteristic tastes . Final proteolysis steps by aminopeptidases, especially from microbial origin, are very important . These enzymes release free amino acids along the process and a substantial increase in the concentration of free amino acids is usually observed.

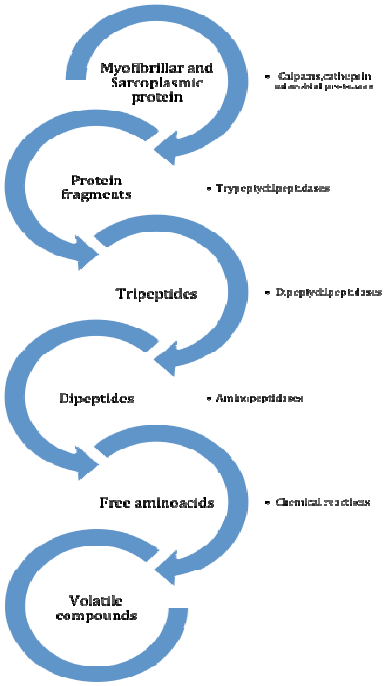


Figure 1.11.2: general scheme of proteolytic chain in dry cured meats.

1.11.2.1 Transformation of Amino Acids

The released free amino acids as a consequence of proteolyisis are then subject of a number of enzymatic and/or chemical transformations that produce different compounds that will affect the sensory characteristics of the product. So, microbial decarboxylation of amino acids may pro- duce biogenic amines. Transamination consists in the transference of the α-amino group of the first amino acid to the α carbon atom from an α-keto acid generating a keto acid from the first amino acid and a new amino acid. Dehydrogenases transform the amino acid in the corresponding keto acid and ammonia. Deamidation also generates ammonia. The microbial degradation of the amino acid side chain by liases may lead to phenol and indole formation (82). The Strecker degradation of amino acids produces branched aldehydes, like 3-methylbutanal, 2-methylbutanal and phenylacetaldehyde from leucine, isoleucine and phenylalanine, respectively, through oxidative deamination-decarboxilation reactions (Table 1.11.2).

Table 1.11.2:Volatile aldehydes from the Strecker degradation (Whitefield, 1992)

Amino acids	Aldehydes
Glycine	Formaldehyde
Alanine	Acetaldehyde
α-Aminobutyric acid	Propanal
Valine	2-Methylpropanal (Isobutyraldehyde)
Leucine	3-Methylbutanal (Isovaleraldehyde)
Isoleucine	2-Methylbutanal
Norvaline	Butyraldehyde
Norleucine	Pentanal
Serine	2-hydroxyethanal
Threonine	2-hydroxypropanal
Methionine	2-Methylthiopropanal (Methional)
Cysteine	2-Mercaptoacetaldehyde or acetaldehyde
Phenylglycine	Benzaldehyde
Phenylalanine	Phenylacetaldehyde
Tyrosine	2-(p-Hydroxyphenyl)ethanal

1.11.3 Lipolysis

Lipolysis consists on the breakdown of tri-acylglycerols by lipases and phospholipids by phospholipases resulting in the generation of free fatty acids. These fatty acids may contribute directly to taste and, indirectly to the generation of aroma compounds through further oxidative reactions. Main lipolytic enzymes, located in muscle and adipose tissue, in combination with microbial lipases, are involved in these phenomena. Although it is difficult to establish a relative role of endogenous and microbial enzymes to lipolysis, the percentage of contribution of endogenous lipolytic enzymes to total fat hydrolysis is estimated around 60 to 80% with the rest due to microbial lipases. The most important lipases located in muscle are the lysosomal acid lipase and acid phospholipase while in adipose tissue are the hormone sensitive lipase and the monoacylglycerol lipase. These enzymes show good stability through the full process . Although their activity also depends on pH, salt concentration and water activity, the conditions found in the sausages favor their action. The generation rate of free fatty acids, especially oleic, linoleic, estearic and palmitic acids, increases during the process. Most of these fatty acids proceed from phospholipids degradation although some of them generate volatile compounds through further oxidative reactions. In the case of adipose tissue, the rate of generation, especially of oleic, palmitic, linoleic, stearic, palmitoleic and myristic acids, is also high.

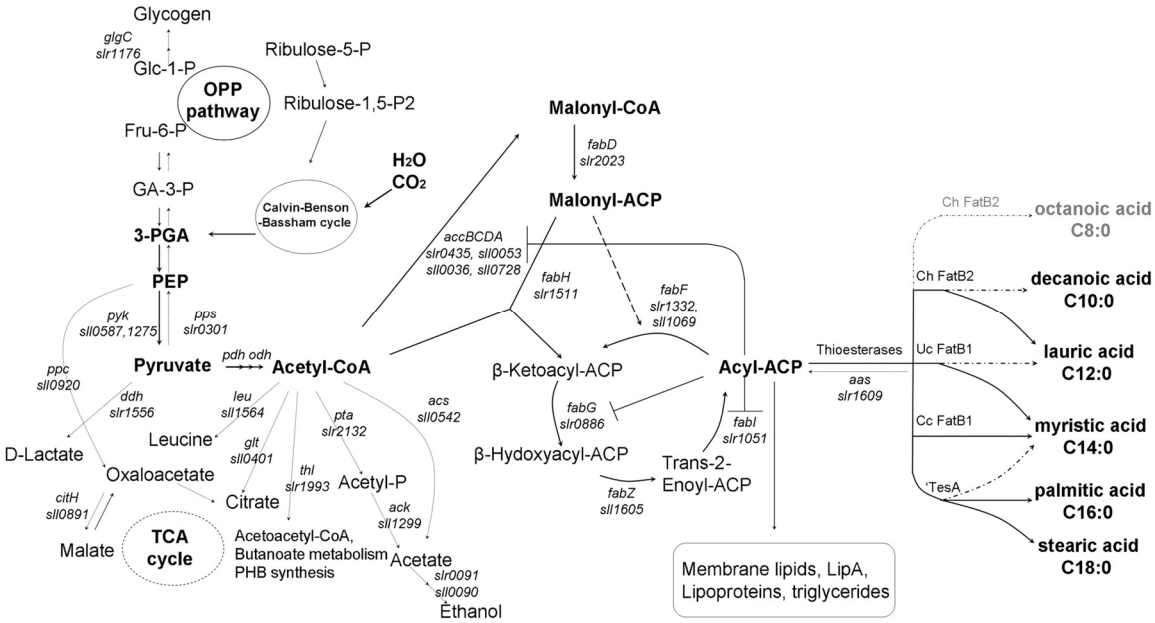


Figure 1.11.3: FFA pathway

1.11.3.1 Oxidation

The generated mono and polyunsaturated fatty acids are susceptible to further oxidative reactions to give volatile compounds. The beginning of lipid oxidation is correlated to an adequate flavor development. On the contrary, an excess of oxidation may lead to off-flavors. In fact, the generation of the characteristic aroma of dry-cured meat products is in agreement with the beginning of lipid oxidation. Free radical formation is catalyzed by muscle oxidative enzymes, like peroxydases and ciclooxygenases, external light, heating and the presence of moisture and/or metallic cations. The next step in oxidation is the formation of peroxide radicals (propagation) by reaction of free radicals with oxygen. The formed hydroxyperoxides (primary oxidation products) are flavorless but very reactive giving secondary oxidation products that contribute to flavor (92). The oxidation is finished when free radicals react each other. Main products from lipid oxidation are aliphatic hydrocarbons (poor contribution to flavor), alcohols (high odor threshold), aldehydes (low odor threshold) and ketones. Alcohols may inter- act with free carboxylic fatty acids giving esters, especially when nitrate is not used.

1.12 The Muscle Proteolytic System

Skeletal muscle contains a lot of good enzymes involved in several metabolic pathways, the most important are related to protein changes. Endopeptidases (calpains and cathepsins) are responsible for protein breakdown, tri and dipeptylpeptidases are involved in generation of small tri and dipeptides and finally, aminopeptidases and carboxypeptidases release free aminoacids (Toldrà, 1992). A general scheme of proteolytical chain was shown above (figure 1.11.2).

Most of the enzymes remain active in postmortem muscle, playing important roles in proteolysis. Some of these proteases, having optimal pH are located in organules like lysosomes whereas others are bound to membrane or free in the cytosol.

1.12.1 Endopeptidases or Proteinases

There are three important groups of endopeptidases or proteinases: cathepsins, calpains and proteasome. Cathepsins B, D, H and L are located in lysosomes. They are small in size (20-40kDa) that allow them to penetrate into the myofibrillar structure and hydrolize important proteins, like myosin and troponins that experience many important changes during dry-curing. The second group of proteinases are two calpains, also known as calcium dependent proteases. Both of them are located in the cytosol, manly in the Z-line area, and they differ in their Ca^{2+} requirement for activation. They have optimal neutral pH, around 7.5, but their activity decreases rapidly at acid pH values such as those found in dry fermented sausages. The third group of proteases is the protosome complex, a large protease with multiple catalytic sites. This enzyme is able to exhibit different activites like chymotrypsin-like activity, trypsin-like activity and peptydil-glutamyl activity. (Coux, Tanaka ad Goldberg, 1995). The 20S proteasome may have a different effect on tenderness for its ability to degrade myofibrils, but this activity is only exhibited at high pH values.

1.12.2 Exopeptidases

Trypeptidylpeptidases (TPP) and dipeptidylpeptidases (DPP) are two important groups of muscle exopeptidases**.** TPP I and II are able to hydrolyze different tripeptides, while DPP III and IV hydrolyze dipeptides and their names can vary depending on the preference for certain amino acids. Aminopeptidases are able to release a free amino acid from the ammino terminus of peptides and proteins. Arginyl, alanyl, pyroglutamyl, leucyl are the most important aminopeptidases in skeletal

muscle. All of the are active at neutral or basic pH.

Moreover there are two carboxypeptidases located in the lysosomes, with optimal activity at acid pH. Carboxypeptidase B has a wide spectrum against any amino acid but carboxypeptidase A is more specific for hydrophobic amino acid.

1.13 The Microbial Proteolytic System

The mechanism for proteolytic breakdown of proteins by microbial enzymes are similar to those described for muscle enzymes. These enzymes are also endopeptidases or proteinases and peptidases. Endopeptidases are predominantly extracellular and peptidases are located inside the cell. Extracellular proteinases may be bound either to the cell wall or to the cell membrane (Visser, 1993). In this way the mode of action may differ if using whole cells or only cell-free extracts. In the case of the whole cells, the aa and peptide transport system is necessary to supply the cells with the aa required for the growth. (Tan, Polman and Konings, 1993). The proteolytic activity associated with the cell wall is the first enzyme to degrade the proteins. The generated peptides are then transported into the cell where they are further degraded by different peptidases to small peptides and free amino acids. Many microorganisms have been used as starters for fermented meats. Some of the most important *are Lactobacillus sakei, L. curvatus, L. carnosus, L. plantarum., Kocuria varians, Staphylococcus xylosus* and the yeast *Debaryomices hansenii* (Toldrà 2004d, 2006).

LAB are widely used in food fermentation; even though the proteolytic system from dairy LAB has been well characterized, limited information is available on meat lactobacilli.

1.13.1 Endopeptidases

Lactic acid bacteria have some endopeptidases or proteinases associated with the cell envelope. These are responsible for the initial breakdown of proteins into oligopeptides. Several in vitro assays have been performed to elucidate the hydrolysis of sarcoplasmic and myofibrillar meat proteins when incubated with whole cells and cell free extract from different *L. casei, L. curvatus, L. plantarum.* The results showed that bacteria proteinases are less active than muscle proteinases in hydrolyzing myofibrillar proteins (Molly et al., 1997). However these lactobacilli can hydrolize the sarcoplasmic proteins, especially *L. plantarum* and *L. casei*, which show the

strongest degradation (Sanz et al, 1996). The substrate specificity is broad and the proteinase activity appears to e extracellurarly located, which is in agreement with the existence of a single cell wall associated proteinase in dairy lactic acid bacteria that is responsible for initial caseins hydrolysis. (Kunji et al.1996). The peptides profiles of the sarcoplasmic and myofibrillar extracts after incubation with these strains show the generations of a large number of hydrophilic peptides: this is very important because they are correlated to desirable cured meat flavors, whereas hydrophobic peptides are correlated to bitterness. (Aristoy and Toldrà, 1995).

1.13.2 Exopeptidases

There are important exopeptidases in *L.sakei*, the most prevalent species in European sausages. The first is the major or general aminopeptidase, which has an optimal neutral pH and is similar to PepL from *L. delbrueckii*. It has a broad range of activity against aminoacids especially alanine and leucine, but is unable to hydrolize basic residues. Second is the arginine aminopeptidase, activated by salt and has preference for basic residues like arginine and lysine (Sanz and Toldrà, 2002).The addition of cell free extracts from *L. sakei, L. curvatus* and *L. casei* to myofibrillar and sarcoplasmic proteins gave a net increase in free amino acids. The increase was very significant for glutamate, alanine and leucine for *L.sakei*; glutamate and alanine for *L. curvatus*; arginine and glutamate for *L. casei* (Fadda et al, 1999b).

Table 1.13.2: main purified peptidases in *L.sakei* (Toldrà,2006)

Enzyme	**Biochemical similarity**	**Activation**	**Substrates**	**Optimal T°C**	**Optimal pH**
Major aminopeptidase	PepL	Ca^{2+},Sn^{2+}, Mg^{2+}, Ba^{2+},Mn^{2+}	Leu-peptide, Ala-peptide	37	7,5
Arginine aminopeptidase	PepN	Reducing agent, salt	Arg-peptide, Lys peptide	37	5,0
X-prolyl-dipeptidylpeptidase	Pep X		X-Pro-peptides Ala-Pro-peptide	55	7,5
Dipeptidase	Pep V		Met-Ala	45	7,8
Tripeptidase	Pep T		Ala-Ala-Ala	40	7,0

1.14 Enhancement and Control of Proteolysis

Fermented sausages are common products throughout Europe. Differences in the composition, ripening and fermentation conditions exist not only in different countries, but also within the same country. Sausage fermentation is a well-known microbial process, and ecological studies during ripening date back to the 1970s (Lucke, 1974). These studies highlighted that two main populations are involved in the process. In the fermentation of sausages, the main transformations that lead to the final product involve the activity of two microbial groups: lactic acid bacteria (LAB) and micro/ staphylococci. The LAB are responsible for the acidification, while the micro/staphylococci produce lipases, eventually releasing short-chain fatty acids that are responsible for the aroma of the fermented sausage (Demeyer et al., 1974). Molecular analysis of microbial changes during fermentation showed that by three days of maturation, these two main groups of organisms were the most abundant in the sausages (Cocolin et al., 2001).

Processing conditions are important for they can increase or reduce the enzyme activity: in he hams the control can be achieved checking the relative humidity and temperature in the curing rooms, because they have an important effect on enzymatic activity (Toldrà and Flores, 1998). The pH achieved in the product during the process, near neutral in the ham and acid pH in fermented sausages, will modulate the enzymatic activity. Furthermore, proteolysis can be controlled by adding an excess of salt, that has an inhibitory effect on cathepsins and other proteases and thus reduces softening. (Toldrà, 2002). Time for ripening is important when intense flavor development is required, as most of the generated amino acids need some time to experience further reactions to aroma volatile compounds.

In summary the ability to steer the proteolysis into the meat products is very important from the economic point of view because reproducible and steerable production processes are needed to produce dry cured meat of high quality (Toldrà and Verplaetse, 1995).

1.14.1 Use of Starter Culture in Fermented Meat

Fermented sausage is prepared from seasoned, raw meat that is stuffed in casings and is allowed to ferment and mature (Campbell-Platt and Cook, 1995; Lucke, 1998). Inoculation of the sausage batter with a starter culture composed of selected lactic acid bacteria (LAB), i.e. homofermentative lactobacilli and/or pediococci, and Gram-

positive, catalase-positive cocci (GCC), i.e. nonpathogenic, coagulase-negative staphylococci and/or kocuriae, improves the quality and safety of the final product and standardizes the production process (Campbell- Platt and Cook, 1995; Lucke, 2000). Nonetheless, small manufacturers continue to use the traditional method of spontaneous fermentation without added starter culture. In the latter case, the required microorganisms originate from the meat itself or from the environment and constitute a part of the so-called house flora (Santos et al., 1998).

Such artisan fermented sausages are often of superior quality compared to controlled fermentations inoculated with industrial starters and possess distinctive qualities, partly due to the properties of the raw material and the characteristics of the technology used (Moretti et al., 2004), but also to the specific composition of the house flora. The flavour- generating, metabolic activity of GCC in artisan chorizo, for instance, has been shown to vary with the manufacturing location (Garcıa-Varona et al., 2000).

It has been suggested that commercial starter cultures in Europe, mainly produced in Northern European countries, are not always able to compete well with the house flora colonizing Southern European meat plants, so that their use often results in losses of desirable sensory characteristics (Samelis et al., 1998). The fitness of commercial meat starter cultures when applied to a particular type of salami is questionable since a culture that performs well in one type of fermented sausage is not necessarily efficient in another type. Appropriate cultures have to be selected according to the specific formulation of the batter and technology of fermentation since environmental factors will interact to select a limited number of strains that are competitive enough to dominate the process (Rebecchi et al., 1998). *Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus pentosus* and *Lactobacillus plantarum*, species sometimes found in commercial starter cultures for meats, are rarely detected in large amounts in spontaneously fermented sausages because of their inferior competitiveness compared to, for instance, *Lactobacillus sakei* or *Lactobacillus curvatus* (Doßmann et al., 1998; Coppola et al., 2000). They initiate the acidification of the meat batter well, but are not always able to prevent spontaneous outgrowth of non-starter LAB with undesirable effects on the end-product (Coventry and Hickey, 1991; Hugas and Monfort, 1997*). Lb. plantarum* may also give rise to a product with overacidity, which is not well perceived by the consumer (Garriga et al., 1996).

Even if the rapid acidification initiated by the starter culture reduces microbial risks in fermented sausages, not all concerns have been solved, mainly so in slightly fermented or ripened varieties. Whereas pseudomonads, *Enterobacteriaceae*, and aerobic sporeformers are usually not of concern (Samelis et al., 1998; Aymerich et al., 2003), the pathogens *Staphylococcus aureus, Escherichia coli, Salmonella, and Listeria monocytogenes* are posing risks to food safety.

In the last years the use of starter cultures of a new generation has been suggested, the so-called Functional starter cultures (De Vuyst, 2000; Leroy and De Vuyst, 2003, 2004) to ensure the consumer microbial safety and offer one or more organoleptic, technological, nutritional, or health advantages.

One of the main challenges is to explore the biodiversity of artisan products and to introduce qualities obtained with wild- type strains in standardized, industrial fermentations. In contrast to ill-adapted industrial starters, wild-type strains that naturally dominate traditional fermentations tend to have higher metabolic capacities which can beneficially affect product quality, for instance with regard to aroma formation or food safety. Natural selection is likely to have forced such strains to be more competitive by endowing them with ecological advantages, i.e. making them less auxotrophic (Ayad et al., 2000) and more able to produce antimicrobials (Maldonado et al., 2002).

1.14.2 Selection of Starter Culture

L. sakei, L. curvatus, L. plantarum, Lactobacillus pentosus, Lactobacillus casei, Pediococcus pentosaceus and *Pediococcus acidilactici* are the species most used as commercial meat LAB starter cultures (Hammes & Hertel, 1998; Hugas & Monfort, 1997). The first stage in designing a starter culture for a meat commodity is to characterize the LAB strains isolated from the meat product in question and then select those best suited. In meat fermentations, the main function of LAB is to obtain a rapid pH drop of the batter, which promotes

iv. product safety by inactivating pathogens,
v. product stability and shelf life by inhibiting undesirable changes caused by spoilage microorganisms or abiotic reactions,
vi. creates the biochemical conditions to attain the new sensory properties of the ripe products through modification of the raw materials (Lücke, 2000).

Currently the use of starters as functional flora is gaining importance; designed starter

cultures have properties additional to those of the more classic type, helping to optimize the sausage fermentation process, and to produce tastier, safer, and healthier products.

The production of organic acids – mainly lactic acid – from carbohydrates is the major role of LAB in sausage fermentation. This depends on several chemical, physical and microbiological reactions. While acidifying the batter, LAB participate in the coagulation of muscle proteins, resulting in the increased slice stability, firmness and cohesiveness of the final product (Hugas & Monfort, 1997; Ordóñez et al.,1999). They also enhance the spontaneous reduction of nitrites to nitric oxide, which reacts with the myoglobin to form nitrosomyoglobin, the compound responsible for the typical pink color of cured sausage (Hugas & Monfort, 1997). Moreover, they contribute to the flavor of the final product through the formation of noticeable acidic and vinegary (acetic acid) tastes. Acidic conditions are also thought to increase the activity of cathepsin D, which is responsible for muscle proteolysis (Molly et al., 1997). The production of organic acids is undoubtedly the determining factor on which the shelf life and the safety of the final product depends. The inhibition of pathogenic and spoilage flora is also dependent on a rapid and adequate formation of these organic acids. Finally, it has been reported that a rapid decrease in pH caused by amine-negative starter cultures can largely prevent biogenic amine (BA) accumulation in sausages (Maijala, Eerola, Aho, & Hirn, 1993).

The immediate and rapid formation of acid at the beginning of the fermentation process, and the production of sufficient amounts of organic acids allowing a pH below 5.1 to be reached, are therefore essential requirements of meat LAB starters. Excessive acid formation, however, is often associated with color defects (due to the inhibition of the CNC) and sometimes with gas formation – one of the most important problems in sausage fermentation (Buckenhüskes, 1993).

The ability of the starter culture to compete with the natural microbiota of the raw material and to undertake the metabolic activities expected is conditioned by its growth rate and survival in the conditions prevailing in the sausage: an anaerobic atmosphere, rather high salt concentrations, low temperatures and low pH. The salt concentration is about 2% (aw=0.94–0.98) in the batter and can reach 15% (aw = 0.85–0.86) in the final product (Lücke & Hechelmann, 1987; Montel, 1999). The manufacturing temperature ranges from 4 to 7°C when preparing the batter (Baracco, Durand, Frentz, Jacquet, & Zert, 1990), from 18 to 24°C during the fermentation

period (Montel, 1999), and from 12 to 15 °C during the drying and ripening period (Montel, 1999). The initial pH of the batter, which is generally around 6.0 decreases during fermentation and reaches values between 4.6 and 5.1. Thereafter, yeasts, mostly *Debaryomyces hansenii*, increase the pH of the product (Cook, 1995), achieving final values ranging from 5.1 to 5.5.

Thus, the growth rate at different temperatures (2–4 to 24°C), the tolerance of salt concentrations of 2–10%, and of pH in the range 4.2–6.0 are limiting factors affecting the persistence and competitiveness of the starter culture over the entire fermentation and ripening process. *L. sakei* can grow at 4 °C, in the presence of 6.5% NaCl, and at pH 4.2 (Ammor, Dufour, Zagorec, Chaillou, & Chevallier, 2005). At 15°C and in the presence of 2% NaCl, it shows growth rates which allow 0.55 generations to be produced per hour (Ammor et al., 2005). Its psychrotrophic character and salt tolerance may be due to its ability to effciently accumulate osmo- and cryoprotective solutes such as betaine and carnitine, and to its cold stress response: *L. sakei* has more putative cold-stress genes than any other lactobacilli (Chaillou et al., 2005). A combination of mechanisms, including modification of carbohydrate metabolism (down regulation of glycolysis) and stimulation of oxidative stress may also increase its resilience to cold (Marceau, Zagorec, Chaillou, Mera, & Champomier-Verges, 2004).

Heterofermentative lactobacilli are not suitable for sausage production because the formation of large amounts of carbon dioxide leads to holes of different sizes in the product (Buckenhüskes, 1993). In addition, these Lactobacilli produce concentrations of acetic acid that cause a pungent off-flavor.

Most lactobacilli are able to form hydrogen peroxide by oxidizing lactate. Hydrogen peroxide can interfere with the organoleptic properties of fermented meat products by increasing rancidity and the discoloration of the final product.

LAB participate in the formation of the typical pink color through the spontaneous reduction of nitrites to nitric oxide. Some meat LAB have also been reported to possess nitrate reductases and heme-dependent and heme-independent nitrite reductases (Hammes et al., 1990; Wolf, Arendt, Pfahler, & Hammes, 1990). These are directly involved in the mechanisms of nitrosomyoglobin formation.

Meat starter cultures are mainly mixtures of LAB and CNC, thus, to perform their expected functions, LAB starters must be able to tolerate or even show synergy with CNC starter components. Hammes et al. (1990) showed some *L. sakei and L. curvatus*

strains to inhibit others used in meat starter cultures (in addition to unwanted flora), such as Kocuria varians.

1.14.3 Use of Starter Culture for a Safer Product

The main antimicrobial effect responsible for safety is evidently the rate of acidification of the raw meat (Lucke, 2000). Nevertheless, certain antimicrobials such as bacteriocins may also play a role, in particular in slightly acidified products or to eliminate undesirable microorganisms that display acid tolerance (e.g. L. monocytogenes).

Bacteriocins produced by LAB are antibacterial peptides or proteins that kill or inhibit the growth of other Gram-positive bacteria (De Vuyst and Vandamme, 1994; Cintas et al., 2001; Cleveland et al., 2001; Diep and Nes, 2002). They often have narrow inhibitory spectra and are most active towards closely related bacteria likely to occur in the same ecological niche (Eijsink et al., 2002). LAB produce a diversity of bacteriocins that are generally active towards other LAB, contributing to the competitiveness of the producer, but also towards foodborne pathogens such as *L. monocytogenes*. The application of bacteriocin-producing LAB in the meat industry offers therefore a way of natural food preservation (Stiles and Hastings, 1991; McMullen and Stiles, 1996; Hugas and Monfort, 1997; Hugas, 1998; De Martinis et al., 2002).

Lactobacilli sausage isolates frequently produce bacteriocins or bacteriocin-like compounds, as has been shown for *L. sakei* (Sobrino et al., 1991; Tichaczek et al., 1992; Garriga et al., 1993; Samelis et al., 1994a; De Martinis and Franco, 1998; Aymerich et al., 2000b; Rosa et al., 2002; Tantillo et al., 2002), *L. curvatus* (Tichaczek et al., 1992; Sudirman et al., 1993; Mataragas et al., 2002), *L. plantarum* (Garriga et al., 1993; Rekhif et al., 1995; Enan et al., 1996; Aymerich et al., 2000b; Messi et al., 2001*), L. brevis* (Benoit et al., 1994), and *L. casei* (Vignolo et al., 1993).

The use of bacteriocin-producing *L. sakei* as starter cultures permits to decrease *Listeria* levels in fermented sausage (Schillinger et al., 1991; Hugas et al., 1995; Hugas et al., 1996; De Martinis and Franco, 1998). Antilisterial effects have also been demonstrated with bacteriocinogenic *L. curvatus* (Hugas et al., 1996; Dicks et al., 2004) and *L. plantarum* (Campanini et al., 1993; Dicks et al., 2004) sausage starter cultures.

The use of bacteriocin producers as new starter cultures may offer considerable food

safety advantages, without risk for human health due to toxicological side effects (Cleveland et al., 2001). It represents a safe way of natural food preservation that, most likely, has always occurred in fermented foods for centuries. Moreover, in situ bacteriocin production does generally not lead to organoleptic or flavour imperfections, as has been shown through taste panels with different strains (Hugas et al., 1995; Coffey et al., 1998). Still, some disadvantages have to be considered related to the fact that bacteriocin activity in situ is lower than may be expected from in vitro experiments (Schillinger et al., 1991; Campanini et al., 1993). Activity may be less effective in the sausage due to low production, genetic instability, the inability to uniformly distribute bacteriocin throughout the product, low solubility of the bacteriocin, inactivation by meat proteases, resistance of the target strain, and interference by meat components, in particular adsorption to fat and meat particles (Knorr, 1998; Ennahar et al., 1999; Cleveland et al., 2001; Aasen et al., 2003; Dicks et al., 2004). It is recommended to use strains that are well adapted to the sausage environment, preferably sausage isolates, for optimal performance and bacteriocin production (Leroy et al., 2002b). For instance, the results obtained during sausage fermentation with *L. plantarum* (Campanini et al., 1993) are less convincing than the results that are generally obtained with *L. sakei or L. curvatus*. In this context, it has been shown kinetically that the sausage isolates *L. sakei* CTC 494 (Leroy and De Vuyst, 1999a), *L. curvatus* LTH 1174 (Messens et al., 2003), and *L. curvatus* L442 (Mataragas et al., 2003) optimally produce bacteriocin under conditions of pH and temperature that prevail during European sausage fermentation. Also, appropriate cultures are to be selected according to the specific formulation and the technology used (Hugas and Monfort, 1997).

Evidently, bacteriocins are not meant to be used as the sole means of food preservation, but should be appropriately integrated in a multi hurdle preservation system, at all times respecting good manufacturing practice.

It is important during strain selection that no undesirable compounds such as toxins, biogenic amines, or D(-)-lactic acid, that could aversely affect health, are formed.

If surface mould growth is desirable, it must be checked if the mould starter culture produces mycotoxins or antibiotics (Sunesen and Stahnke, 2003). The use of moulds that are free of mycotoxin production as starter cultures could be useful in outcompeting mycotoxin-producing strains from the house flora. Moulds may also produce green or dark spots that are not acceptable to most consumers or have a

negative impact on flavour and taste (Sunesen and Stahnke, 2003).

During the ripening of fermented sausages, biogenic amines such as tyramine, histamine, tryptamine, cadaverine, putrescine, and spermidine, may be formed by the action of microbial decarboxylases on amino acids that originate from meat proteolysis (Komprda et al., 2004). Microbial decarboxylation reactions may be ascribed to both the microorganisms that were introduced via the starter culture and the ones that constitute part of the natural population of the meat. In general, starter bacteria have limited tyrosine-decarboxylating activity, but contaminant non-starter LAB, in particular enterococci, are believed to be responsible for tyramine production (Ansorena et al., 2002). The use of decarboxylase-negative starter cultures that are highly competitive and fast acidifiers prevents the growth of biogenic amine producers and leads to end-products nearly free of biogenic amines (Bover-Cid et al., 2000a, b; Suzzi and Gardini, 2003), as long as the raw material is of sufficient quality (Bover-Cid et al., 2001). Also, the introduction of starter strains that possess amine oxidase activity might be a way of further decreasing the amount of biogenic amines produced in situ (Leuschner and Hammes, 1998; Martuscelli et al., 2000; Fadda et al., 2001; Gardini et al., 2002; Suzzi and Gardini, 2003). Although it is known that the superficial inoculation with *P. camemberti* in cheeses increases the concentration of certain amines, the production of amines by moulds in fermented sausages does not appear significant but has not been fully studied yet (Bruna et al., 2003).

The nature of the lactic isomer produced by the LAB strains is of concern, since high levels of the D(-)-lactic acid isomer are not hydrolyzed by lactate dehydrogenase in humans and are thus capable of causing acidosis. Therefore, strains producing L(+)-lactic acid should be preferably selected (Holzapfel, 2002).

Antibiotic resistance is a worldwide public health problem that continues to grow. Limiting the transmission of antibiotic resistance genes to unrelated pathogenic or opportunistic bacteria is essential. The food chain has been recognized as one of the main routes for the transmission of antibiotic resistant bacteria between animal and human populations (Witte, 2000). European Authorities have recently concluded that some bacteria used for or in feed production might pose a risk to human and animal health because of harboring strains with transferable resistance genes (European Commission, 2005). Fermented meats that are not heat-treated before consumption provide a vehicle for such bacteria and can act as a direct link between the indigenous microflora of animals and the human GIT. Recently, food-associated bacteria such as

L. sakei, L. curvatus, Leuconostoc mesenteroides, and P. pentosaceus have been isolated from human feces, suggesting their ability to survive passage through the human gastrointestinal tract (GIT) (Walter et al., 2001).

Several studies have reported antibiotic resistance in LAB from meats and meat products; a few strains involved in sausage fermentation such as *L. sakei, L. curvatus and L. plantarum* have been found to show such resistance (Gevers, Danielsen, Huys, & Swings, 2003; Holley & Blaszyk, 1997; Teuber & Perreten, 2000). Although most of these resistances have been characterized as intrinsic, some genetic determinants such as chloramphenicol acetyltransferase (cat-TC), erythromycin [erm(B)] and tetracycline [tet(M), tet(S)] resistance genes have been identified, suggesting that horizontal gene transfer may have occurred (Ahn, Collins-Thompson, Duncan, & Stiles, 1992; Gevers et al., 2003; Lin, Fung, Wu, & Chung, 1996; Tannock et al., 1994) .A recent study has shown that seven out of 62 lactobacilli strains might harbor transferable resistance genes on the basis of their resistance levels to chloramphenicol, erythromycin/clindamycin, tetracycline and oxacillin (Danielsen & Wind, 2003). Therefore, before launching a starter culture or probiotic product, it is important to verify that the bacterial strains involved do not content transferable resistance genes.

1.15 Microrganisms Involved in Sausage Fermentation

The microorganisms that are primarily involved in sausage fermentation include species of LAB, GCC, moulds, and yeasts.

In spontaneously fermented European sausages, facultative homofermentative lactobacilli constitute the predominant flora throughout ripening. *L. sakei* and/or *L. curvatus* generally dominate the fermentation process (Rebecchi et al., 1998; Samelis et al., 1998; Santos et al., 1998; Andrighetto et al., 2001; Cocolin et al., 2001; Aymerich et al., 2003; Papamanoli et al., 2003; Rantsiou et al., 2004, 2005). *L. sakei* appears to be the most competitive of both strains, frequently representing half to two thirds of all LAB isolates from spontaneously fermented sausage, whereas *L. curvatus* is frequently found in amounts up to one fourth of all LAB isolates. Other lactobacilli that may be found, albeit generally at minor levels, include *L. plantarum, Lactobacillus bavaricus* (now reclassified as *L. sakei or L. curvatus*), *Lactobacillus brevis, Lactobacillus buchneri, and Lactobacillus paracasei* (Hugas et al., 1993; Rebecchi et al., 1998; Aymerich et al., 2003; Papamanoli et al., 2003). Recently, the

new species *Lactobacillus versmoldensis* has been isolated from German, quick-ripened, salami-style sausages (Krockel et al., 2003).

Pediococci are less frequently isolated from European fermented sausages but occasionally occur in small percentages (Santos et al., 1998; Papamanoli et al., 2003). They are more common in fermented sausages from the United States where they are deliberately added as starter cultures to accelerate acidification of the meat batter.

Enterococci are sometimes associated with fermented meat products, in particular artisan products from Southern Europe, where they increase during early fermentation stages and can be detected in the end-product at levels of 10^2-10^5 ufc/g (Rebecchi et al., 1998; Samelis et al., 1998; Aymerich et al., 2003; Franz et al., 2003; Papamanoli et al., 2003). They are ubiquitous in food processing establishments and their presence in the gastrointestinal tract of animals leads to a high potential for contamination of meat at the time of slaughter (Franz et al., 1999). Opinions about their significance vary, as they may enhance food flavour but also compromise safety if opportunistic pathogenic strains proliferate or antibiotic resistance is spread (Franz et al., 1999, 2001, 2003; Vancanneyt et al., 2002; Cocconcelli et al., 2003; De Vuyst et al., 2003).

Coagulase-negative staphylococci and kocuriae are GCC that participate in desirable reactions during ripening of dry fermented sausages, such as color stabilization, decomposition of peroxides, proteolysis, and lipolysis. They are poorly competitive in the presence of actively growing aciduric bacteria, often not growing more than one log cfu/g during ripening (Samelis et al., 1998). The non- pathogenic, coagulase-negative staphylococci are dominated by *Staphylococcus xylosus, Staphylococcus carnosus, and Staphylococcus saprophyticus*, but other species occur too (Papamanoli et al., 2002; Gardini et al., 2003; Blaiotta et al., 2004; Mauriello et al., 2004). In addition to staphylococci, *Kocuria varians*, formerly known *as Micrococcus varians*, or other kocuriae are sometimes isolated in small quantities from naturally *fermented sausage (*Coppola et al., 1997; Papamanoli et al., 2002; Gardini et al., 2003*).* Moulds, usually *Penicillium nalgiovense and Penicillium chrysogenum*, are used in mould-ripened sausages, particularly in Southern Europe (Lopez-Dıaz et al., 2001; Sunesen and Stahnke, 2003). A yeast population, dominated by *Debaryomyces hansenii*, may also be found on the sausage surface and originates from the house flora or is sometimes added as starter culture (Samelis et al., 1994b; Coppola et al., 2000; Encinas et al., 2000; Olesen and Stahnke, 2000).

1.15.1 The Role of Microorganisms in Meat Curing

Studies on fermented meat at the beginning of the 20th century showed that in traditional meat fermentation yeasts, moulds, lactic acid bacteria and staphylococci/micrococci are involved (Table 1.15).

Intensified studies lead to the introduction of starter cultures which enable to control the fermentation process in regard to achieving a high standard in sensory quality and hygiene as well as reduction of production time and costs. They also ensure to achieve the reduction of the added nitrate/nitrite to safe low limits. Yeasts affect flavor (Olesen and Stahnke, 2000), and may improve color through creation of anaerobic conditions, which are required for effective nitrate reduction. The species employed in starter culture are not or only weakly fermenting and also do not reduce nitrate (Nakase and Suzuki, 1985). Moulds grow on the surface, affect flavor (Sunesen and Stahnke, 2003).

Microorganisms play a crucial role in maintaining the global nitrogen cycle (Figure 1.15). The nitrate reducing reactions of the cycle take also place in meat fermentation. The most efficient nitrate reducing organisms are staphylococci and micrococci (Gøtterup et al., 2008), which affect also further important attributes of fermented meat. Lactic acid bacteria can also reduce nitrate and nitrite but their potential has not been used in commercial starter cultures. The occurrence of greyish/brownish discoloration and oxidative reactions causing off-flavour caused by accumulation of H2O2 during fermentation is prevented by their strong catalase activity of staphylococci and micrococci (Hammes and Knauf, 1994). The aroma is further positively affected by their lypolytic and free fatty acid degrading activities (Hammes and Hertel, 1998). Finally the formation of nitrite and further of RNIs contributes to hygienic safety.

Under anaerobic conditions staphylococci use nitrate as the final electron acceptor in nitrate respiration. This was shown for a set of strains of *Staphylococcus carnosus* and the closely relate *Staphylococcus piscifermentans* (Hartmann et al., 1995). It was observed that the growth yield of anaerobic cultures depended on the concentration of nitrate and was optimal at 20 mmol/L.

Some typical features need to be taken in consideration in determining the technological conditions prevailing during meat fermentation.

iii. as dissimilative nitrate reduction provides bacteria with energy and is inhibited by oxygen, it is essential that anaerobic conditions prevail during

fermentation;

iv. as nitrite is temporarily accumulated and will be reduced by staphylococci to the oxidation level of ammonia after nitrate has been used up, the primary curing agent NO originates mainly from chemical reactions. These are enabled by the use of lactic acid bacteria that provide acidic conditions and/or the use of reducing additives such as ascorbate or erythorbate.

The reactions that occur are the following:

- KNO3 potassium Nitrate → KNO2 nitrite (action of bacteria)
- KNO2 → HNO2 nitrous acid (in acid medium, pH 5.2 - 5.7)
- HNO2 → NO nitric oxide
- NO → myoglobin → nitrosomyoglobin (pink color)

Potassium Nitrate worked wonderfully at 4-8°C (40-46°F) which was fine as refrigeration was not very common yet. If the temperatures dropped below 4°C (40°F) the bacteria that was needed to force Nitrate into releasing nitrite would become lethargic and the curing would stop. Potassium Nitrate was a slow working agent and the meat for sausages had to be cured for 72-96 hours.

Lactic acid bacteria fulfill numerous tasks in meat fermentation. Of superior importance is the decrease of pH that results in acid taste, preservation, hygienic safety, texture and support of reddening. They also produce aroma and may exert additional effects such as reduction of nitrate and nitrite as well as destruction of peroxides. *Lactobacillus pentosus* forms ammonia from nitrite (Wolf et al., 1990) in a process named fermentative nitrate reduction or ammonification. In this type of reaction the electrons are not used for energy conservation in a respiratory chain but to regenerate reduction equivalents and thereby gaining additional energy. Nitrate reduction was also found in strains of *L. plantarum* and *Pediococcus pentosaceus*. Strains of *L. sakei* and *L. farciminis* exhibit nitrite reductase activity which is haeme independent and results in release of NO and N_2O as intermediates of the denitrification process. *L. sakei* exhibits catalase activity in the presence of a haeme source that is abundantly available in meat.

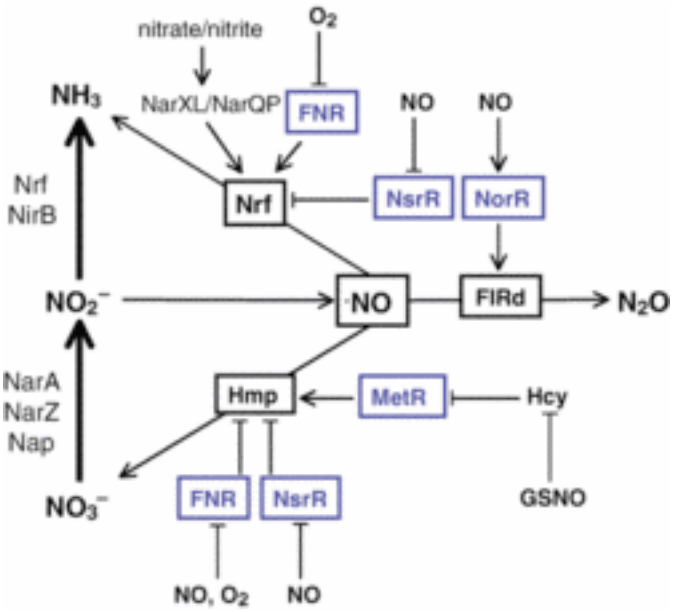


Fig. 1.15 Pathways for NO synthesis and consumption

Under anaerobic conditions, nitrate is reduced to nitrite by nitrate reductase (NarA, NarZ or Nap), and nitrite is reduced to ammonia by a respiratory or NADH-linked nitrite reductase (Nrf and NirB, respectively). Maximal rates of nitrate and nitrite reduction occur in cultures grown anaerobically in the presence of nitrate or nitrite (the regulatory mechanisms involved are not shown, but involve FNR and the two-component systems NarXL and NarPQ). Nitrite can be converted to NO by biological reduction (by nitrate and/or nitrite reductase) or by disproportionation. Under anaerobic conditions, NO is reduced to ammonia by Nrf, or to nitrous oxide by the flavorubredoxin (FlRd). In the presence of oxygen, NO is oxidized to nitrate by flavohaemoglobin (Hmp). The relevant regulators are shown along with their signals. Positive regulation is denoted by arrows, negative regulation by perpendicular lines. Hcy, homocysteine.

1.15.2 Lactic Acid Bacteria (Lab)

The primary contribution of LAB to flavor generation is ascribed to the production of large amounts of lactic acid and some acetic acid, although they also produce volatiles through fermentation of carbohydrates (Molly et al., 1996). They usually do not possess strong proteolytic or lipolytic properties, although a degree of peptidase and lipase activity has been observed for some meat strains.

Exopeptidases from meat lactobacilli contribute, in conjunction with muscle aminopeptidases, to the generation of free amino acids, contributing to flavour (Demeyer et al., 2000). LAB isolated from Greek sausages exhibited high in vitro leucine and valine aminopeptidase activities (Papamanoli et al., 2003). However, lactobacilli and pediococci display low catabolism of branched-chain amino acids, and hence do not play a major role in the formation of typical sausage aroma compounds such as 3-methyl butanal, as it is the case for staphylococci (Larrouture et al., 2000).

Little information is available about the lipolytic activity of lactobacilli during sausage fermentation, but some in vitro activity has been documented for *L. sakei, L. curvatus*, and *L. plantarum* (Hugas and Monfort, 1997; Lopes et al., 1999; Papamanoli et al., 2003). However, lipases from lactobacilli often display little or no activity under conditions found in fermented sausages (Kenneally et al., 1998a; Demeyer et al., 2000), although for some the production of lipase appears to be significant under conditions relevant for sausage ripening (Lopes et al., 1999).

In addition to lactobacilli, other LAB may be added to the sausage batter to influence flavour. The major contribution of LAB to flavour seems, however, to be limited to their carbohydrate catabolism, mainly the production of organic acids, whereas GCC appear to be more appropriate for the generation of specific aroma compounds.

Lactobacillus sakei, L. curvatus and L. plantarum are the species most widely isolated from acid-fermented meat products (Hammes et al., 1990; Hugas et al., 1993; Parente et al., 2001; Samelis et al., 1994; Schillinger and Lucke, 1987; Aymerich et al., 2003). They are responsible for lactic acid production, for the ‘‘tangy’’ flavor of sausages, and for the small amounts of acetic acid, ethanol, acetoin, carbon dioxide, and pyruvic acid that are produced during fermentation, depending on the starter applied, the carbohydrate substrate, and the sources of meat proteins and additives (Bacus, 1986; Demeyer, 1982; Thornill and Cogan, 1984).

In a study, (Rantsiou and Cocolin, 2005) natural fermentations were followed in three European countries with a long tradition in sausage production: Greece, Hungary and Italy. Differences in the LAB dynamics between the countries studied were noted and could be explained by considering the recipe used for the production as well as the fermentation conditions used in the first days. On a total number of 358 strains that were isolated, 324 were identified as *Lactobacillus spp*., representing the 90.5% of the entire population isolated and identified. Among them, three species, *L. plantarum, L.*

curvatus and L. sakei, counted for the 91.0% with a total number of isolates equal to 295. Twenty-seven strains of *L. plantarum*, 100 strains of *L. curvatus* and 168 strains of *L. sakei* were subjected to molecular characterization by RAPD-PCR and cluster analysis of the profiles. For almost all of the strains, grouping was dependent on the provenience. The majority of the clusters were formed by isolates coming from one specific country or mainly constituted by strains of only one country with a few others from one or both the other countries considered in the study. This evidence leads to the conclusion that there is a geographic distribution of LAB population among the three countries.

Another work from Bonomo et al., (2008) about the molecular characterization of the microflora present in a typical Italian sausage, showed that *L. sakei* was the predominant species (67%) followed by *Pediococcus pentosaceus* (16%), *Leuconostoc carnosum* (8%), *L. plantarum* (4%), *L. brevis* (2%) and *Leuconostoc pseudomesenteroides* (2%). The technological characterization revealed that most of the isolates had good acidifying and proteolytic properties. Moreover*, L. sakei* strains showed antimicrobial ability, while *Leuconostoc* strains the highest reduction of nitrates.

1.15.3 Micrococcaceae

Coagulase negative staphylococci (CNS) are commonly found as natural flora of fermented meat products, in relatively high number also in dry fermented sausages produced without addition of starter cultures (Miralles, Flores, & Perez-Martinez, 1996).

CNS play a major role in the development of sensory properties of fermented sausages, by reduction of nitrates to nitrite and then to nitrous oxide, by preventing rancidity through peroxide decomposition, and by proucing flavor and aroma compounds through proteolysis and lipolysis (Hammes & Hertel, 1998; Sondergaard & Stahnke, 2002).

Staphylococcus xylosus is the dominating CNS species in many Italian sausages (Cocolin, Manzano, Aggio, Cantoni, & Comi, 2001; Rossi, Tofalo, Torriani, & Suzzi, 2001) and in the Spanish sausage Chorizo (Garcıa-Varona, Santos, Jaime, & Rovira, 2000). *S. saprophyticus* and *S. carnosus* the dominating species in traditional Greek sausages (Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2002; Samelis, Metaxopoulos, Vlassi, & Pappa, 1998) and in Naples type salami (Coppola,

Mauriello, Aponte, Moschetti, & Villani, 2000). Many other CNS species (*S. haemolyticus, S. warneri, S. equorum, S. cohnii, S. epidermidis, S. hominis, S. capitis, S. intermedius*) have been reported to occur in one or more sausage types.

Staphylococcus species are commonly used as starters cultures for fermented sausages. Actually Italian law permits the use of *S. xylosus, S. carnosus and S. simulans* as starter for fermented sausage production (Repubblica Italiana, 1995) and knowledge of their technological properties is a matter of considerable interest. Nitrate reductase and catalase activities are considered to be the most important properties of staphylococci to be used as starter cultures for fermented sausages production (Weber, 1994). Mauriello et al. (2004) reported that all staphylococcal strains belonged to *S. xylosus, S. equorum and S. lentus* species were able to reduce nitrate to nitrite at 30 °C and a similar result was reported by Miralles et al. (1996) who found three strains of *S. xylosus* and one strain of *S. epidermidis* able to reduce nitrates to nitrites.

Coppola, Iorizzo, Sorrentino, Grazia (2004) studied the capability of staphylococcal strains to reduce nitrate in nitrate broth, at the temperature adopted during the ripening of the sausages: they found different strains belonging to the species of *S. xylosus*, *S. equorum* and *S. simulans* able to reduce nitrates to nitrite at 18 and 30 °C.

In relation to the proteolytic abilities, authors reported different results for CNS: it seems to be strain dependent and highly correlated to the processing conditions of every different sausage. Moreover the final proteolysis in fermented sausages is often a result of the enzymatic exogenous activity of microorganism and the endogenous activity of meat enzymes.

Staphylococci, in particular *S. xylosus* and *S. carnosus*, modulate the aroma through the conversion of amino acids (particularly the branched-chain amino acids leucine, isoleucine, and valine) and free fatty acids (Stahnke et al., 2002; Beck et al., 2004; Olesen et al., 2004; Tjener et al., 2004a,b). Aroma generation depends however on sausage technology and variety. For fast-ripened sausages, increasing inoculum levels of staphylococci may increase methyl-branched aldehyde production, whereas in slow-ripened sausages the situation is more complex (Tjener et al., 2004b). In the latter case, aroma production is particularly pronounced (Tjener et al., 2004a) and high inoculation levels favour the formation of methyl-branched acids and sulphites, whereas low levels favour diacetyl and ethyl ester production (Tjener et al., 2004b). It is thus possible to modify sausage aroma profiles by changing the inoculation level of

the Staphylococcus starter culture. In addition, additives such as nitrate, nitrite, or ascorbate, precultivation parameters, and environmental factors clearly influence the generation of aroma compounds (Olesen and Stahnke, 2003, 2004; Olesen et al., 2004).

The use of well-selected strains that generate high amounts of aroma components could permit to achieve improved sensory qualities and/or to accelerate the meat fermentation process. Selection of appropriate staphylococci in view of the application will be crucial. Strains of *S. xylosus*, for instance, predominate in Southern European salamis, which are characterized by a rounded aroma and a less acidic taste, and have been recommended when production of very aromatic sausage is intended (Samelis et al., 1998). The species has been shown to produce, amongst others, 3-methyl-1-butanol, diacetyl, 2-butanone, acetoin, benzaldehyde, acetophenone, and methyl-branched ketones (Stahnke, 1999a; Søndergaard and Stahnke, 2002).

The presence of an incomplete h-oxidation pathway in staphylococci explains the formation of methyl ketones (2- pentanone and 2-heptanone) as being derived from intermediates of this pathway (Montel et al., 1996; Stahnke, 1999a; Engelvin et al., 2000; Fadda et al., 2002a). h-Ketoacyl-CoA esters are deacylated into h-ketoacids by a thioesterase and then decarboxylated to the methyl ketone.

Besides contributing to flavour, GCC also prevent the formation of off-flavours and can be used to control the oxidation of unsaturated fatty acids, due to their nitrate reductase and antioxidant activities (Montel et al., 1998; Barrie`re et al., 2001a,b). In conclusion, selected *S. carnosus or S. xylosus* strains, with specific peptide uptake systems and branched- chain amino acid converting and fatty acid oxidising activities, could be used as functional starter cultures to obtain a tastier end-product. For instance, it has been shown that sausages with *S. carnosus 833* mature more than 2 weeks faster than control sausages (Stahnke et al., 2002). Maturity correlates significantly with higher amounts of branched-chain aldehydes and alcohols arising from the breakdown of branched-chain amino acids, and branched- and straight-chain methyl ketones in turn derived from microbial h-oxidation of fatty acids (Stahnke et al., 2002).

1.15.4 Molds and Yeasts

Moulded sausages are very common in the Mediterranean area. It has been shown that the superficial inoculation of the sausage with atoxigenic moulds, e.g. *Penicillium* or

Mucor species, contributes to sensory quality (Bruna et al., 2000, 2001, 2003; Garcıa et al., 2001). This contribution is mediated by lactate oxidation, proteolysis, degradation of amino acids, lipolysis, lipoxidation, the delay of rancidity, and reduced water loss due to slower evaporation (Sunesen and Stahnke, 2003; Benito et al., 2004; Sunesen et al., 2004). Moreover, molds contribute to the overall attractiveness of the end-product due to their characteristic white or greyish appearance, to the stabilization of color through catalase activity, oxygen consumption and protection against light, and to easy skin peeling. A characteristic popcorn odor in mold-fermented sausages has been ascribed to 2-acetyl-1-pyrroline, which may be caused by conversion of proline, often found in sausage collagen casings, by the molds (Sunesen and Stahnke, 2003). However, as with bacterial starter cultures, the selection of mold starter strains should be done carefully since the proteolytic and lipolytic capabilities, and hence the effect on the end-product, can significantly differ between strains and depend on the applied technology (Selgas et al., 1995, 1999; Sunesen and Stahnke, 2003).

The involvement of yeasts in dried fermented sausage ripening has been widely recognized over several decades (Encinas, Lopez-Diaz, Garcia-Lopez, Otero and Moreno, 2000; Flores, Toldrà 2004) Gardini et al., 2001; Geisen, Lucke, & Krockel, 1992; Hammes & Knauf 1994;). Many yeasts isolated from dry-fermented sausages are lipolytic and therefore can attack the fatty tissue and contribute to the development of taste and flavor (Geisen et al., 1992). Moreover, some authors (Papon et al., 1990) have demonstrated that dry-fermented sausages inoculated with yeasts have a greater lipolysis resulting in a stronger odor. However, the role of yeasts in sausage flavour formation is not sufficiently well characterized. In model sausage minces, Olesen and Stahnke (2000) observed that *Candida utilis* was able to produce several volatile compounds, in particular esters and alcohols, many of which were probably derived from branched-chain amino acids, whereas *Debaryomyces hansenii* had very little effect on the production of volatile compounds. In other studies, *Debaryomyces spp.* affected proteolysis (Durà, Flores and Toldra , 2004). Flores et al. (2004) showed that an appropriate inoculum level of *Debaryomyces* spp. influenced volatile production by inhibiting lipid oxidation due to its antioxidant effect and by promoting the generation of ethyl esters. However, large amounts of *Debaryomyces spp*. resulted in high quantities of acids (e.g. 2-methyl-propanoic and 2- and 3-methyl-butanoic acid) that masked the positive effect.

Studies carried out with different yeast species (mainly *Debaryomyces hansenii* and its imperfect form *Candida famata*) have shown that they can positively contribute to the stabilization of the reddening reaction (by removing oxygen) and the development of a characteristic yeast flavor, due to their ability to degrade peroxides, lipolytic and, to a lesser extent, proteolytic activities (Olesen & Stahnke, 2000). Moreover, yeasts have been reported to increase the ammonium content and reduce the amounts of lactic and acetic acids, with the concomitant diminution of the acid taste (Dura´ et al., 2004; Gehlen, Meisel, Fischer, & Hammes, 1991). The protection of sausages from the adverse effects of light is also attributed to this microbial group (Lucke & Hechelmann, 1987). However, the contribution of yeasts to fermented sausage characteristics is affected by the presence of spices and the species of starter cultures used (Flores et al., 2004). The yeast microbiota mainly found in sausages belongs to the genera *Debaryomyces, Rhodotorula, Hansenula* (synonym of *Pichia)* and *Torulopsis* (synonym of *Candida*) (Comi & Cantoni, 1980; Gardini et al., 2001). Studies on salami indicate *D. hansenii* to be the most commonly isolated yeast species. Due to its occurrence in the traditional products and its positive effects on flavour and color, *D. hansenii* is used in starter preparations and should be added to sausage mixtures at a concentration of 6.0 log cfu/g (Hammes & Knauf, 1994). However, Dura´ et al. (2004) stressed the necessity to use yeasts in combination with microorganisms having nitrate reductase activity to counteract the color defect attributable to their inhibition of the indigenous staphylococci.

1.15.4.1 The ‘Non-Conventional’’ Yeast *Yarrowia Lipolytica*

Yarrowia lipolytica is one of the most extensively studied ‘‘non-conventional’’ yeasts, being a strictly aerobic microorganism capable of producing important metabolites and having an intense secretory activity, which justifies efforts to use it in industry (as a biocatalyst), in molecular biology and in genetics studies. It is considered as nonpathogenic and several processes based on this organism were classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA).

Y. lipolytica has been considered an adequate model for dimorphism studies in yeasts, since it has an efficient system for genetic engineering transformation, and is easy to distinguish between its morphological forms, in contrast to *Saccharomyces cerevisiae,* which does not produce true filaments and exhibits pseudo-hyphae growth under

nitrogen-limited conditions. The yeast-to-mycelium transition is associated with unipolar growth, asymmetric division, large polarly located vacuoles and repression of cell separation after division. It is believed that yeast dimorphism is related to a defense mechanism to adverse conditions, such as temperature and nutritional changes. (Coelho et al, 2011).

One of the most important products secreted by this microorganism is lipase, which is an enzyme that attracts the interest of scientists and industrial researchers because it can be exploited for several applications in the detergent, food, pharmaceutical, and environmental industries. Being strictly aerobic yeast, its growth and metabolite secretion are affected by the amount of oxygen available in the culture medium. (Coelho et al, 2011).

Another ability of *Yarrowia lipolytica* strains is to grow on Olive Mill Wastewater (OMW) based medium and produce high-value compounds. In fact, this yeast has been used for bioremediation applications due to its cell wall characteristics and surfactant production. (Lanciotti *et al.*, 2005).

Y. lipolytica is unique strictly aerobic yeast with the ability to degrade efficiently hydrophobic substrates such as n- alkanes, fatty acids, fats and oils for which it has specific metabolic pathways (Fickers *et al.*, 2005) The genome sequence of the fungus has revealed that the organism is distantly related to the conventional yeast *Saccharomyces cerevisiae*.

Rodrigues and Pais have shown that *Y. lipolytica* is capable to use acetic, lactic, propionic, malic, succinic, citric and oleic acids as the sole carbon and energy source, this capacity being, in most cases, independent of the pH of the culture media. Diauxic growth was observed when the yeast was grown in glucose and citric or lactic acid suggesting that the utilization of these two acids is subjected to glucose repression. Propionic, butyric and sorbic acids also had inhibitory effects on yeast growth.

Most strains of *Y. lipolytica* grow very efficiently on acetate as sole carbon source. Concentrations up to 0.4% sodium acetate are well tolerated, higher concentrations reduce the growth rate and, concentrations above 1.0% inhibit the growth (Barth et al, 2007).

Y. lipolytica uses ethanol as carbon source at concentrations up to 3%. Higher concentrations of ethanol are toxic. Several NAD+- and NADP+-dependent alcohol dehydrogenases were observed in *Y. lipolytica*. (Barth *et al*, 2007).

The yeast *Yarrowia lipolytica*, the perfect form of *C. lipolytica*, has also frequently been isolated from fresh beef and sausages (Gardini et al., 2001). Due to its lipolytic and proteolytic activities, this species can have a high technological potential. In fact, its use as co-starter for the production of some cheese varieties has already been proposed by several authors (van den Tempel & Jakobsen, 2000). However, these authors stressed the need for an accurate strain selection based on the characteristics it is desired to impart to the products, the formulation, the production flow sheet adopted and the ripening conditions. In fact, it is well established that the microbial growth and/or the enzymatic activities can be completely inhibited by specific combinations of physico-chemical and environmental factors. Sorensen (1997) presented evidence that the lipolytic activity of several *D. hansenii* strains can be completely repressed at the pH and temperatures normally used in salami ripening. The wide intra-species variability of *D. hansenii* and *Y. lipolytica* in the lipolytic and proteolytic patterns of milk fat and proteins is well documented (Guerzoni et al., 2001; van den Tempel & Jakobsen, 2000). However, these authors presented evidence that the physico-chemical environment and composition of the system can generate more pronounced differences in the expression and activity of specific enzymes of these species than those existing among different strains of the same species.

In a recent study, Patrignani showed that the manufacture of dried fermented sausages using *D. hansenii* and *Y. lipolytica* strains did not affect the pH evolution, while positively influencing the aw decrease of the samples during ripening. Moreover the sausages made by inoculation with selected yeast strains showed more marked and earlier aw reductions; they showed at the end of ripening more pronounced proteolysis and lipolysis. In particular, the use of *D. hansenii* induced an earlier degradation of meat proteins. However, this strain showed less lipolytic potential than *Y. lipolytica* strains. The lipolytic patterns of the products were affected not only by the yeast strain but also by the degree of mincing of the meat mixture. However, important qualitative and quantitative differences in the content of FFA, that can be attributed to the yeast strain activities, were detected in the final sausages and therefore will have a high impact on the final sensory properties of the products. These results demonstrate the necessity to accurately evaluate the technological characteristics of yeast strains to be used as starters in relation to the type of sausage, the ripening time and conditions (Patrignani et al, 2006).

Martin et al investigated about the proteolytic activity of *Penicillium chrysogenum*

during ripening of meat. He showed that inoculation of meat products with *P. chrysogenum* Pg222 promoted hydrolysis of myofibrillar proteins increasing the concentrations of peptides and most amino acids. The increase in free amino acids observed in the batches inoculated with *P. chrysogenum* Pg222 may have a strong influence on the flavour of the ripened product, not only by the direct effect of the amino acids on flavour, but also through the volatile compounds that can be formed from them. This can be very important for meat products such as dry cured ham, where ripening requires several months (Cordoba et al., 1994b). Moreover this effect could contribute to shortening ripening time and improving flavor of meat products of low surface/volume ratio that require an extremely long ripening time.

Table 1.15: Species involved in meat starter cultures (Hammes et al, 2003).

Bacteria

Lactic Acid Bacteria

*Lactobacillus acidophilus,*a *Lb. alimentarius,*b *Lb. paracasei*,a *Lb. rhamnosus*, *Lb. curvatus, Lb. plantarum, Lb. pentosus, Lb. sakei, Lactococcus lactis, Pediococcus acidilactici, P. Pentosaceus*

Actinobacteria

Kocuria varians,c *Streptomyces griseus, Bifidobacterium spp*.a

Staphylococci

Staphylococcus xylosus, S. carnosus ssp. *carnosus, S. carnosus* ssp. *utilis, S. Equorum*

Halomonadaceae

*Halomonas elongata*b

Fungi

Penicillium nalgiovense, P. chrysogenum, P. camemberti

Yeasts

Debaryomyces hansenii, Candida famata

1.16 The Genus *Lactobacillus*

Lactobacilli are Gram-positive, non-spore-forming microorganisms. Considering cellular shape, they can occur as rods or coccobacilli. They are fermentative, microaerophylic and chemo-organotrophic, requiring rich media to grow. They are catalase negative, even if pseudocatalase activity can sometimes be present in some strains. Considering DNA base composition of the genome, they usually show a GC content of lower than 54 mol% (Felis and Dellaglio, 2005).

They are almost ubiquitous: they are found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), respiratory, GI and genital tracts of humans and animals, and in sewage and plant material.

According to *Taxonomic Outline of the Prokaryotes* (Release 5.0, Garrity *et al*., 2004), the genus *Lactobacillus* belongs to the phylum *Firmicutes,* class *Bacilli,* order *Lactobacillales*, family *Lactobacillaceae* and its closest relatives, being grouped within the same family, are the genera *Paralactobacillus* and *Pediococcus*.

The main discrepancy in the taxonomy of the genus *Lactobacillus* is the non-correlation between phylogenetic placement and metabolic properties. The historical subdivisions of the genus *Lactobacillus* based on the type of fermentation have been excellently reviewed by Pot *et al*. (1994), who have underlined how terms such as 'homofermentative', 'heterofermentative', 'obligately homofermentative', 'facultatively heterofermentative' and 'obligately heterofermentative' have been given different meanings by different authors and may be misleading. The accepted 'modern' definition is that given by Hammes and Vogel (1995): obligately homofermentative lactobacilli are able to ferment hexoses almost exclusively to lactic acid by the Embden–Meyerhof–Parnas (EMP) pathway while pentoses and gluconate are not fermented as they lack phosphoketolase; facultatively heterofermentative lactobacilli degrade hexoses to lactic acid by the EMP pathway and are also able to degrade pentoses and often gluconate as they possess both aldolase and phosphoketolase; finally, obligately heterofermentative degrade hexoses by the phosphogluconate pathway producing lactate, ethanol or acetic acid and carbon dioxide; moreover, pentoses are fermented by this pathway.

1.16.1 *Lactobacillus sakei*

Lactobacillus sakei is a Gram-positive anaerobic bacterium commonly found living on fresh meat and fish. This bacterium is valuable in the fermentation of meat products and exhibits properties that allow for better preservation and storage of fresh meats and fish. It is the predominant bacteria used for meat fermentation in Europe, whereas *Pediococcus pentosaceus* tends to be widely used in the United States. *Lactobacillus sakei* took its name from rice alcohol, or sake, which was the product that it was first described in. Sequencing *Lactobacillus sakei*'s genome was important in determining how this bacterium is so well adapted to meat. A team of INRA (Institut National de La Recherche Agronomique) researchers was able to determine its genome and found that its effectiveness in fermentation and food storage is indicative of its ability to sustain life even under challenging environmental conditions, its ability to produce toxics to kill other bacteria, and its capability to use nutrients in meat for self growth. The entire genome of *Lactobacillus sakei* strain 23K was determined to be a circular chromosome containing 1,884,661 base pairs. It consisted of 1,883 protein coding genes, seven rRNA gene clusters, and had a G + C content of 41.25%, (Chaillou, 2005). The presence of "one prophage remnant and 12 complete insertion sequences (IS) mostly localized in two diametrically opposed A+ T-rich regions, suggesting hotspots for genome evolution," was also found. Although raw meat provides *Lactobacillus sakei* nutrients for growth, it contains limited amounts of carbohydrates. Out of the few sugars found in meat and raw fish, *Lactobacillus sakei* can utilize only glucose and ribose (Stentz, 2001). It is no surprise that upon examination of its genome, very small transport systems are present for sugar uptake Because sugars are rapidly exhausted in meat, *Lactobacillus sakei* is also able to catabolize nucleosides such as inosine and adenosine for energy source (Chaillou, 2005). *Lactobacillus sakei* interacts with other bacteria present on meat products. Among them are the pathogenic bacteria such as *Escherichia coli* and *Listeria monocytogenes* those can be very dangerous to humans. Other bacteria include food-spoiling bacteria like *Psuedomonas fragi* and *Brochothrix thermosphacta* that do not necessarily pose danger to health, however, could damage meat products (Chaillou, 2005). *Lactobacillus sakei* has the ability to produce ribosomally-synthesized antimicrobial peptides called bacteriocins that inhibit growth of some of these bacteria. For instance, it was found in a research study that the bacteriocin-positive strain of *Lactobacillus sakei* was able to obstruct the growth of

Listeria monocytogenes in rainbow trout fillets in specific environmental conditions, whereas bacteriocin-negative strain of *Lactobacillus sakei* provided no inhibition (Husar, 2004). *Lactobacillus sakei* is able to produce bacteriocin called sakacin P that inhibits growth of several pathogenic and food-spoiling bacteria present in meat and fish products. It is no wonder that *Lactobacillus sakei* is widely used for meat fermentation. Several research studies have been performed on *Lactobacillus sakei* to further determine optimal environmental conditions, as well as its capacity to ferment meat. *Lactobacillus sakei* is able to grow under both anaerobic and aerobic conditions. With this characteristic, a research study was performed to determine the proteins and genes involved when *Lactobacillus sakei* is growing anerobically. Using two-dimensional electrophoresis, they found that the protein that is over-expressed during anaerobic growth was a peptidase and its corresponding gene is pepR. After performing several experiments on this gene, they found that pepR was indeed responsible for the *Lactobacillus sakei*'s ability to live in an anaerobic environment (Champomier-Vergès, 2002). In another study, it was found that four genes of *Lactobacillus sakei* namely ctsR, asnA2, LSA1065, and LSA1194 were involved during fermentation of raw sausage (Hufner, 2007).

1.16.2 *Lactobacillus casei*

Lactobacillus casei is one of the many species of bacteria belonging in the genus *Lactobacillus*. It is a mesophilic bacteria that is gram positive, rod shaped, nonsporing, nonmotile, anaerobic, and contains no cytochromes. *L. casei* can be found in various environments such as raw and fermented dairy products, intestinal tracts and reproductive systems of humans and animals, and fresh and fermented plant products (Holzapfel, 2001). The optimum pH for *L. casei* is 5.5. The lactic acid produced by *L. casei* through fermentation is very important since it can be used to make cheeses and yogurts, reduce cholesterol levels, enhance immune response, control diarrhea, alleviate lactose intolerance, inhibit intestinal pathogens, and serve as probiotics (Mishra, 2005). Probiotics are viable microorganisms that promote or support a beneficial balance of microbes to live in the gastrointestinal tract (Holzapfel, 2001). There are many strains/isolates of *L.casei* from different origins and geographical locations. That is why molecular typing of *L.casei* is crucial to understanding the evolutionary adaptation of this species to different ecological niches (Cai et al, 2001). Another reason for having *L. casei's* genome sequenced is to

determine the phylogenetic relationships between various groups of bacteria in *Lactobacillus*. *L. casei, L. paracasei, L. rhamnosus*, and *L. zeae* form a closely related taxonomic group within *Lactobacillus*. Sometimes the classification *L. casei* is cloosely applied to strains of any of these species by commercial companies. By having the genome sequenced, species boundaries could be drawn and names can be attached to those species (Desai, 2006). The genome of *Lactobacillus casei* strain ATCC 334 is composed of one circular chromosome and one plasmid. The chromosome has 2.9 million base pairs and the plasmid has 0.029 million base pairs. The chromosome encodes for 2,751 proteins and the sequencing was completed at the US DOE Joint Genome Institute and The Lactic Acid Bacteria Genome Consortium and Fidelity Systems, Inc. Currently, the genome of the plasmid of *Lactobacillus casei* is being sequenced. One of *L. casei's* qualities is its ability to live in various diverse ecological niches. Research through comparative genomic analyses has suggested that extensive gene loss and gene acquisitions during the evolution of lactobacilli, presumably via bacteriophage or conjugation-mediated horizontal gene transfers have facilitated *L. casei's* adaptation to diverse ecological niches (Desai, 2006). The study used 40 different strains of *L. casei* showing that there is a high degree of recombination and phylogenetic diversity among the species. Another feature in the genome of *L.casei* is the *csp-A* gene. This gene codes for a cold shock protein Csp A (66 amino acid residues) which allows the bacteria to adapt to low temperatures (Sauvageot et al, 2006). It is a facultatively anaerobic organism that gets its energy through fermentation. Most *L. casei* strains can ferment galactose, glucose, fructose, mannose, mannitol, N-acetylglucosamine, and tagatose (Cai et al, 2001). The ability to ferment lactose is less common in strains isolated from plant materials than in those from cheese and human gastrointestinal tracts . The conditions of fermentation such as temperature, pH, the type of growth media, oxygen, and some neutralizers also play a role in the growth activity of *L. casei* (Ha et al, 2003). The most important compound that *L. casei* produces is lactic acid. It is obtained by fermenting glucose and lactate formation. Lactic acid is a hydroxy acid that can be produced chemically from acetaldehyde and hydrogen cyanide or by microbial fermentation. It is used for numerous industrial processes such as chemical and biological production of organic acids, the use as a flavoring in food, the manufacturing of cosmetics, and the production of biodegradable plastics. *Lactobacillus casei* has the ability to adapt to a variety of ecological niches. One of

these niches is the gastrointestinal tract. *L. casei* functions as a probiotic in the gastrointestinal tract. Probiotics are originally defined as microorganisms promoting the growth of other microorganisms (Holzapfel et al, 2001). The characteristics of a successful probiotic are acid and bile tolerance, antimicrobial activity against intestinal pathogens, and ability to adhere and colonize the intestinal tract (Mishra et al, 2005). In order for the probiotics to carry out their functions, the probiotic live cells must not be lower than $10^6/10^7$ cfu/g (Nebesny, 2007). The strains of *L. casei* that live in the intestines are sensitive to the intestinal conditions by having high bile salt concentrations and have the permeabilization and release of intracellular lactase to produce lactic acid (Holzapfel et al, 2001). *L. casei* is very important in regulating the immune system of the gastrointestinal tract. *L. casei* will bind to the luminal surface of gastrointestinal cells and stimulate gut-associated lymphoid tissue . This will strengthen the innate immune response and give local and systemic immunity to the body. To fight off the pathogens that may invade the immune system, *L. casei* can compete for nutrients or adhesion site against the pathogens. They can also inhibit the growth of pathogenic bacteria by a pH reduction through the production of organic acids such as acetic, propionic, or lactic acid, or by producing hydrogen peroxide (Millette et al, 2007). Furthermore, *L. casei* can secrete bacteriocins, antimicrobial peptides of cationic, amphiphilic molecules, to get rid of the pathogens in the body. Another interesting characteristic of *L. casei* is its ability to adapt to colder temperatures, cold shock response. Research has shown that cold shock can cause a sudden growth stop or significantly reduced growth rate by decreasing membrane fluidity, and arrest or decrease of the synthesis of most housekeeping proteins. The cold shock would turn on the *csp-A* gene to make cold shock proteins (CSP A) to help the cell adjust to its colder environment. The research has also shown that CSP is needed not only for cold shock response, but for optimal growth in normal, unstressed cells (Sauvageot, 2006). *L. casei* is generally considered nonpathogenic and safe. However, cases of sepsis, meningitis, and infections localized in organs have been reported (Salvatore, 2007). *Lactobacillus casei* produces lactic acid which is used in various applications in biotechnology since it has numerous beneficial effects such as an increase in immune system response, a decreased risk for bladder cancer, and reduced cholesterol levels. Most of the biotechnology applications are related to the food industry.

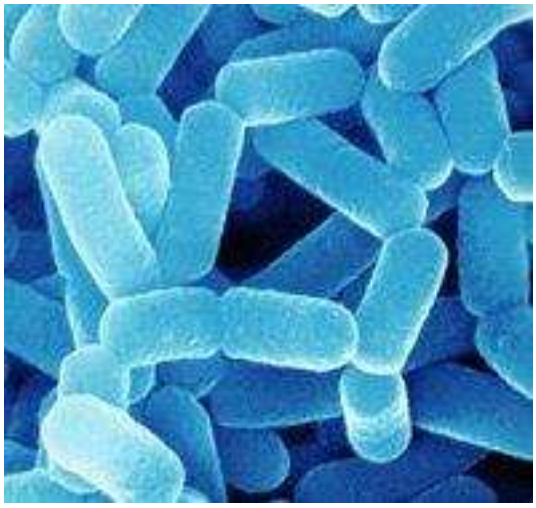


Figure 2.1.2 : *L.casei*

1.16.3 *Lactobacillus rhamnosus*

Lactobacillus rhamnosus is a bacterium that was originally considered to be a subspecies of *L. casei*, but later genetic research found it to be a species of its own. Some strains of *L. rhamnosus* are being used as probiotics.

Probiotics, as defined by the Food and Agricultural Organization of the United Nations, are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." *L. rhamnosus* was first isolated in 1983 in the intestines of a healthy human subject by scientists Barry Goldin and Sherwood Gorbach, when it was shown to have remarkable tolerance for the harsh acids normally found in the stomach and digestive tract. The "GG" in the title of the strain *L. rhamnosus* GG is derived from the last names of the two scientists. Like other probiotics, *L. rhamnosus* has properties that are beneficial to the intestinal tract. It is also believed to be of considerable assistance with the immune system, particularly in combating intestinal and urinary tract pathogens. It is also used as a natural preservative in yogurt-based products, where the bacterium attaches to the lining of the intestines, where it encourages the growth of helpful organisms that aid in digestion.

Figure 2.1.3: *Lactobacillus rhamnosus*

Lactobacillus rhamnosus is a probiotic bacterium that helps eliminate and prevent the growth of harmful bacteria in the intestines. Many consumers may be familiar with lactobacillus probiotics, which are touted today by some yogurt manufacturers as an aid in digestion and in promoting regular bowel activity. In fact, lactobacilli have been used for centuries to aid in the fermentation of dairy products. During the 20th century, researchers began evaluating these organisms and their positive effects on the human body and its ability to naturally ward off disease and infection. The *Lactobacillus rhamnosus* bacterium was first isolated by researchers in 1983, when it was shown to have remarkable tolerance for the harsh acids normally found in the stomach and digestive tract (Conway et al, 1987). The following are some of the well noted benefits of *L. rhamnosus:*

vi. Helps Fight Intestinal Tract Illnesses: according to studies published by Goldin and Gorbach, *L. rhamnosus* is said to be able to survive the highly acidic conditions of the human stomach, as well as the intestinal tract. It is also believed to be bile-stable (Conway et al, 1987). This makes the probiotic highly desirable in its ability to conquer intestinal ailments.

vii. Suppresses Bacterial Infections in Renal Patients: in 2005, it was demonstrated that with patients experiencing kidney-related illnesses, *L. rhamnosus* is capable of interrupting the gastrointestinal transportation of the variety of enterococcus that is resistant to the antibiotic vancomycin.

viii. Assists in Prevention of Urinary Tract Infections: according to an article published in the November 2009 issue of Renal and Urology News, daily ingestion of *L. rhamnosus* Gr-1 may be effective in helping postmenopausal women who suffer from chronic urinary tract infections. While dosage of trimethoprim-sulfamethoxazole is considered to be a standard treatment for a

UTI, *L. rhamnosus* is a viable alternative when antibiotic resistance is a consideration (Schieszer, 2009). The probiotic seems to be capable of safeguarding the urogenital tract by its ability to excrete biosurfactants. This enables the tract to limit the adhesion of pathogens.

ix. Helps Build a Superior Immune System: while blood cells are certainly a major agent in managing the body's immune system, the gut is also a huge contributor in this area. Because of the ability of *L. rhamnosus* to survive in extremely acidic environments such as the digestive system, the probiotic can thrive in the gut. It stimulates the production of antibodies and also assists in the process of phagocytosis, a means by which the body combats dangerous invasive bacteria.

x. Decreases Duration of Diarrhea: research conducted in 2000 in several European countries indicated that the administration of *L. rhamnosus* GG to children suffering from rotavirus shortened the duration by at least one day of the pervasive diarrhea associated with the illness. (Guandalini et al., 2006) Another study showed that ingestion of *L. rhamnosus* GG was helpful in reducing the extent of diarrhea when it exists as a side effect of antibiotic use to combat *H. pylori* infections.

Lactobacillus rhamnosus is one of the most widely studied probiotics, noted and valued for its ability to survive and even thrive in the harsh conditions of the digestive and urinary tracts. Multiple clinical trials have determined the bacterium to be especially beneficial in promoting and maintaining digestive tract health. It is extremely well tolerated by men and women, and has been associated with only very rare side effects. Studies have shown that, taken regularly, *L. rhamnosus* can be an effective supplement in promoting and maintaining digestive tract health.

1.16.4 *Lactobacillus plantarum*

L. plantarum is a gram positive bacterium that is found in a variety of niches. These niches include dairy, meat, and much vegetable fermentations, it is also found in the human gastrointestinal tract. It is a facultative heterofermentative lactic acid bacterium that utilizes an extensive range of fermentatable carbon sources. Lactic acid bacteria are Gram-positive and they are non-spore forming, fermentative bacteria that grow anaerobically. The main function of these bacteria is the fermentative

conversion of sugars present in raw materials into lactic acid. *L. plantarum* also produces anti-microbial peptides and exopolysaccharides. It has the ability to maintain a pH gradient between the inside and outside of the cell in the presence of large amounts of acetate or lactate. (De Vries et al, 2006). *L. plantarum* is one of the most common microbes used as a silage inoculant. Silage is a fermented fodder that can be fed to ruminants or used as a biofuel feedstock for anaerobic digesters. (Giraud et al, 1994). *L. plantarum* has one of the largest genomes among lactic acid bacteria. In its circular chromosome it contains 3,308,274 base pairs. The genome was sequenced by using whole genome sequencing as assembly approach. The overall GC content of its chromosome is 44.5%, the plasmids tend to have a lower percent GC content. Putative biological functions have been given to 2,120 of the predicted proteins. One particular interesting region of the chromosome is the 213-kb region from 3,072,500 – 3,28,500, which encodes proteins for sugar transport, metabolism, and regulation. This region has a lower percent CG content (41.5%), leading researchers to believe that horizontal gene transfer has acquired many of these genes (Kleerebezem et al, 2007). *L. plantarum* has three plasmids, pWCFS101, pWCFS102, and pWCFS103. The plasmid sizes are as follows: pWCFS101 contains 1,917 bp, pWCFS102 contains 2,365 bp, and pWCFS103 contains 36,069 bp. Plasmid pWCFS101 is believed to contain replication proteins. Plasmid pWCFS102 is believed to contain replication proteins as well as proteins that function as copy number controls. Plasmid pWCFS103 contains genes that are predicted to be involved in arsenate and/or arsenite resistance as well as cadmium resistance; it also has genes that are believed to encode replication proteins, resolvases, DNA-damage-inducible proteins, and oxidases. *L. plantarum* contains two apparently complete prophage genomes, as well as some prophage remnants (Kranenburg et al, 2005). The *L. plantarum* chromosome reveals that this microbe has a major focus on carbon catabolism. The sequence of its chromosome also supports its extreme flexibility, versatility, and ability to adapt to different environmental conditions (Kleerebezem et al, 2007). *L. plantarum* has a rod shaped structure with rounded ends. This microbe is a gram positive bacteria meaning there is a high concentration of peptidoglycan in the cell wall, and lack an outer cellular membrane. The organism is also Auxotrophic meaning that it synthesizes few organic compounds, when it has the ability to break down sugars and pyruvate. It is also a facultative heterofermentative lactobacilli microorganism, this means that the organism takes carbon from sugars and pyruvate

and the byproduct is either alcohol or lactic acid. This process happens in an aerotolerant environment meaning that oxygen is not present. When oxygen is present it is released as H_2O_2 which can be used as a weapon that kills off other bacteria. Do to the inability to handle oxygen the organism uses a manganese dependent process. This process uses metal as a pseudo catalase and lowers oxygen concentration that is favorable to the aero tolerant environment. Sugar is a key source of energy for the microorganism to degrade (Dudley et al, 2001). During the degradation of sugar carbon is released and becomes a source of energy for the *L. plantarum*. When it is exhibiting a pyruvate metabolism it is similar to homolactic fermentation. This happens when growth occurs on glucose that is degraded to pyruvate though an EMP pathway. Once the pyruvate is formed it is converted to d and l-lactate though sterospecific lactate dehydrogenase enzymes (Dudley et al, 2001). Researchers believe that the sequence of the *L. plantarum* genome has certain features that allow this microbe to be versatile and adaptive to different environments (Kleerebezem et al, 2007). It can grow in-between 15-45 °C and can grow at pH levels of 3.2 and greater. This versatility allows *L. plantarum* isolates to be found in human saliva, fermenting dairy products, plant material, silage, and even certain waste waters. It gains its energy through the fermentative conversion of sugars to lactic acid, as long as is able to go through this process, most environments will allow the growth of this microbe. Experts believe that the high number of regulatory genes causes this microorganism to be so adaptable. The most common habitat is in a protein enriched environment such as dairy because of its primary protein-degradation which produces peptides. A study showed that there are 144 N-terminals that can be used for peptidase cleavage. Another key part to describe the adaptability of this microorganism is its ability to perform horizontal gene transfer. This process is accomplished though natural competition, bacteriophage infection and more. *L. plantarum* can perform these transformations because it can bind DNA and uptake that DNA (Kleerebezem et al, 2007). Recently *L. plantarum* has been identified as a probiotic. Probiotics are non-pathogenic microorganisms that can have a positive impact on human health when they are digested. They are becoming a very popular dietary supplement to many people, especially those who have gastrointestinal problems (Adrian et al, 2008). In this case, *L. plantarum* can be considered a human symbiotic. When the probiotics are ingested regularly it is possible that the composition of microflora in the intestinal tract can be manipulated. This

manipulation may allow an improvement of microbe balance, stabilization of digestive enzyme patterns, and immunomodulation by activating and regulating mucosa-associated and systemic immune system responses. The microflora found in the intestinal tract are thought to provide protection from pathogens. Some companies currently sell bottles containing *L. plantarum* as a probiotic to help with intestinal problems including IBS and IBD, stating that these bacteria help to "balance the intestinal ecosystem". Along with its possible use as a probiotic, there is another very important use for *L. plantarum,* in fact it is currently being explored to convert lignocellulosic biomass to biofuel and bioproducts. Current research into this idea is looking at a strain of *L. plantarum* which has certain genes inactivated to eliminate undesirable fermentation products (Liu et al, 2006). It is also able to degrade cassava raw starch. Its ability to degrade raw starch is useful because it could potentially be used as a starter in certain traditional fermentation processes. There are also potential uses for *L. plantarum* to be used in treatments of certain wastewater due to its ability to degrade phenolic compounds, such as those in olive mill wastewaters (Bronze et a, 2008). The ability of the this microbe to adapt and thrive in a range of environments, its ability and capacity to be genetically manipulated, as well as its ability to ferment and degrade different materials makes *L. plantarum* a very interesting and important bacteria to study.

1.16.5 *Lactobacillus reuteri*

Lactobacillus reuteri is a Gram-positive, rod-shaped, and anaerobic. This heterofermentatic lactic acid bacterium naturally inhabits the gut of a wide range of organisms, including humans, pigs, chickens and mice (Morita et al, 2008). It can also be isolated from human breast milk. In vitro, *Lactobacillus reuteri* grows optimally on MRS media at 37°C (Morita et al, 2008). They have also been found to grow in biofilms.

L. reuteri produces reuterin, an antimicrobial that inhibits growth of harmful bacteria, fungi, and protozoa. Due to these probiotic properties, *L. reuteri* is believed to be a promising therapy for the alleviation and reduction of certain illnesses related to gastrointestinal health, oral health, and urogenital health, including infantile colic, eczema, and H. pylori infection.

The genome of the strain JCM1112 was fully sequenced at Kitasato Institute for Life

Sciences by April 21, 2008. The genome contains one circular chromosome and does not contain any plasmids. The entire genome is 2,039,414 nucleotides long, with a GC content of 38%. The genome contains 1901 genes, 83% of which are protein coding. The chromosome contains 1,820 open reading frames (ORFs), 53 of which are phage related. *L. reuteri* is a Gram-positive rod that forms chain arrangements and does not produce endospores. Some strains have also been shown to form relatively thin biofilms (5-7µm thick.) (Jones et al, 2009). *L. reuteri* is a mutualistic host-associated microbe, living in the guts of animals. As such, it requires a host habitat and is a mesophilic, facultative anaerobe with a preference for acidic environments. It is an obligate heterofermentative microbe, producing carbon dioxide, ethanol, acetate, and lactic acid from glucose fermentation. It can also anaerobically metabolize glycerol, producing the antimicrobial reuterin (3-hydroxypropionaldehyde) (Morita et al, 2008). *L. reuteri* has also been shown to produce folate and cobalamin, also known as vitamin B12, nutrients that many animals, including humans, require.

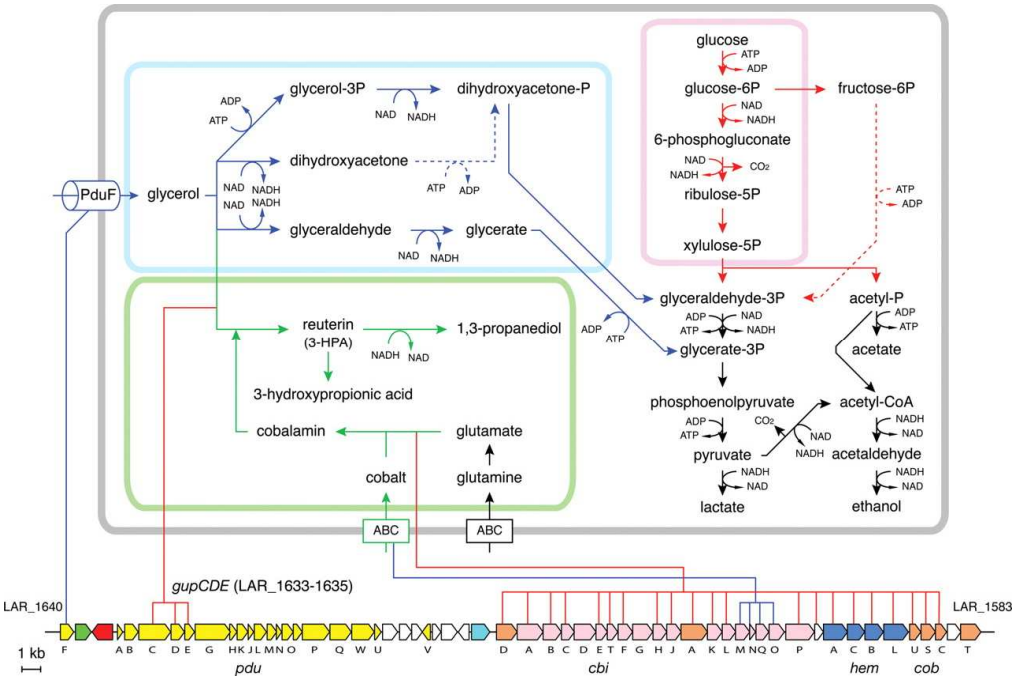


Figure 2.1.5: Proposed metabolic pathways of *Lactobacillus reuteri*

Lactobacillus reuteri is a naturally occurring component of the animal stomach and intestine microflora. It has not been known to be pathogenic; rather, it has been studied as a probiotic organism; it produces reuterin by fermenting glycerol. (Morita et al, 2008). Reuterin is a broad-range antibiotic, affecting both Gram-positive and Gram-negative species, that most pathogenic intestinal bacteria are sensitive to. Studies have shown reuterin produced by *L. reuteri* is useful against a wide array of

harmful bacteria such as pathogenic *E. coli* strains, *Clostridium difficile, Salmonella enterica, Vibrio cholerae*, and many others (Cleusix et al, 2007). While it is known how reuterin is produced, the mechanism of how reuterin inhibits growth of such a broad range of pathogenic microbes is still unclear. Its antimicrobial ability is also contributed by its colonization of the epithelial cells, preventing other bacteria from colonizing. Studies have shown that some strains may prevent *Helicobacter pylori* infection, the main cause of gastric ulcers, via colonizing competition (Mucai et al, 2001). *L. reuteri's* ability to produce the nutrients folate and vitamin B12 has also been studied as a possible nutritional supplement. Humans require daily intakes of both of these nutrients, and vitamin B12 deficiency has been linked to heart disease, anemia, cancer, and other problems. Vitamin B12 deficiency is particularly prevalent in people with strict vegetarian diets, where supplements of *L. reuteri* would be useful.

1.16.6 *Lactobacillus fermentum*

L. fermentum is a closely related species of *L. reuteri* based on a sequence analysis of the 16S ribosomal RNA gene (95% identity) (Ennahar, 2003) and on phenotypic properties, including being an obligate heterofermentative organism. Previously, both these species were classified as a single species (Ennahar, 2003), but were subsequently separated based primarily on DNA hybridization and GC content (Klein, 1998). Despite many similar phenotypic characteristics, probiotic effects have primarily been observed for *L. reuteri*, with a few studies suggesting probiotic properties for *L. fermentum* (Strompfova et al, 2006; Mikelsaar et al, 2009). The use of gut microbes as probiotics in food is aimed towards preventing and treating various health problems. Among these health problems allergies, neoplastic growth, and inflammatory bowel disease are included. Recent areas of study have focused on the influence of probiotics on metabolic functions of their host. One area has been the metabolism of cholesterol by LABs acting as probiotics. Research has shown that *Lactobacillus* species have been proven to remove cholesterol in vitro through various ways such as assimilation, binding to the surface cells, and incorporation into cellular membranes (Mikelsaar et al, 2009). Recent studies have shown that *L. fermentum* has antibiotic resistances. DNA was isolated from *Lactobacillus fermentum* and tested for antibiotic resistance against clinically important agents by using broth dilution tests.

Different strains of *Lactobacillus fermentum* demonstrated uniform resistance patterns demonstrating resistance to glycopeptide vancomycin and to tetracycline (Klein et al, 2011). This is in contrast with other studies, where it's shown that *Lactobacillus fermentum* is sensitive to some common antibiotics such as gentamicin, cefazolin, penicillin, trimethoprim/sulfamethoxazole, ampicillin, carbenicillin, erythromycin, amikacin, and cholorampehnicol (Zheng et al, 2010).

1.17 The Lab Metabolism and Its Importance in Fermented Foods.

Lactic acid bacteria (LAB) are the predominant microorganisms in a majority of food fermentations and their metabolic activity determines and maintains the quality of fermented foods. The application of starter cultures has become the state of the art in a majority of industrial food fermentation as it allows process standardization, mitigation of hygienic risks, and product diversification (Gänzle, 2009).

Strains suitable as starter cultures exhibit a combination of several desirable properties. Generally, strains must be suitable for large scale production and culture preservation. Metabolites that make an important contribution to food quality in one application may constitute a spoilage event in another (Gänzle, 2009).

1.18 Antimicrobial Activity

Food preservation by lactic fermentation generally relies on the removal of fermentable carbohydrates, the consumption of oxygen, the formation of organic acids and the concomitant decrease in pH (table 2.3.1). The preservative effect can be increased by the strain-specific formation of specific inhibitors. Diacetyl, acetaldehyde or H_2O_2 contribute to the inhibitory effect of LAB but these compounds strongly affect the sensory properties of food when present in inhibitory concentrations (De Vuyst and Vandamme, 1994; Vandenbergh, 1993). Bacteriocin application in food can be considered a mature technology and bacteriocin producing LAB are primarily used to inhibit or to eliminate *Listeria monocytogenes* from ready-to-eat meats and dairy products (Stiles, 1996; Montville et al., 2001; Drider et al., 2006).

Table 2.3.1. Metabolic activities of lactic starter cultures

UNSPECIFIC FACTORS	SPECIFIC INIHIBITORS
Substrate utilization	Diacetyl, acetaldehyde
Occupation of surfaces	Short chain fatty acids, hydroxy-fatty acids
Consumption of oxygen	Reuterin, reutericyclin
Acid formation (lactate, acetate, propionate)	Bacteriocins, antifungal peptides, and cyclic dipeptides (cyclo[Phe-Pro], cyclo[Phe-OH-Pro], cyclo[Gly-Leu])
Decrease of pH	H_2O_2
Decrease of redox potential	Benzaldehyde, (hydroxy-phenyllactate, Benzoate

Although fungi represent major spoilage organisms on fermented foods, food applications of antifungal compounds from LAB were only recently described. Antifungal metabolites from LAB include propionate, phenyllactate, hydroxyphenyllactate, several cyclic dipeptides, and 3-hydroxy fatty acids (Table). Phenyllactate and hydroxyphenyllactate are produced from phenylalanine and tyrosine, respectively (Lavermicocca et al., 2000 and 2003). Fungistatic cyclic dipeptides are produced by several strains of *Lactobacillus plantarum* (Ström et al., 2002; Schnürer and Magnusson, 2005; Broberg et al., 2007). Minimum inhibitory concentration of phenyllactate and cyclic dipeptides are in the range of 5–50 g/L but the concentrations generated in food fermentations are well below this MIC (Vermeulen et al., 2006a). These compounds are thus active only when acting in concert with other antimicrobial agents (Schwenninger et al., 2008).

1.19 Acidifying Activity

The energy yield in glucose metabolism of obligate heterofermentative lactobacilli is dependent on the availability of hydrogen acceptors for NADH regeneration (Gänzle et al., 2007). Oxygen, fructose, and oxidized glutathione are used as hydrogen acceptors and are reduced to H_2O mannitol, and thiols, respectively, with concomitant oxidation of NAD(P)H to NAD(P).

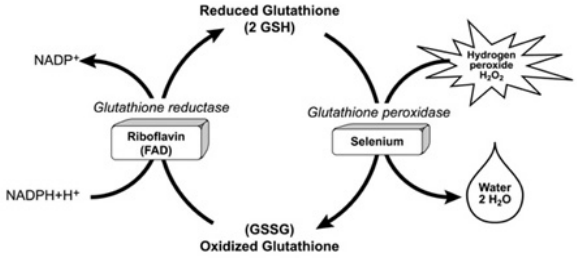


Figure 2.4: Overview on the effect of intracellular, NADH dependent glutathione reductase (GshR) on intra- or intermolecular disulfide bonds: one molecule of hydrogen peroxide is reduced to two molecules of water, while two molecules of glutathione (GSH) are oxidized in a reaction catalyzed by the selenoenzyme, glutathione peroxidase. Oxidized glutathione may be reduced by the flavin adenine dinucleotide (FAD)-dependent enzyme, glutathione reductase.

1.20 Carbohydrate Metabolism

Most obligatory heterofermentative lactobacilli but not *L. sanfranciscensis* metabolize pentoses (Hammes and Hertel, 2003). Simultaneous metabolism of maltose and pentoses was observed *for L. brevis, L. fermentum and Lactobacillus hilgardii* during growth in sourdough (Gobbetti et al., 1999). Sucrose is metabolized by fructosyltransferases in some strains of *L. sanfranciscensis* .

Lactate, ethanol and CO2 are the major products of hexose metabolism unless co-substrates are present that enable the regeneration of reduced cofactors. In the presence of electron-acceptors, acetyl-phosphate is converted to acetate with the yield of an additional molecule of ATP. Glycerol and erythritol are alternative metabolites of *L. sanfranciscensis* from glucose.

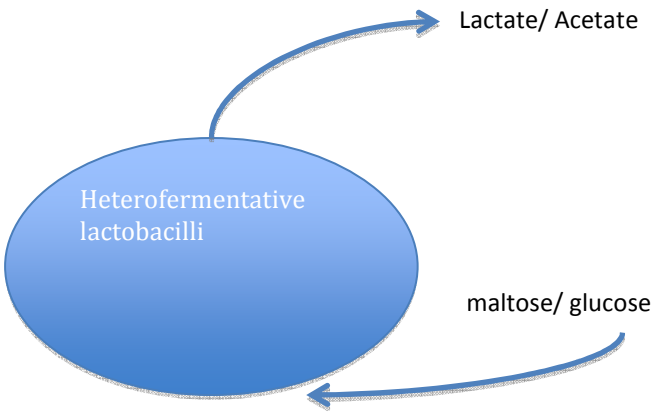


Figure 2.5.1 : the heterofermentative pathway of lactic acid bacteria

Obligate homofermentative and facultative heterofermentative lactobacilli degrade

hexoses via the Emden-Meyerhoff pathway, in these organisms, maltose and fructose utilization generally occurs only after glucose depletion. Maltose is hydrolyzed by a-glucosidase activity. Pentose metabolism by LAB is repressed by glucose (Lokman et al., 1997; Titgemeyer and Hillen, 2002) but simultaneous metabolism of maltose and arabinose was observed during growth of *L. plantarum* in sourdough (Gobbetti et al., 2000).

Hexose metabolism via the Emden-Meyerhoff pathway leads to lactate production with pyruvate as central intermediate of metabolism. Numerous alternative pathways exist in lactobacilli for alternative fates of pyruvate, and metabolites other than lactate may be the major metabolites of hexose metabolism at conditions of glucose-limitation or in the presence of oxygen as electron acceptor (Cocaign-Bousquet et al., 1996; Axelsson, 2004). However, lactate is the major end product of homofermentative hexose fermentation in many fermented foods.

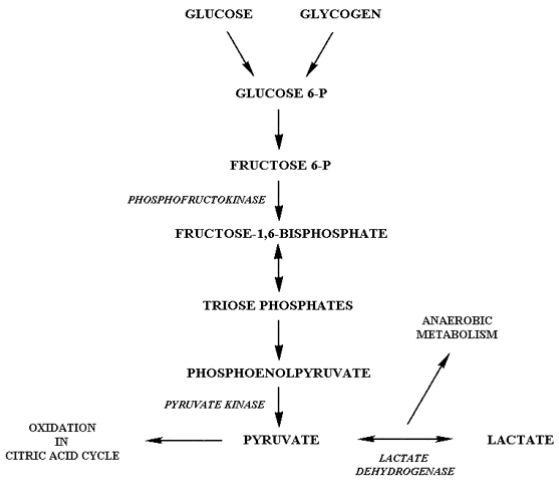


Figure 2.5.2: Emden-Meyerhoff pathway

The initial steps for citrate metabolism in LAB are transport by the citrate permease and the citrate-lyase reaction. Two alternative routes exist for oxaloacetate conversion, one pathway results in succinic acid formation, the other resulting in the decarboxylation to pyruvate (Cselovszky et al., 1992; Ferain et al., 1996). In *Lactococcus lactis*, pyruvate is partially converted to α-acetolactate when electron acceptors such as citrate are present that create a pyruvate surplus relative to the need of NADH regeneration via the lactate dehydrogenase reaction. α-acetolactate is enzymatically reduced to acetoin or converted non-enzymatically to the flavour

compound diacetyl (for review, see Hugenholtz et al., 2002). *L. sanfranciscensis* strains use the pyruvate branch to convert citrate to lactate and acetate (Gobbetti and Corsetti, 1996); the cofactor regeneration in the lactate dehydrogenase reaction enables the additional formation of acetate. Malate and fumarate are converted to lactate by *L. sanfranciscensis. L. reuteri and L. pontis* do not utilize citrate and convert malate and fumarate to succinate (Stolz et al., 1995a, b). The growth of *L. sanfranciscensis* in sourdough is limited by the pH (Ganzle et al., 1998) and lactate formation from citrate does not decrease the pH. Therefore, the use of citrate results in increased lactate and acetate levels.

1.21 Peptide and Amino Acid Metabolism

The majority of sourdough LAB does not exhibit cell-wall associated proteinase activity (Pepe et al., 2003; Vermeulen et al., 2005). Generally, a comparable extent of protein degradation is observed in wheat sourdough and in chemically acidified dough (Thiele et al., 2002, 2003, 2004; Loponen et al., 2004). However, several strains of sourdough LAB strains exhibiting proteolytic activity were characterized (Gobbetti et al., 1996; Di Cagno et al., 2002; Pepe et al., 2003) and a contribution of selected LAB to proteolysis could be demonstrated by analysis of the degradation of albumins, globulins, and gliadins in wheat sourdoughs (Di Cagno et al., 2002; Pepe et al., 2003; Zotta et al., 2006). LAB predominantly use peptides to meet their demand of complex nitrogen (Kunji et al., 1996). Comparable to *Lc. lactis and L. plantarum, L. sanfranciscensis* expresses transport systems for oligo and dipeptides (Vermeulen et al., 2005) and peptides are hydrolysed by intracellular peptidases, several of which were characterized on biochemical or genetical level (Gobbetti et al., 1996; Gallo et al., 2005; Vermeulen et al., 2005). Vermeulen et al. (2006b) compared phenylalanine turnover by *L. plantarum* and *L. sanfranciscensis* using phenylalanine and defined dipeptides as substrates. In N-limited media, both strains produced higher levels of phenyllactate from phenylalanine when the dipeptides FL, FS or PF were offered as substrate. Thus, transport limitations to the metabolism of amino acid in *L. sanfranciscensis* and *L. plantarum* are overcome by the addition of peptides as substrate. These finding are in keeping with previous observations in *Lc. lactis* and *L. helveticus* that the transamination of amino acids can be enhanced by cell lysis or permeabilization (Martinez-Cuesta et al., 2002; Valence et al., 2000).

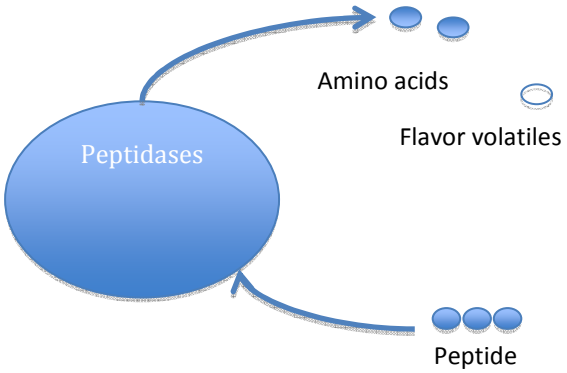


Figure 2.6: peptide hydrolysis and amino acid metabolism

Fadda and Vignolo studied the acidogenic metabolism of a strain of *L.plantarum* isolated from fermented sausage (2008). They focused on the hydrolysis of sarcoplasmic protein during fermentation in a meat model system under controlled pH and Temperature. Results showed that the hydrolysis of sarcoplasmic protein at pH 4, as during a sausage fermentation, was carried out from the synergic action of meat proteases, LAB proteolytic system and lactic acid promoted soluble-protein hydrolysis, while at pH 6 the hydrolysis was carried out exclusively from the proteolytic system of the *L. plantarum*. Moreover they observed that at acid pH (4 or lower) all the aminopeptidases are inhibited, that's a limiting factor for amino acids release.

1.22 Glutamine and Glutamate Metabolism

Proteolysis and amino acid metabolism by starter cultures are of particular relevance for food quality as amino acid metabolites are active as flavour compounds, biogenic amines, taste compounds, or antifungals. For example, the strain specific conversion of phenylalanine may yield phenylethanolamine, a biogenic amine, phenylacetate or phenylacetaldehyde, two flavour compounds which impart a "flowery" or "honey like" flavour impression, or phenyllactate, a compound with antifungal activity. The spectrum of metabolites produced by LAB from amino acids varies greatly among species and within different strains of a species (Liu et al., 2008). Recent advances in the selection of starter cultures are particularly related to the biochemical and genetic characterization of glutamine and glutamate metabolism.

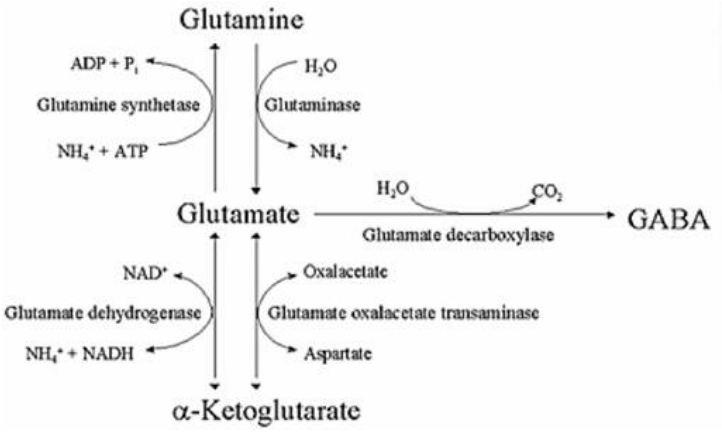


Figure 2.7: glutamine and glutamate metabolism in LAB.

An overview of the metabolism in Lactic acid bacteria is reported in figure 2.7.

vii. Amino acids are taken up as peptides and cleaved by an array of intracellular peptidases (Gänzle et al., 2007).

viii. Glutamine is converted to glutamate by glutaminase, an enzyme activity that was reported in *L. rhamnosus* (Weingand-Ziade et al., 2003), *L. sanfranciscensis* and *L. reuteri (*Vermeulen et al., 2007b).

ix. Strain specific conversion of glutamate to γ-aminobutyrate (GABA) was reported for *L. brevis, L. paracasei, L. delbrueckii, L. plantarum and L. lactis* (Yokoyama et al., 2002; Siragusa et al., 2007).

x. NADH-dependent conversion of glutamate to α-ketoglutarate (αKG) by glutamate dehydrogenase is a strain specific property of *L. plantarum, L. sanfranciscensis, L. paracase*i and other lactic acid bacteria (Tanous et al., 2002 and 2005; Vermeulen et al., 2006a).

xi. LAB generally exhibit transaminase activity and convert αKG to glutamate in the presence of other amino acids as amino donors.

xii. The conversion of αKG to GABA was reported for *Oenococcus oenii* (Radler and Bröhl, 1984).

Glutamate contributes to the umami taste of fermented foods (Drake et al., 2007). LAB liberate glutamate from peptides, or by conversion of glutamine. Glutamine conversion is particularly relevant during growth of LAB in cereal substrates as glutamine is the predominant amino acid in cereal prolamins (Wieser, 2004). *L. rhamnosus, L. reuteri* and *L. sanfranciscensis* exhibit glutaminase activity and convert glutamine or glutamine containing peptides to glutamate (Weingand-Ziade et al., 2003; Vermeulen et al., 2007b).

GABA possesses multiple physiological functions and some studies indicate that dietary GABA has antihypertensive properties (Inoue et al., 2003). GABA is a non-proteinogenic amino acid formed by glutamate decarboxylase activity. A strain of *Lactobacillus brevis* quantitatively decarboxylated glutamate to GABA in cereal mashes (Yokoyama et al., 2002). LAB exhibit glutamate dehydrogenase activity in a strain specific manner. The enzyme catalyses the NAD(P)H-dependent recycling of glutamate to a-ketoglutarate, and consequently increases the flux through the transaminase reaction (Rijnen et al., 2000; Tanous et al., 2002). Remarkably, the cofactor-dependency of the glutamate dehydrogenase linked glutamate recycling and the metabolic flux through the transaminase reaction in *L. sanfranciscensis* DSM20451 to NAD(P)H regeneration in central carbohydrate metabolism (Vermeulen et al., 2006b). The conversion of hydrophobic and branched chain amino acids in LAB is initiated by transamination and requires a α-ketoacid as amino group acceptor. The preferred acceptor in lactococci and lactobacilli is α- ketoglutarate (αKG, Fernández and Zúñiga, 2006).

α-Ketoglutarate serves as amino acceptor in the transamination reaction of leucine, phenylalanine and other amino acids, correspondingly, the addition of α-ketoglutarate strongly increases amino acid conversion *of L. sakei, L. plantarum and Lc. lactis* (Yvon et al., 1998; Larrouture et al., 2000; Rijnen et al., 2000). Glutamate conversion to αKG occurs via transamination with oxaloacetate as amino acceptor, and simultaneous addition of citrate and glutamate increased the conversion of phenylalanine and leucine to the corresponding α- ketoacids (Tanous et al., 2005). NADH and NADPH dependent glutamate dehydrogenase (GDH) activity is an alternative pathway for αKG formation in lactococci and lactobacilli and differences in the conversion of phenylalanine and leucine by transamination were explained by the strain-dependent differences in GDH activity (Tanous et al., 2002).

1.23 Role and Importance of Glutamate in Foods.

It is merely one decade that we recognize the taste of glutamate as one of the basic tastes termed as “umami”. The recognition of glutamate as an excitatory neurotransmitter in mammalian nervous system as well as umami as the fifth basic taste has an interesting historical background. Historically, glutamate was first isolated as glutamic acid from acid hydrolysate of wheat gluten, by the German

scientist Ritthausen in 1866 and thus named it as “glutamic acid”. The history of glutamate in food is older than the history of science of nutrition. Practice of adding large seaweed (*Laminaria japonica*) to soup stocks has been in use in Japan for last 12 centuries. This seaweed markedly increases the taste of the soup. But what was unknown that it contained high amount of glutamate. It was not until 1908 that the link between the seaweed and glutamate was discovered. The brown crystals left behind after evaporation of a large amount of kombu broth, was scientifically identified as glutamate by Prof Ikeda of Tokyo University. He termed this unique flavour as “umami” (Ikeda, 1908).

Glutamate is one of the most abundant amino acids in nature. Since glutamate is a building block of protein and free glutamate exists in organs and tissues, it is found naturally in virtually all foods such as milk, vegetables, seafood, poultry, meats, traditional seasonings like fish sauce and soy sauce, and many other foods (Yoshida, 1998). It has long been used around the world to enhance the palatability of foods before the discovery of its taste. Foods rich in free glutamate, such as tomatoes, cheese and mushrooms have been used in cooking for their flavour favoring qualities. Glutamate also has been a component of traditional seasonings such as fish and soy sauces. More than 1200 years ago, in ancient Rome, fish sauce called “Garum” was used. Fish and soy sauces have been used in South Eastern Asian countries, China and Japan for more than several centuries. Mother’s milk the first food for babies, and is the only food when they are just born. It has to give them the entire nutrient they need. It was reported that glutamate is the most abundant amino acid in mother’s milk in all the species analyzed (Kare and Kawamura, 1998).

MSG, sodium salt of glutamate, is widely used as a flavor enhancer all over the world and also one of the most studied food ingredient, in history. After marketed in Japan in 1909, MSG had been used as a food ingredient in many countries for half century, into the late 1960’s. Although there was not much safety data, MSG was generally regarded as a safe substance, similar to GRAS (Generally Recognized As Safe) status in the United States (Giacometti, 1979) in part because glutamate is one of the most abundant amino acids found in nature and a component of all protein. It was also thought that intake of glutamate from added MSG was much less than that normally ingested from foods. The range oral intake varies from 0.4 g/person in Italy to 3 g/person in Taiwan (Giacometti, 1979).

Glutamate is the principal excitatory neurotransmitter in the CNS. During recent advances in the field it is realized that it is much more than a conventional neurotransmitter. It is not only the predominant excitatory neurotransmitter in the mature neurons but also it can influence immature neural cell proliferation, migration, differentiation and survival processes (Schlett K., 2006). Extracellular glutamate level has been shown to be high in embryonic CNS. Glutamate and its receptors are essential for the normal functioning of the CNS. However their excessive activation by glutamate is thought to contribute to neuronal damage in many neurological disorders ranging from hypoxic–ischemic and traumatic brain injuries to chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS (Platt SR.).

Glutamate is important and indispensable for the functioning of the CNS and important in food. The physiological control mechanisms of our body keep a check on its excitotoxic properties (Mallick, 2007).

Glutamate is a dietary essential amino acid and its level in the diet can affect the oxidation of some essential amino acids, such as leucine. The human body metabolizes added glutamate in the same manner it metabolizes glutamate found naturally in many foods. Glutamate is a multifunctional amino acid involved in taste perception, intermediary metabolism, and excitatory neurotransmission.

In Western societies, there is a general trend to an increased consumption of flavored convenience food. Theoretically, this change in behavior might lead to an increased glutamate intake, which is used in these products as flavor enhancer. However, the food industry steadily increases the number of glutamate-free products due to an enhanced reservation of the consumer against food additives. Therefore glutamate enriched protein hydrolysates are currently used in food production to replace the use of glutamate as additive and to enable the reduction of salt in processed foods.

Then the research and study of bacterial strains able to convert glutamine in glutamate, during several fermentations, become very important to ensure the consumer's health and improve the taste of processed foods.

1.24 GABA: γ-Amino Butyric Acid

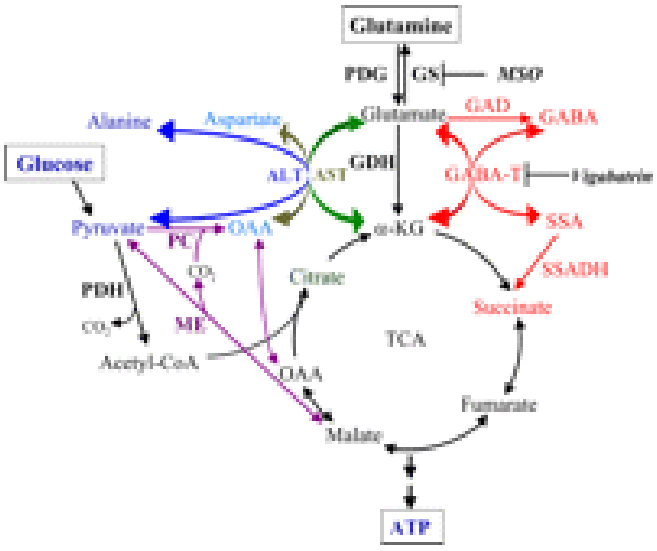


Figure 2.9: Metabolic pathway depicting the synthesis and catabolism of γ-aminobutyric acid (GABA) in the GABA shunt and its relationship to glucose metabolism and the tricarboxylic acid (TCA) cycle.

γ-amino butyric acid (GABA) is the major inhibitory neurotransmitter in mammalian brain and is found widely throughout the central and peripheral nervous systems. In the neocortex GABAergic neurons are plentiful, constituting 15–30% of all neurons. GABA serves both metabolic and trophic functions, in addition to its role as a neurotransmitter, influencing the migration of neurons and astroglia to their target locations in the cortex. During early brain development GABA elicits excitatory (depolarizing) rather than inhibitory (hyperpolarizing) postsynaptic responses. Later in development GABA influences the synaptic organization and fine-tuning of local circuits (Behar, 2009).

Due to the physiological functions of GABA, development of functional foods containing GABA at high concentration has been actively pursued (Tsushida and Murai, 1987; Saikusa et al., 1994). GABA enrichment has been achieved in anaerobic-incubated tea (gabaron tea) (Tsushida and Murai, 1987) and in rice germ soaked in water (Saikusa et al., 1994). GABA production by various microorganisms has been reported, including bacteria (Smith et al., 1992; Maras et al., 1992), fungi (Kono and Himeno, 2000), and yeasts (Hao and Schmit, 1993).

Screening various types of LAB that have GABA-producing ability is important for the food industry, because individual LAB have specific fermentation profiles, such as acid production and flavor formation ability. Because quality of fermented foods, such as taste and flavor, depends on the fermentation profiles of the LAB, such

profiles are considered an important factor in the use of LAB as starters in the production of fermented foods (Kato et al., 2001; Gran et al., 2003). Glutamate decarboxylase (GAD) is considered responsible for GABA production in GABA-producing strains of LAB. GAD has been isolated from a wide variety of sources, and its biochemical properties have been characterized (Nomura et al., 1999, 2000). Although GAD is widely distributed in LAB, GABA-producing ability varies widely among LAB. GABA-producing LAB is expected to enhance development of functional fermented foods containing GABA.

Komatsuzaki et al (2005), based on the hypothesis that GABA production during cultivation of the strains can be improved by adjusting the culture conditions suitable for GAD reaction, adjusting culture conditions, and adding a coenzyme of GAD during cultivation of screened LAB that have high GABA-producing ability, showed that *L. paracasei* isolated from a traditional fermented fish (funa sushi) produced GABA at high level (4300mM), and that cultivation conditions suitable for GABA production can be improved by utilizing the biochemical characteristics of GAD.

CHAPTER 2

OBJECTIVES

The role of microorganisms in fermented meats is well known, even if there are several models of fermentation and the different types of salami are well distinguished ecosystems. However, the role of yeasts and lactic acid bacteria in dry-cured meats (i.e. speck and ham) is often underestimated.

The ripening process of these type of products can last up to 24 months and their water activity (aw) values can decrease reaching values of 0,76. Only Staphylococci, micrococci, yeasts and moulds can colonize the superficial parts of these products thus being considered as ripening index (Munez *et al.,* 1996).

Yeasts are one of the predominant microbial groups during the ripening phase of several food products of intermediate moisture, such as Iberian dry-cured meat products. In particular *Rhodotorula rubra, Hansenula holstii* and *Hansenula sydowiorum* are the dominant microbial species during the first stage of ripening, while *Debaryomyces* spp. can be found at the end of ripening in Iberian hams (Monte *et al.,* 1986; Huerta *et al*., 1988) and "Prosciutto di Parma" (Comi *et al.*, 1990).

In particular, depending on the part of the products that is analyzed, yeasts can reach values of 10^7 cfu/g in some parts, while attain lower levels, i.e. 10^4-10^5 cfu/g, in some others at the end of ripening.

The position and particularly the presence of superficially spread fat in several positions seem to play an important role on the distribution of different biotypes of *Debaryomyces hansenii* in the product (Munez *et al.,* 1996). However, the principal aim of this study was the ecological characterization of the products rather than the assessment of the role of yeasts during ripening processes that is still not well known.

Andrade *et al*., (2009) tried to find a relationship between the molecules produced *in vitro,* i.e. in a designed model culture medium under conditions representative of dry-cured ham processing, by different biotypes of of *Debaryomyces* spp. and other dominant microbial species isolated from Iberian hams and the volatiles involved in the dry-cured flavour. According to their findings most of the biotypes produced brached aldehydes and alchools, which derive from valine, leucine and isoleucine. Such compounds are very important molecules as they contribute to the aromatic profiles of hams and all fermented and not fermented meat products. On the other hand the same metabolites are produced by lactic acid bacteria too.

The principal aim of this research project has been the evaluation of the specific role of yeasts in ripening processes of dry-cured meat products, i.e. speck and in salami produced by adding *Lactobacilli.* produced by adding *Lactobacilli* starter cultures, i.e. *L. sakei, L. casei, L. fermentum, L. rhamnosus, L.sakei* in combination with *S.xylosus*.

In particular the role of the predominant yeasts on the hydrolytic patterns of meat proteins has been studied. As yeasts are not used as starter cultures for these products, the first part of the work has been focused on the identification and characterization of the yeasts strains isolated from speck and salami during the ripening process. Also the evolution of their cell loads and changes in the composition of the microflora has been monitored. Afterwards, their contribution to the proteolytic activity of the products has been evaluated.

Therefore this research work has been developed according to the following activities:

8. Characterization of the yeasts and lactic acid bacteria in samples of speck produced by different farms and analyzed during different production and ripening stages
9. Characterization of the superficial or internal yeasts population in salami produced with or without the use of lactobacilli as starter cultures.
10. Molecular characterization of different strains of yeasts and detection of the dominant biotypes able to survive despite environmental stress factors, (such as smoke, salt i.e.)
11. Study of the proteolytic profiles of speck and salami during the ripening process and comparison with the proteolytic profiles in meat model system of a relevant number of yeasts isolated from speck and salami.
12. Study of the proteolytic profiles of *Lactobacilli* starter cultures in salami
13. Comparative statistical analysis of the proteolytic profiles to find a relationship between specific bands and peptides and specific microorganisms.
14. Evaluation of the aromatic characteristics of speck and salami, during the production process, to detect metabolic differences and find relationships among the metabolites released by starter cultures or dominant microflora.

CHAPTER 3

MATERIAL and METHODS

Table 3.1: List of the strains used

FUA Nr	Microbial species	Source
Lbcd	*Lactobacillus casei*	Milk whey
MR13	*Lactobacillus fermentum*	Cassava
C249	*Lactobacillus rhamnosus*	Milk
DSM-20531	*Lactobacillus amylovorus*	Meat
63	*Lactobacillus plantarum*	Sausage
BB12	*Lactobacillus sanfranciscensis*	Sourdoughs
	Lactobacillus sakei	Commercial starter
CLCD	*Yarrowia lipolytica*	Salami
CSLF2	*YarrowialLipolytica*	Salami
CLF1	*Yarrowia lipolytica*	Salami
CLR1	*Yarrowia lipolytica*	Salami
CsLR4	*Yarrowia lipolytica*	Salami
C1	*Yarrowia lipolytica*	Salami
C4	*Yarrowia lipolytica*	Salami
1 II YL 1a	*Yarrowia lipolytica*	Speck
1 II YL 3b	*Yarrowia lipolytica*	Speck
1 II YL 4a	*Yarrowia lipolytica*	Speck
1 II YL 6b	*Yarrowia lipolytica*	Speck
1 II YL 7a	*Yarrowia lipolytica*	Speck
1 II YL 8a	*Yarrowia lipolytica*	Speck
1 II YL 9a	*Yarrowia lipolytica*	Speck
4B	*Yarrowia lipolytica*	Irradiated poultry meat
16B	*Yarrowia lipolytica*	Irradiated poultry meat
5D	*Yarrowia lipolytica*	Irradiated poultry meat
27D	*Yarrowia lipolytica*	Irradiated poultry meat
LM2	*Yarrowia lipolytica*	Pecorino cheese
PO1	*Yarrowia lipolytica*	Superficial waters of Po river
PO11	*Yarrowia lipolytica*	Superficial waters of Po river
PO17	*Yarrowia lipolytica*	Superficial waters of Po river
PO19	*Yarrowia lipolytica*	Superficial waters of Po river
PO14	*Yarrowia lipolytica*	Superficial waters of Po river
PO23	*Yarrowia lipolytica*	Superficial waters of Po river
RO3	*Yarrowia lipolytica*	Commercial Light butter
RO9	*Yarrowia lipolytica*	Commercial Light butter
RO12	*Yarrowia lipolytica*	Commercial Light butter
RO19	*Yarrowia lipolytica*	Commercial Light butter
RO22	*Yarrowia lipolytica*	Commercial Light butter
RO25	*Yarrowia lipolytica*	Commercial Light butter
Y9	*Yarrowia lipolytica*	Chilled food
Y10	*Yarrowia lipolytica*	Chilled food
Y14	*Yarrowia lipolytica*	Chilled food
Y22	*Yarrowia lipolytica*	Chilled food
Ly56	*Lysyeria monocytogenes*	Fish
G	*Pseudomonas fulva*	Soy derived products
BT	*Pseudomonas fragi*	Soy derived products
PA22	*Leuconostoc lactis*	Soy derived products

T2	*Enterococcus faecium*	Soy derived products

Table 3.2: Composition of M17 media for *Lactococci*

Ingredients	**Quantity**
Tripton	5 g/l
Meat extract	5 g/l
Yeast extract	2.5 g/l
Soy Peptone	5 g/l
Acid ascorbic	0.5 g/l
Magnesium sulphate	0.25 g/l
Di-sodium-glycerol sulfate	19 g/l

Add 50ml/l of sterile lactose after autoclave.

Table 3.3 : Composition of MRS media for Lactobacilli

Ingredients	**Quantity**
Universal peptone	10 g/l
Lab-lemco	10 g/l
Yeast extract	5 g/l
Glucose	20 g/l
K_2HPO_4	2 g/l
sodium acetate anhydrous	5 g/l
tri-ammonium citrate	2 g/l
$MgSO_4 \cdot 7H_2O$	0.2 g/l
SO_4 (4 H_2O)	0.05 g/l
Tween 80	1 g/l

Table 3.4: Composition of YPD media for yeast

Ingredients	Quantity
Universal peptone	10 g/l
Glucose	20 g/l
Yeast extract	5 g/l

Table 3.5: Composition of PCA media for Total Bacterial Growth

Ingredients	Quantity
Universal peptone	5 g/l
Glucose	1 g/l
Yeast extract	2.5 g/l

3.1 Meat Model System Development For Proteolytic Activity Assessment

The meat model system, which was used for the *in vitro* evaluation of the proteolytic activity of both yeasts and lactic acid bacteria strains, was prepared according to the protocol described by Vignolo *et al.,* (2004).

3.1.1 Culture media

- MRS broth culture medium for lactic acid bacteria
- YPD broth culture medium for yeasts
- PCA broth culture medium for Total Bacterial Growth

3.1.2 Soluble muscle extracts

10) Weigh 10g of lean muscle in a stomacher bag
11) Add 90 ml of 20mM phosphate buffer, pH7
12) Homogenize in a stomacher 400 blender for 3 minutes
13) Centrifuge the protein solution (12000g ,4°C for 20minutes)
14) Adjust the pH to 6.5 with 1N NaOH
15) Add 1% (w/v) of glucose
16) Filter the supernatant containing the proteins through Whatman paper
17) Filter-sterilize this solution by using a 250 ml capacity filter (Millipore) with a vacuum-pump
18) Add 0.1% of Tween-80 previously sterilized

3.1.3 Sterility Control of the soluble muscle extract

4) To quantify the total aerobic organism, inoculate in duplicate Petri dishes with 0.5 ml of protein extract
5) Add 10 ml of the melted PCA and homogenize appropriately
6) After incubation at 37°C for 96 hours, check for the growth of microorganisms that must be negligible, i.e. lower than $1*10^2$ CFU/ml

3.2 Proteolytic activity of the soluble muscle extract by LAB strains

3.2.1 Culture condition of the strains

4) Inoculate *Lactobacillus* spp. strains in MRS broth media and incubate at 37°C overnight
5) Harvest exponential-phase cells and wash twice with 20mM phosphate buffer, pH7
6) Remove the supernatant and resuspend the pellet in 20mM phosphate buffer, pH7

3.2.2 Growth in the Soluble Muscle Extract

8) Inoculate 30 ml of the soluble muscle extract with 30 ml of an overnight culture to yield an initial cell number of 10^5 CFU/ml corresponding to an OD_{680} OF 0.15
9) Incubate at 30°C for 96 hours
10) Withdrawn samples every 24 hours for pH, bacterial growth, and take proteolytic activity measurements
11) Measure the pH values with a pH meter
12) Determine bacterial cell counts using 0.1% peptone water as the diluent
13) Plate 0.5 ml of each dilution in Petri dishes adding the MRS media
14) Incubate at 30°C for 48 hours

3.3 Cell Wall Extraction Of *Lactobacilli* for Meat Proteins Hydrolysis

3.3.1 Total Meat Protein Extraction

Total proteins were extracted from 2 g samples with 40 ml of 1.1 M potassium iodide, 0.1 M sodium phosphate, pH 7.4 buffer (Cordoba, 1994). The samples were homogenized for 3 minutes. The extracts were centrifuged at 8000g for 15 min at 4

°C (Beckman Coulter Avanti J-10, Fullerton, CA, USA), and the supernatants were filtered through a 0.22 μm filter.

Sarcoplasmic proteins were extracted from 2 g samples with 40 ml of 0.02 M, pH 6.5 sodium phosphate buffer (Fadda *et al*., 1999) following the steps indicated for the above extraction. The protein content of the sarcoplasmic extract was 0.80 mg/ml.

3.3.2 Lab strains growth conditions

The strains of *Lactobacillus casei* lbcd, *L. rhamnosus C249, L.sanfranciscensis BB12, L. fermentum* M13*, L amylovorus* DSM-20531 and *L. plantarum 63* isolated from different food sources (table3.1) were used for the proteolytic assay. All the strains were usually grown in MRS broth at 37°C for 24 hours and maintained at -80°C in 15% (v/v) glycerol. For enzymatic assays liquid medium was inoculated (1%, v/v) with the selected microorganism, previously sub-cultured once and incubated overnight at 37°C.

3.3.3 Preparation of Cell Suspensions and Extracts

Cells were harvested by centrifugation (10000g for 20 minutes at 4°C), washed twice in 0,085% (w/v) NaCl, containing 20 mM $CaCl_2$ and resuspended in 2% initial volume of 50 mM Tris-HCl, pH 6.5. This was designated as whole-cell suspension (WCS) (Sanz *et al*, 1999).

Cell free extracts (CFE) were obtained for each strain by the procedure of Sanz and Fadda (1999). Cells were collected as above, washed twice in 20 mM phosphate buffer (pH 7.0) and resuspended in the same buffer (10% of initial volume) supplemented with lysozyme (1mg/ml). After incubation at 30°C for 1 hour, the cell wall fraction was removed by centrifugation (15000g for 20 minutes at 4°C). The pellet was washed twice in 20 mM phosphate buffer (pH 7.0) resuspended in the same buffer and sonicated for 15 mnutes. Cell debris was removed by centrifugation (10000 g for 20 minutes at 4°C).

3.3.4 Enzymatic Mixtures

Three independent assays were carried out for the protein extracts (i.e. total protein or meat soluble muscle extract) using as enzymatic sample either whole cell suspension, CFE, or a combination of both (1:1). The reaction mixture consisted of 6ml of whole

cell suspension or CFE aseptically added at 30ml of protein extract. For the combined mixture, 3ml of whole cell suspension and 3ml of CFE were added to 30ml of protein extract. The mixtures were incubated at 37C for 96 hours (Sanz and Fadda, 1999). Bacterial counts were determined on MRS agar plate and pH values were monitored. The hydrolysis of muscle proteins was monitored by SDS-PAGE analysis, using a 4-15% polyacrylamide precast gels (BioRad).

3.4 Hydrolysis Of Meat Protein extracts by *Yarrowia lipolytica*

3.4.1 Yeasts growth conditions

Several strains of *Yarrowia lipolytica* isolated from different food sources were grown in YPD broth at 28°C for 72 hours.

Cells were separated from culture broth by centrifugation at 7000 rpm for 15 minutes. The supernatants were used for the hydrolysis with total meat proteins.

Reaction mix was:

- 12,5 ml of meat protein (1,25 g/ml)
- 2,5 ml of supernatant
- 0,1 µl of a sodium azide solution (0,002%)

The mix was incubated at 28°C for 24 and 48 hours; the enzymatic reaction was then stopped by heating at 100°C for 5 minutes.

The samples were stocked at -20°C and then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

3.5 Protein Quantification By Bradford Assay

The Bradford assay was used to determine the total protein concentration of the samples. The method is based on the proportional binding of the dye Coomassie to proteins. Coomassie absorbs at 595 nm. The protein concentration of a test sample is determined by comparison to those of a series of protein standards which are known to give rise to a reproducible linear absorbance profile. Bovine Serum Albumin (BSA) was used as standard protein.

Procedure

- Prepare a 4-fold dilution of a 2 mg/mL BSA sample by adding 50µl of 2 mg/mL BSA to 150 µl of distilled water to make 200 µl of 0.5 mg/mL BSA.
- Generate test samples for the reference cell, blank, BSA standards and the protein sample to be tested according to Table 1 in disposable cuvettes.
- Allow each sample to incubate at room temperature for 10-30 minutes.
- Measure the absorbance of each sample at 595nm using a UV-visible spectrophotometer.
- Plot the absorbance of each BSA standard as a function of its theoretical concentration.
- Determine the best fit of the data to a straight line in the form of the equation

  "y = mx + b"

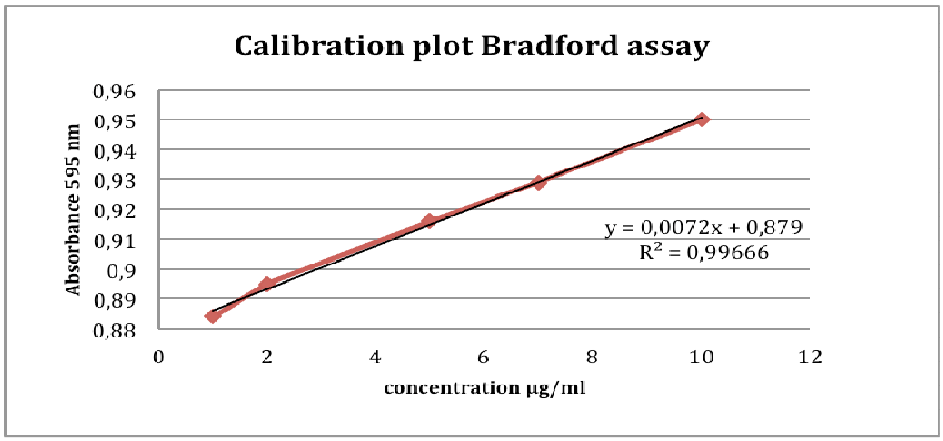
  where y = absorbance at 595 nm

  and x = protein concentration.
- Use this equation to calculate the concentration of the protein sample based on the measured absorbance.

Table 3.6: preparation of the test samples for the Bradford protein assay

Test Sample	Sample volume (µl)	Water volume (µl)	Bradford Reagent Volume (µl)
Blank	0	800	200
BSA Standard-5 µl /ml	10	790	200
BSA Standard-10 µl /ml	20	780	200
BSA Standard- 15µl ml	30	770	200
BSA Standard-20 µl /ml	40	760	200
BSA Standard-25 µl /ml	50	750	200
Protein Sample	100	700	200

Figure 3.1 : Calibration plot for Bradford assay



3.6 SDS Page Electrophoresis

The hydrolysis of muscle proteins was monitored by sodium dodecyl sulfate gel (SDS)-polyacrylamide electrophoresis (PAGE).

For the denaturation of each protein fraction, 50 µl of extract were mixed with 25 µl of Laemmli sample buffer (Bio-Rad Laboratories, Milan, Italy) containing β-mercaptoethanol. The mixture was incubated at 100 °C for 5 minutes.

The SDS-PAGE analysis was then carried out with two different methods: by using precast gels or 15% polyacrylamide gels.

As far as pre casted gels, Ready Tris-HCl Gel, 10–20% resolving gel, 4% stacking gel supplied by Bio-Rad Laboratories were used. 10 µl of a Precision Plus Protein Standard Unstained (Biorad) was used as standard.

The wells of the electrophoresis gels were loaded with 20 µl of the denatured protein samples.

Gels were run in a Mini Protean Cell System with a 10% SDS buffer, at 250 V for 30 minutes.

Staining was 1 hour in the following solution

Bromophenol blue	0.1%
Methanol	50%
Glacial acetic acid	7%

De-staining was made for 2 hours in a solution of

Methanol	50%
Glacial acetic acid	10%

Several washes were then made with distilled water, until obtaining a clear gel.

3.6.1 PREPARATION OF 15% POLYACRYLAMIDE GELS

Running gel (30 ml) 15%:

Water	6.9 ml
30% acrylamide and bis-acrylamide solution, 19:1 (BioRad)	15 ml
1.5 M Tris (pH8.8)	7.5 ml
10% SDS	0.3 ml
10% APS	0.3 ml
TEMED	0.012 ml

Stacking gel (10ml) 5%:

Water	6.8 ml
30% acrylamide and bis-acrylamide solution, 19:1 (BioRad)	1.7 ml
0.5 M Tris (pH6.8)	1.25ml
10%SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.01 ml

10 µl of a Broad Range (Bio-Rad Laboratories, Milan, Italy) were used as standard. The protein denaturation was performed according to the above described procedure. The wells of the electrophoresis gels were loaded with 25 µl of the denatured proein samples.

Gels were run in a buffer tank supplied by Bio-Rad with a 10% SDS buffer.

Voltage was at 90V for 60 minutes and 250 V for 3 hours.

The gels were then stained overnight in a staining solution made of:

Comassie Blue	0.3mM
Methanol	40%
Glacial acetic acid	7%

Destaining solution was:

Methanol	5%
Glacial acetic acid	7%

3.7 Antimicrobial Activity Of Meat Hydrolyzates.

Antimicrobial activity of meat protein extracts hydrolyzeds by *Yarrowia lipolitica* and *Lactobacillus* spp. strains was determined with the MIC method, using 96 wells sterile plates with lid (Corning Incoporated, Corning, New York). Each plate was set up as follows: column 1, 100µl of culture media plus 50µl of stock solution, i.e. meat protein extract hydrolyzate (positive control), column 2-12: 100µl of media plus 50µl of an overnight inoculum of different target microorganisms: *Lysyeria monocytogenes* Ly56*, Pseudomonas fulva* G*, Pseudomonas fragi* BT*, Leuconostoc lactis* PA22 and *Enterococcus faecium* T2. BHI was used as media for *Lysyeria monocytogenes, Pseudomonas fulva* G and *Pseudomonas fragi,* while MRS for *Leuconostoc lactis* and M17 for *Enterococcus faecium.* The inoculum of each microorganism was about 10^3 cfu/ml. 100µl of a stock solution (meat protein extract hydrolysate) were added at column 3, mixed with micropipette and transferred into the column 4. The same procedure was followed until column 12. Microtiter plates were incubated at 37°C for 24 hours and examined for microbial growth by observing the presence of turbidity. To determine the Minimum Bactericidal Concentration (MBC), 10µl of the mixture used in MIC plates was collected from the wells of each target microorganism that showed very little growth, and inoculated onto sterile nutrient agar. The plates were then be incubated at 37^{o}C for 24 hours. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria is observed on the nutrient agar plates. Therefore the concentration that showed no visible growth after incubation has been considered as MBC (Akinjogunla *et al.,* 2010; Petrus *et al.,* 2011).

3.8 Nitrate Reductase Activity

Nitrate reductase activity was determined as described by Miralles *et al.* (1996) on YTA (1.0% tryptone, 0.5% yeast extract, 1.5% agar, pH 7.0) supplemented with 1 g/l of KNO_3 (Sigma Aldrich, Milan, Italy).

The cell pellet of an overnight culture was resuspended in 10 ml of 50 mM phosphate buffer pH 7.0 and 3 spots of 10 µl loaded in YT agar plates (16g/l Bacto Tryptone, 10g/l Bacto Yeast Extract, 5g/l NaCl, adjusted at pH 7.0 with 5N NaOH). After incubation at 30°C for 24 and 72 hours, the plates were flooded with 1 ml of a 1:1 solution of NIT1 (0.8 g of sulphanilic acid in 100 ml of 5 N acetic acid) and NIT 2 (0.6 g of N-N-dimethyl-1-Naphthylamine in 100 ml of 5 N acetic acid) for the detection of nitrite. The appearance of red haloes surrounding the spots indicates the presence of nitrate reductase activity. It was considered high if the assay was positive for all the 3 spots, medium if positive for 2, low for 1 and absent if the assay was negative.

3.9 Gas-Chromatographic Analysis Of Amino Acids

This technique allows the determination of the amminoacidic fraction of the samples with EFC. The derivatization makes the sample volatile, thus allowing their detection by a gas-chromatograph couples with a mass spectrometer GC-MS).

3.9.1 Derivatization Method Of Meat Protein Extract Hydrolyzates Samples

13. Take 1000 µl of sample and add 10 µl of standard (decanoic acid 500ppm)
14. Add 200 µl of NaOH 0.1 N and mix by vortexing for 20 seconds
15. Add 169 µl of methanol and mix by vortexing for 20 seconds
16. Add 36 µl of pyridine and vortex 20 seconds
17. Add 22 µl of ECF, wait for the heat dispersion, and vortex 20 seconds
18. Repeat point 5
19. Add 400 µl of chloroform and vortex 20”
20. Add 400 µl of $NaHCO_3$ 50mM and mix by vortexing for 20 seconds
21. Separate the superior phase from the lower organic one
22. Add a spatula of Na_2SO_4 and vortex Transfer into a clean conic tube
23. Inject 1 µl of derivatized solution into the GC-MS.

24. Inject 1 µl of derivatized solution into the GC-MS.

Samples were analyzed with an Agilent Hewlett–Packard 7890 GC gas-chromatograph equipped with a mass spectrometer detector 5975 MSD (Hewlett–Packard, Geneva, Switzerland) and a 60 m ·250 µm i.d. fused silica capillary column coated with a 5% difenil dimethylpolysiloxane (Supelco, Palo Alto, Ca, USA). The conditions were as follows: injection temperature, 250 °C; detector temperature, 230°C; carrier gas (He); flow rate, 1 ml/min; splitting ratio, 1:20 (v/v). The oven temperature was programmed as follows: 80 °C for 1min; from 80°C to 240 °C, at 4°C/min then holding for 14 min.

3.10 Salami Manufactures

For the salami preparations, two mixtures were used: a basic salami mixture (20kg) and a mixture with added the commercial starter currently used by the company (20kg).

The basic salami mixture comprised (% w/w) lean pork (70%) and lard (30%), plus KNO_3 (0.015%), $NaNO_2$ (0.015%), NaCl (3.0%), sucrose, dextrose, spices and ascorbic acid. After mincing, one mixture was inoculated with *S. xylosus* e *L. sakei* at a level of 6.0 log cfu/g (mixture with starter). Four species of Lactobacilli were selected for the fermentations of the other one: *L. rhamnosus* C243, *L. casei* lbcd, *L. fermentum* MR13 and *L.sakei* (commercial). They were pre-grown twice in MRS broth (Oxoid, Basingstoke, UK) at 37 °C for 24 hours. After the second incubation period, cells were harvested by centrifugation (8500 g, 10 min, 4 °C), washed and suspended in sterile physiological solution for their utilization.

Five types of salami were produced with the basic mixture and the co-addition of the Lactobacilli species (table 3.7).

After inoculation, the mixtures were stuffed into synthetic gut (diameter of about 5.0 cm), obtaining salami of about 500g. They were kept in a fermentation chamber at about 20 °C for the first 10 days, with the temperature down to 18°C for the following 3 days, at 15°C for one day and then was kept at 13°C until the end of ripening (table 3.7).

Table 3.7: experimental plan for the microfermentations

inoculum / mixture	No inoculum	*L. rhamnosus* C243	*L. casei* lbcd	*L. fermentum* MR13	*L. sakei*	*S. xylosus* and *L.sakei*
Meat with starter	C	CLR	CLC	CLF	CLS	CS

Table 3.8 : fermentation plan for the sausages

Stage	Time (hours)	Temperature (°C)	U.R. %
1	3	18	
2	3	20	
3	3	22	
4	3	24	
5	12	22	50-60%
6	4	21	
7	20	20	55-90%
8	4	18	
9	20	20	55-90%
10	4	18	
11	30	18	55-90%
Ripening	24	13-15	
Ripening	1752	12-13	

3.10.1 Microbiological Analysis

About 10 g of sample were transferred aseptically to 90ml of sterile water and homogenized in a stomacher (BagMixer, interscience, France) for 2 min at low speed at room temperature. Serial decimal dilutions in physiological solution were prepared and 1 ml or 0.1 ml samples of appropriate dilutions were poured or spread on total count and selective agar plates. Total aerobic mesophilic flora was determined on Plate Count Agar (Oxoid), incubated at 30 °C for 72 h; Lactococcus spp. on M17 agar (Oxoid, CM0785) to which 10% v/v lactose was added, incubated at 37 °C for 48 h; Lactobacillus spp. on de Man Rogosa Sharpe (MRS) Agar (Oxoid), incubated at 30°C for 72 h under anaerobic conditions (Gas generating kit, Oxoid, BR0038); staphylococci on Baird Parker Agar (Oxoid) incubated at 37 °C for 48 h; yeasts on Sabouroud Agar (10g/l Universal peptone, 20 g/l glucose), incubated at 30°C for 48 hours.

3.10.2 Physico-Chemical And Chemical Analysis

The measurements of pH were performed by diluting 5 g of sample in 5 ml of distilled water and using a Hanna Instruments 8519 (Incofar, Modena, Italy) pH- meter.

Water activity (aw) was measured using an Aqualab Series 3 instrument (Decagon Device, Inc., Pullman, Washington, USA).

3.10.3 Volatile Profiles

Volatile compounds were monitored at the end of ripening using a gas-chromatographic–mass spectrometry coupled with a solid phase microextraction (GC–MS-SPME) technique. For each sausage, 5 g samples (a slice about 0.5 cm thick broken in small pieces) were placed in 10 ml sterilized vials, and the vials were sealed by PTFE/silicon septa. The samples were then equilibrated for 20 min at 60 °C and volatiles adsorbed on a fused silica fibre covered by 65 lm polydimethylsiloxane-divinyl benzene (PDMS- DVB), (Supelco, Steiheim, Germany). Adsorbed molecules were desorbed in the gas-chromatograph for 5 min. For peak detection, an Agilent Hewlett–Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett–Packard, Geneva, Switzerland) and a 50 m · 0.32 i.d. fused silica capillary column coated with a 1.2 lm polyethylenglycol film (Chrompack CP-Wax 52 CB, Middelburg, The Netherlands), as stationary phase were used. The conditions were as follows: injection temperature, 250 °C; detector temperature, 220°C; carrier gas (He) flow rate, 1 ml/min; splitting ratio, 1:20 (v/v). The oven temperature was programmed as follows: 50 °C for 2min; from 50°C to 65°C, at 1°C/min; from 65°C to 220 °C, at 5 °C/min, then holding for 22 min. Volatile peak identification was carried out by computer matching of mass spectral data with those of the compounds contained in the Agilent Hewlett–Packard NIST 98 and Wiley vers. 6 mass spectral database.

3.10.4 Strains Isolation

During sausages manufacture and ripening isolations of different microorganisms were made: yeasts on YPD agar, lactobacilli on MRS agar and lactococci on M17 agar.

Colonies from countable plates were initially selected for morphologic differences.

Briefly, DNA extraction was carried out from a single colony by using an Insta Gene Matrix (Bio-Rad Laboratories, Hercules, CA).

3.10.5 Rapd PCR

Strains were analyzed with RAPD-PCR, using M13 as universal primer. Amplification was carried out with RAPD-PCR on 10 µl of genomic DNA, using the thermocycler T3000 (Biometra). The reaction mix had 25 µl of volume:

- Taq polimerasi 0,2 µl
- buffer $MgCl_2$ 1X 1,875 µl
- dNTPs 2 µl
- primer M13 1,25 µl (MWG, Milano, Italy)
- dH_2O steril 17, 675 µl

- DNA 2 µl

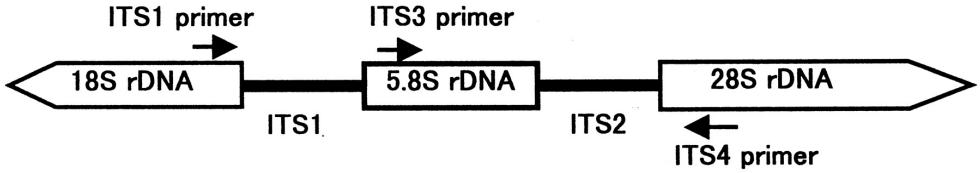
Denaturation was at 94°C for 1 minute; annealing, 45°C for 20 seconds; extension, 72°C for 2 minutes; final extension, 72°C for 5 minutes.

The PCR products were separated on 1,5% agarose gel (Sigma-Aldrich, Milano, Italy) with 0,5 mg/ml of ethidium bromide. DNA was analysed with UV trans illumination and the profiles acquired with a Gel Doc EQ System (Bio-Rad, Germany). RAPD patterns were compared using a Fingerprinting II Informatix TM Software (Bio-Rad).

3.11 Yeast identification and PCR fingerprinting

For the yeast identification, restriction analysis of rDNA region with gene 5.8 r RNA was made and the 2 not codificant regions Internal Transcribed Spacers (ITS) ITS1 and ITS2.

The primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') were used to amplify the region 5.8 rDNA and the 2 next regions ITS1 and ITS2.



Amplifications were made in a reaction volume of 30µl. 2 µl of DNA were added at the PCR mix, containing 0.5µM of every primer, 200µM of every deoxynucleotide 1.5mM $MgCl_2$ and 1X di Buffer and 1U of Ex*Taq* DNA polymerase (Takara Biomedicals). Amplifications were made in a Personal Cycler Vers 3.26 Biotron (Biometra) at the following conditions:

- initial denaturation at 95°C for 5 min,
- 35 denaturation cycles at 94°C for 1 min,
- annealing at 55.5°C for 2 min and extension at 72°C for 2 min
- final extension at 72°C for 10min.

Amplification products were digested with endonucleases of restriction HaeIII, HinfI, CfoI and Taq I (Promega). 10 µl of every sample were added in a mix made of 2µl ultrapure dH_2O, (Eppendorf), 2µl Buffer and 1µl of enzyme. Then the samples were put for 2 hours at 37°C except for enzyme1 Taq I, where the temperature was 65°C.

For the polymorphism analysis of DNA a RAPD-PCR was made using two universal primers M13 (5' GAGGGTGGCGGTTCT 3') and RF2 (5' CGGCCCCTGT 3') (Andrighetto *et al.*, 2000). The 2 amplification reactions were made in a 25µl volume. 2 µl of DNA were added at the PCR Mix containing 1X Buffer, 1.5mM $MgCl_2$, 200µM of deoxynucleotides, 1µM of primer and 1U of Ex*Taq* DNA polymerase (Takara Biomedicals). The conditions of amplification were as follow:

- for the primer M13: initial denaturation at 95°C for 5 min, 35 cycles at 94°C for 1 min, 45°C for 20 sec and 72°C for 2 min; final extension at 72°C for 5 min;
- for the primer RF2: initial denaturation at 95°C for 1 min 30s, 35 cycles at 94°C for 30s, 36°C for 1 min and 72°C for 1min 30s; and final extension at 72°C for 5 min;

RAPD –PCR profyles were analysed with the software Fingerprinting II (Biorad).

The calculation of similarity between the bands was based on the Pearson coefficient profiles and grouped in a dendrogram, with clustering methods using unweighted arithmetic average of the groups.

3.11.1 Electrophoresis On Agarose Gel

Amplifications products were separated by Electrophoresis on an agarose gel (1,5%), in a TAE 1X Buffer (2M Tris, 1M acetate, 100mM EDTA). The length of the bands was evaluated using a 100bp ladder (Promega).

RAPD profyles were separated on a 1,5% agarose gel and the bands compared with a 1kb ladder (Promega).

After the electrophoresis, gels were stained with an ethidium bromure solution (0,5µg/ml) (Sigma) and visualized with UV light. Imagine was acquired with the program Gene Genius Bio Imaging System (SynGene).

3.12 Proteolysis Assessment

The evolution during ripening of the proteolysis products was monitored by SDS-PAGE. Low ionic strength-soluble proteins were extracted from 2 g samples with 40 ml of 0.03 M, pH 7.4 sodium phosphate buffer (Cordoba et al., 1994). The samples were homogenized. The extracts were centrifuged at 8000g for 15 min at 4 °C (Beckman Coulter Avanti J-25, Fullerton, CA, USA), and the supernatants were filtered through a 0.45 µm filter. Total proteins were extracted from 2 g samples with 40 ml of 1.1 M potassium iodide, 0.1 M sodium phosphate, pH 7.4 buffer following

the steps indicated for the above extraction. For denaturation of each protein fraction, 10 μl of extract were mixed with 20 μl of Laemmli sample buffer (Bio-Rad Laborato- ries, Milan, Italy) containing β-mercaptoethanol. The mixture was incubated at 100°C for 5 min. The proteins were then characterized by SDS-PAGE.

CHAPTER 4

RESULTS

4.1 Salami Characterization

During the ripening of six different types of salami, obtained by inoculating commercial starters (*S. xylosus* and *L. sakei*) or strains of *L. sakei*, *L. casei*, *L. fermentum* and *L. rhamnosus*, the following parameters have been evaluated :

- Evolution of the microflora
- pH
- Aw
- Chrome and color

As shown in fig. 4.1 both the starter culture (*L.sakei+S.xylosus*) and the other *Lactobacillus* spp. used as starters reached levels of 9 log cfu/g in all the salami , while the controls (with no starter added) showed a slower growth dynamics and lower final extents.

Micrococci (fig 4.2) reached the highest value of 7 log cfu/g in the products with the commercial starters (*L.sakei+S.xylosus*) after 15 days of ripening and in samples with added *L.sakei* after 20 days of ripening.

Lactococci (fig 4.3) were characterised by a fast development in samples with the starter cultures of *L.rhamnosus, L.casei* and *L.fermentum;* especially in the presence of *L.rhamnosus*, lactococci attained a value of 9.6 log cfu/g after 20days of ripening.

As expected, yeasts (fig 4.4) never get values higher than 6log cfu/g. Their maximum cell load was observed after 15 days of ripening, but it was followed by a fast cell counts reduction.

During the first part of ripening (10-15 days of ripening), about 20 colonies of yeasts were isolated and identified: most of them resulted to be *Debaryomices hansenii,* 7 *Yarrowia lipoytica* and the others *Pichia pastoris.* These strains were subsequently characterized particularly for their proteolytic activity.

The first principal effect of the yeasts' growth is the consumption of lactic acid. Indeed pH (fig 4.5), which lowered down to 5 during the first 8-15 days, reached values of about 6, in all the different salami after 45 days of ripening.

Aw values (fig 4.6) reduced during the ripening, getting the lowest level of 0,88 in samples with added *L.rhamnosus.*

Figure 4.1: Evolution of *Lactic acid bacteria* during ripening of salami obtained by using different LAB species as starters. C=Control (no starters added); Cs= commercial starters (*S.xylosus*+*L. sakei*); CLC=*L. casei*; CLR=*L. rhamnosus*; CLS= *L. sakei*; CLF= *L. fermentum.*

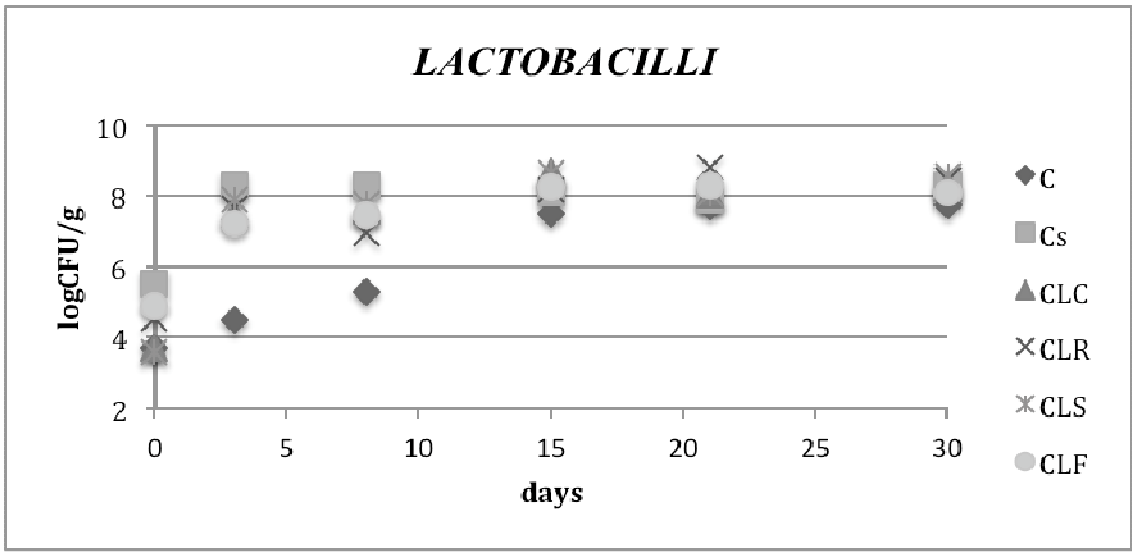


Figure 4.2: Evolution of *Micrococci* during ripening of salami obtained by using different LAB species as starters. C=Control (no starters added); Cs= commercial starters (*S.xylosus*+*L. sakei*); CLC=*L. casei*; CLR=*L. rhamnosus*; CLS= *L. sakei*; CLF= *L. fermentum.*

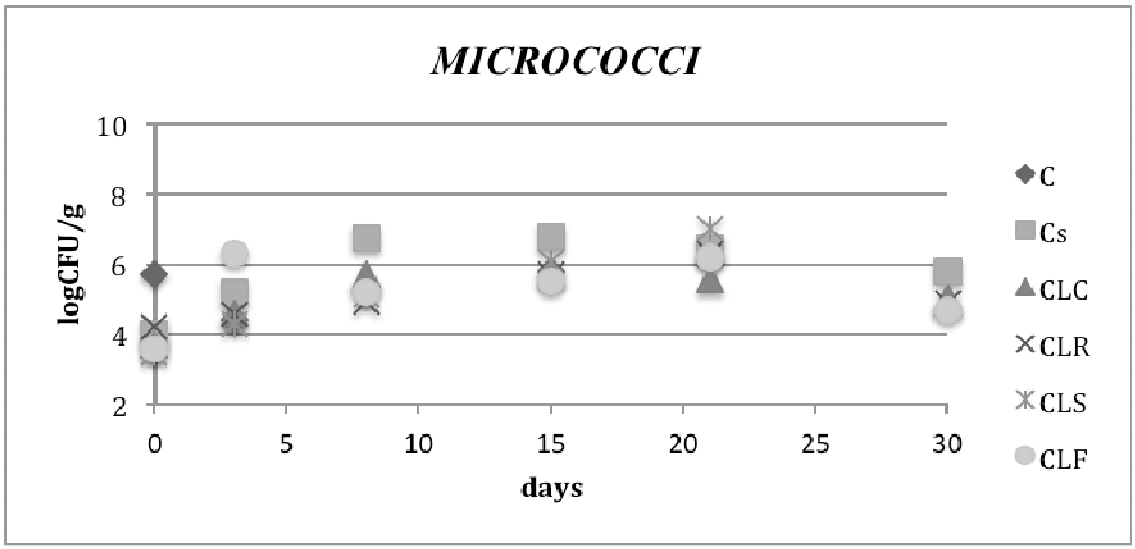


Figure 4.3 Evolution of *Lactococci* during ripening of salami obtained by using different LAB species as starters. C=Control (no starters added); Cs= commercial starters (*S.xylosus*+*L. sakei*); CLC=*L. casei*; CLR=*L. rhamnosus*; CLS= *L. sakei*; CLF= *L. fermentum.*

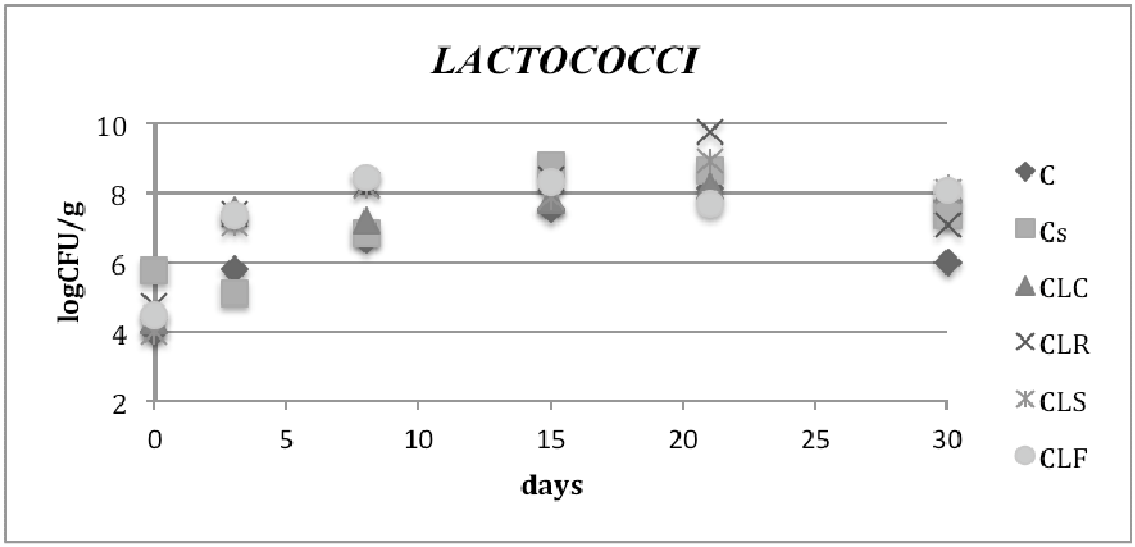


Figure 4.4 Evolution of *Yeasts* during ripening of salami obtained by using different LAB species as starters. C=Control (no starters added); Cs= commercial starters (*S.xylosus*+*L. sakei*); CLC=*L. casei*; CLR=*L. rhamnosus*; CLS= *L. sakei*; CLF= *L. fermentum.*

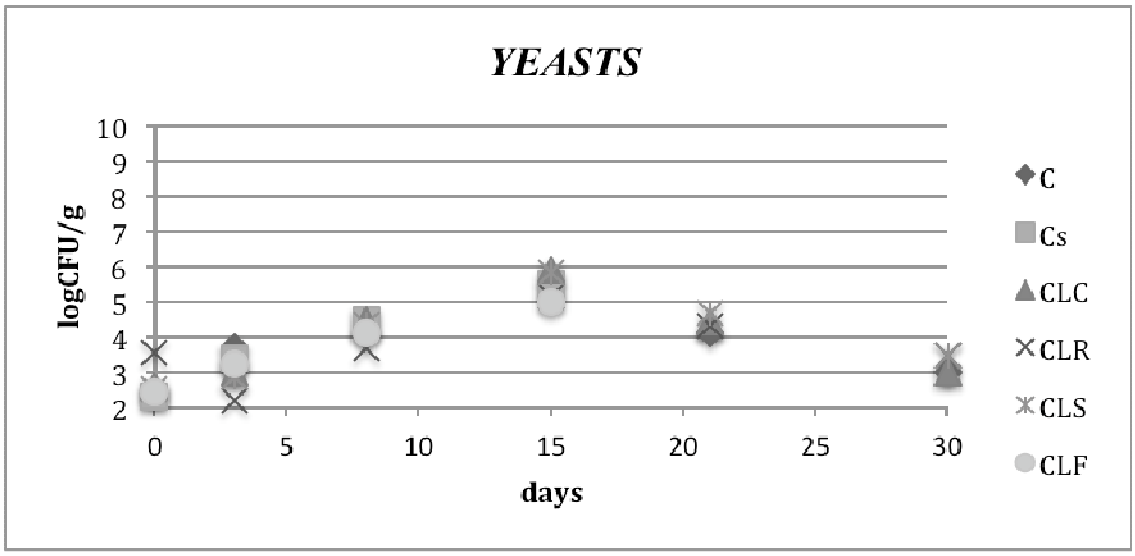


Figure 4.5 Evolution of pH during ripening of salami obtained by using different LAB species as starters. C=Control (no starters added); Cs= commercial starters (*S.xylosus*+*L. sakei*); CLC=*L. casei*; CLR=*L. rhamnosus*; CLS= *L. sakei*; CLF= *L. fermentum.*

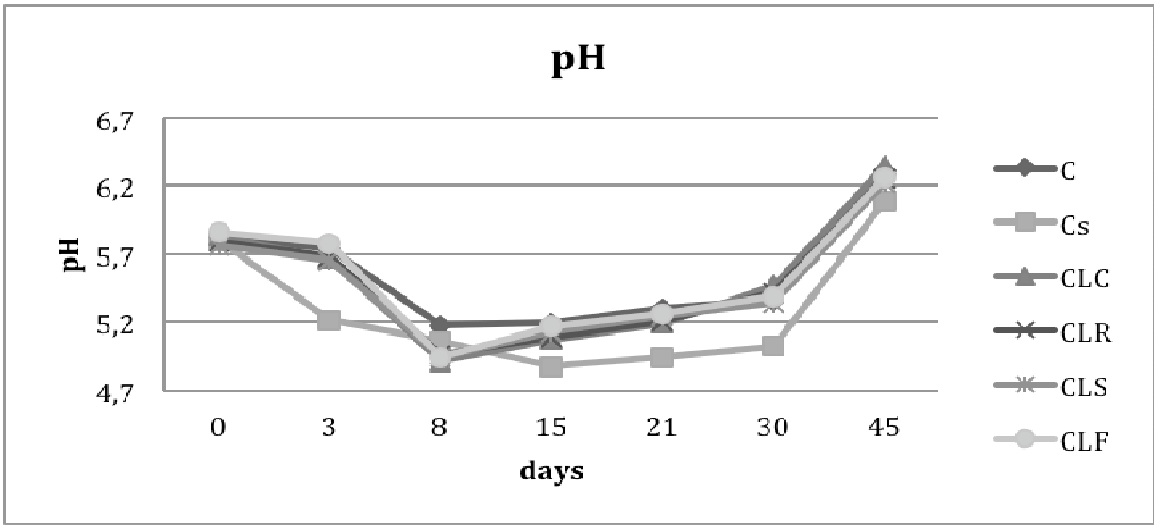
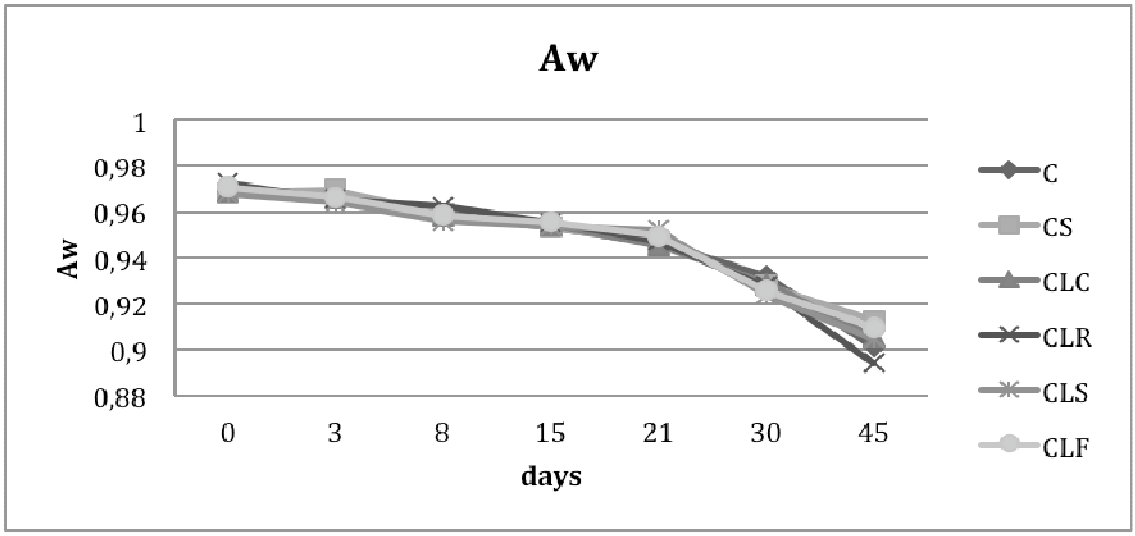


Figure 4.6 Evolution of Aw during ripening of salami obtained by using different LAB species as starters. C=Control (no starters added); Cs= commercial starters (*S.xylosus*+*L. sakei*); CLC=*L. casei*; CLR=*L. rhamnosus*; CLS= *L. sakei*; CLF= *L. fermentum.*

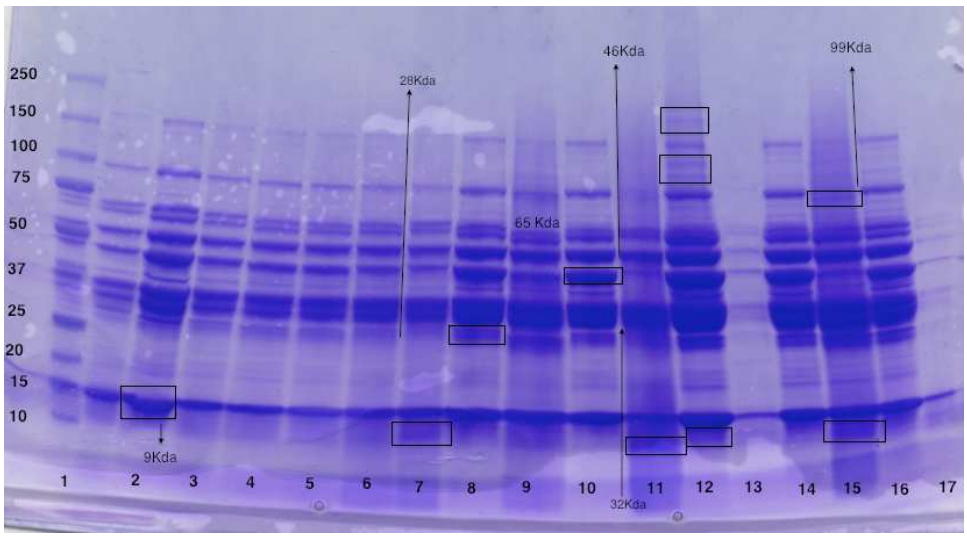


4.2 Proteolytic Characterization Of *Yarrowia lipolytica, Debaryomyces hansenii* And Starter Lactic Acid Bacteria Strains Isolated From Salami

Figures 4.7 4.8 and 4.9 show the electrophoretic profiles of meat total protein extracts hydrolysed by *Yarrowia lipolytica* strains isolated from salami. In particular hydrolyzates were obtained by enzymatic reaction of meat preoteins with n overnight culture of each strain incubated for 48 hours at 28°C.

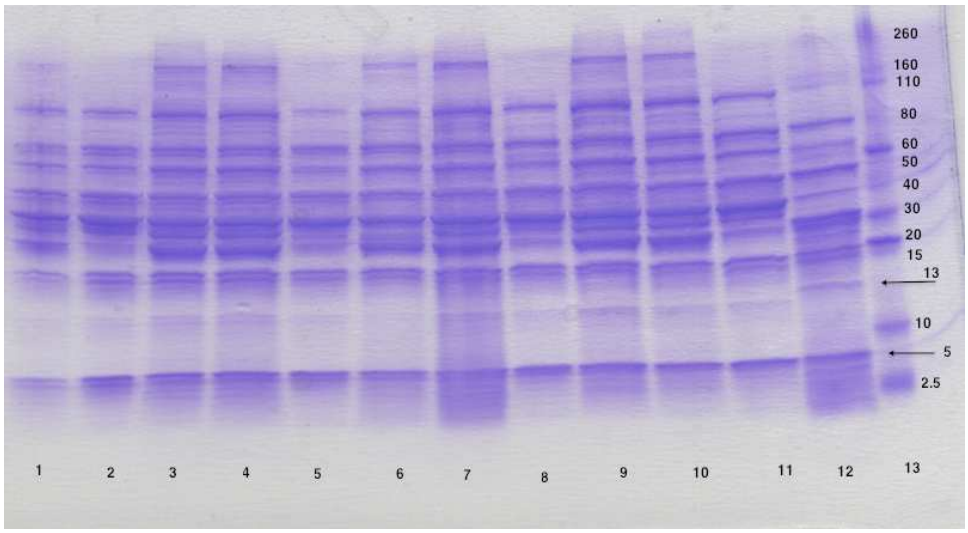
Several differences among strains can be noted, particularly for the peptides with the highest (> 100 KDa) and the lowest (<10 KDa) molecular weights. It is well known that *Yarrowia lipolytica* produces a trans-glutaminase able to crosslink the peptides.

Figure 4.7: SDS-PAGE of meat protein extracts hydrolyzed by different strains of *Yarrowia lipolytica* at 0 and 48 hours of incubation.



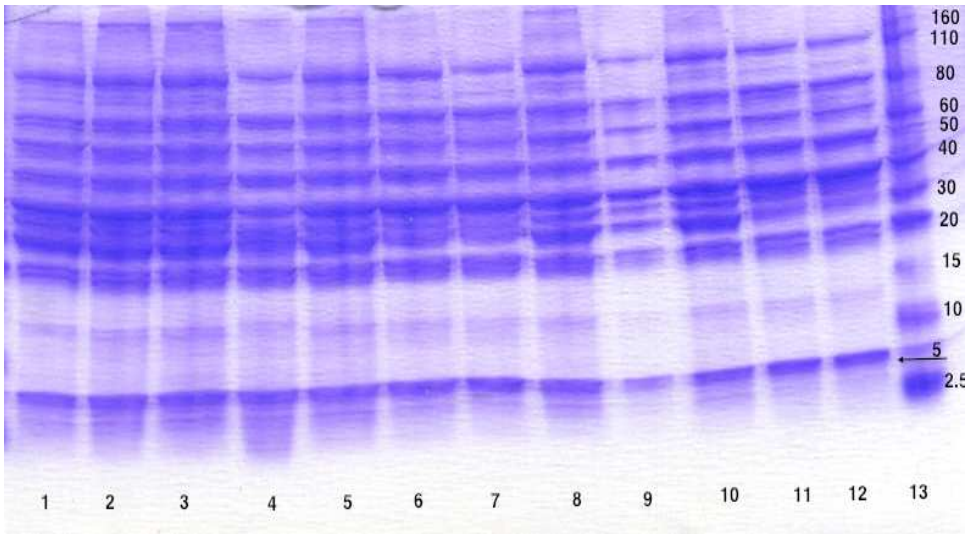
1. Marker
2. 3IIYL3b 0h
3. 3IIYL3b 48h
4. LM2 0h
5. LM2 48h
6. CLCd 0h
7. CLCd 48h
8. CslF2 0h
9. CslF2 48h
10. ClF1 0h
11. ClF1 48h
12. 5D 0h
13. 5D 48h
14. 3IIYL6b 0h
15. 3IIYL6b 48h
16. Y9 0h
17. Y9 48h

Figure 4.8: **SDS-PAGE of meat protein extracts hydrolyzed by different strains of *Yarrowia lipolytica* at 48 hours of incubation**



1. Lbcd
2. LM2
3. RO12
4. PO12
5. YL1A
6. 27D
7. YL3
8. Meat protein extract
9. PO23
10. PO23 dd
11. 4B
12. PO19
13. Marker

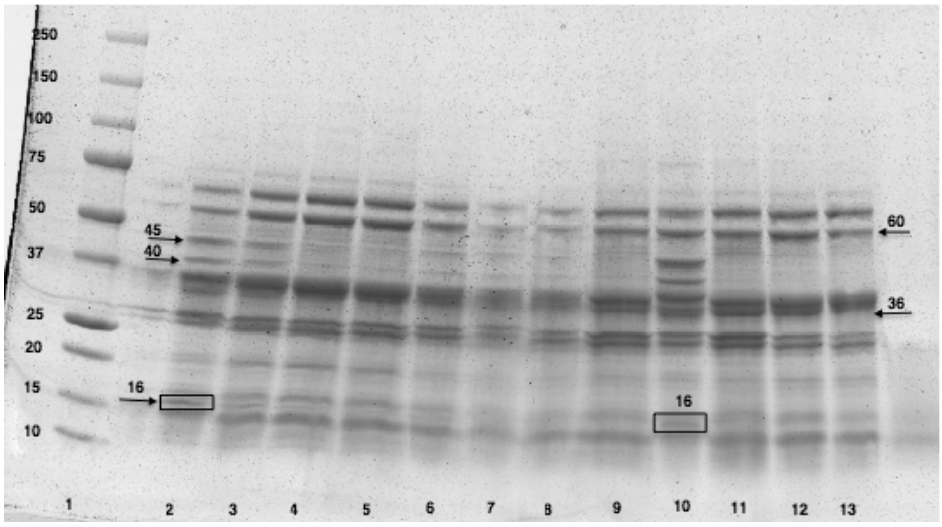
Figure 4.9 SDS-PAGE of meat protein extracts hydrolyzed by different strains of *Yarrowia lipolytica* at 48 hours of incubation



1. RO23
2. 1IIL8A
3. PO14 dd
4. YL1A
5. Meat protein extract
6. RO3
7. PO11
8. R025
9. R022
10. R03
11. RO12
12. YL3
13. marker

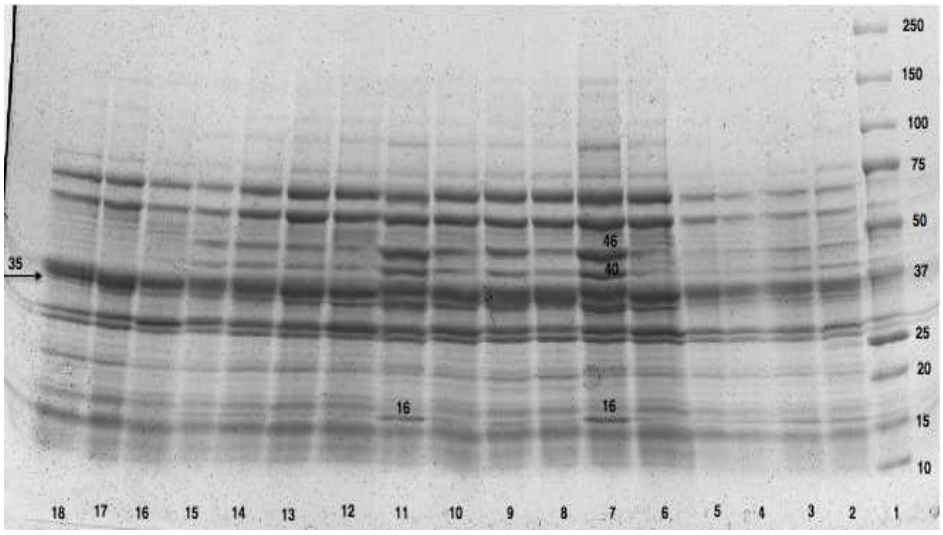
Figures 4.10 and 4.11 show electrophoretic profiles of salami made with different starter cultures that resulted to be colonized by several strains of *Yarrowia lipolytica*. The individual role of each starter can be evidenced by the absence of several bands with molecular weight ranging between 37 and 60 KDa in salami produced with *L.casei.* On the contrary such bands are present in all the other salami.

Figure 4.10:SDS-PAGE of salami produced with different LAB starters and analysed over ripening.



1. Marker
2. *L.rhamnosus* T0
3. *L.rhamnosus* T15 days
4. *L.rhamnosus* T30 days
5. *L.rhamnosus* T45 days
6. *L.sakei* T0
7. *L.sakei* T15 days
8. *L.sakei* T30 days
9. *L.sakei* T45 days
10. *L.casei* T0
11. *L.casei* T15 days
12. *L.casei* T30 days
13. *L.casei* T45 days

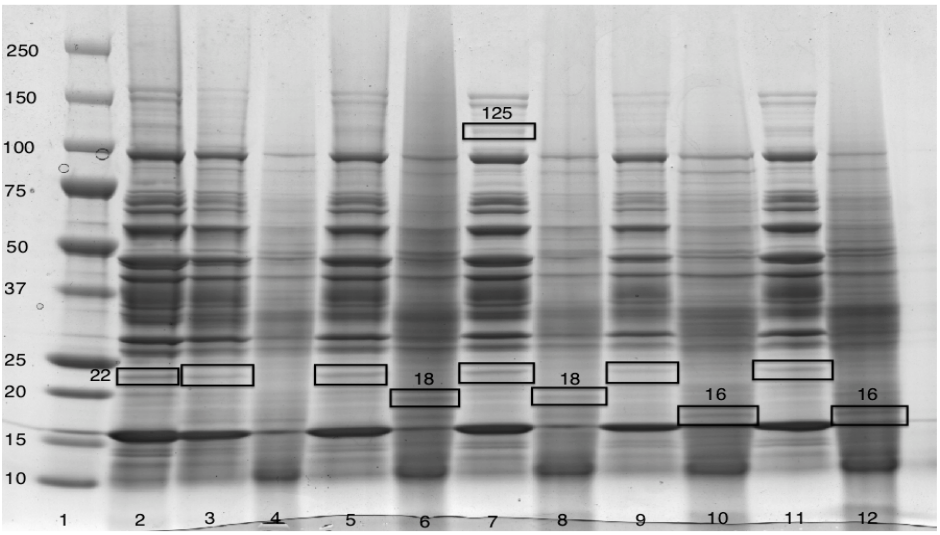
Figure 4.11: SDS-PAGE of salami produced with different LAB starters and analysed over ripening.



1. Marker
2. Control T0
3. Control T15 days
4. Control T30 days
5. Control T45 days
6. Commercial starter (*S. xylosus* +*L. sakei*) T0
7. Commercial starter (*S. xylosus* +*L. sakei*) T15 days
8. Commercial starter (*S. xylosus* +*L. sakei*) T30 days
9. Commercial starter (*S. xylosus* +*L. sakei*) T45 days
10. *L.fermentum* T0
11. *L.fermentum* T15 days
12. *L.fermentum* T30 days
13. *L.fermentum* T45 days
14. *L.fermentum* T45
15. *L.casei* T0
16. *L.casei* T15 days
17. *L.casei* T30 days
18. *L.casei* T45 days

To better understand the role of the LAB in the proteolysis and ripening of salami, a meat model system has been prepared as reported by Vignolo *et al.,* (2004).
As it can be seen in figure 4.12, several differences were detected in relation to the different strains. In fact some peptides with molecular weight of 22KDa, which were present in all samples at at time 0 of incubation, disappeared after 96 hours of incubation at 37°C. On the other hand, the band corresponding to 18 KDa was is present only in samples hydrolysed by *L.fermentum* and *L.rhamnosus.*
Bands with high (about 150 KDa) molecular weight decrease at the end of the period of incubation.

Figure 4.12: SDS-PAGE of meat model system hydrolysed by LAB strains at 0 ad 96 hours of incubation.



1. Marker
2. Meat protein extract
3. Commercial starter (*S. xylosus* + *L. sakei*) 0h
4. Commercial starter (*S. xylosus* +*L. sakei*) 96h
5. *L.fermentum* 0h
6. *L.fermentum* 96h
7. *L. rhamnosus* 0h
8. *L. rhamnosus* 96h
9. *L. sakei* 0h
10. *L.sakei* 96h
11. *L.casei* 0h
12. *L.casei* 96h

4.3 Analysis Of The Electrophoretic Profiles By Heat Maps

In order to better understand the individual contribution of the strains inoculated or not in salami, all the bands detected in the various electrophoretic patterns of salami and meat model systems have been grouped in a single matrix. In particular, grouping has been made on the basis of the molecular weight of the bands, type of samples (i.e. salami or meat model systems), microbial species (LAB and yeasts) and the origin of the strains of *Yarrowia lipolityca* i.e. meat products, fermented meats, salami, chilled foods, water of the Po river, cheeses..

The different colours in the heat map are a measure of the significance of the correlation. In particular in figure 4.13 (heat map 1) it can be noted that profiles of salami made with *L.rhamnosus, L.sakei, L.casei L.fermentum* and with the commercial starters analysed during ripening clustered altogether, and similarity among such samples was due to the bands having the following molecular weights:

- 100 KDa
- 75 KDa
- 65 KDa
- 55KDa
- 40KDa
- 35KDEa
- 47KDa

The second cluster corresponds to the hydrolytic profiles of meat model systems by yeasts isolated from salami and speck. Bands with significant influence are

:

- 160KDa
- 100KDa
- 75KDa
- 65KDa

Less significant is the cluster based on the bands at 55, 37, 30 and 35 KDa. In facts these peptides belong to the proteolytic profiles obtained both *in vitro* model system with yeasts and with salami.

Rather interesting is the behaviour of a strain of *Yarrowia lipolytica* isolated from salami made with *L.casei* as starter culture, whose band at 150 KDa is present only in salami made with *L.casei*, *L.sakei* and *L.rhamnosus.*

The third cluster is made of the electrophoretic profiles of salami ripened with *L.casei, L.sakei, L.rhamnosus* and the commercial starters. They share some bands with the yeasts of different origins, especially the band at 150 KDa and to a lesser extent the bands at 65 and 60 KDa.

The forth cluster include the profiles of meat protein model hydrolyze by yeasts with different origins. In all of them the band with molecular weight of 100 KDa is present; in addition the most important bands are those at 99, 95,80, 90 and 75 KDa, which are also sporadically detectable in all the other samples.

Very common is the band at 68 KDa.

The starters used for both salami production and *in vitro* hydrolysis of meat protein extracts show significantly different profiles, except for some peptides related to the salami. The most significant bands are at 90, 80, 65, 60, 44 and 30 KDa. They are absent in the " *in vivo* system" (salami) except for the band at 100 KDa.

Finally, the meat model profiles related to the yeasts strains isolated from different food sources and the yeast YLCD (isolated from salami ripened with *L.casei*) cluster altogether. Some bands like those at 260 and 120 KDa are related to these samples and the yeast PO23dd, while others, e.g. 99, 93, 80 and 75 KDa, are common only to yeasts isolated from sources different by meat.

Based on this cluster, it can be suggested that *Yarrowia lipolytica* not deliberately inoculated in the product, but simply present on the surface of salami or on its internal parts, seems to contribute to proteolysis of salami in a major and different way from lactic acid bacteria.

This suggestion is supported by the comparison of the proteolytic profiles of salami with those of the hydrolysates obtained in meat model system with different strains of *Yarrowia lipolytica* isolated from cheese, chilled foods and waters of the Po river.

The second heat map (figure 4.14) shows the specific role of different bands on the clustering of the proteolytic patterns in relation to the sample type (salami or meat model system) and the microbial species responsible for them. Particularly the bands at 65, 80, 100, 130 and 160 KDa contribute to the grouping of hydrolytic patterns due to the yeasts from speck and salami with those related to the yeasts isolated from salami during the first stage of ripening.

The bands at 260, 180 and 125 KDa are responsible for the cluster the yeasts from other sources.

Another group is made of all the starter cultures used for the salami production and used for an *in vitro* hydrolysis (meat model system) after 96 hours of incubation at 37°C.

Bands at low molecular weight (about 15 KDa) significantly affect the clustering of the profiles of all the salami regardless the lab starter used.

The last group of bands, having molecular weight of 39, 40, 62 and 63 KDa, is common to the proteolytic profiles produced by the yeasts of different sources and to the yeast strain CLCD which has been isolated from salami produced by using *L.casei* as starter culture.

Figure 4.13 : heat map 1

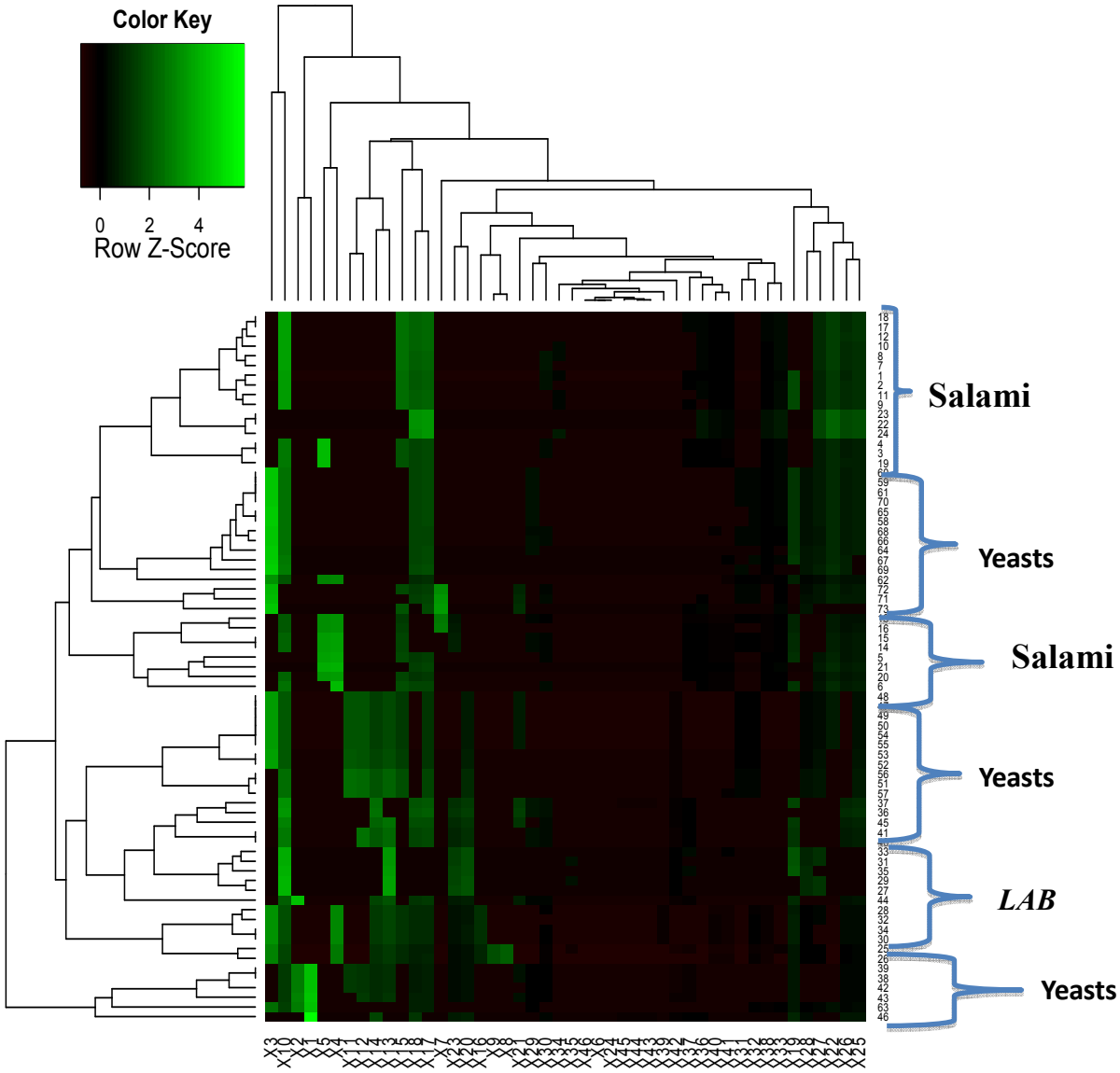
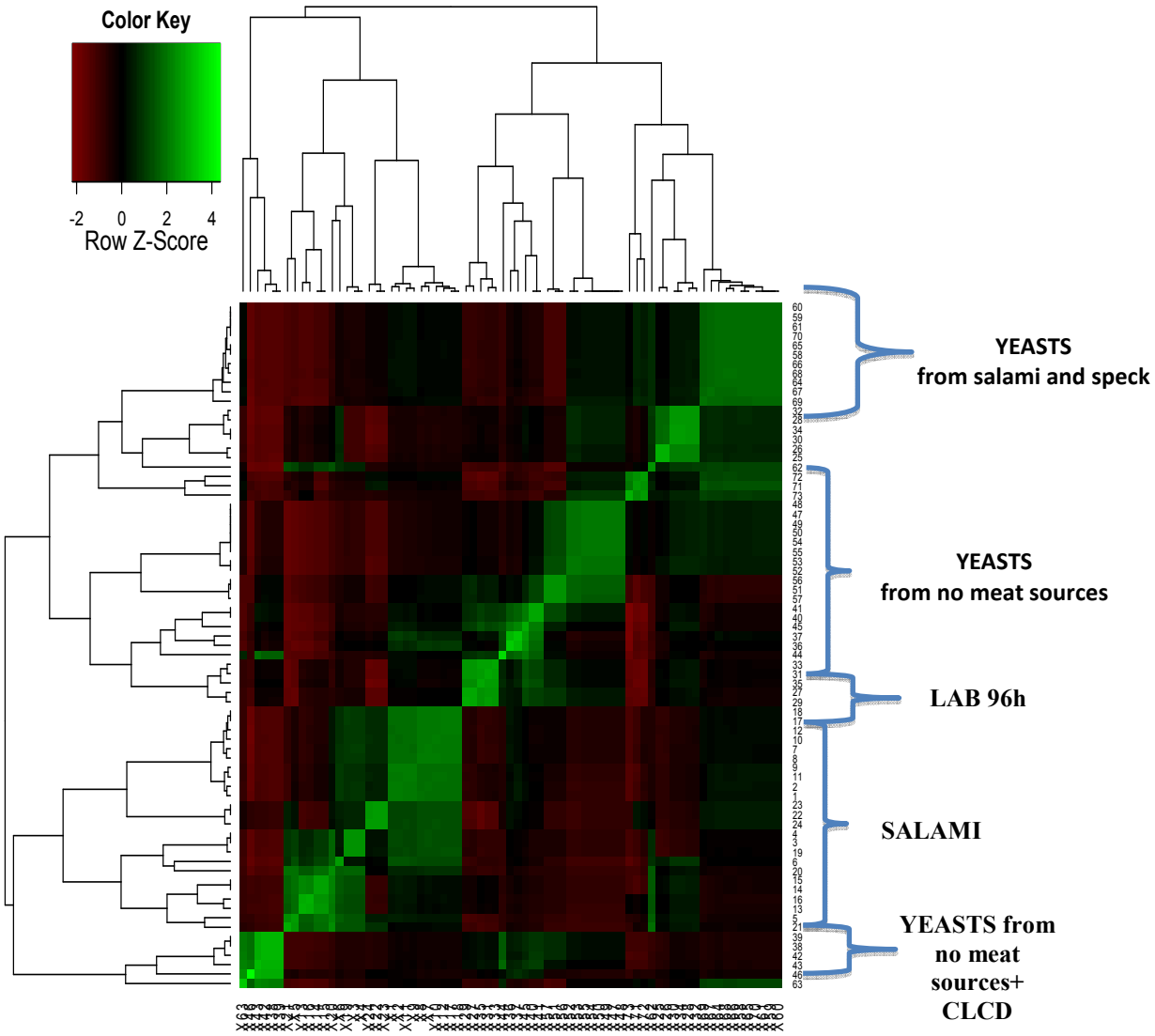


Figure 4.14: heat map2



4.4 Aromatic Characterization Of Salami

Graphs reported in figures 4.15 4.16, 4.17, 4.18 show the evolution of volatiles during the ripening process of salami and namely organic acids, alchools, ketones and aldehydes.

The comparison between all the figures shows that the various molecules detected by GC-MS/SPME have similar behaviours despite their different dynamics..

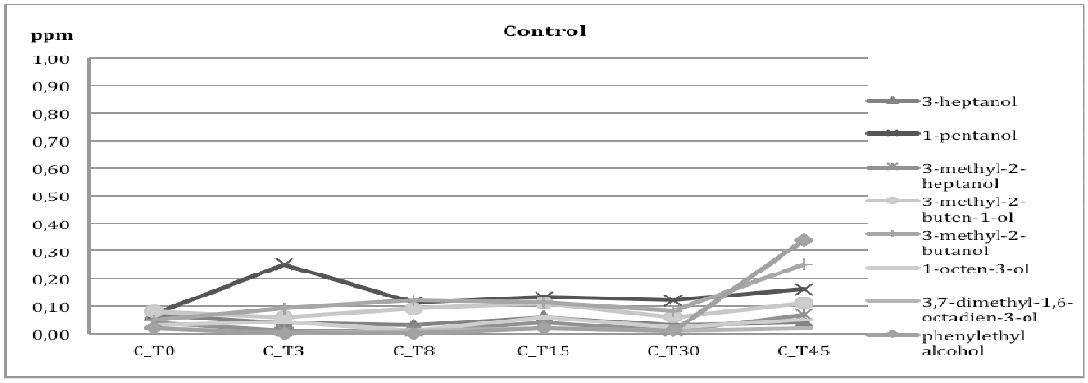
As far as organic acids, acetic acid, butanoic and eptanoic acids, which have a great impact on sensory profile of the products, have been detected (fig4…..)

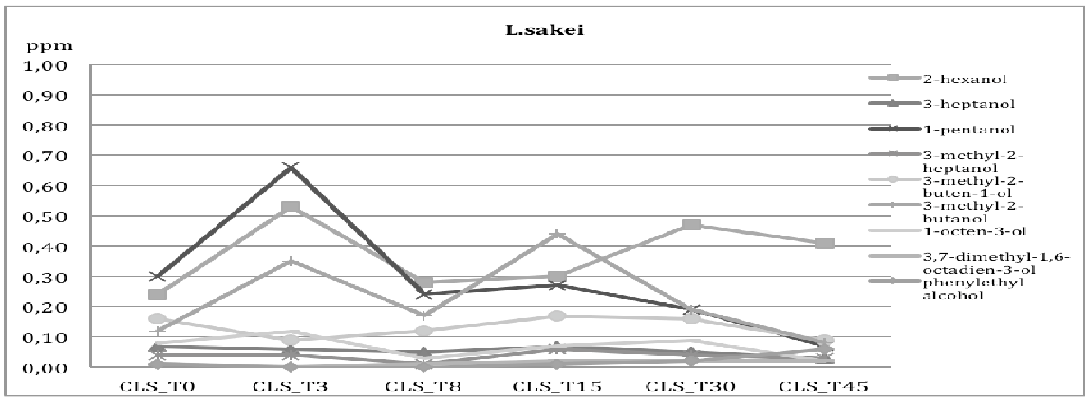
Alchools show different evolution patterns in samples with or without the addition of starter cultures of lactic acid bacteria (fig 4…). In particular the earliest molecule detected in salami with *L.fermentum* is penthanol, while the latest one is hexanol (fig).

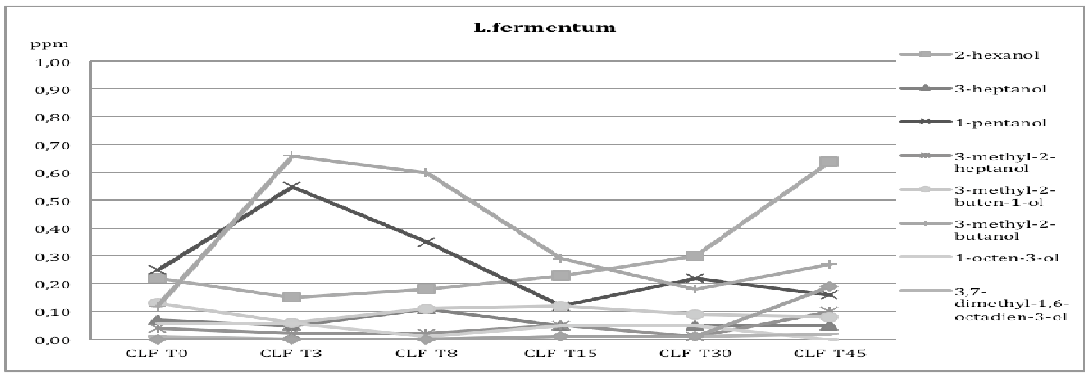
Among aldehydes, hexanal is the earliest molecule, mostly produced in salami ripened with *L.sakei* and *L.casei*, however hexanal is converted to hexanol during the ripening process.

Ketones are present at low concentrations in most of the samples; in salami with *L.rhamnosus* and *L.fermentum* as starters it can be noted a significant higher initial content which decreases during the following phases of ripening.

Figure 4.15: GC Evolution of organic determination of a Figure 4.16 Evolution of Alchools detected by GC-MS/SPME during ripening of salami produced with no starter added or with *L. sakei*, *L. fermentum* and *L. casei* as starters.







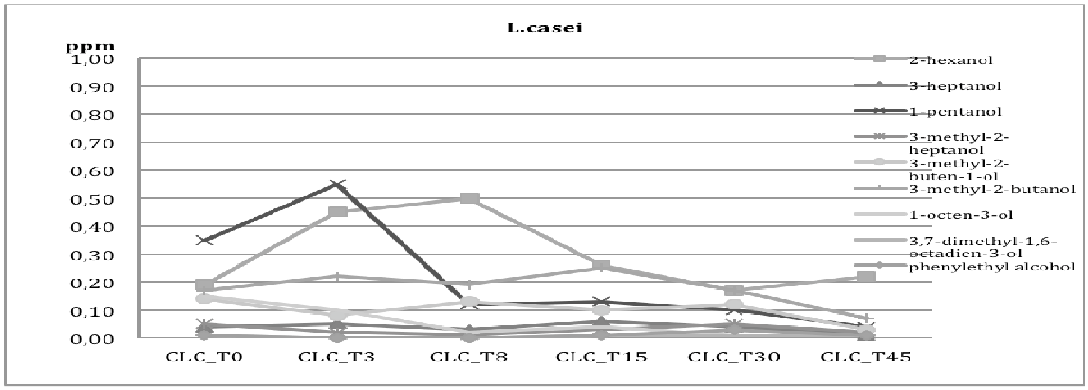


Figure 4.16 Evolution of Alchools detected by GC-MS/SPME during ripening of salami produced with no starter added or with *L. sakei*, *L. fermentum* and *L. casei* as starters.

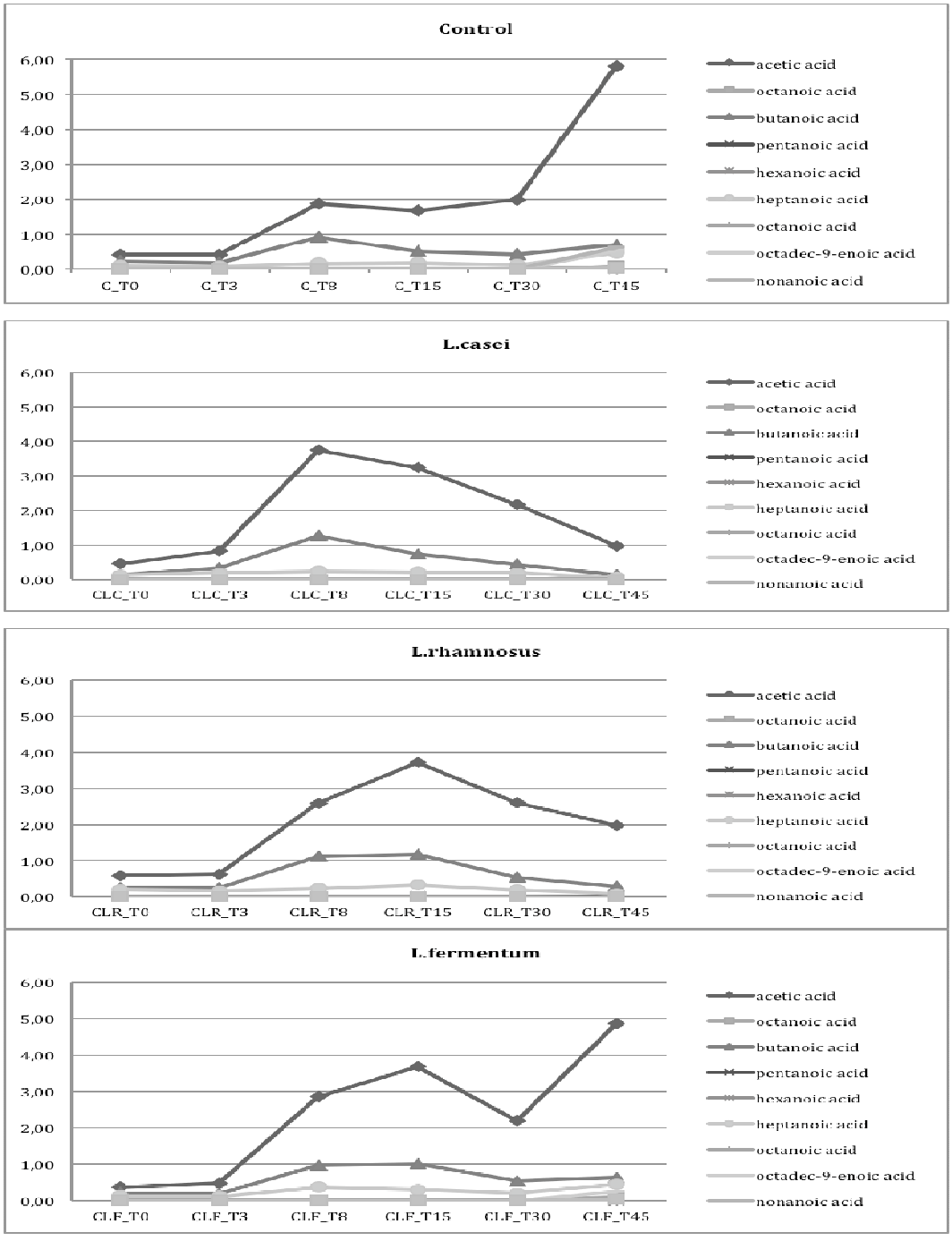


Figure 4.17 Evolution of Aldehydes detected by GC-MS/SPME during ripening of salami produced with no starter added or with *L. casei, L. sakei* and *L. fermentum* as starters.

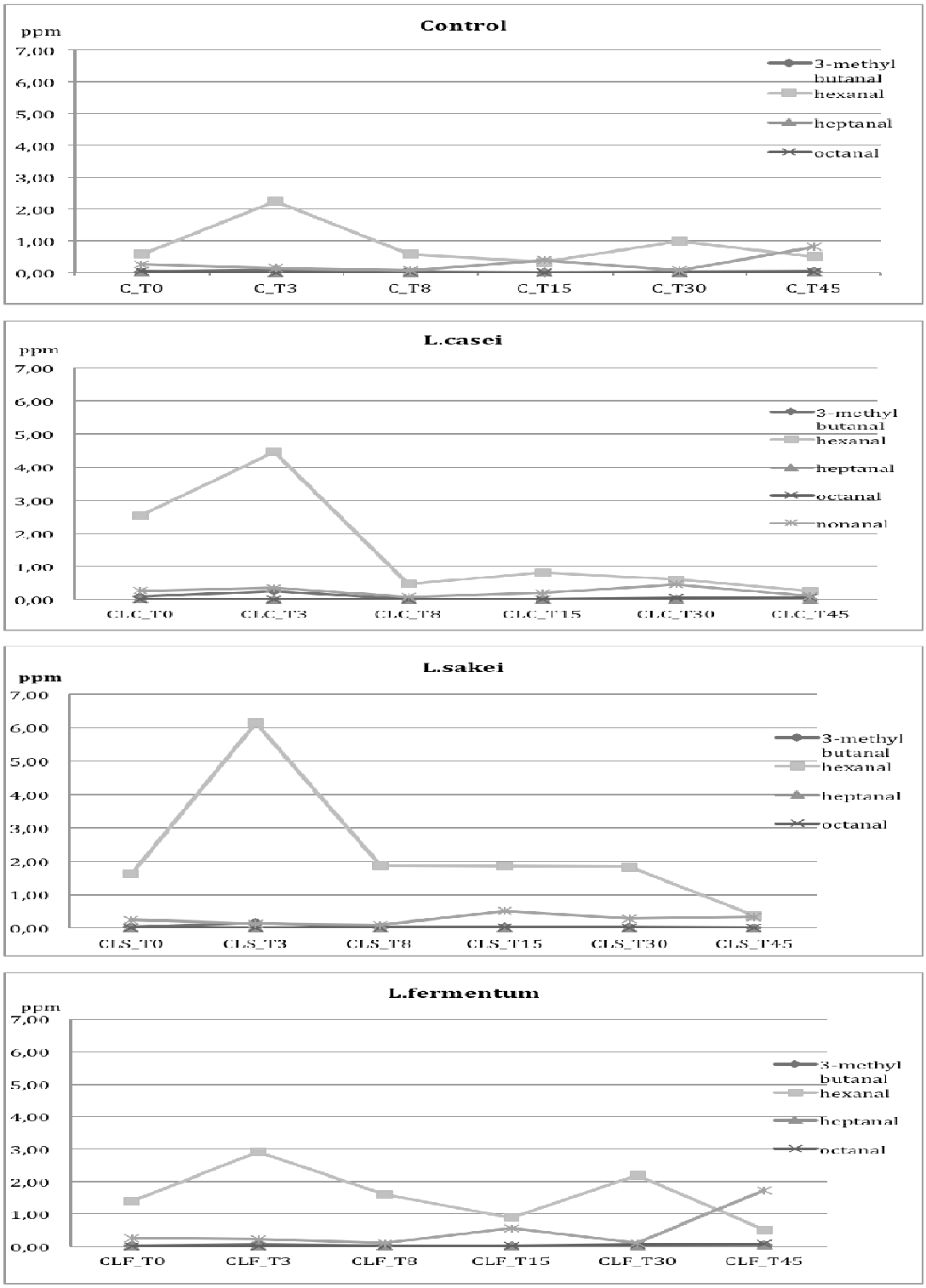
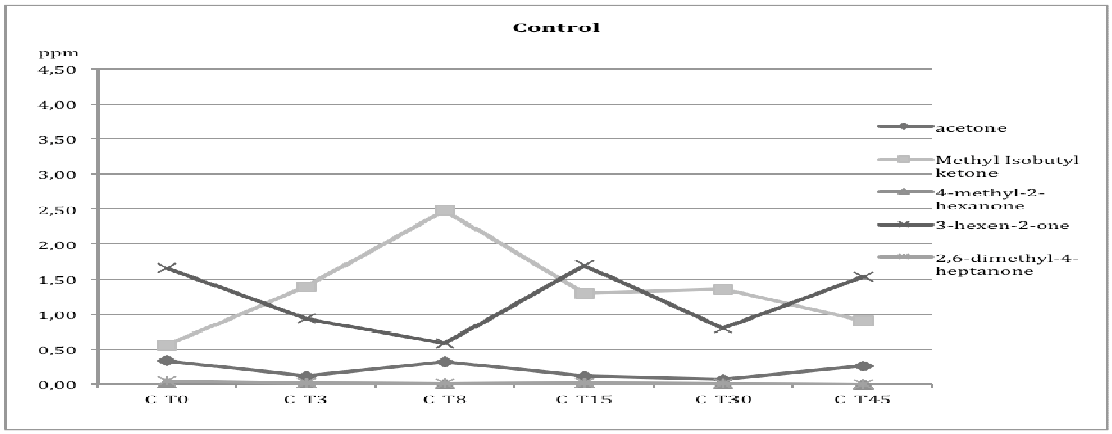
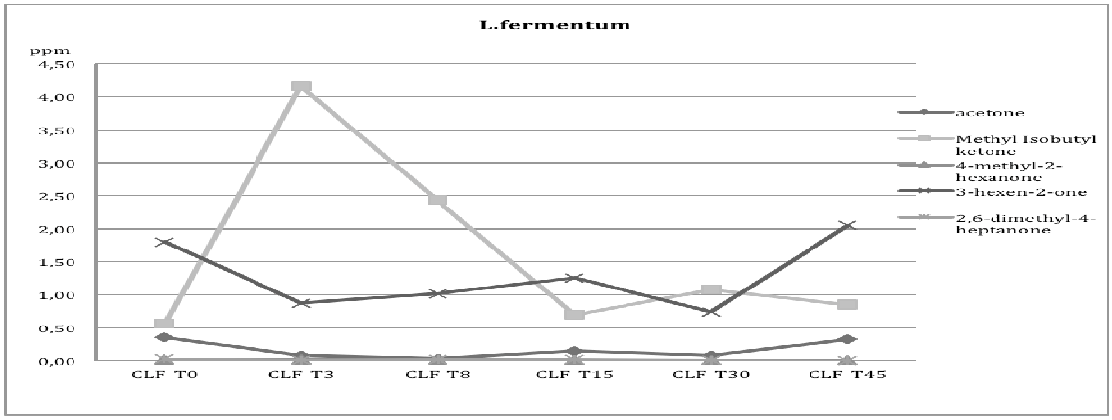
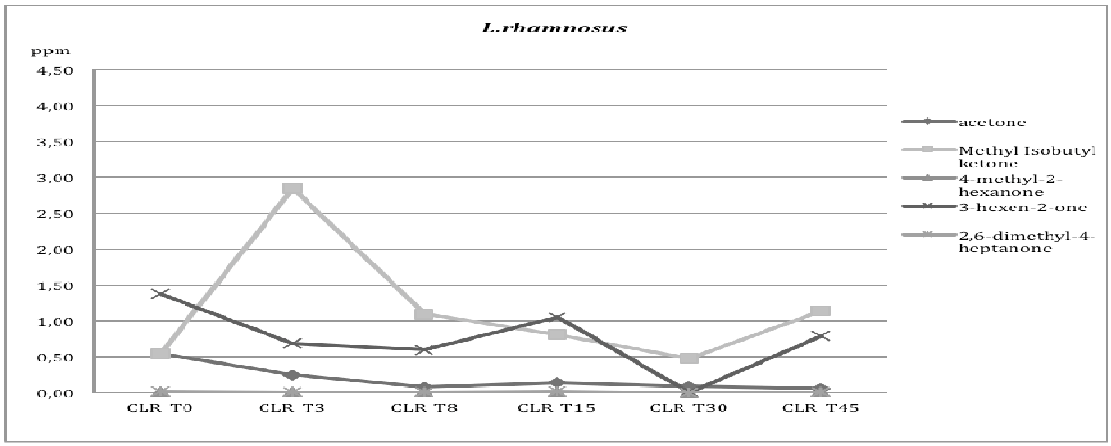


Figure 4.18 Evolution of Ketones detected by GC-MS/SPME during ripening of salami produced with no starter added or with *L. fermentum* and *L. rhamnosus* as starters.







CHAPTER 5

CONCLUSIONS

The identification and assessment of the contribution of both biotic and abiotic factors to the characteristics of the final products is rather hard in food systems such as salami or speck. This is mainly due to the complexity of these foods in terms of raw materials, formulation and processing parameters as well as physico-chemical parameters that change over processing and ripening.

The metabolites associated to the fermentation phase are not always specific for each microbial species. Therefore, it is quite difficult to relate their presence to a microorganism rather than to another one. For instance, both yeasts and lactic acid bacteria produce aldehydes, acids, ketones, alchools and short or medium chain fatty acids.

In meat products without added starters, e.g.speck and ham, the role of several microbial species is underestimated as their presence is detected only onto the product surface. Consequently, several Authors reported that the proteolytic activity in these products has only an endogenous origin.

In this experimental work I have identified several peptides from meat protein extracts, most of which can be assigned to the activity of different strains of *Yarrowia lipolytica* and *Debaryomyces hansenii*, often found in the ecosystem of Speck. On the other hand these peptides were not detected in the hydrolysates of *Yarrowia lipolytica* isolated from other sources such as chilled foods, cheeses, water of the Po river, either in hydrolysates obtained by the lactic acid bacteria strains used as starter cultures for salami production.

Characteristic peptides found in the various salami (i.e. produced with the different starters) were not produced by the same lactic acid bacteria when used in a meat model system (in vitro).

The characterization given by *Yarrowia lipolytica* to salami made with different starter cultures of lactic acid bacteria suggests that this microbial species has ability of cellular penetration. Moreover, due to its ability to release extracellular enzymes, I can hypothesize that a migration of these enzymes in the internal parts of the matrix of the meat occurred.

An example is given by the yeast CLCD, isolated from salami fermented by *L.casei.* Such a yeast wasable to produce in the *in vitro* model the same peptides produced in the salami, where it was isolated, even if total yeasts counts were not really high, i.e. about 6 log cfu/g. Furthermore it should be considered that *Yarrowia lipolytica* is a dimorphyc yeast and originates aggregates during its growth. Moreover, it is highly

hydrophobic as it can be isolated in emulsions at the interface between the aqueous and lipid phase.

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