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DOTTORATO DI RICERCA IN
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Tesi di dottorato

A proteomic approach for the characterization of typical meat products: definition of molecular markers of industrial and artisanal Naples-type salami

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PREFACE

The characterization of the metabolites generated during ripening of the Naples type salami is interesting from either the technological or the commercial point of view. In the first case, the knowledge of the components originated during the maturation of the Naples type salami can be the starting-point for the definition of optimal parameters for the production process and transformation. On the other hand, thanks to marketing strategies, Naples-type salami has become the best known and exported product.
The increasing interest from the producers of Campania to the valorization of this product through acknowledgement PDO (Protected Designation of Origin), pushes to the deeper definition of the characteristics of the product.
The disciplinary of production of the Protected Designation of Origin of "Naples Salami" has been published in the Italian Official Gazette of the 13-07-2004, n. 162. The request for acknowledgement of the P.D.O. to this product, according to the Council Regulation (EC) N. 510/2006 of 20 March 2006 (ex EC 2081/92), is still under evaluation by the M.I.P.A.F. This acknowledgement would imply exclusive feature of the production of the salami of Naples for the Regione Campania: a link that the brand exercises on all the steps of the process of production from the breeders to the confectionery. This will be able to increase the investments in Campania, while the certainty of the controls, guaranteed from the brand, could open new perspectives of market.

The structural characteristics define the nutritional and organoleptic quality of the Naples-type salami.

Naples-type salami supplies proteins with high biological quality from nutritional standpoint, moreover it is rich in iron, zinc, thiamine and riboflavin. The organoleptic and sensory properties are due to the degradation events (proteolysis, lipolysis) occurring during the maturation of salami. During ripening, in fact, the lipid fraction undergoes hydrolytic and oxidative changes, involving liberation of free fatty acids (FFA) and oxidation of unsaturated fatty acids, particularly polyunsaturated acids, with production of carbonyl compounds (Demeyer et al., 1974). The free fatty acid (FFA) during enzymatic and chemical oxidative reactions form directly volatile compounds and precursor of odorous molecules. Additionally endogenous enzymes, such as calpains and cathepsins, are primarily responsible for the initial degradation of the sarcoplasmic and myofibrillar proteins, even if recently it has been demonstrated that the most commonly found *Lactobacillus* species in dry fermented meats are able to hydrolyse myofibrillar and sarcoplasmic muscle proteins in vitro (Fadda et al., 1999). The peptides produced can influence the final taste of the salami and moreover are object of the activity of endogenous and exogenous amino peptidases releasing amino acids which represent precursors of aromatic compounds. The characterization of sarcoplasmic proteins and peptides as well as the analysis of aromas could be used for identification of molecular markers of quality and typicality in order to obtain the P.D.O. mark, to differentiate an artisanal salami from an industrial one and to allow traceability of the products according to EC Regulation 178/2002.
In literature we can already find studies in which a peptide class has been proposed as marker of a defined process, for example the phosphopeptides have been used which markers of the process of maturation of the "Grana Padano" cheese, in particular has been put in evidence the possibility to use like ripening index the phosphopeptides 7-28 of β-casein, 61-79 of αs_1-casein and 7-21 of αs_2-casein (Ferranti et al, 1997); then an innovative method based on MALDI-TOF mass spectrometry has been developed and successfully applied to fish authentication: the signals generated from proteins with molecular weights of about 11 kDa have been selected as specific biomarkers for unambiguous discrimination. This method is also suitable for verifying commercial product authenticity and to rapidly discriminate species subjected to fraudulent substitutions (Siciliano et al, 2008).

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CHAPTER 1: STATE OF ART

1-STATE OF ART

1.1 Meat composition

In the broadest sense, meat is the edible post-mortem component originating from live animals such as domesticated cattle, hogs, sheep, goats, and poultry, as well as wildlife such as deer, rabbit, and fish. Excluding the skin, the carcass component of live animals basically consists of three parts: muscle, fat and bone. Of these, muscle is the most important, constitutes the majority of the weight, and often is considered unequivocally synonymous with "meat".

From the standpoint of histology, the skeletal muscles are made up of:
- Connective tissue organized in epimysium (membrane that surrounds the muscle mass, inside of which originates from the perimysium, which incorporates the muscle, blood vessels and nerves) and endomysial (dense connective network that starts from the perimysium and around each single muscle fiber).
- Muscle tissue: Muscles are composed of tubular cells called myocytes or myofibers. Myofibers are composed of tubular myofibrils. A myofibril is a basic unit of a muscle and composed of long proteins such as actin, myosin, and titin, and other proteins that hold them together. These proteins are organized into thin filaments and thick filaments, which repeat along the length of the myofibril in sections called sarcomeres:

-Thin filaments consist primarily of the protein actin, coiled with nebulin filaments.
-Thick filaments consist primarily of the protein myosin, held in place by titin filaments.

In striated muscle, such as skeletal and cardiac muscle, the actin and myosin filaments each have a specific and constant length on the order of a few micrometers, far less than the length of the elongated muscle cell (a few millimetres in the case of human skeletal muscle cells). The muscle cell is nearly filled with myofibrils running parallel to each other on the long axis of the cell. The sarcomeric subunits of one myofibril are in nearly perfect alignment with those of the myofibrils next to it. This alignment gives rise to certain optical properties which cause the cell to appear striped or striated. In smooth muscle cells, this alignment is absent, hence there are no apparent striations and the cells are called smooth.
The protein complex composed of actin and myosin is sometimes referred to as "actomyosin". Muscles contract by sliding the thin (actin) and thick (myosin) filaments along each other. Actomyosin motors are important in muscle contraction.

Appearance

The names of the various sub-regions of the sarcomere are based on their relatively lig hter or darker appearance when viewed through the light microscope. Each sarcomere is delimited by two very dark coloured bands called Z-discs or Z-lines (from the German zwischen meaning between). These Z-discs are dense protein discs that do not easily allow the passage of light. The T-tubule is present in this area. The area between the Z-discs is further divided into two more faint bands at either end called the I-bands, and a darker, grayish band in the middle called the A band. The I bands appear lighter because these regions of the sarcoma mainly contain the thin actin filaments, whose smaller diameter allows the passage of light between them. The A band, on the other hand, contains mostly myosin filaments whose larger diameter restricts the passage of light. A stands for anisotropic and I for isotropic, referring to the optical properties of living muscle as demonstrated with polarized light microscopy. The parts of the A band that abut the I bands are occupied by the both actin and myosin filaments (where they interdigitate as described above). Also within the A band there is a relatively brighter central region called the H-zone (from the German helle, meaning bright) in which there is no actin/myosin overlap when the muscle is in a relaxed state. Finally, the A band is bisected by a dark central line called the M-line (from the German mittel meaning middle).

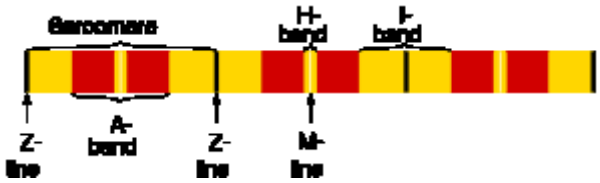

Fig. 1- Structure of sarcomere

Action

When a muscle contracts, the actin is pulled along myosin toward the centre of the sarcomere until the actin and myosin filaments are completely overlapped. The H zone becomes smaller and smaller due to the increasing overlap of actin and myosin filaments, and the muscle shortens. Thus when the muscle is fully contracted, the H zone is no longer visible (as in the bottom diagram, left). Note that the actin and myosin filaments themselves do not change length, but instead slide past each other. This is known as the sliding filament theory of muscle contraction

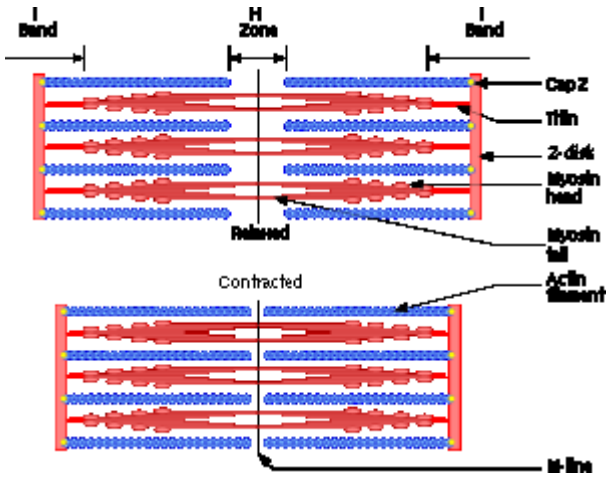

Fig. 2- Sliding filament theory of muscle contraction

1.2- Molecular Composition of muscle

Protein is the basic building material for making cells and its adequate intake can be of particular benefit for those growing or in adults where muscle tissue is being rebuilt, such as athletes or those recuperating post surgery. Meat is a good source of protein and it contains all the essential amino acids. Meat protein has a higher biological value than plant protein as some of the amino acids are limiting in plant protein. For example, lysine is the limiting amino acid in wheat, tryptophan is the limiting amino acid in maize and sulphur- containing amino acids are limiting in soybean. It is necessary for vegans and vegetarians to eat a wide variety of vegetable protein foods to provide the necessary amounts of each amino acid. Meat is a rich source of taurine. Taurine is considered to be an essential amino acid for newborns, as they seem to have a limited ability to synthesise it.
On average, most muscles should contain about 1% ash (primarily represented by the elements potassium, phosphorus, sodium, chloride, magnesium, calcium, and iron), 1% carbohydrate (primarily glycogen ante mortem, and lactic acid post-mortem), 5% lipid, 21% nitrogenous compounds (predominantly proteins), and the rest (72%) as moisture.
There are a host of chemical compounds in muscles. They include free fatty acids, glycerol, triglycerides, phospholipids, non-protein nitrogenous components such as DNA, RNA, ammonia, amine groups, and vitamins. There are glycogen granules and ATP. Myoglobin is present. Several minerals are present in minute quantities.

Most important from a quantitative perspective, there are the various proteins of each fiber. These proteins are classified into four groups, the largest of which is myofibrillar. Myofibrillar proteins represent about 60% of the total proteins, whereas sarcoplasmic proteins represent 29%, stroma proteins 6%, and granular proteins 5%.
The myofibrillar proteins are responsible for the contractile mechanisms and thus shorten or lengthen the muscle for movement and support functions. There are nine known major myofibrillar proteins, quantitatively the one most important protein is myosin that represents 43% of the myofibrillar proteins, 26% of all muscle proteins, 23% of all nitrogenous compounds, and 5% of the fresh muscle mass. Myosin is the thick strand of protein that appears in the sarcomere structure. Actin represents about 22% of myofibrillar proteins and is the thin filament within this same contractile formation. Myosin is composed of six subunits: two identical heavy chain with a molecular weight of 200 kDa spiral wound (heavy meromyosin HMM) and four light subunit with a molecular weight of 16 kDa and 20 kDa giving rise to a double globular head (light meromyosin LMM). Actin exist as a long helicoidal polymer (F fibrous actin) of a globular protein (G actin). The G actin monomer is a molecule with two domains with a molecular weight of 42 kDa. The other seven proteins represent much smaller compositional fractions, but play equally important roles in contraction. Titin represents 8% and has by far the largest molecular weight and is considered more structural than metabolic in function. Tropomyosin (MW 37-40 kDa) and troponin C, I and T (MW18 kDa 37 , kDa and 37 kDa respectively) contribute about 5% and can be found attached to the actin molecule and are primarily responsible for initiating contraction after calcium has been released by the sarcoplasmic reticulum. All the other proteins combined represent less than 20% of the weight.

Sarcoplasmic proteins are primarily represented by enzymes and myoglobin. Sarcoplasmic protein (32% of total protein) are soluble in water or dilute salt solutions. They are responsible for the performance of major life activities of the cell and consist of a complex mixture of 50 components many of which are glycolytic cycle such as: calpains consist of two neutral proteases that require Ca for activation, acting on contractile proteins and those of cytoskeleton; cathepsins or lysosomal enzymes that are acid proteases and divided into: cathepsins B, C, D, H end L.

Stroma proteins originate from the connective tissue structure found as a part of muscle, the most important quantitatively being collagen. About one-third of collagen's amino acid residues consist of glycine, whereas another one-fifth is proline and hydroxyproline. It is the only protein known which contains hydroxyproline, with the possible exception of reticulin. Hydroxyproline analysis is often used as a measure for determining total connective tissue in muscles. Another stroma protein of less concentration is elastin. It is even more resistant to degradation: to degrade, it must be subjected to high temperatures in the presence of strong bases or acids. Elastin contains about one-third of its amino acid residues as glycine and over one-tenth as proline. Reticulin is the other major stroma protein. Its amino acid composition is similar to that of collagen, and it is often considered a form of collagen that contains lipids and carbohydrates.

In addition to the proteins, there are other important nitrogenous constituents in muscle. First are the vitamins, which are divided into two classes based on their solubility in either aqueous or non-aqueous solutions. The lipid-soluble vitamins are minimal because of the small quantities of fat normally deposited in most muscles. However, water-soluble vitamins, primarily the B vitamins, are present in substantive enough quantities to serve as appropriate sources to meet daily dietary requirements for humans. They include thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, and B12. Ascorbic acid [vitamin C] (as well as calcium) is essentially absent in muscles, and because of this, muscles are not considered a perfect food from a nutritional perspective. The nitrogenous, no protein extractives include creatine, nucleotides, ammonia, methylamines, free amino acids, and other derivatives of proteins. Two of the components in highest concentrations are carnosine and anserine. Other extractives include volatile organic carbonyls, such as acetyl aldehyde, acetone, carbon dioxide, and formaldehyde, all of which have been found in muscles. Various sulfur compounds include hydrogen sulfide, methylmercaptans, and methyl sulfides. Some minute quantities of sulfur are present in the form of the amino acids cystine, cysteine, and methionine. Inorganic ions include calcium, magnesium, sodium, potassium, chlorine, phosphorus, and iron, but their contributions to mass are minimal.

1.3. Sarcoplasmic proteins

The sarcoplasmic proteins comprise 30-35% of the total protein in muscle cells. They are involved in such diverse functions as protein synthesis and degradation, fatty acid oxidation, electron transport, phosphorylation, glycolysis, glycogenesis and glycogenolysis.

The sarcoplasmic proteins can be separated into four different fractions based on differential centrifugation: the nuclear fraction, the mitochondrial fraction, the microsomial fraction and the cytoplasmic supernatant .
The nuclear fraction consists of those proteins that are pelleted at 500-1000 g and contains the large molecules, such as DNA, RNA, and other nucleoproteins and lipoproteins. The mitochondrial fraction comprises the next heaviest group of proteins, which are centrifuged out between 1000 and 10000 g. this group consists of about 30 lipoproteins. It contains the mitochondria, the tricarboxylic acid (TCA) cycle enzymes, the enzymes involved in fatty acid oxidation, and the components of the electron transport system, including the flavoproteins and cytochromes, as well as the lysosomes and peroxisomes. The microsomial fraction, which is precipitated by centrifuging at 100000g, containsthe microsomes, the sarcoplasmic reticulum (SR), and ribosomes as well as some other small structural elements present in muscle. The components that remain in solution above 100000g comprise the cytoplasmic supernatant and contain the glycolytic enzymes, the soluble muscle and blood pigments, myoglobin and hemoglobin, as well as some other cytoplasmic enzymes including the calcium-activated factor (CAF).

1.3.1 Cytoplasmic supernatant

Most of the intermediary metabolism and protein synthesis required for cell growth and maintenance takes place in the cytosol, which contains thousands of enzymes that catalyze glycolysis and gluconeogenesis as well as biosynthesis of sugar, fatty acids, nucleotides, and amino acids.
Other constituents of the cytoplasmic supernatant are the muscle pigments such as myoglobin and hemoglobin that play important roles in meat color and oxidation.

1.3.1.1 Glycolysis and gluconeogenesis

Glycolysis involves the splitting of a glucose molecule with six carbon atoms into two molecules of pyruvate, each containing three carbon atoms.
The conversion process involves nine different enzymatic reactions that proceed by way of a series of phosphate-containing intermediates:
Phosphorylation of glucose (reaction 1) is catalyzed by the enzymes hexokinase; conversion of glucose-6- phosphate to fructose-6-phosphate (reaction 2) is catalyzed by the enzymes glucose-6-phosphate isomerase; the formation of fructose 1-6-bisphosphate from fructose-6-phosphate (reaction 3) is catalyzed by 6-phosphofructokinase this reaction is accelerated by a high concentrations of ADP and AMP and inhibit by ATP, citrate, and long chain fatty acids. Step 4 results in formation of 2 mol glyceraldehyde 3-phosphate for every mole of fructose1-6-bisphosphate and is catalyzed by fructose –bisphosphate aldolase that contains several free SH groups that are specific for activity. Also essential in this step is the enzymatic conversion of dihydroxyacetone phosphate to glyceraldehydes-3-phosphate catalyzed by triose-phosphate isomerase.
The oxidation of glyceraldehyde 3-phosphate in 1,3-diphosphoglycerate (reaction 5) is catalyzed by the enzymes glyceraldehyde 3-phosphate dehydrogenase and requires two mol NAD^+. Reaction 6 results in conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate, being catalyzed by the enzyme phosphoglycerate kinase. In reaction 7 phosphoglycerate mutase catalyzes the transfer of the phosphate group from the 3 to the 2 position on glyceric acid, with formation of 2-phosphoglycerate from 3-phosphoglycerate. Reaction 8 results in 2-phosphoglycerate forming phosphoenolpyruvate and is catalyzed by the enzyme enolase. The final reaction in glycolysis results in formation of pyruvate from phosphoenolpyruvate (reaction 9) and is catalyzed by pyruvate kinase. This reaction results in formation of 2 mol ATP for each mole of glucose or other hexose going through the glycolytic pathway.

1.3.1.2. Citric acid cycle

In the citric acid cycle, pyruvate enters through its condensation with acetyl-CoA catalyzed by pyruvate dehydrogenase (reaction 1). An aldol condensation between the methyl group of acetyl-CoA and the carbonyl group of oxaloacetate to form citrate (reaction 2), is catalyzed by citrate synthase. Reaction 3 is catalyzed by aconitase and results in formation of isocitrate from citrate. Reaction 4, catalyzed by isocitrate dehydrogenase, is a rate-limiting step and results in conversion of isocitrate to α-ketoglutarate. The enzyme requires Mg^{2+} or Mn^{2+}. Reaction 5 results in conversion α-ketoglutarate to succinyl-CoA and is catalyzed by α-ketoglutarate dehydrogenase that requires thiamin pyrophosphate, lipoic acid, CoA, FAD, and NAD^{+} as coenzymes. Reaction 6 is catalyzed by succinate thiokinase, and in it succinyl- CoA loses its CoA group to form succinate. The conversion of succinate to fumarate (reaction 7) is catalyzed by succinate dehydrogenase. Reaction 8 results in conversion of fumarate to L-malate and is catalyzed by the fumarate hydratase. The final conversion of the citric acid cycle involves oxidation of malate to oxaloacetate, which is catalyzed by malate dehydrogenase.

1.3.1.3. Other cytosolic enzymes

Numerous other enzymes are present in the cytoplasmic supernatant, many of these are proteases anima be involved in the dynamic systems that function in protein accretion and degradation in muscle.
The proteases in muscle con be divided into three main groups: alkaline proteases, acidic proteases or cathepsins and neutral proteases activated by Ca^{2+} (CAF). The alkaline proteases are apparently bound to myofibrillar proteins and degrade the muscle proteins, hemoglobin, serum albumin, casein and their activity is enhanced by thiol reagents.
Neutral proteases (CAF) hydrolyze troponin T, troponin I, C-protein and tropomyosin; immunofluorescence studies demonstrated that CAF is localized only in the Z-disk of the myofibril (Dayton e Schollmeyer, 1981).
The acid proteases exhibit optimal activity at acidic pH such as cathepsins classified as sarcoplasmic proteins. Cathepsins A, B, C, D and L have been isolated from skeletal muscle (Obinata et al.,1981). Although cathepsins A and C can degrade synthetic peptides, they do not degrade native proteins however they play a role in breakdown of proteolytic fragments in muscle.
Cathepsins B has been shown to be active in degradation of myosin and actin (Schwartz e Bird, 1977); cathepsins D hydrolyzes myosin, actin and hemoglobin, then cathepsins L breaks down myosin actin, α-actinin, troponin T and troponin I but does not degrade troponin C or tropomyosin (Okitani et al., 1980; Matsukura et al., 1981).

1.3.1.4. Respiratory pigments

All of the respiratory pigments are heme proteins, consisting of a porphyrin nucleus attached to a protein prosthetic group. The pigment responsible for most of meat color is the protein myoglobin. This protein consists of a globular protein of about 153 amino acids with a molecular weight of 16,800 Da, housing a porphyrin ring structure held in a pocket of the protein. At the ring's core is a cavity large enough to hold a transition metal ion, in myoglobin the metal is iron. The combination of iron and porphyrin is called heme. Myoglobin is chemically very similar to the blood protein hemoglobin, which also contains iron bound in porphyrin. Muscle contains some hemoglobin, but its hemoglobin content is much lower than that of myoglobin. Because myoglobin is the dominant pigment in muscle, measurements of iron concentration, myoglobin concentration, and color are all strongly correlated. If one muscle appears redder than another, it very likely contains more myoglobin and, thus, more iron. The porphyrin ring structure held in the confines of the myoglobin protein accounts for four of the six coordination sites available on the iron atom. These four sites are the nitrogen atoms of the porphyrin's pyrrole groups. A fifth coordination site is a strategically placed histidine molecule resident in the globular protein. The sixth coordination site is available for binding oxygen or other small molecule that qualifies. Binding at the sixth site is largely responsible for the various colors of meat, mainly red, but also purple, brown, and other colors. In its role as an oxygen store, the sixth coordination site on the iron in myoglobin's porphyrin ring reversibly binds molecular oxygen (Oxymyoglobin). When

bound, the color of myoglobin changes from a purple-red to a bright red. The iron atom at the business end of the myoglobin molecule in these two forms is in the ferrous oxidation state. For this reason, myoglobin in the ferric state—called metmyoglobin—cannot bind oxygen but binds a molecule of water instead. Metmyoglobin's inability to bind oxygen is crucially important because metmyoglobin slowly forms from oxymyoglobin, both in live muscle and in meat. In the case of the live animal, metmyoglobin formation must be avoided because metmyoglobin is useless as a oxygen store. In the case of meat, metmyoglobin formation must be avoided because this pigment is brown and not the attractive bright red that consumers value.
Hemoglobin functions as the carrier of oxygen from the lungs to the tissues (muscle) where the oxygen is exchanged with CO_2. Hemoglobin contains four heme groups per molecule and has an approximate molecular weight of 67000 Da. It undergoes the same reactions with oxygen as myoglobin, forming oxyhemoglobin and methemoglobin.
Catalase destroys hydrogen peroxide and prevents it from reaching toxic levels in the cell. It has a molecular weight of about 250000 and contains approximately 0.09%. The iron in catalase is normally in the ferric state. Peroxidase has a molecular weight of about 44000 Da and also catalyzes the breakdown of hydrogen peroxide.

1.4 Description and composition of fat

Lipids include that group of non-polar compounds soluble in organic solvents but insoluble in water. Pure lipids are colorless, odorless, and flavorless and can be classified as follows:
1. Simple Lipids are esters of fatty acids with certain alcohols such as glycerol. If lipids are solid at room temperature, they are called fats; if liquid, oils. Waxes are simple lipids that are esters of fatty acids with long-chain aliphatic alcohols or with cyclic alcohols. Examples of waxes include esters of cholesterol and the vitamins A and D.
2. Compound or Conjugate Lipids are esters of fatty acids that, on hydrolysis, yield such substances as phosphoric acid, amino acids, choline, carbohydrates, and sulfuric acid, in addition to fatty acids and an alcohol. Examples include phospholipids, glycolipids, sulfolipids, and lipoproteins.
3. Derived Lipids are formed in the hydrolysis of simple or compound lipids. Examples include saturated and unsaturated fatty acids, aliphatic alcohols, sterols, alcohols containing the Beta-ionone ring, aliphatic hydrocarbons, carotenoids, squalene, and the vitamins D, E and K.
Fat is found in nearly every anatomical location imaginable, but the great majority of it occurs subcutaneously, inter- and intramuscularly, in the mesentery, on the walls of the thoracic, abdominal and pelvic cavities, and in the bone marrow (intra-skeletal).
Fat is deposited in the udders of females and in the scrotal sacs of male castrates. Fat is deposited in brain, liver, and kidney, and the quantity may be excessive under abnormal conditions.
Lipids are found in some form in all body cells because phospholipids contribute to the structure of every cell wall. Blood and lymph contain lipids, the quantity varying greatly with time after an animal consumes a fatty meal. All dietary fats are transported to body tissues via one of these routes. Although adipose tissue is ubiquitous, it is not evenly and universally distributed in obesity, but is deposited in certain preferential sites while others are spared. For example, feet, eyelids, nose, ears, and genitalia seldom accumulate excess fat.

1.5 Muscle Metabolism

Glucose and fatty acids enter muscle cells by escape from the capillaries, diffusion through the extracellular space, and active transport across the muscle cell membrane. Glycogen is a polymer of glucose that is stored in muscle in preparation for ATP generation. Resting muscle or muscle undergoing mild activity relies primarily on fatty acid metabolism for ATP synthesis. However, intense work may require more ATP-generating capacity than can be provided by lipolysis and fatty acid transport from the bloodstream (Brooks, G.A 1998). There is a small supply of triglycerides in the muscle cell that could liberate fatty acids, but this pathway does not appear to be significant for producing ATP.
Both the glycolysis from glycogen and glucose as well as degradation of fatty acids result in the generation of pyruvate. In living muscle, pyruvate is usually transported to the mitochondria for further oxidation to carbon dioxide and water. The mitochondrial pathway yields a much larger amount of ATP than the glycolysis steps. The greatest rate of ATP utilization in the muscle cell occurs during contraction. However, there is a continual need for ATP to power the calcium and

sodiumpotassium pumps in the cell. An additional backup source of high-energy phosphate bonds can be provided by creatine phosphate (CP). The enzyme creatine phosphokinase catalyzes the reaction of ADP plus CP to generate ATP plus creatine (C). When the ATP and C levels are high, the enzyme operates in the reverse direction to generate new CP. ATP levels are typically about 5 mMolar in the muscle cell, but CP levels may reach 20 to 30 mMolar in quiescent muscle. The transfer of the phosphate from CP to ATP is very rapid. Historically there was much controversy about whether ATP really was required for muscle contraction because no decline in ATP levels could be detected during a twitch, the creatine phosphokinase reaction restores ATP to resting levels so quickly that the ATP level stays essentially constant. However, the limited quantity of CP (probably no more than 5-fold greater than the ATP levels) means that it rapidly becomes depleted during short periods of intense work.

1.6 Postmortem protein changes in muscle

Muscle tissue is complex structurally and is nonhomogeneous in its protein composition and metabolic emphasis. The rate and extent of metabolic changes postmortem have important effects on the color of meat, its texture, and its usefulness for inclusion in processed meat products. A number of postmortem changes in the muscle proteins have been identified. The myofibrillar proteins desmin, troponin T, titin, nebulin, and vinculin all become partially or completely degraded during the first week postmortem (Boehm, M.L. et al., 1998.). Although the proteolytic enzymes responsible for this degradation have not been unequivocally identified, the patterns of fragments generated and the proteolytic susceptibility in vitro all suggest that the calpains are involved. Calpains are calcium-activated proteases originally described by Dayton and coworkers (Dayton, W.R. et al.,1976). Three different isoforms of the calpains have been identified in muscle m-calpain, μ-calpain, and P94-calpain. Millimolar levels of calcium activate m-calpain, and μ-calpain requires only micromolar concentrations for activity. The recently described P94-calpain is slightly larger than the other two isoforms; its role in protein degradation is unknown since to date it has been not possible to isolate the enzyme from muscle in an enzymatically active form. The current hypothesis regarding postmortem protein degradation suggests that the calcium in the sarcoplasmic reticulum leaks into the cytosol and activates the calpains after the muscle ATP is depleted (Koohmaraie, M. 1996.). A number of pieces of evidence support this hypothesis. First, soaking muscle strips in calcium solutions or in-jecting muscle with calcium results in increased proteolytic degradation. Second, animals such as the callipyge lamb that have higher muscle levels of calpastatin (the natural inhibitor of the calpains) have lower rates of postmortem protein degradation (Geesink, G.H. et al., 1999). Third, the proteins that are degraded by the calpains in the test tube are the same ones that are broken down in postmortem muscle. The calpains are maximally active near pH 7, so they would be expected to have much lower activity at the usual ultimate pH of around 5.5. The activity of μ-calpain declines rapidly post mortem; m-calpain is more stable (Bendall, J.R, 1973.). Calpastatin activity also declines after death, so it remains unclear which of these components are most important in controlling postmortem protein degradation.

1.7 Sausages

Sausages are a unique type of comminuted meat products that are usually spiced or seasoned to obtain various flavor intensities and profiles. The development of sausages was initially driven primarily by economic factors, i.e., it utilizes low-quality meats such as trimmings, head and shoulder meat, and edible by-products. Convenience and variety are other important reasons why sausages are widely consumed in modern society. In the United States, about 4 billion kg of sausage products are produced annually, and the per capita consumption is estimated to be 15 kg per year. Based on the product characteristics and the specific processing method used, sausages can be classified into three major groups: fresh sausages, cured sausages, and fermented sausages.

Technologically, sausage making consists of several common steps—comminution to reduce meat and fat particle size (grinding, mincing, chopping, or flaking), mixing with ingredients, stuffing into a specific casing, linking to obtain specific lengths, and finally, packaging.

Ingredients

- Raw Meat

A proper selection of meat ingredients is essential for the production of sausages of uniform quality. Raw meats used for sausages are generally low-valued materials, but they must be fresh, i.e., with very low microbial counts. These include cuts high in connective tissue or fat, tough meat from mature animals, carcass trimmings, mechanically separated meat, and edible animal by-products. The function of each selected raw meat ingredient may be unique. Meats used for binding should have a sufficiently high protein content and the proteins should be readily extracted and form gels during cooking. Skeletal muscle from cows, bulls, and sows is an excellent meat binder, whereas high-fat trimmings are generally poor binders. There is another group of meats that are included in sausage formulations to "fill" the void space in sausages. Filler meats have little or no binding ability, and they include offal meats (e.g., tripe, snouts), skin, and partially defatted beef and pork tissue.

The rapidly increased use of poultry meat in the sausage industry is worth particular mentioning. Poultry meat has been blended into pork and beef sausages. The increased use of poultry meat in sausage production has resulted mainly from the relatively low cost for poultry meat (particularly turkey), and increased consumer demands for "light meat," which is perceived as more healthy than red meat.

- Salt and Nitrite

Salt is the single most critical nonmeat ingredient. The main form of salt utilized in sausage production is sodium chloride. Its principal function is to solubilize and extract the myofibrillar proteins needed to form a bind during cooking. Of course, it also imparts flavor and has antimicrobial effects. Thus, salt is responsible for the textural characteristics and integrity of finished sausage products. Most commercial sausages contain 1.5% to 2.5% added salt. Phosphates at a level up to 0.5% in finished products are used to improve water- binding capacity of meat by increasing fiber swelling and solubilizing proteins. Phosphates may also help to stabilize flavor and color in finished product, presumably by sequestering transitional metal ions (Fe and Cu), thus reducing oxidation.

Many sausage products are cured with nitrite. Sodium nitrite is commonly used, although in certain cases it may be substituted for by potassium nitrite. The maximum level of nitrite allowed in sausage is 156 ppm. The use of nitrate is more restricted; it can be used only in dry and semi-dry fermented sausages. Nitrite is used in conjunction with the reducing agent ascorbate or erythorbate, and phosphates.

- Water and Extenders

Water, sometimes together with ice, is added in sausage making to help distribute nonmeat ingredients and increase the product yield. Along with the addition of water, nonfat dry milk, whey protein concentrate, sodium caseinate, wheat gluten, cereal flours, tapioca dextrin, soy flour, soy protein concentrate, and more recently, polysaccharide gums, at limited levels, are used as extenders or fillers in sausages. Their main functions are to improve functional properties related to product texture and flavor, and to aid in meat particle binding and water retention.

- Seasonings

Unlike most other processed meats, sausages are seasoned products. Different spices and flavorings are added in sausages, and their use levels are primarily dictated by product identity standards and not by regulations. Spices are aromatic vegetable substances in whole, broken, or ground form. Spices may be added as natural spices or spice extracts. In the latter case, they must be labeled as "flavoring". Flavorings refer to extractives that contain flavor constituents from fruits, vegetables, herbs, roots, meat, seafood, poultry, eggs, dairy products, and other food sources. Flavoring compounds can also be synthesized. A good example is monosodium glutamate, which is a potent flavor enhancer. Most flavorings are oil-based extracts. Because of their high flavor intensity, they can be more accurately applied in sausage to obtain desired flavor intensity than their natural counterparts (spices). Sugars in a variety of forms sucrose, dextrose, corn syrup, and so on, are most commonly used in sausages. Almost all sausage products contain sugar in one form or another.

Peppers are used as spice in greater amount in sausages. There are two families of peppers: vine peppers and capsicum (red) peppers. Vine peppers are grown in tropical areas and yield black

pepper, white pepper, and long pepper. The different kinds of pepper vary in aroma, size, pungency, and peppercorn color.
Black pepper is used in most countries in basic cooking. It contains piperine, a compound that stimulates the flow of gastric juices. The flavor is the result of a nonvolatile resinous substance. The pepper tastes strongest when it is freshly ground, but usually, preground pepper is used in seasonings for convenience. White pepper comes from the same plant as black pepper, but the berries are picked ripe instead of green. White pepper is used mainly in dishes in which black pepper specks are undesirable.
Capsicum peppers are in the same family as chili peppers, red peppers, sweet bell peppers, cayenne peppers, and paprika. The peppers differ greatly in size and degree of pungency. Chilies and red pepper are used mostly in foods of South India, Mexico, and Asia. Cayenne pepper is very hot and ranges in color from orange to red. Capsicum peppers are known to have a very hot bite, sometimes to the point of being overwhelming. Chili powder is a blend of several peppers and spices. Paprika is usually used for its red color.

1.7.1 Fresh Sausages

As the word "fresh" indicates, fresh sausages include a variety of uncooked sausages, such as breakfast sausage and sausage patties, whole hog sausage, bratwurst, Italian-style sausage, and Polish-style sausage, all of which can have a fat content up to 50% of the raw product weight. They are salted but not cured with nitrite, and are generally coarsely ground and not emulsified. Particle size reduction is achieved through extrusion and cutting in a screw auger operating in a horizontal chamber. The ground meat is mixed with salt, seasonings, and other ingredients by blending in a mixer or similar equipment. Stuffing is done by extrusion of the batter into natural or synthetic casings through a smallopening tube called the horn. Natural casings are small intestines from hog and sheep inverted and thoroughly washed in a dilute chlorine solution (0.5%) followed by water rinsing. Synthetic casings are made of edible collagen materials or inedible cellulose. Fresh sausages are sold uncooked, and require either refrigeration or freezing.

1.7.2 Cured Sausages

Nitrite-cured sausages are mostly finely chopped and emulsified. The most popular products in this group are frankfurters and bologna; most frankfurters are a blend of beef and pork, with or without poultry, mildly seasoned with paprika and other spices, and smoked. The processing technology for bologna is similar to that for frankfurters; however, bologna is much larger in diameter for the purpose of sandwich preparation, and the spices used may be different.
In a typical cured sausage processing, the mixture of meat and ingredients is finely chopped and emulsified. The chopping process creates sufficient shear to comminute meat and fat into fine particulates. Because myofibrillar proteins are extracted in the presence of added salt and phosphate during chopping, they will form coatings on the surface of the fat droplets and produce protein matrices surrounding the emulsified fat particles, thereby reducing the surface tension. The finely chopped meat system is completely stabilized during cooking, where three-dimensional gel structures are formed and fat particles are imbedded in the gel matrices. Most cured sausages are also smoked. One of the most critical factors in the production of emulsified meats is the temperature of meat batters during chopping. This temperature should be maintained low enough to prevent emulsion collapse, but not too low to keep fat soft. Finely chopped sausages are stuffed mostly in synthetic casings. Therefore, after cooking, casings are removed by peeling prior to vacuum packaging. Natural casings described above are also used for certain finely chopped products, and they are not removed after cooking.

1.7.3 Fermented Sausages (Salami)

The first use of fermentation in meat is lost in the mists of antiquity but may date back to the Babylonian culture around 1500 B.C. Fermented sausages can be divided into two main groups based on the processing procedure and product characteristics: dry and semi-dry. For both groups, lactic acid is produced; thus, meat is "fermented." Dry and semi-dry sausages, as their names imply, differ in moisture content, which averages between 30% to 40% and 40% to 50%, respectively. The most crucial processing step in sausage fermentation is to timely lower the pH of fresh meat (which averages about 5.6 to 5.8 post rigor) so as to curtail the growth of spoilage

microorganisms. The final pH of fermented sausages typically ranges from 4.8 to 5.2, depending on tanginess, firmness, and other product characteristics desired. Lactic acid bacteria, which produce lactic acid through glycolysis, can be introduced into meat either by "chance inoculation" (natural fermentation) or by inoculating a starter culture. In natural fermentation, lactic bacteria are inoculated by chance from the processing environment. Sometimes, a portion of already fermented meat from a previous batch is added to a new batch to start fermentation. This procedure, called "back slopping," reduces the incubation time for the bacteria to reach a productive level. Natural fermentation has been used for centuries but it has many obvious disadvantages. The fermentation usually takes a long time (e.g., more than one week). The population and type of lactic acid bacteria in fresh meat are difficult to control. If the initial population of lactic bacteria is small and the meat pH cannot be rapidly lowered, spoilage microorganisms will predominate and the product will fail. Moreover, pathogens can grow well in meat when the pH is not sufficiently low, especially when they do not have to compete with lactic bacteria.

Many lactic bacteria from chance inoculation are heterofermentative, i.e., in addition to producing lactic acid, they also produce acetic acid, alcohol, gas, etc. Because different bacteria species may be introduced each time, batch-to-batch variability in product flavor, acidity, and textural characteristics can be very high.

Today, almost all commercial production of fermented sausages is done by using selected starter cultures. Starter cultures are available in two forms: frozen concentrate and lyophilized dry powder. Starter cultures available commercially are typically blends of two or more different microorganisms and sometimes different strains of the same microorganism. The most commonly used microorganisms are *Lactobacillus*, *Pediococcus*, *Lactococcus* (all three are homofermentative), and *Micrococcus* (used to reduce nitrate to nitrite). Specific examples are *L. plantarum, P. acidilactici*, and *L. lactis* sub sp. *lactis*. Fermented sausages are salted and cured, and both nitrate and nitrite can be used. Salt is needed to facilitate dehydration and impart flavor. Fermented sausages are usually heavily spiced, making the product particularly palatable. Organic acidulants, such as encapsulated glucono-delta-lactone and lactic acid, are sometimes mixed with fresh meat at the beginning of fermentation. They are used to quickly establish an acidic environment that will stimulate the growth of lactic acid bacteria and inhibit spoilage microorganisms. In fact, dry or semi-dry sausages can be produced by direct acidification with proper acidulants such as lactic acid and glucono-delta-lactone, a slow acid-releasing compound. Sausages prepared by direct acidification have a characteristic tangy flavor closely resembling that of fermented products, and they are most widely used in pepperoni production for pizza toppings.

In order to produce lactic acid during fermentation, sugar must be present, which serves as substrate for glycolytic enzymes inside the bacterial cells. Simple sugars, such as sucrose and dextrose, are preferred because they can be readily transported through the bacterial cell wall. The amount of sugar added to dry or semi-dry sausages are typically in the range of 0.5% to 2.0%. The lower the desired pH, the more sugar will be needed.

After ground meat is blended with all ingredients, including the starter culture, the mixture is stuffed and subsequently incubated in a closed chamber (sometimes a smoke- house) to allow fermentation to take place. Temperature of the incubator is typically maintained at 21° to 24°C with a 75% to 80% relative humidity for dry sausage, and 30° to 37°C with a 75% to 80% relative humidity for semi-dry sausage. The fermentation time, however, is longer for dry sausage (1 to 3 days) than for semi-dry sausage (8 to 20 hours) (Terrell, 1977). For dry sausage production, the fermented meat is placed in a drying room to allow further dehydration and flavor development. As a general recommendation, the temperature of the drying room should be controlled to 7° to 13°C and the relative humidity to 70% to 72%. The air of the drying room should be changed periodically to ensure air quality and prevent moisture buildup on the surface of sausage. The drying time varies considerably, depending on the size (diameter) and type of product. Most dry sausages are aged for somewhere between 10 days to 3 months. Dry sausages are not cooked, and most are not smoked. They do not require refrigeration after manufacture. The low moisture content (aw ~ 0.91) and low pH conditions in the sausage are effective to preserve the product. Semi-dry sausages, however, are generally cooked to an internal temperature of at least 68°C following fermentation. Semi-dry sausages have a relatively high moisture content (aw ~ 0.95) and hence, require refrigeration to prevent microbial spoilage. Most semi-dry sausages are smoked.

1.8 Flavour: measurement and development

In general, the term flavour is defined as the overall impression perceived via the chemical senses from a product in the mouth. Flavour includes the sensation of taste and aroma as well as trigeminal feelings, such as astringency, the pain from hot spices, metallic note from blood, etc. Texture, appearance and the sounds of the food during chewing have an influence on the perceived flavour as well, but they are not commonly included in the definition of flavour (Meilgaard et al., 1991). However, one must be aware that the temporal order of the sensation has a great influence on the total flavor impression, i.e., the order of stimulation is very important for how the food is perceived and liked. During eating the consumer is first of all confronted with the appearance and colour of the food and later on with its odour. This gives rise to certain expectations on how the food will taste. Finally, during the chewing process, the consumer is confronted with texture, taste and aroma, which together will create the final impression of the flavour (Rothe, 1988).
The sensation of taste is caused by primarily non-volatile compounds in the food interacting with the taste buds on the surface of the tongue as well as in the mucosa of the palate and areas of the throat. The sensation of aroma is caused by volatiles in the food evaporating from the food during the chewing process and travelling through the nasopharynx to the nasal cavity, where they react with the olfactory receptors producing an electrical signal, which is transmitted to the olfactory bulb in the front brain (Rothe, 1988). The discrepancy between taste and aroma should be kept in mind when analysing flavour either by sensory or instrumental means. However, it is also true that sub-threshold concentrations of non-volatile compounds may affect sensitivity to an aroma compound (Dalton et al., 2000). Such 'taste-olfaction integration' of senses is apparent from the aroma enhancement due to the glutamate-umami taste and peptides in fermented sausages may have a similar effect.

1.9 Aroma in salami

The raw sausage mince does not contain any volatile compounds of sensory importance since it has little or no aroma. On the other hand, it contains a large number of aroma precursors, which during the fermentation, drying and maturation steps are converted by endogenous enzymes, microbial activity and chemical reactions into a large number of volatile compounds of both sensory and non-sensory importance. The volatiles present in fermented sausage consist of a wide variety of compounds from many different chemical classes depending on ingredient levels, spices, meat origin, smoke, starter cultures, processing conditions, packaging conditions. Until now more than 200 compounds have been identified, but not all of them are of sensory relevance. In particular, compounds such as the alkanes and straight chain alcohols have sensory threshold values much too high for them to have any influence on fermented sausage flavour (Grosch, 1982).
In dry-sausages volatile compounds derive principally from: added spices, enzymatic and chemical fatty acids oxidation, glucidic and protein catabolism. Generally, the nature and amount of spices (pepper, garlic, and others) used in fermented sausages characterize the aroma.
Pepper compounds were quantitatively the largest group of volatiles. They accounted for between 51 and 81% of the total content (Meynier, Novelli, Chizzolini, Zanardi & Gandemer, 1998; Viallon et al., 1996), in particular majority the terpens (limonene, α-fellandrene, α-pinene). Volatile lipid oxidation products made up between 3% and 18% of the total amount of volatiles (L.O. Sunesen et al., 2001), most significant classes are alkani, alkeni, alcohols, aldehyde, ketones and some acids. A lot of aldehydes were lipid oxidation products such as the hexanal, which imparts a green odour (Stahnke, 1994) and that was at all times the most abundant aldehyde together with nonanal, or they derive from amino acids degradation (Forss, 1972, Montel M. C. et al., 1998). Some alcohols are generated as reaction products of lipid oxidation (Shahidi, 1994). The most abundant alcohol 1-octen-3-ol imparts a mushroom note. Among alcohols may be present also 1-propanol and 2-heptanol which were found after fermentation. Propanol may arise from carbohydrate metabolism via propanal (Halvarson, 1973). Methyl ketones are products of microbial β-oxidation of saturated fatty acids followed by β-keto acid decarboxylation (Okumura & Kinsella, 1985). The compounds more abundant are 2-pentanone, 2-hexanone, 2-heptanone (Berdague et al., 1993; Stahnke, 1995a, b; Montel et al., 1996). Hydrocarbons included n-alkanes and n-alkenes with 7 and 8 carbons, they are formed from the rearrangement of the alkylic radical formed by β-splitting of the alcoxy-radical (Frankel, 1980).

The degradation of branched-chain amino acids such as isoleucine and leucine, trough a non enzymatic Strecker reaction, form important aromatic compounds 2- and 3 methylbutanal and their corresponding alcohols and acids (Halvarson, 1973; Berdaguè, 1993). Finally ethyl esters such as ethyl octanoate, ethyl hexanoate, ethyl acetate are present in fermented meat products and their aromatic characteristics contribute to the fruity note of the products (Stahnke, 1994; Montel et al., 1996).

1.9.1 The origin of the aroma compounds

1.9.1.1 Aroma compounds from carbohydrate catabolism

During the fermentation period most of the added carbohydrate is converted into lactic acid and different amounts of side products depending on the applied lactic acid bacteria, the type and content of carbohydrate, temperature and other processing parameters. The additional starter cultures of, for example, staphylococci or yeast probably exert some effect in converting sugars to products other than lactic acid, but are of course in strong competition with the lactic acid bacteria. Volatile compounds in fermented sausage, generally considered to be derived from carbohydrate catabolism, are acetic, propionic and butyric acids, acetaldehyde, diacetyl, acetoin, 2,3-butandiol, ethanol, acetone, 2-propanol and more (Gottschalk, 1986; Demeyer, 1982; Stahnke, 1999). However, the compounds are derived from pyruvate, which may originate from many sources other than carbohydrate during microbial metabolism (Demeyer et al., 1986).

1.9.1.2 Aroma compounds from protein degradation

The most important taste-active components of meats are amino acids, peptides, organic acids, nucleotides and other flavor enhancers, among others (Shahidi, 1989). It has further been concluded that high-molecular-weight fibrillar and sarcoplasmic proteins have little effect on the development of meat flavor volatiles.
During maturation extensive proteolysis takes place in fermented sausages creating peptides and free amino acids by the microorganisms and converted into numerous aroma compounds by different pathways. Some of the more important are the biochemical conversions of the amino acids leucine, isoleucine, valine, methionine and phenylalanine into the sensory important branched aldehydes and corresponding secondary products, such as acids, alcohols and esters (Montel et al., 1998; Stahnke et al., 2002). Although such findings suggest that most of the significant aroma compounds are derived from the protein fraction of the sausage, it is known that intensity of proteolysis reflects release of peptides affecting taste, rather than aroma (Nishimura et al., 1988) as shown for cheese (Fox, 1989) and raw ham (Hansen-Møller et al., 1997). Work on proteolysis in dry sausage has involved the initial degradation of myosin and actin (Verplaetse et al., 1992; Verplaetse, 1994b; Molly et al., 1997; Harnie et al., 2000). The use of antibiotics and paucibacterial meat incubations has clearly established that initial proteolytic changes mainly involve myosin and actin degradation through the action of cathepsin D-like enzymes. The contribution of bacteria in further endo- and, mainly, exoproteolytic changes increases down to ammonia production, the end of the proteolytic chain.
The microorganisms responsible for those conversions are primarily species from the *Micrococcaceae* family. It has been shown, both in model experiments and in sausages, that different staphylococci and micrococci produce 2- and 3-methylbutanal, 2-methylpropanal, 2- and 3-methylbutanoic acid, 2-methylpropanoic acid, 2- and 3-methylbutanol, ethyl-2- and 3-methylbutanoate, methional, phenylacetaldehyde, phenylethanol and many more (Berdague´ et al., 1993; Stahnke, 1994, 1999; Montel et al., 1996; Masson et al., 1999; Larrouture et al., 2000). The amount of the compounds is highly influenced by the processing conditions. In minimal media, model minces and in sausages it has been shown that parameters such as temperature, pH, glucose, salt, nitrite, nitrate and ascorbate all influence the amount of aroma compounds in one way or the other (Stahnke, 1995b, 1999; Masson et al., 1999; Larrouture et al., 2000). Results indicate that for Staphylococcus the reactions are negatively correlated with their growth, i.e., it seems as if the organisms produce more of the abovementioned compounds when they are in the resting phase than when in active growth but this still needs to be studied further (Stahnke, 1999). It has been suggested that the branched-chained aldehydes could also arise from the nonenzymatic Strecker reactions between the corresponding amino acids and a diketone, such as

diacetyl (Stahnke, 1995b; Barbieri et al., 1992). Apart from aldehydes, the Strecker degradation results in various keto-amines that dimerize into different pyrazines (Hurrell, 1982). This would explain the presence of different pyrazines in unspiced fermented sausages (Stahnke, 1995b; Johansson et al., 1994; Berdague´ et al., 1993). Indeed, studies have shown that the amount of 2- and 3-methylbutanal and 2-methylpropanal was of the same magnitude in sausages with or without microbial growth (Stahnke, 1994). However, the Strecker degradation is favoured by high temperature and very low water activity, i.e., conditions not prevailing in fermented sausage (Hurrell, 1982).

1.9.1.3 Aroma compounds from lipid degradation

Flavour is an important quality attribute of muscle foods and comprises mainly the two sensations of taste and aroma or smell. Although both of these factors affect the overall acceptability of foods, the aroma or flavor volatiles are of utmost importance because they influence the judgment of the consumer even before the food is eaten.
The role of lipids in meat flavor generation has been the subject of extensive studies. Lipids may contribute both to desirable and undesirable flavors of meat from different species. Their effect on generation of desirable aromas in cooked meats may arise from mild thermal oxidative changes which produce important flavor compounds; they may also react with components of lean tissues to afford other flavor compounds and may act as a carrier for aroma compounds, thus affecting their sensible threshold values. Other potentially desirable flavor components that might be formed in processed meats are free fatty acids and related compounds which are prevalent in dry-cured-ham. These compounds are formed in such products via fermentation reactions. The flavor characteristics of dry-cured ham has been reported (Toldra et al., 1997). During the fermentation and maturation periods the lipid fraction of the sausage mince is partly hydrolyzed by lipolytic reactions in which triglycerides and phospholipids are liberating free fatty acids. Residual mono- and diglycerides have also been detected and it was shown that more unsaturated fatty acids were liberated preferentially, probably because of a preferential membrane phospholipid degradation as well as a positional and/or fatty acid specificity of the meat lipases (Demeyer et al., 1974; Molly et al., 1997). One should bear in mind that aroma compounds are present in the ppb to ppm levels whereas the level of free fatty acids are between 0.5 to 7% depending on sausage type (Nagy et al., 1989; Dominguez and Zumalaca´rregui,1991; Stahnke, 1994; Johansson et al., 1994; Navarro et al., 1997).
Lipolysis is caused both by microbial enzymes and endogenous enzymes in the meat and fat and there has been much debate about which mechanisms are the dominant. However, the most recent results from sterile model minces and sausages with added antibiotics show that the major part of the lipolytic breakdown is attributed to endogenous enzymes even if strongly lipolytic strains of *Staphylococcus* are used as a starter culture (Molly et al., 1997; Stahnke, 1994). The pH of the sausage mince may be decisive for the degree of lipolysis arising from microorgansims since pH is a major factor influencing the amount of *Micrococcaceae*, their production of lipases and their activity (Søndergaard and Stahnke, 2002; Hierro et al., 1997; Sørensen and Jakobsen, 1996). It has also been shown in sausages that the amount of free fatty acids is increased by high fermentation temperature and reduced salt levels (Stahnke, 1995a). The partial glycerides and/or the free fatty acids produced during lipolysis may oxidize via different pathways chemically or microbially. It is not clear whether free fatty acids are oxidized faster than intact glycerides. Although addition of lipases increased lipid oxidation during maturation (Ansorena et al., 1998), other work has shown that increased lipolysis was not associated with increased rancidity (Nagy et al., 1989; Fernandez and Rodriguez, 1991). Chemical autoxidation of unsaturated fatty acids produces a whole range of volatile aldehydes, ketones, alchols, etc., some of which are very potent aroma compounds. Gas chromatography olfactometry technique has shown that compounds such as hexanal, octanal and 1-octene-3-one are important for the overall flavour (Meynier et al., 1999; Schmidt and Berger, 1998; Stahnke, 1995c). In general, the influence of autoxidation processes will increase during maturation and storage depending on the sausage ingredients. It has been shown that ascorbate prevents autoxidation (Houben and Krol, 1986) and that nitrate may increase it (Stahnke, 1995b). Additionally, species belonging to the genus *Staphylococcus* have been reported to prevent autoxidation, possibly due to their capability of forming catalase and superoxide dismutase that degrade hydrogen peroxide (H_2O_2) and superoxide (O_2^-), respectively (Barriere et al., 2001; Talon et al., 2000).

As mentioned previously, methyl ketones (2-alkanones) can be formed during microbial metabolism, either directly by decarboxylation of free β-keto acids or by β-oxidation of free fatty acids. Their sensory threshold values are quite high though, compared to other lipid oxidation compounds (Grosch, 1982). The 2-alkanones may be further reduced to 2-alkanols by alcohol dehydrogenase in the microorganism. The level of methyl ketones increases steadily over time in Mediterranean fermented sausages (Sunesen et al., 2001; Croizet et al., 1992) and the *Penicillium* growing on the surface may be responsible. However, North European non-moulded sausages also contain methyl ketones, be it in slightly lower amounts (Stahnke et al., 1999). Model experiments show that both *Staphylococcus* and *Penicillium* species are capable of producing methyl ketones (Stahnke, 1999; Montel et al., 1996; Larsen, 1998).

1.9.2 Sensory measurement of aroma, taste and flavor

The aroma (or odour), taste and flavour of dried sausage is commonly measured by hedonic methods or descriptive analysis, either using a point scale, a ranking scale or a continuous line scale (Mendoza et al., 2001; Hagen et al., 2000; Sanz et al., 1997; Diaz et al., 1997; Dellaglio et al., 1996). Typical taste attributes are: acidity, saltiness, sweetness, metallicness, bitterness, umami and acidic aftertaste. Preferably taste should be measured while the panellists have a clip on their nose in order to prevent air from travelling through the nasopharynx to the nasal cavity and confusing the flavour impression with the tasting sensation (Bingham et al., 1990). Aroma (or odour) and flavour attributes that are frequently used are: overall intensity, meat type (pork, beef, etc.), fresh meat, sour-sweet, acid, vinegar, tanginess, sour socks, spices, pepper, flowery, nutty, garlic, maturity, cured, dry sausage, butter, cheese, sourdough, fatty, rancid, nauseous, burned, solvent, smoked (Stahnke et al., 2002; Hagen et al., 2000; Stahnke et al., 1999; Zalacain et al., 1997; Viallon et al., 1996; Dellaglio et al., 1996; Stahnke, 1995c; Berdague´ et al., 1993; Acton et al., 1972). During training of panellists for dry sausage evaluation, chemical standards may be included to exemplify the qualitative description of the various attributes. This was recently done by Erkkila¨ et al., (2001) who used lactic acid, acetic acid, arginine, alanine and salt to describe the flavour of lactic and acetic acid, bitterness, sweetness and saltiness, respectively.

1.9.3 Instrumental measurement of aroma and taste compounds

A huge number of methods have been developed for analysing the flavor compounds of meat products and other foods, but one should realise that the composition of the final aroma sample is highly reflected by the choice of method. Also, the reproducibility of flavour analyses is in general lower than for other analytical methods, in particular on complex matrices such as meat products. Standard deviations within the same sausage range between 5 and more than 10% for some volatiles and between-batch variability may exceed50% (Schmidt and Berger, 1998, Hinrichsen and Pedersen 1995; Mateo and Zumalaca´rregui 1996; Meynier et al., 1999, Stahnke et al., 2002). Flavour research has been primarily confined to the study of the volatile and the semivolatile compounds since they are the most important contributors to the characteristic flavour of most foods (Cronin, 1982). The following paragraphs will therefore focus on the measurement of volatile compounds and only slightly on the non-volatile. The basic principles include four steps: Collection of the flavour compounds, concentration, separation and detection (Bett and Grimm, 1994).

- Collection of compounds: depending on the type of flavour compounds, their polarity, volatility, etc., and the kind of matrix in which they are embedded (raw meat/cooked meat, lipid content, structure, etc.) different collection methods are preferred and basically, three different methods exist; (i) direct extraction with a solvent (organic solvent, water, super-critical fluids), (ii) distillation combined with a transfer of the volatiles into a small amount of solvent (e.g., steam distillation, Likens-Nickerson, high vacuum distillation of extracts) and (iii) headspace collection, in which the volatiles in the air above the sample are sampled and collected in a cold-trap or on an adsorbent (dynamic headspace sampling, purge and trap sampling, solid phase micro-extraction, etc.).

- Concentration: depending on the collection method and the concentration of compounds, the aroma sample may have to be concentrated to a smaller volume. For extracts the solvent is

commonly evaporated on a Vigreux column in a thermostatted water bath or by blow down of an inert gas, usually nitrogen, on the surface of the extract. In headspace methods using adsorbent traps the aroma sample is located in the trap, which may be extracted with a minute amount of solvent or desorbed by heat. During the concentration step there is a risk of highly volatile compounds being lost due to evaporation together with the solvent (Parliament, 1997; Hartman et al., 1993; Burgard and Kuznicki, 1990).

-Separation: the separation of the flavour sample is primarily performed by gas-chromatography (GC) (for volatiles) or, for non-volatiles and compounds with low volatility by high pressure liquid chromatography (HPLC). Volatiles collected on adsorbent traps are often injected directly into the GC by a thermal desorber. Aroma extracts are preferably injected directly on-column at as low a temperature as possible since high temperatures may decompose labile compounds and produce artefacts (Wampler, 1997; Merritt and Robertson, 1982).
Analysis of the peptide fraction merits special mention: early work studying proteolysis during dry sausage fermentation has used semi-quantitative SDS-PAGE (Verplaetse et al., 1989; Verplaetse, 1994b; Verplaetse et al.,1992). Such a method is limited however to the molecular weights (MW) > 5 kDa. Size exclusion chromatography (gel filtration) by HPLC can be used to isolate smaller MW fractions for further analysis by reversed phase HPLC (Lambregts et al., 1998).

-Detection: the detection of flavour compounds in gas chromatographic analysis is mostly performed by mass spectrometry (MS) and by flame ionization detection (FID), which responds to any compound that is combustible in a hydrogen flame. Element-selective detectors are available for organic compounds containing halogens, nitrogen, sulphur and phosphorus, but are rarely used in flavor research. The advantage of the mass spectrometer compared to the other detectors is based on its ability both to quantify and identify the compounds at the same time. However, in many applications the sensitivity and the linear range of the MS are much less than the other detectors (Rood, 1999). A highly efficient, commonly used detection principle in instrumental flavor analysis method is olfactometry. In gas chromatography olfactometry (GCO) all or part of the effluent from the column is led to an outlet outside the GC-oven, where a human subject sniffs the compounds as they elute. The odours are rated qualitatively and sometimes also quantitatively and make it possible to identify the more important odorous compounds in the food sample. While instrumental detectors quantify the individual components of the food sample, the peak areas do not necessarily correspond to the flavour intensity. The human nose is much more sensitive than the instrumental detector to many flavour compounds (Bett and Grimm, 1994). Different protocols for analysing flavour samples by GCO have been developed (Blank, 1997) and modified versions are commonly used (Meynier et al., 1999; Stahnke, 1995c; van Ruth and Roozen, 1995). By using gas chromatography olfactometry (GCO), it has been shown that the aroma compounds creating the basic cured sausage flavour are most likely to consist of compounds from microbial degradation of fatty acids and of the amino acids valine, leucine, isoleucine and methionine together with compounds from carbohydrate catabolism. More specifically, different branched aldehydes and acids, ketones, various sulfides, diacetyl, acetaldehyde, acetic acid and perhaps also certain ethyl esters (Stahnke et al., 2002; Stahnke 2000; Meynier et al., 1999; Stahnke et al., 1999; Stahnke 1994, 1995c; Schmidt and Berger 1998; Montel et al., 1996; Berdague´ et al., 1993). Compounds originating from chemical autoxidation of lipids such as hexanal, octanal, 1-octene-3-one, etc., are of great importance but may not be involved with the cured flavour attribute, but rather contribute to the rancid notes.
The newest principle for detection of volatile flavour compounds is the ‘electronic nose’, a device based on an array of sensors each having a partial specificity for each volatile compound in the gas phase thus producing an odour fingerprint that can be identified by a pattern recognition system without need for prior separation (Strike et al., 1999). The main advantage of electronic noses is rapid analysis, enabling quick decisions making, e.g., in relation to quality control. Electronic nose sensing was shown to be sufficiently accurate as an approximation of human olfaction apparatus, but further development is necessary although its successful use in discriminating between sausage types has been reported (Vernat-Rossi et al., 1996; Eklov et al., 1998).
For analysis of peptides by reversed phase HPLC, detection sensitivity may be a limiting factor, necessitating the use of mass spectrometry. Analogous to GCO, the liquid fractions from gel filtration or preparative HPLC on non-volatile flavor compounds may be tasted by a sensory

panel and the taste of the eluted compounds evaluated (Henriksen and Stahnke, 1997) but this approach has not been much used by flavour researchers.

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CHAPTER 2: AIM OF THE STUDY

2 AIM OF THE STUDY

Naples-type salami is a popular salami native to the Campania, today produced throughout Italy. Thanks to marketing strategies, Naples-type salami has become the best known and exported product of this kind. It supplies proteins with high biological quality from nutritional standpoint, moreover it is rich in iron, zinc, thiamine and riboflavin.

Naples-type salami is a dry fermented sausage with medium-size grana made of coarsely minced lean pig, mixed with fat similarly cut into small pieces, with addiction of salt, pepper and various spices, stuffed into natural or artificial casings and is subjected to fermentation and dry-ripening for 30-60 days. During these phases, physical, chemical, physicochemical, biochemical and microbiological changes occur, all influenced by product formulation, the raw materials used, the starter culture and process conditions (Hughes et al., 2002; Pérez-Álvarez, 2006). These changes affect the organoleptic properties and the shelf life of the product (Fernández-López, Sendra, Sayas-Barberá, Navarro, & Pérez-Álvarez, 2008; Lizaso, Chasco, & Beriain, 1999; Toldrá, 2002a).

The typical flavour of dry cured fermented sausages is the result of a careful balance between volatile (alcohol, ketones, aldehydes and furans) and non-volatile compounds (amino acids, peptides, sugars and nucleotides), these coming from raw materials (meat, spices, nitrites and other additives) or generated from biochemical reactions occurring during fermentation and ripening (Flores, Marcus, Nieto, & Navarro, 1997; Toldrá, 1998; Stahnke, 2002). In fact, proteolysis and lipolysis, by both microbial and endogenous enzymes, are the main biochemical reactions in the generation of flavour or flavour precursors (Ordoñez, Hierro, Bruna, & de la Hoz, 1999; Toldrá, 1998).

Many of non-volatile compounds such as peptides and amino acids involved in the characteristic flavour development are produced during meat protein hydrolysis. In this regard, meat LAB have a direct or indirect participation on this phenomenon, so its presence determines to a large extent, the sensory characteristics of the final product.

Recently, proteomic approaches have been applied to correlate proteolytic profiles with technological parameters in view to detect valuable biomarkers to monitor and predict meat quality (Lametsch et al., 2003; te Pas, Jansen, Broekman, Reimert, & Heuven, 2009). Certain peptidic fractions originated from meat have been identified to be related to sensory attributes (Sentandreu et al., 2003, 2007; Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009). Also, texture is to a large extent due to protein changes during ripening (Toldrá, 2002b). At present, scientific studies aimed to define proteic fraction of Naples-type salami are limited to use of electrophoretic techniques and no substantial information about global characteristics of products nor about the exhaustive characterization of generated peptides during meat fermentation are available.

Even though the flavour impression is probably the most important component of the eating quality of fermented sausage, research on the matter has been rather scarce and until quite recently it was not directly focused on the analysis of flavour compounds. The first study on the aroma profile of a fermented sausage appeared in 1990 and since then different sausages from all over Europe have been analysed by various analytical methods (Berger et al., 1990; Berdague´ et al., 1993; Johansson et al., 1994; Stahnke, 1994, 1995b; Mateo and Zumalaca´rregui, 1996; Schmidt and Berger, 1998; Stahnke et al., 1999; Viallon et al., 1996; Meynier et al., 1999; Sunesen et al., 2001; Stahnke et al., 2002).

On the other hand much research has been aimed at understanding the chemistry of cooked meat flavour (Mottram, 1998). But one should be careful not to confuse results from meat flavour studies with fermented sausage flavour since flavour compounds in cooked meats primarily are derived through thermal processes, whereas compounds in raw dried meat products such as salami and dry ham mainly arise from both endogenous and microbial enzyme reactions taking place during the fermentation and drying steps.

Lipolysis has been extensively studied over the years since free fatty acids are believed to be important precursors for oxidation products of relevance for flavour. Nevertheless, a direct correlation between lipolysis and maturity development has not been established (Montel et al., 1998). Recent results indicate that methyl ketones from microbial β-oxidation of free fatty acids may be important for maturity (Stahnke et al., 2002) but perhaps the amount of free fatty acids is so plentiful that increased amounts of this precursor do not influence the flavour profile. Lipids are affected by lipolytic and oxidative reactions, (Gandemer, 2002) through the generation of free fatty acids (FFA) that are further subjected to lipid oxidation producing a large variety of volatile

compounds (Zanardi, Ghidini, Battaglia, & Chizzolini, 2004). Furthermore lipids are important not only for the generation of flavour compounds but also as a solvent for aroma compounds (Leland, 1997). The free fatty acid (FFA) during enzymatic and chemical oxidative reactions form directly volatile compounds and precursor of odorous molecules. Monoglyceride and diglyceride formed are able to bind the liposoluble fat fraction with proteic hydrosoluble fraction employing the structural stability and the texture of the final product. Traditionally, lipolysis has been mainly related to bacterial lipase activity whereas oxidative changes of unsaturated fatty acids, resulting in the production of lipid peroxides and carbonyl compounds, have been related to both chemical reactions and bacterial metabolism (Demeyer et al., 1974). Recently, the importance of endogenous meat and fat lipases in lipolysis during dry sausage ripening has been suggested by Talon et al. (1992) and Garcia et al. (1992).

Carbohydrates also affect the process by their fermentation and further accumulation of lactic acid, causing pH values to fall to near the isoelectric point of meat proteins (Hierro et al., 1997). Mould growth on the sausage surface also affects the organoleptic properties. Their enzymes participate in the proteolysis, amino acid degradation, lipolysis, β-oxidation, and lactate oxidation (Grazia, Romano, Bagni, Roggiani, & Guglielmi, 1986). Moulds also retard rancidity and stabilize the colour by hindering oxygen and light penetration, and through catalase activity (Bruna et al., 2001).

Trends in meat industry are focalizing in products with high organoleptic standards, long shelf-life and containing specific nutrients to cover special consumer requirements. In parallel, consumers are increasingly demanding pathogen free foods, with minimal processing and few preservatives and additives. Thus, biopreservation has gained increasing attention as means of naturally controlling the shelf-life and safety of meat products.

The aim of this PhD thesis has been to characterize the metabolites formed during ripening of Naples-type salami in order to identify molecular markers of quality. In particular has been first made a metabolomic analysis of the raw materials, the pork meat, and then a comparison between industrial and artisanal salami analyzing the modification occurred in sarcoplasmic proteins, lipids and aroma on different steps of both products. Finally the analysis has been extended to industrial and artisanal salami procured on the market.

As mentioned above, at present scientific studies aimed to define the protein fraction of Naples-type salami are limited to use of electrophoretic techniques and no substantial information about generated peptides during meat fermentation are available so far, therefore in this PhD thesis has been taken an proteomic approach based on the combined use of high resolution liquid and gas-chromatography and advanced mass spectrometry techniques. The characterization of sarcoplasmic proteins and peptides as well as the analysis of aromas could be used for identification of molecular markers of quality and typicality in order to obtain the P.D.O. mark, to differentiate an artisanal salami from an industrial one and to established a comprehensive system of traceability for meat products in according EC regulation no. 178/2002 of the European Parliament.

2.1 References

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CHAPTER 3: RESULTS AND DISCUSSION

3 RESULTS AND DISCUSSION

3.1 Proteomic analysis of sarcoplasmic fraction of Naples-type salami

The structural characteristics define the nutritional and organoleptic quality of the Naples type salami. Naples-type salami supplies proteins with high biological quality from nutritional standpoint, moreover it is rich in iron, zinc, thiamine and riboflavin. The organoleptic and sensory properties are due to the degradation events (proteolysis, lipolysis) occurring during the maturation of salami. Endogenous enzymes, such as calpains and cathepsins, are primarily responsible for the initial degradation of the sarcoplasmic and myofibrillar proteins, while bacterial peptidases contributed significantly to the release of free amino acids, in fact it has been demonstrated that the most commonly found *Lactobacillus* species in dry fermented meats are able to hydrolyse myofibrillar and sarcoplasmic muscle proteins in vitro (Fadda et al., 1999). Moreover many small hydrophilic peptides produced in fermented sausages originate from both the sarcoplasmic and myofibrillar proteins (M.C. Hughes et al., 2002). Finally muscle endogenous and exogenous amino peptidases may play an important role on flavour releasing amino acids which represent precursors of aromatic compounds.
It has been suggested that the basic meaty aroma of beef, pork and mutton is the same and is derived from the water-soluble fraction of the muscle which is a reservoir of low-molecular-weight compounds (Hornstein and Crowe, 1960; 1963). The peptides produced can influence final taste of the salami and moreover are object of the activity of endogenous and exogenous amino peptidases releasing amino acids which represent precursors of aromatic compounds. The characterization of sarcoplasmic proteins and peptides as well as the analysis of aromas could be used for identification of molecular markers of quality and typicality in order to obtain the P.D.O. mark, to differentiate an artisanal salami from an industrial one and to allow traceability of the products.

3.2 Materials and methods

3.2.1 Samples

In this section we compared the sarcoplasmic fraction of two Naples-type salami productions, an industrial (Quattromani) and an artisanal (Arzano), that differ in amount of pepper added (0.5% and 0.2% respectively), and in use of starter: in particular the industrial salami was produced with microbiological starter containing *S.xylosus* and *L. plantarum* while the artisanal salami was manufactured without starter cultures. (Fig. 3)
Analyses were carried out on six different steps of each production: meat, minced meat, mixture and Naples-type salami ripened respectively 7, 21 and 30 days.
Finally the analysis has been extended to industrial (Galbani and Franchi) and artisanal (S. Anastasia and Avellino) salami procured on the market.
Solvents were HPLC-grade from J.T.Baker (Holland). Acetic acid was obtained from Sigma-Aldrich Chemical Co. (USA) and TFA, methyl alcohol and chloroform were supplied by Carlo Erba (Milan, Italy).

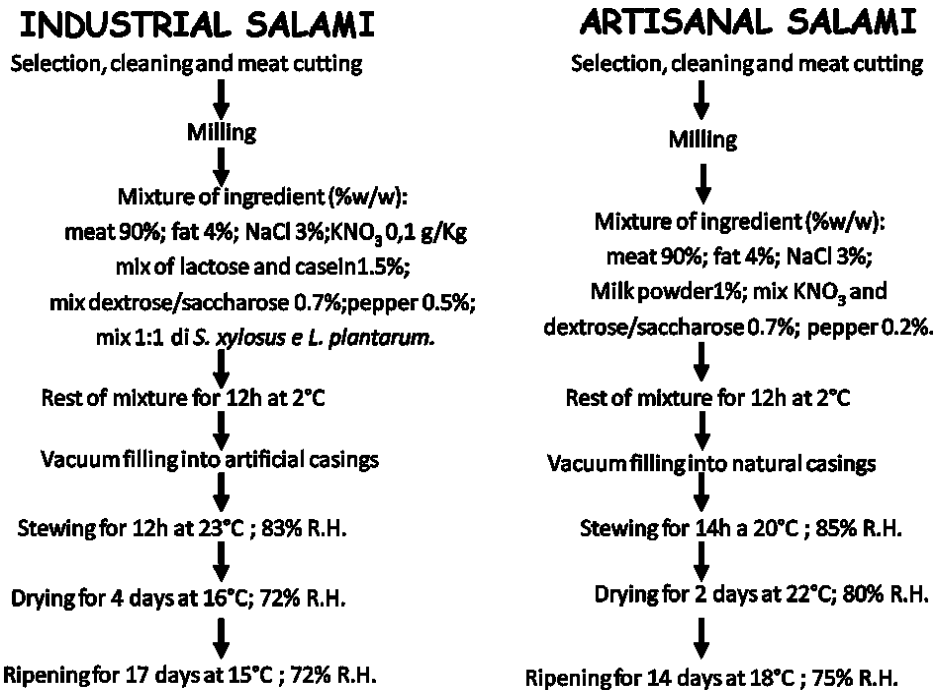

Fig. 3 Production flow chart of two typologies of Naples-type salami.

3.2.2 Extraction procedure

Samples (5.0 g) were freed of connective and adipose tissue and homogenized with an Ultra-Turrax T8 homogenizer (Ika, Staufen, Germany) with 20 ml of water Milli-Q (Millipore) for 30 min. The homogenate was centrifuged under refrigeration at 4°C and 4500 x g (Biofuge centrifuge, Hereaus Instruments,Germany) for 20 min to extract the water soluble fraction: the supernatant contained the sarcoplasmic fraction and the pellet contained the myofibrillar and connective tissue proteins. The supernatant was then filtered on a 0.45 μm membrane (Millex-HA, MCE, Millipore) to remove coarse impurity, and sarcoplasmic proteins were precipitated overnight by addition of 50 ml of –20°C cold acetone and concentrated in a Speed-Vac-Concentrator (SAVANT Instruments, USA). The supernatant was charged on cut-off 3 kDa diafiltration membrane (Millipore Co Badford MA, USA) to separate the proteic fraction from peptide at low molecular weight by centrifugation under refrigeration at 15°C and 4500 x g (Biofuge centrifuge, Hereaus Instruments,Germany) for 60 min.

3.2.3 Solid phase extraction (SPE) of peptide fraction

The peptide fraction was separated from lipid and glucic compounds and from salts through a Solid Phase Extraction (SPE) using reversed-phase C18 Sep-pak cartridges (Millipore, Bedford, USA). The resin was started with 10 ml di methanol, purified with distilled water and balanced with 10 ml of Milli-Q water containing 0.1% (v/v) trifluoracetic acid (TFA). 2 ml of peptide extract were loaded on to cartridge and, after washing with H2O Milli-Q + 0.1%TFA, were eluted by 5 ml of aqueous 70% (v/v) acetonitrile/0.1% (v/v)TFA. The eluate was concentrated in a Speed-Vac-Concentrator (SAVANT Instruments, USA).

3.2.4 Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC) of sarcoplasmic proteins and peptides

The solutions of sarcoplasmic protein (1mg/ml H2O+ 0.1%TFA) and peptide extracts were loaded on to a 218TP54, 5 μm reversed-phase C18, 250 x 4.6 column (Vydac, Hesperia, CA, USA). Solvent A was water containing 0.1% (v/v) trifluoracetic acid (TFA) and solvent B was acetonitrile containing 0.1% (v/v) trifluoracetic acid (TFA). A linear gradient from 10 to 40%

solvent B was applied over 60 min following by a linear gradient from 40 to 60% solvent B applied over 30 min, at constant flow rate of 0.2ml/min. UV detection was carried out at 220 nm. Each fractions were manually collected and concentrated in a Speed-Vac-Concentrator (SAVANT Instruments, USA) for mass spectrometric analysis.

3.2.5 MALDI-TOF analysis of protein and peptide fractions

Mass spectra were acquired on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N2 laser (λ=337nm). The instrument operated with an accelerating voltage of 20 kV for peptide analysis and of 25 kV for protein analysis. Peptide samples were mixed with the matrix α-cyano-4-hydroxy-cinnamic acid prepared by dissolving 5 mg in 1 ml of aqueous 50% (v/v) acetonitrile/0.1% (v/v)TFA, while protein samples with sinapinic acid prepared by dissolving 5 mg in 1 ml of aqueous 50% (v/v) acetonitrile/0.1% (v/v)TFA, on the MALDI target and air-dried. Mass spectra were acquired in the linear and reflector mode using Delay Extraction (DE) technology for peptide analysis, while for protein analysis mass spectra were acquired in the linear mode. External mass calibration was performed with protein mixtures (BSA and Lisozyme) and with peptide standards supplied by PerSeptive BioSystems. The mass spectra were analysed using the software Data Explorer furnished by PerSeptive BioSystems

3.3 Results and discussion

3.3.1 Proteomic analysis

In this work a preliminary protein analysis of the raw materials, the fresh pork meat, followed by a comparison between industrial and artisanal salami has been performed by analyzing the modification occurred in sarcoplasmic proteins on different steps of both products. In Fig.4 it was showed the comparison between MALDI-TOF spectra of sarcoplasmic protein of fresh pork (panel a) and MALDI-TOF spectra of sarcoplasmic protein of salami samples after 30 days of ripening, respectively an industrial salami (Quattromani panel b) and artisanal one (Arzano panel c). In fresh meat, being in an early stage of the tenderization process (48-72h), have been identified many sarcoplasmic protein such as myoglobin, troponine C, triose phosphate isomerase, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, fructose biphosphate aldolase, β-enolase, lactic dehydrogenase and piruvate kinase. In salami samples the proteolysis produced a different final proteic pattern probably due to the action of different microflora; the proteins less susceptible to proteolysis were carbonic anhydrase 3, glyceraldehyde-3-phosphate dehydrogenase , β-enolase 3 and triosephosphate isomerise. Creatine kinase, phosphofructokinase and phosphoglicerate kinase 1 were completely proteolyzed in artisanal salami and in industrial one, instead myoglobin, fructose bisphosphate aldolase A and phosphoglicerate mutase were hydrolyzed only in industrial salami, while troponine C 2, pyruvate kinase and glucose-6-P-isomerase in artisanal one. In Table 1 were summarized the identification of sarcoplasmic proteins of fresh meat and those of industrial and artisanal samples.

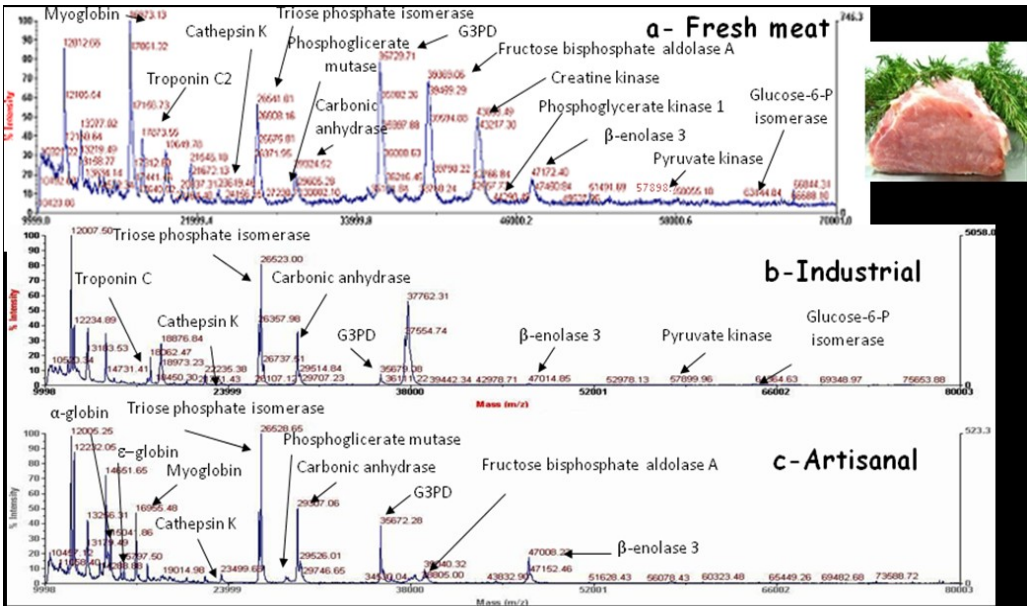

Fig 4- MALDI-TOF-MS spectra of samples of: a-fresh meat; b-industrial salami after 30 days of ripening; c-artisanal salami after 30 days of ripening.

Tab 1- Identification of sarcoplasmic proteins analysed by RP-HPLC coupled to MALDI-TOF/MS, ESI-Q-TOF/MS.

MW measured	MW theoretical	Protein	Fresh meat	Artisanal salami after 7 days of ripening	Artisanal salami after 30 days of ripening	Industrial salami after 7 days of ripening	Industrial salami after 30 days of ripening
15041.8	15039.1	Alpha globin (P01965)		x	x		
15807.0	15963.4	Epsilon globin (P02101)		x	x	x	
16955.5	16953.4	Myoglobin (P02189)	xxx	x	x		
18019.8	18024.9	Troponin C 2 (P02587)	xx				x
23619.0	23593.6	Cathepsin K (Q9GLE3)	x		x		x
26522.9	26598.4	Triosephosphate isomerase (Q29371)	xxx	xxx	xxx	xxx	xxx
28727.5	2872.8	Phosphoglycerate mutase (Q3SZ62)	x		x		
29307.0	29280.2	Carbonic anhydrase 3 (Q5S1S4)	xx	x	x	xx	xx
35724.0	35704.8	G3PD (P00355)	xxx	x	xx	x	x
36093.9	36489.4	L-Lactate dehydrogenase A chain (P00339)		x			
39248.2	39211.7	Fructose bisphosphate aldolase A (P00883)	xxx	x	x		
43056.1	43059.0	Creatine kinase M-chain (Q5XLD3)	xxx				
44528.9	44427.4	Phosphoglycerate kinase 1 (Q7SIB7)	x				
47078.8	46998.9	β-Enolase 3 (Q1KYT0)	xx	xx	xx	x	x
57898.7	57817.7	Pyruvate kinase (A5D984)	x			x	x
62872.5	62994.8	Glucose-6-P isomerase (P08059)	x			x	
76794.4	76701.4	NADPH cytocrome p450 reductase (P04175)				x	
	85195.6	Phosphofructokinase	x				

( ) Primary accession number of data base UniProtKB/Swiss-Prot

3.3.2 Peptide characterization

The low molecular weight peptide fraction isolated from the fresh meat sample which was the raw material for preparation of either industrial or artisanal products was analysed with the same mass spectrometry approach described for the protein fraction. As this sample was in an early stage of the tenderization process (48-72 h), a few peptides were present. These peptides derived mainly from the proteolysis of specific proteins such as phosphofructokinase, piruvate kinase and b-enolase. Some of the peptides identified, such as 257-268 of G3PD and 65-80 of β-enolase, have been already reported to be formed during tenderization of bovine meat (Stoeva S. et al, 2000).

In artisanal salami the proteolysis was less pronounced with respect to the industrial one and the peptides derived from the hydrolysis of proteins different from those of the industrial product. This difference was due to the use of microbial starter in the industrial salami, which broke down the sarcoplasmic proteins faster than the endogenous microflora acting on the artisanal one.

In Fig 5 was reported the comparison of MALDI-TOF spectra of peptides, respectively of industrial and artisanal salami after 7 days of ripening; in the industrial sample the peptides derived mainly from hydrolysis of β-enolase 3 and creatine kinase while in the artisanal sample most peptides derived from β-enolase 3 and pyruvate kinase, in both samples there were the same peptides such as 198-202 and 421-429 of β-enolase 3.

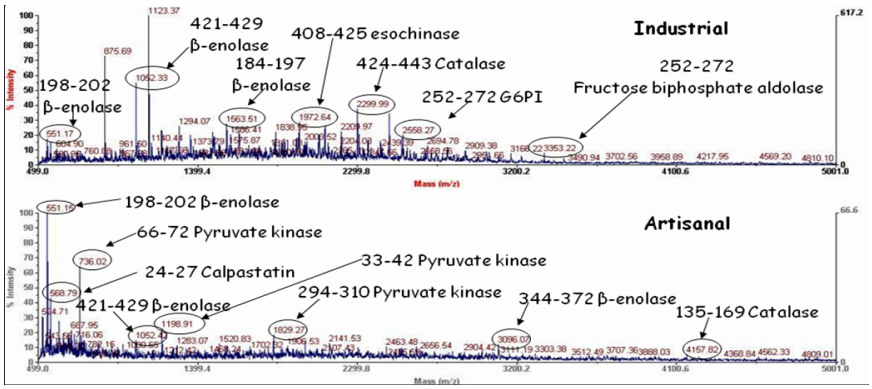

Fig.- 5 MALDI-TOF-MS spectra of peptides of industrial and artisanal salami after 7 days of ripening

In Fig 6 was reported the comparison of MALDI-TOF spectra of peptides, respectively of industrial and artisanal salami after 21 days of ripening in the industrial salami they derived mainly from pyruvate kinase and β-enolase. In the sample of artisanal salami after 21 days of ripening, the peptides derived mainly from creatine kinase and calpastatin.

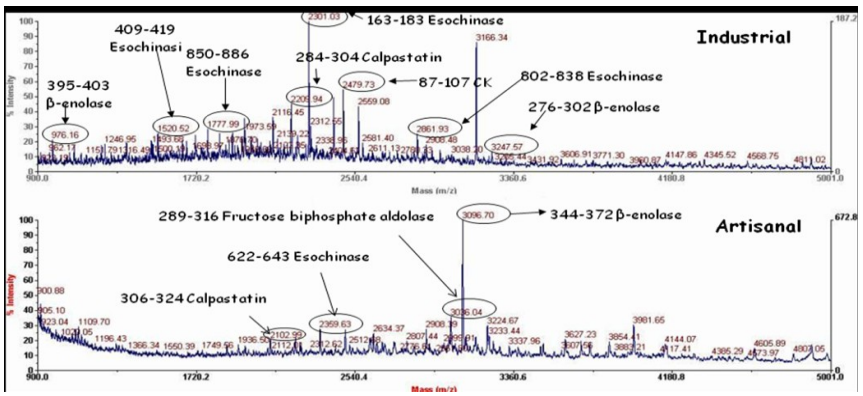

Fig. 6- MALDI-TOF-MS spectra of peptides of industrial and artisanal salami after 21 days of ripening

Finally in industrial salami after 30 days of ripening the proteolysis degree resulted more intensive than artisanal sample (Fig.7); this could be due to inoculated microflora which break down sarcoplasmic protein faster, with the production of a high number of novel peptides that derived mainly from G3PD, pyruvate kinase and fructose bisphosphate aldolase A, while in artisanal salami they derived mainly from esokinase, creatine kinase and β-enolase3.
Some peptides common to both type of salami were also found: the peptide 127-132 of β-enolase 3, already reported by A. Di Luccia et al, 2005 in cured ham, present at 21 days of industrial salami and 21 and 30 days of artisanal one and the peptide 421-429 of β-enolase present at 7 days of industrial salami and 7 and 30 days of artisanal one.
In industrial salami some peptides were common to the three different ripening times, such as the peptide at 2209.9 Da (104-126 of β-enolase3) and the peptide at 2479.5 Da (87-107 of creatine kinase), while another peptide at 1357.7 Da (1-13 of G3PD) was present in the samples at 21 and 30 days of ripening. The persistence of these peptides was justified by the fact that they were quite resistant to proteolysis or because they were continuously formed during ripening.

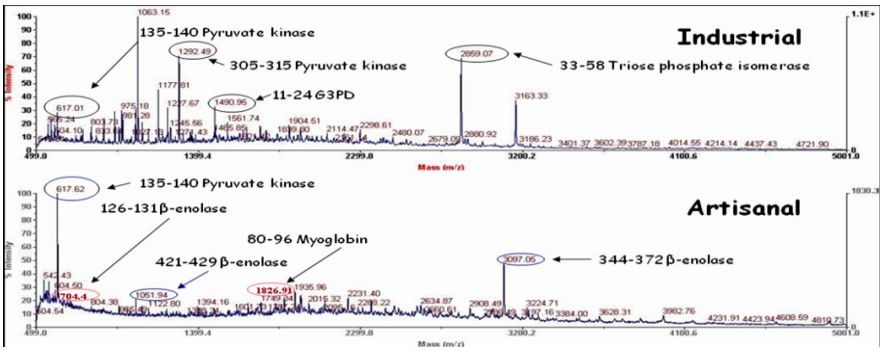

Fig. 7- MALDI-TOF-MS spectra of peptides of industrial and artisanal salami after 30 days of ripening

The protein and peptide studies have been finally extended to industrial (Galbani and Franchi) and artisanal (S. Anastasia and Avellino) salami procured on the market, the analysis were carried out by the same approach described for the protein fraction and the results are summarised in Table 2.

Even though few peptides are present in all salami samples analysed (such as the peptide 2166.66 Da that is 401-422 piruvate kinase, 2685.96 Da that is 488-512 phosphofructokinase, 3241.45 Da that is 276-302 β-enolase 3), there are some peptides present only in the three industrial samples (Fig.8) such as the peptide at 2207.69 Da (104-126 β-enolase 3) and at 1253.83 Da (408-419 esokinase II); some peptides common only to Quattromani and Franchi industrial salami such as the peptide at 1172.80 Da (490-500 pyruvate kinase), 1559.93 Da (88-101 fructose biphosphate aldolase) and 1673.03 Da (367-381 creatine kinase) and some other common only to Galbani and Franchi salami such as the peptide at: 1931.49 Da (153-170 creatine kinase), 2079.54 Da (117-135 creatine kinase), 2510.09 Da, 2943.46 Da, 3760.73 Da, 4049.09 Da, 4127.75 Da and 4138.24 Da.

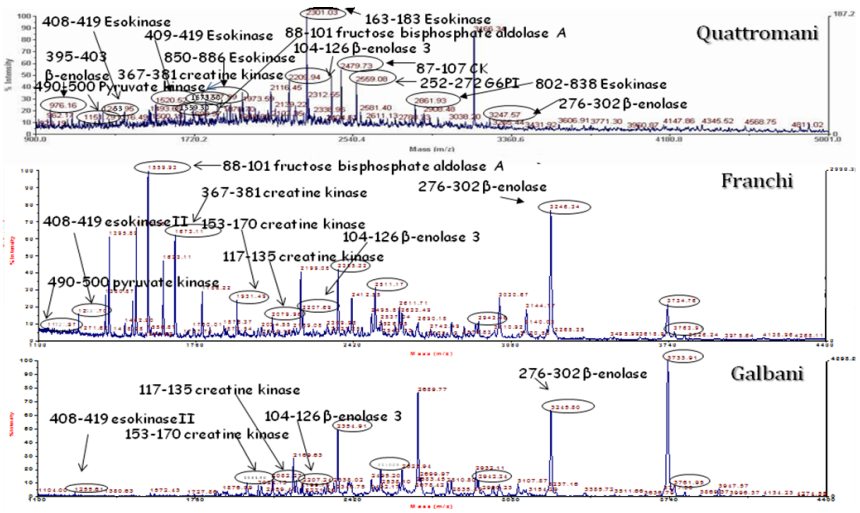

Fig.8- MALDI-TOF spectra of peptide fractions of industrial salami Quattromani, Franchi and Galbani respectively

On the other hand there are some peptides common to only two commercial artisanal salami (S. Anastasia and Avellino) such as the peptide at 1103.43 Da (199-207 calpastatin), 2033.55 Da, 2696.91 Da and 3106.15 Da (307-332 G3PD).(Fig. 9)

All these peptides could be used as molecular markers of manufacturing and technological process in order to differentiate an industrial salami from an artisanal one. Furthermore the characterisation of the peptides formed during ripening could be used to evaluate the degree of proteolysis in a given meat sample as well as to correlate the rheological and sensory characteristics with formation of typical compounds.

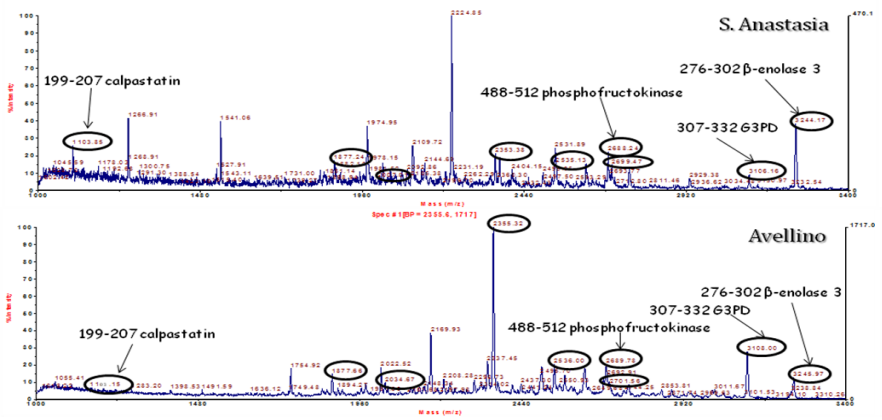

Fig.9- MALDI-TOF spectra of peptide fractions of artisanal salami S. Anastasia and Avellino respectively.

Table 2- Peptides of industrial and artisanal salami after 30 days of ripening identified with direct MALDI-TOF analysis

Fractions 0-10kDa Peptide mass (m/z) Da (peptide sequence)	**Artisanal salami**			**Industrial salami**		
	Arzano	S.Anastasia	Avellino	Quattromani	Galbani	Franchi
667.99 (89-93 phosphofructokinase)		x			x	x
749.21			x			
872.38			x			x
916.41 (120-126 troponin C)		x				
1103.43 (199-207 calpastatin)		x	x			
1172.80 (490-500 pyruvate kinase)				x		x
1207.47					x	
1253.83 (408-419 esokinase II)				x	x	x
1267.89 (97-107 creatine kinase)		x			x	x
1395.89 (406-418 eso-kinase)	x					x
1490.72		x	x	x	x	x
1509.06 (157-170 creatine kinase)						x
1541.06 (305-316 creatine kinase)		x				x
1559.93 (88-101fructose biphosphate aldolase)				x		x
1570.14					x	
1623.11 (301-314 catalase)						x
1673.03 (367-381 creatine kinase)				x		x
1751.28	x		x		x	

(264-278 pyruvate kinase)						
1786.22 (342-358 creatine kinase)						x
1860.60			x		x	
1905.49			x			
1931.49 (153-170 creatine kinase)					x	x
1973.43 (10-25 creatine kinase)	x	x			x	
2022.52 (57-74 glucose-6-P-isomerase)		x	x		x	
2033.55		x	x			
2079.54 (117-135 creatine kinase)					x	x
2109.72 (31-50 β-enolase 3)	x	x				
2166.66 (401-422 piruvate kinase)		x	x		x	x
2178.62		x				
2199.08						x
2207.69 (104-126 β-enolase 3)				x	x	x
2224.85 (108-129 phosphofructokinase)		x				
2306.77		x				
2338.29			x			
2351.76		x	x		x	x
2510.09					x	x
2533.81		x	x			x
2629.67	x	x				
2685.96 (488-512 phosphofructokinase)		x	x		x	x
2696.91		x	x			
2809.11					x	
2928.01 (185-210 phosphofructokinase)		x			x	
2943.46					x	x
3031.41 (290-317 fructose biphosphate aldolase)						x
3106.15 (307-332 G3PD)		x	x			
3143.65						x
3230.37 (16-46 catalase)	x				x	x
3241.45 (276-302 β-enolase 3)		x	x		x	x
3729.72		x	x		x	x
3760.73					x	x
4049.09					x	x
4127.75					x	x
4134.24		x	x		x	x
4138.24					x	x

4192.88					x	
4237.74		x				
4395.16					x	
4463.39		x				
4649.65					x	
4785.82					x	
5077.96						x
5093.86						x
5149.03						x
5161.19		x				
5207.00						x
5296.02						x
6991.98		x				

3.4 Conclusions

A remarkable contribution to characterisation of the sarcoplasmic protein fraction of Naples-type salami was reached by proteomic approach using RP-HPLC, MALDI-TOF-MS and ESI-MS techniques.
The results are here summarized:

-Proteolysis resulted less proceeded in artisanal salami than industrial one, because the latter has been manufactured using selected starter cultures.

-Final protein pattern of industrial salami resulted different from artisanal one because essentially endogenous muscle enzymes, such as calpains and cathepsins, act during ripening of artisanal salami together with enzymes of inside microflora. These differences can be put in evidence by creation of data banks could be achieved to quickly identify and safeguard typical products.

-The proteins less susceptible to proteolysis and common to both salami were carbonic anidrase, glyceraldehyde-3-phosphate dehydrogenase and triose phosphate isomerase.

-Moreover some peptides common to industrial samples after 7, 21 and 30 days of ripening, have been identified and some other common to artisanal salami in order to use them as molecular markers as indicators of manufacturing and technological processes.

-Furthermore the characterisation of the peptides formed during ripening could be used to evaluate the degree of proteolysis in a given meat sample as well as to correlate the rheological and sensory characteristics with formation of typical compounds.

-The selection of suitable starters could improve quality and sensory properties of this typical product, furthermore the peculiar features of artisanal salami, manufactured without use of starter cultures, could be developed with the selection of suitable productive standards.

-The characterization of sarcoplasmic proteins and peptides could be used for identification of molecular markers of quality and typicality in order to obtain the Protected Designation of Origin (P.D.O.) mark, to differentiate an artisanal salami from an industrial one and to allow traceability of the products.

3.5 References

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3.6 Metabolomic analysis of lipid and aromatic components

Flavour is an important quality attribute of muscle foods and comprises mainly the two sensations of taste and aroma or smell. Although both of these factors affect the overall acceptability of foods, the aroma or flavor volatiles are of utmost importance because they influence the judgment of the consumer even before the food is eaten.
The role of lipids in meat flavor generation has been the subject of extensive studies. Traditionally, lipolysis has been mainly related to bacterial lipase activity whereas oxidative changes of unsaturated fatty acids, resulting in the production of lipid peroxides and carbonyl compounds, have been related to both chemical reactions and bacterial metabolism (Demeyer et al., 1974). Recently, the importance of endogenous meat and fat lipases in lipolysis during dry sausage ripening has been suggested by Talon et al. (1992) and Garcia et al. (1992).
During ripening the lipid fraction undergoes hydrolytic and oxidative changes, involving liberation of free fatty acids (FFA) and oxidation of unsaturated fatty acids, particularly polyunsaturated acids, with production of carbonyl compounds (Demeyer et al., 1974). The free fatty acid (FFA) during enzymatic and chemical oxidative reactions form directly volatile compounds and precursor of odorous molecules.
Synergistic interactions between various compounds can influence the perception of meat flavour (Calkins et al., 2007) and the characteristic aroma and taste of salami are associated to a complex mixing of spices, acidification products and enzymatic reactions involving mainly fats and proteins (Taurino A.M. et al., 2003). Only little is known about the naturally occurring microflora in Italian artisanal salami and its role determining the specific biosynthetic pathways which produce a number of volatile metabolites responsible of aroma and taste.

3.7 Materials and methods

3.7.1 Aroma extraction

The SPME analysis was carried out from 25 g of salami sample homogenized in 100 mL of water and 20 g of NaCl in a hermetically closed vial (130 mL), the fibre (CAR/DVB/PDMS) was placed in the headspace (HS) until equilibrium was reached. With analogous conditions the SPME analysis was carried out on the aged minced used in the making process (50 g) and on some added ingredients (pepper added and milk powder; 3 g).

3.7.2 GC-MS aroma analysis

Thermal desorption of the analytes from the fibre inside the GC injection port was carried out at a desorption temperature of 250 °C for 1 minute.

All samples were analysed with an HP6890 GC (Agilent Technologies) coupled to a 5973N quadrupole HP mass spectrometer. The gas chromatograph was equipped with an HP-5ms capillary column (30 m x 0.25 mm ID) and the carrier gas used was helium.
For the analysis of free compounds the GC oven temperature was programmed from 40 °C (hold for 7 minutes) to 180 °C at 5 °C/min. The masses were scanned on m/z range of 45-350 amu. For the identification of odorous components the NIST library and/or comparison with spectra and retention times of standards were used.
The quantitative determinations were carried out through calibration in matrix in the range of verified linearity; multiple replicates (n=3-6) of the samples were analysed.

3.7.3 Lipid compounds analysis

The extraction of free fatty acids and glycerides was carried out on the fat separated from minced meat and from artisanal and industrial salami. 0,4 g of fat have been subjected to fractional extraction through three steps: 1.5 ml of methanol, methanol/chloroform (1:1) and chloroform. The methanol extract was first directly analyzed by GC/MS for the detection of short-chain fatty acids unstable in assay conditions utilized. On the three extracts was performed transesterification with methanolic potassium 2N. After transesterification methyl esters were extracted with hexane and analyzed by GC/MS.

3.8 Results and discussion

In order to observe possible differences in the composition of volatile metabolites related to the different technological choices of production (spontaneous fermentation due to microorganisms originated from the meat itself, the environment, "house flora", the fermentation with selected starter cultures, the variability of quantity and variety of used spices, etc.) a SPME-GC/MS analysis was carried out in the headspace of the two typologies of Naples-type salami samples (artisanal and industrial salami).
Only 39 different volatile and semi-volatile compounds were isolated from the analysed Naples-type samples (Table 3).
From a qualitative point of view, aldehydes, alcohols, esters, ketones, carboxylic acids, terpenes and their derived compounds, sulfides and phenols were the chemical families present in the headspace. The terpene compounds were the dominant chemical family: at least 80% of the total chromatogram area, and their qualitative and quantitative composition (α-pinene, caryophyllene, β-pinene, β-myrcene, 3-carene, linalool, limonene, α-phellandrene, copaene, α-guajene, β-selinene, cedrene etc.) derived mainly from the specific variety and quantity of the added pepper (Table 3, Fig. 10; Mamatha et al., 2008), in fact some terpenes were not detected simultaneously in the both Naples-type salami typologies (e. g. camphene, guajene; Table 3).

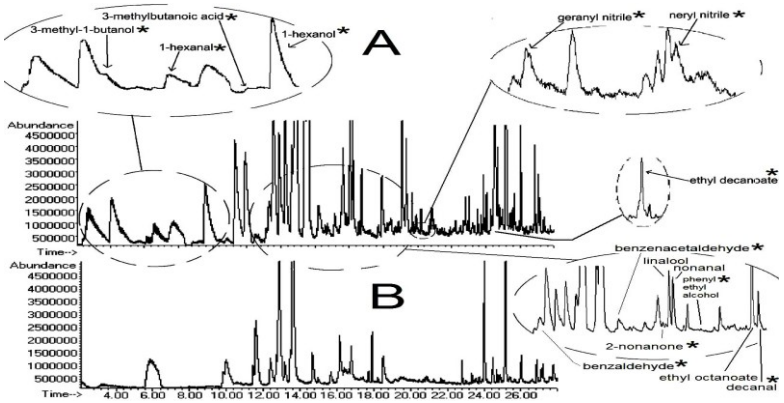

Fig. 10- TIC chromatograms obtained by means of headspace-SPME-GC/MS analysis on industrial (A) and artisanal (B) Naples-type salami samples respectively. Potential process markers are indicated with asterisk (*).

Terpene derivatives such as the cariophyllene oxide (which derived reasonably from the oxidation of caryophillene, sesquiterpenoid compound present in the added pepper) were also detected but only in industrial salami sample; in addition some terpene nitrile compounds, such as neryl nitrile and geranyl nitrile were also detected in the salami samples. The occurrence in salami samples of organic nitrile compounds was already reported in some previous studies (Stahnke, 1995; Mottram et al., 1984): they can be formed during the lipid oxidation involving nitrite (Stahnke, 1995). These compounds also could act as possible process and quality markers, taking in account that the addition of nitrites can play also a role in the fermentation process: these substances have a peddling effect on lactic acid bacteria, whereas "atypical streptobacteria" show relevant resistance. Taurino et al (2003) indicated that this empirical selection can be induced by producers, in addition to other tools, increasing the number of area typical strains accounting for the recognised area tipicity of registred origin product designation (Taurino A. M. et al., 2003).
Although the major sources of terpenes are related to the use of spices in the production process, some terpene compounds were also detected, but only in traces, in the used raw materials and other added ingredients (i.e. meat, milk powder, spices etc.; traces of limonene and some other terpenes, for example, appeared already present in the milk powder used for the Naples-type salami: these hydrophobic compounds deriving from animal feeding can remain adsorbed in the milk powder components, some terpene compounds were also detected in the minced meat, deriving, in this case also, from the animal feeding).

Tab. 3 Qualitative comparison of some volatile metabolites in industrial and artisanal Naples-type salami samples.

	Tr (min)		Artisanal salami	Industrial salami	Odor descriptor	Metabolic pathway
Aldehydes	2.8	3-methyl-1-butanal		+	malty, bit fruity	Catabolism of leucine (Beck et al., 2004)
	4.6	Hexanal		+	green	Lipidic auto-oxidation, microbial beta-oxidation or amino acid degradation (Montel et al., 1998)
	8.9	Heptanal		+	green	Lipidic auto-oxidation, microbial beta-oxidation or amino acid degradation (Montel et al., 1998)
	15.2	Nonanal	+	+	pelargonium, rancid	Lipidic auto-oxidation, microbial beta-oxidation or amino acid degradation (Montel et al., 1998)
	22.7	2,4-decadienal		+		Lipidic auto-oxidation, microbial beta-oxidation or amino acid degradation (Montel et al., 1998)
	19.5	Decanal		+	pelargonium, rancid	Lipidic auto-oxidation, microbial beta-oxidation or amino acid degradation (Montel et al., 1998)
	11.2	Benzaldehyde		+	almond	From phenylalanine by means of microbial activity (Montel et al., 1998)
	14.2	Benzene acetaldehyde		+	floral	From phenylalanine by means of microbial activity (Montel et al., 1998)
Alcohols	2.9	3-methyl-1-butanol	+	+	fruity	Catabolism of leucine (Beck et al., 2004)
	7.5	1-hexanol		+	green	Lipidic oxidation followed by reduction by means of alcohol dehydrogenase (Montel et al., 1998)
	11.9	1-octen-3-ol	+	+	mushroom	Lipidic oxidation followed by reduction by means of alcohol dehydrogenase (Montel et al., 1998)
	18.2	Decen-1-ol	+			Lipidic oxidation followed by reduction by means of alcohol dehydrogenase (Montel et al., 1998)
	17.2	Phenyl ethylalcohol		+	floral	From phenylalanine by means of microbial activity and/or oxidative deamination (Montel et al., 1998)
Esters	19.1	Ethyl octanoate	+	+	pineapple	Chemical or microbial esterification (esterase activity of different microorganisms, for example *S. carnosus and xylosus*)(Toldrà, 1998)
	24.5	Ethyl decanoate		+	fruity	Chemical or microbial esterification (esterase activity of different microorganisms, for example *S. carnosus and xylosus*)(Toldrà, 1998)
	13.3	Ethyl hexanoate		+	apple	Chemical or microbial esterification (esterase activity of different microorganisms, for example *S. carnosus and xylosus*) (Toldrà, 1998)
Ketones	15.9	2-nonanone		+	hot milk	Auto-oxidation, microbial beta-oxidation of fatty acids (Montel M.C. et al., 1998) followed by beta-keto acid decarboxylation
Carboxylic acids	6.9	3-methylbutanoic acid		+	sweat socks	Catabolism of leucine (Beck et al., 2004)
	13.0	hexanoic acid	+	+	pungent, cheese	Hydrolysis of triglycerides and phospholipids (Toldrà, 1998)
	19.0	octanoic acid	+	+	rancid, fruity	Hydrolysis of triglycerides and phospholipids (Toldrà, 1998)
	24.4	decanoic acid	+	+	pungent, cheese	Hydrolysis of triglycerides and phospholipids (Toldrà, 1998)

Terpene nitrile compounds	19.9	Geranyl nitrile		+		From lipid oxidation involving nitrite (Stahnke, 1995; Mottram et al., 1984)
	20.9	Neryl nitrile		+		From lipid oxidation involving nitrite (Stahnke, 1995; Mottram et al., 1984)
Terpene compounds and derived molecules	11.6	β-pinene	+	+	pine oil	Pepper
	10.5	Camphene		+	pepper	Pepper
	12.3	β-myrcene	+	+	spice	Pepper
	12.6	α-phellandrene	+	+	pepper	Pepper
	13.2	cymene	+	+	floral	Pepper
	13.5	Limonene	+	+	lemon	Pepper
	14.6	Carene	+	+	lemon	Pepper
	16.0	Linalool	+	+	orange peel	Pepper
	23.8	Copaene	+	+	spice	Pepper
	25.6	α-Guajene		+		Pepper
	26.0	Caryophyllene	+	+	spice	Pepper
	26.8	β-Selinene		+	herb	Pepper
	27.3	Cariophyllene oxide		+	herb	Pepper
	27.7	Cedrene		+		Pepper
Sulfides	2.0	Dimethylsulfide	+	+	putrid odor	From sulphurated amino acids (Montel M.C. et al., 1998)
Synthetic antioxidant phenol	27.5	BHT	+	+		From animal feeding

The qualitative and quantitative composition of the other volatile compounds deriving from specific metabolic pathways, such as proteolysis and lipolysis (due to proteases, peptidases and lipases which derived from muscles and microorganisms) and/or from chemical transformations (lipid oxidation, Maillard reactions, Strecker degradation, etc.), contributed to differentiate, through some other "molecular markers", the artisanal and industrial Naples-type salami samples. In Table 3 a qualitative comparison of the volatile component composition of both salami products is reported and in Fig. 11 a quantitative comparison among data obtained for some representative aroma compounds is also shown. All quantified volatile compounds (with odour thresholds which ranged from some units of ppb, for example in the case of terpenes, up to about fifty ppm for some carboxylic acids; Montel et al., 1998) could be potential odour-active molecules (also considering that, because of the specific texture interactions present in the meat matrices, the odour threshold values could be particularly high in comparison for example with water; Montel et al., 1998); in fact the quantified molecules (Fig. 11) appeared in concentration above the odour threshold reported in literature (Marco A. et al., 2007; Montel et al., 1998). The observed differences in the composition of the volatile fraction appeared to indicate some potential "process markers"

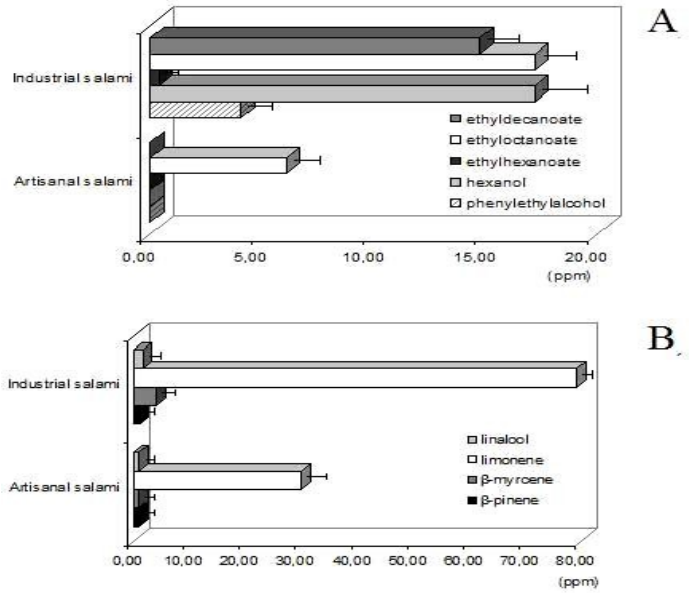

Fig. 11- Quantitative composition in industrial and artisanal salami samples obtained for: (A) some representative volatile metabolites derived from microbial and/or chemical pathways, and (B) terpene compounds from the added ingredients (pepper).

Among the volatile metabolites deriving from amino acids in the industrial salami, benzaldehyde and benzeneacetaldehyde, formed from phenylalanine through a microbial enzymatic conversion (Montel et al., 1998), were detected. In addition, 3-methyl-1-butanol, 3-methyl-1-butanal and 3-methyl butanoic acid, derived from leucine catabolisme, whose presence is possibly related to the presence of *S. xylosus* which was added in the industrial salami product (3-methyl butanoic is considered one of characteristic sausage aroma compound with "sweaty" flavour; Leroy et al, 2006; Beck et al., 2004) were not detected only in the case of the artisanal salami. C6 compounds (hexanal, hexanol, with herbaceus descriptor, present in the initial sausage batter), which can be oxidized to hexanoic acid, were absent in the artisanal sample while their quantity decreased during the ripening in the aroma profile of the industrial salami sample in comparison with the minced meat. The absence of added starters in the artisanal sample appeared to produce minor concentrations of some compounds such as ethyl esters (present already, but only in traces, in the minced aged meat used in the initial process step and in the used pepper) which were formed through chemical esterification of alcohols and carboxylic acids or through microbial esterase activity of different microorganisms such as *S. carnosus* and *S. xylosus* (Toldrà, 1998; Talon et al., 1998).

Short chain fatty acids such as octanoic, decanoic, dodecanoic acids were present in major quantity in the industrial salami, from the lipolysis during the ripening process of salami which appeared more advanced in this latter salami typology. These compounds, as final products of microbial lipase hydrolysis of tissue, seem to give an important contribute to the salami flavour (Taurino et al., 2003).

Fatty acids (deriving from hydrolysis of triglycerides and phospholipids; Toldrà, 1998) appeared initial substrate for oxidation reactions with formation of final odorous products (for example, 2-nonanone, among the different possible metabolic pathways, can be formed through the beta-oxidation of decanoic acid derived from lipolysis and in second step through beta-ketoacid decarboxylation due to microbial enzymes, *S. carnosus* and *S. xylosus*; Montel et al., 1998) or formation of oxidized forms on the double bound of unsaturated fatty acids with production of hydroxylated forms present in traces in the chromatograms obtained after esterification with methanolic potassium. Among the identified aldehydes, hexanal and 2,4-decadienal are volatile oxidation products from linoleic acid, while nonanal is an oxidation product from oleic acid (oleic and linoleic acids are the two most abundant unsaturated fatty acids in pork) (Moretti V. M. et al., 2004). Some aldehydes, such as heptanal and decanal, derived from hydrolysis of triglycerides by exogenous and endogenous lipases, and consequent fatty acid oxidation and/or microbial beta-oxidation, or from amino acid degradation (Montel et al., 1998) were not detectable in the artisanal salami sample.

3.9 Conclusions

The results indicated that the use of starters could modify the quantitative and qualitative composition of volatile fractions of the Naples-type salami; if the use of selected starters can permit to obtain the desired optimal qualitative and/or functional characteristics (Leroy et al., 2006), on the other hand they can potentially modify and then affect the specific typicalness characteristics of salami products (and consequently their contribute on cultural and gastronomic identity of each different regional area), which derive from the characteristics of each ingredient and their specific natural source of interesting wild-type microorganisms (Mateo et al., 1996; Meinier et al., 1999; Procida et al., 1999). Further studies on the sensorial acceptance of these different typologies of Naples-type salami depending on the molecular composition, should be carried out, in view of the qualitative improvement of this typical product. It is important to consider that the presence of higher amounts of volatiles in the industrial salami could affect not only positively but also negatively the salami flavour (Moretti et al., 2004).

The knowledge and study of the metabolic pathway characteristics of these wild type microorganisms on molecular basis could be useful to reinforce the qualitative attributes of local products and eventually consider the use of wild-type microorganisms of artisanal specialities in industrial products.

Some volatile "molecular markers" related to the used process (such as C6 compounds, hexanal and hexanol, heptanal, decen-1-ol, 3-methyl-1-butanal, neryl nitrile, geranyl nitrile etc.) were observed by comparing the volatile fraction of the two salami typologies considered.

Further studies are also necessary to investigate the release of volatile compounds in this kind of fermented meat products by different autochthonous strains in order to support the possible use of some metabolites as quality and process molecular tracers.

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ABSTRACT

Naples-type salami is dry fermented sausages with medium-size grana made of coarsely minced lean pig, mixed with fat, salt, pepper and various spices, stuffed into natural or artificial casings and ripened for 30-60 days, native to the Campania, today produced throughout Italy. The organoleptic and sensory properties of this product are due to the degradation events (proteolysis, lipolysis) occurring during the maturation: the lipid fraction undergoes hydrolytic and oxidative changes, involving liberation of free fatty acids (FFA), volatile compounds and precursor of odorous molecules. Endogenous enzymes, such as calpains and cathepsins, are primarily responsible for the initial degradation of the sarcoplasmic and myofibrillar proteins, then the most commonly found Lactobacillus species in dry fermented meats are able to hydrolyse myofibrillar and sarcoplasmic muscle proteins in vitro. The peptides produced can influence final taste of the salami and moreover are object of the activity of endogenous and exogenous amino peptidases releasing amino acids which represent precursors of aromatic compounds. The aim of this work has been to characterize the metabolites formed during ripening of Naples-type salami, in particular have been evaluated structural and aromatic differences between industrial and artisanal products.

Analyses were carried out on two Naples-type salami experimental productions, an industrial and an artisanal, that differ in amount of pepper and for the use of microbiological starter in industrial salami and on four salami samples procured on the market. At first a proteomic analysis has been carried out on sarcoplasmic fraction of Naples-type salami by RP-HPLC technique coupled to MALDI-TOF-MS and ESI-MS, later on a metabolomic analysis of lipid and aromatic components using HS-SPME-GC-MS techniques.

The proteolysis produced a different final proteic pattern probably due to the action of different microflora a microbial starter in industrial salami and autochthon microflora in artisanal one; the proteins less susceptible to proteolysis were carbonic anhydrase 3, glyceraldehyde-3-phosphate dehydrogenase , β-enolase 3 and triosephosphate isomerase; in both salami samples were observed the total proteolysis of creatine kinase, phosphofructokinase and phosphoglicerate kinase, instead myoglobin, fructose bisphosphate aldolase A and phosphoglicerate mutase disappeared only in industrial salami and troponine C 2, pyruvate kinase and glucose-6-P-isomerase in artisanal one. The fresh meat sample presented a few peptides because in an early stage of the tenderization process (48-72 h), however they derived mainly from the proteolysis of phosphofructokinase, piruvate kinase and β-enolase 3. In the sample of industrial salami after 30 days of ripening the proteolysis resulted very pronounced with production of a high number of novel peptides that derived mainly from glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and fructose bisphosphate aldolase A. Moreover some peptides common to industrial samples after 7, 21 and 30 days of ripening, such as the peptide 284-304 of calpastatin and the peptide 87-107 of creatine kinase, have been identified and some other common to artisanal salami in order to use them as molecular markers as indicators of manufacturing and technological processes.

Final proteic pattern of industrial salami resulted different from artisanal one because essentially endogenous muscle enzymes, such as calpains and cathepsins, act during ripening of artisanal salami together with enzymes of inside microflora. These differences can be put in evidence by creation of data bank could be achieved to quickly identify and safeguard typical products. Furthermore the characterisation of the peptides formed during ripening could be used to evaluate the degree of proteolysis in a given meat sample as well as to correlate the rheological and sensory characteristics with formation of typical compounds.

The aromatic profile of industrial salami resulted more complex than artisanal one for the use of starter cultures. Pepper compounds were quantitatively the largest group of volatiles identified in the aromatic profile of industrial salami at 30 days of ripening. The absence of microbial starter in artisanal salami determine a minor concentration of compounds such as ethyl esters and methyl ketones related to activity of *S. xylosus* and *carnosus* species. Among volatile and semivolatile odorous molecules identified in salami samples, were present short chain fatty acids such as octanoic acid, decanoic, dodecanoic. These molecules are present in greater amount in the industrial salami to indicate a more advanced lipolytic process in this last one respect to artisanal salami.

The characterization of sarcoplasmic proteins and peptides as well as the analysis of aromas could be used for identification of molecular markers of quality and typicality in order to obtain the Protected Designation of Origin (P.D.O.) mark, to differentiate an artisanal salami from an industrial one and to allow traceability of the products.

RIASSUNTO

Il salame tipo Napoli è il prodotto della fermentazione lattica di carne cruda di grana media, tritata e salata, miscelata con lardo in cubetti o tritato, addizionata di varie spezie, insaccata e pressata in contenitori costituiti da budello naturale o sintetico e stagionata per 30-60 giorni. E' un insaccato originario della Campania, oggi prodotto in tutta Italia.

Le proprietà organolettiche e sensoriali di questo prodotto sono dovute agli eventi di degradazione (proteolisi, lipolisi) che si verificano durante la fase di maturazione: la frazione lipidica subisce cambiamenti idrolitici e ossidativi, che coinvolgono la liberazione di acidi grassi liberi (FFA), composti volatili e precursori di molecole odorose. Gli enzimi endogeni, calpaine e catepsine, sono i principali responsabili della degradazione iniziale delle proteine sarcoplasmatiche e miofibrillari, in seguito alcune specie di Lactobacilli ritrovate più comunemente nelle carni fermentate, sono in grado di idrolizzare le proteine miofibrillari e sarcoplasmiche del muscolo. I peptidi prodotti possono influenzare il gusto finale del salame e per di più sono oggetto dell'attività di amminopeptidasi endogene ed esogene che rilasciano amminoacidi liberi che rappresentano i precursori di composti aromatici.

Lo scopo del seguente lavoro è stato quello di caratterizzare i metaboliti generati durante la maturazione del salame tipo Napoli. In particolare sono state analizzate le differenze strutturali ed aromatiche tra prodotti industriali ed artigianali. Le analisi sono state effettuate su due produzioni sperimentali di salame tipo-Napoli che si differenziano per quantità di pepe aggiunto e per l'uso di starter microbici e su quattro campioni di salame reperiti in commercio.

Dapprima è stata effettuata l'analisi proteomica sulla frazione sarcoplasmatica del salame tipo Napoli mediante tecnica RP-HPLC e MALDI-TOF-MS e ESI-MS, poi si è passati ad un'analisi metabolomica delle componenti lipidiche e aromatiche mediante tecnica HS-SPME-GC -MS .

La proteolisi ha prodotto un diverso profilo proteico per l'azione della diversa microflora : lo starter microbico nei salami industriali e la microflora autoctona in quelli artigianali; le proteine meno suscettibile alla proteolisi sono state l'anidrasi carbonica, la gliceraldeide-3-fosfato deidrogenasi e la β-enolasi 3 e la triosofosfato isomerasi, in entrambi i campioni di salame è stata osservata la totale proteolisi di creatina chinasi, fosfofruttochinasi e fosfoglicerato chinasi; inoltre la mioglobina, la fruttosio bifosfato aldolasi A e la fosfoglicerato mutasi sono state proteolizzate solo nei salami industriali, mentre la troponina C 2, la piruvato chinasi e la glucosio-6-P-isomerasi solo in quelli artigianali. Il campione di carne fresca presenta pochi peptidi, perché è in una fase iniziale del processo di tenderizzazione (48-72 h), tuttavia essi derivano principalmente dalla proteolisi di fosfo-fruttochinasi, piruvato chinasi e β-enolasi 3. Nel campione di salame industriale dopo 30 giorni di stagionatura la proteolisi è risultata molto pronunciata con la produzione di un elevato numero di nuovi peptidi che derivano principalmente dalla gliceraldeide-3-fosfato deidrogenasi, la piruvato chinasi e fruttosio bisfosfato aldolasi A. Inoltre sono stati identificati alcuni peptidi comuni ai campioni industriali dopo 7, 21 e 30 giorni di stagionatura, come il peptide 284-304 della calpastatina e il peptide 87-107 della creatina chinasi, ed altri comuni ai salami artigianali al fine di usarli come marcatori molecolari dei processi tecnologici. In definitiva il profilo proteico del salami industriali è risultato diverso da quelli artigianali essenzialmente a causa degli enzimi endogeni del muscolo, come le calpaine e le catepsine, che agiscono durante la maturazione del salame artigianale insieme con gli enzimi della microflora autoctona. Inoltre la caratterizzazione dei peptidi formati durante la fase di maturazione potrebbe essere utilizzata per valutare il grado di proteolisi in un dato campione di carne e per correlare le caratteristiche reologiche e sensoriali, alla formazione di composti tipici.

Il profilo aromatico del salame industriale è risultato più complesso di quello artigianale per l'uso di colture starter. I composti del pepe sono stati quantitativamente il più grande gruppo di sostanze volatili identificate nel profilo aromatico dei salami industriali a 30 giorni di maturazione. L'assenza di starter microbici nel salame artigianale ha determinato una minore concentrazione di composti come gli esteri etilici e metilici e i chetoni derivanti dall'attività delle specie *S. xylosus* e *carnosus*. Tra le molecole odorose volatili e semivolatili identificate ritroviamo gli acidi grassi a catena corta come acido ottanoico, decanoico, dodecanoico, presenti in maggiore quantità nel salame industriale ,ciò indica che in esso vi è stato un processo lipolitico più avanzato rispetto al salame artigianale. La caratterizzazione di proteine sarcoplasmatiche e di peptidi così come l'analisi degli aromi potrebbero essere utilizzate per l'identificazione di marcatori molecolari di qualità e tipicità al fine di ottenere la Denominazione di Origine Protetta e per consentire la tracciabilità dei prodotti.