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PROTEOLYSIS IN DRY-CURED HAMS: SALT REDUCTION AND BIOACTIVE PEPTIDES

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Summary

1 ABSTRACT 7

2 INTRODUCTION 9

2.1 DRY-CURED HAMS 9

2.1.1 PRODUCTION PROCESS 9

2.2 PROTEINS AND PROTEOLYSIS 11

2.2.1 Muscle composition. 11

2.2.2 Muscle proteins 12

2.2.3 Proteolysis 12

2.3 THE ROLE OF SODIUM CHLORIDE AS CURING AGENT 14

2.3.1 Sodium and health implications 14

2.3.2 Salt reduction in dry-cured ham 14

2.3.3 Salt reduction in dry-cured ham: strategies 15

2.4 PEPTIDES RELEASED BY PROTEOLYSIS 16

2.4.1 NPADs 17

2.5 BIOACTIVE PEPTIDES 19

2.5.1 Antihypertensive peptides 19

2.5.2 Antioxidant peptides 22

2.5.3 Models of gastrointestinal digestions 25

2.5.4 Peptides transport across the intestinal barrier 26

2.6 METHODS FOR THE STUDY AND THE CHARACTERIZATION OF PEPTIDES 28

2.6.1 High-performance liquid chromatography (HPLC) 28

2.6.2 Mass spectrometry 29

2.7 BIBLIOGRAPHY 32

3 TRADITIONAL AND REDUCED SALT DRY-CURED HAM: STUDY OF THE PEPTIDE FRACTION 41

3.1 INTRODUCTION 41

3.2 MATERIALS AND METHODS 42
3.2.1 Reagents and solvents 42
3.2.2 Samples 42
3.2.3 Extraction of the peptide fraction (MW<10 kDa) 42
3.2.4 SDS-PAGE analysis on extracted samples 43
3.2.5 In-gel digestion 43
3.2.6 HPLC/ESI-MS/MS analyses 44
3.2.7 LTQ-Orbitrap analysis 44
3.2.8 Synthesis of NPADs 45
3.2.9 Data statistical analysis. 47
3.3 RESULTS AND DISCUSSION 47
3.3.1 Extraction method 47
3.3.2 Characterization of the peptide fractions extracted from “traditional” dry-cured hams 48
3.3.3 Conclusions 70
3.4 ACKNOWLEDGEMENTS 71
3.5 BIBLIOGRAPHY 71
4 BIOACTIVE PEPTIDES RELEASED BY THE GASTROINTESTINAL-DIGESTION OF DRY-CURED HAMS 73
4.1 INTRODUCTION 73
4.2 MATERIALS AND METHODS 74
4.2.1 Samples 74
4.2.2 Reagents and solvents 74
4.2.3 Extraction of the peptide fraction (MW<10 kDa) 75
4.2.4 Simulated in vitro gastro-intestinal digestion 75
4.2.5 SDS-PAGE analyses 76
4.2.6 Peptide transport studies 77
4.2.7 UPLC-ESI-MS analysis 78

4.2.8 HPLC/ESI-MS/MS analyses 79
4.2.9 LTQ-Orbitrap analyses 79
4.2.10 Solid phase peptide synthesis 80
4.2.11 Synthesis of NPADS 80
4.2.12 Ace-inhibitory activity 83
4.2.13 Antioxidant activity. 84
4.3 RESULTS AND DISCUSSION 85
4.3.1 Simulated in vitro gastro-intestinal digestion 85
4.3.2 Efficiency of digestion process 87
4.3.3 Chromatographic profile of ham samples before and after the digestion process. 88
4.3.4 Characterization of the peptide profile of the digested samples 89
4.3.5 NPADS 95
4.3.6 Peptide profile of the digesta as a function of the ageing time 96
4.3.7 Study of peptides transport across the intestinal barrier 97
4.3.8 ACE-inhibitor activity 106
4.3.9 Antioxidant activity 119
4.3.10 Linoleic acid assay 122
4.4 CONCLUSIONS 123
4.5 ACKNOWLEDGEMENTS 124
4.6 BIBLIOGRAPHY 124
5 GENERAL CONCLUSIONS and PERSPECTIVES 129
6 SUMMARY 131
6.1 BIBLIOGRAFY 133
7 CURRICULUM VITAE 135

1 ABSTRACT

This PhD thesis is focused on the study of the evolution of the proteolytic phenomenon in dry-cured hams as a function of the technological process (with particular emphasis on the possible technological solution aimed at reducing salt content) and on the evaluation of the functional properties linked to the occurrence and possible formation of bioactive peptides (in particular, antioxidant and antihypertensive activity). The characterization of the peptide profile and the study of its evolution is carried out by advanced methods of mass spectrometry hyphenated with chromatographic and electrophoretic separations. The evaluation of the functional properties is performed by in vitro tests, taking into account also the effect of the gastrointestinal digestion on the possible release of active peptides sequences encrypted in meat proteins. For this aspect, the project is also aimed at obtaining structure-activity relationships for the identified peptides and to perform the synthesis of the most significant structures in order to better characterize their properties.

Riassunto

Lo scopo di questo progetto di dottorato è quello di studiare l'evoluzione del fenomeno proteolitico in prosciutti crudi stagionati in funzione delle caratteristiche tecnologiche di produzione (con particolare riferimento alle soluzioni tecnologiche che permettano di ridurre il contenuto di sale) e di valutare le proprietà funzionali legate alla presenza e/o alla formazione di peptidi bioattivi (in particolare, attività antiossidante e antiipertensiva). La caratterizzazione del profilo peptidico e lo studio della sua evoluzione con la stagionatura viene effettuata mediante l'utilizzo di tecniche avanzate di spettrometria di massa accoppiate a metodi di separazione cromatografici e/o elettroforetici. La valutazione delle proprietà funzionali sarà condotta mediante test in vitro, considerando anche l'effetto della digestione gastrointestinale sul possibile rilascio di sequenze peptidiche bioattive incluse nelle proteine della carne. Per questo aspetto, il progetto ha anche l'obiettivo di studiare le correlazioni struttura-attività dei peptidi identificati e di realizzare la sintesi delle sequenze più significative per studiarne meglio le proprietà.

2 INTRODUCTION

2.1 DRY-CURED HAMS

There are many types of dry-cured hams whose main characteristics depend on the pig crossbreed, age, composition of the feed and type of production process. Some of the most important dry-cured hams are: Spanish Iberian and Serrano hams, Italian Parma and San Daniele hams and French Bayonne ham (Toldrà, 2006).

Traditional dry-cured ham is a high-quality product with a typical and characteristic texture and flavour. These parameters require a long process of production involving salting, post-salting, and ripening steps, which could last up to 24 months or even more.

2.1.1 PRODUCTION PROCESS

Dry-cured hams are manufactured with posterior thighs of domestic heavy pigs.

The production process of dry-cured hams may be divided in two main phases as a function of applied temperatures: the so called “cold phase”, generally a 4°C, which is divided in salting and post-salting steps and a second phase, the “hot phase”, generally between 14°C-20°C (the maturation step). The typical workflow of a dry-cured ham production process is summarized in Figure 1:

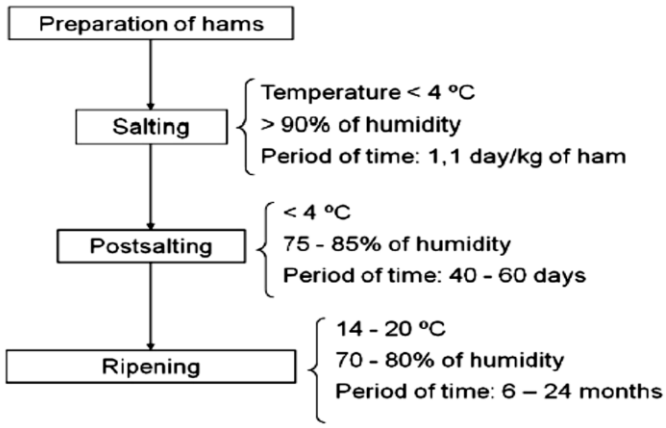

Figure 1. Typical workflow of dry-cured ham processing (Mora, 2013).

1. *Salting.* Hams are bledded on a steel belt with pressing rollers and receive the adequate amount of cure salt on the outer surface. During salting, that is kept under refrigeration (temperatures between 2 and 4°C), hams are placed on shelves and they are left to stand for 10–14 days to allow salt penetration into the ham (Toldrà, 2006).
2. *Post-salting.* In the next stage, named post-salting, the temperature is kept below 4°C for a period of time not less than 20 days but not exceeding 2 months. This step allows salt diffusion into the full piece.
3. *Ripening.* During this step several variable conditions such as time, temperature, relative humidity and air velocity could change as a function of the final characteristics of the product. In general, hams are placed in natural or air-conditioned chambers and subjected to different time-temperature-relative humidity cycles. Temperature is usually held between 14 and 20°C with a relative humidity decreasing from 90 to 70%. Hams ripening (or ageing or maturation time) might take from 6 to 18 months. The external part of the ham, which mainly corresponds to the muscle Semimembranosus (SM), as shown in Figure 2, dries up during the process due to the dehydration. On the other hand, internal muscles such as Biceps femoris (BF) present higher residual moisture content that allows a better enzyme activity for longer periods of time, thus inducing larger biochemical changes (Toldrà, 1997).

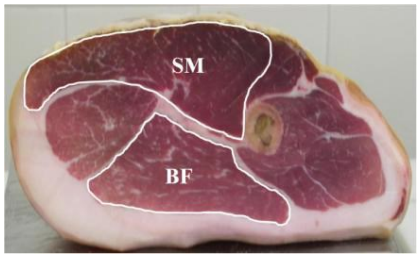

Figure 2. Muscles in dry cured hams: biceps femoris and semimembranosus muscle.

Many biochemical reactions take place during this process and are responsible for the final characteristic texture and flavour properties of the product (Toldrà, 2002). In order to properly understand them, it is essential to deeply know muscle structure and composition.

2.2 PROTEINS AND PROTEOLYSIS

2.2.1 Muscle composition.

Meat derives from the biochemical processes that take place in the skeletal muscles of slaughtered animals upon resolution of rigor mortis.

Skeletal muscle is composed of long, thin cells arranged in mutually parallel fibers. Each fiber is surrounded by connective tissue called endomysium. The fibers are then arranged in bundles surrounded by large sheets of thin connective tissue known as perimisium. Finally, more units of muscle bundles are held together by a large and thick layer of connective tissue called epimysium. The membrane that surrounds each individual fiber is called sarcolemma. Each fiber is comprised of many smaller myofibrils that are the constitutive contractile elements. The myofibrils are composed of actin and myosin filaments repeated in units called sarcomeres, the basic functional units of the muscle fiber. The sarcomere is responsible for skeletal muscle's striated appearance and forms the basic machinery necessary for muscle contraction. A sarcomere has two distinct bands – an 'I' band and an 'A' band. The 'I' band corresponds to an area where the actin proteins are located. The 'A' band corresponds to the area in which actin and myosin overlap. An 'H' zone marks the location of myosin proteins only. The boundary of each sarcomere is marked by 'Z' lines.

The myofibrils are constituted in turn by bundles of protein filaments: the thin filaments, consisting of the actin protein, together with minor amounts of tropomyosin and troponine, and the thick filaments, that are very large and elongated aggregates of myosin.

The bands “I” have only thin filaments while in the “H” bands only thick filaments occur; both types of filaments are present in other parts of the band “A” (Toldrà, 2010).

Meat is basically composed of water, protein, lipid, minerals and trace amounts of carbohydrate. Lean muscle tissue contains approximately 74% water, 21% protein, 4% fat and 1% ash. This proportion may change depending on the amount of fattening, especially if adipose tissue is included. The percentages of protein and water decrease when fat increases.

2.2.2 Muscle proteins

Proteins are the major constituent of skeletal muscle and make up approximately 15-22% of its weight. Muscle proteins may be classified in three main groups: myofibrillar, sarcoplasmic and stromal proteins.

- *Myofibrillar proteins.* These proteins are soluble in high ionic strength buffers. They are the main constituents of the structure of the myofibrils, and, in fact, myosin and actin provide the structural backbone of the myofibril. Tropomyosin and troponin are regulatory proteins associated with muscle contraction. The proteins in Z line serve as bridges between the thin filaments of adjacent sarcomeres. Titin and nebulin are two very large proteins, running in parallel to the long axis of myofibril, that contribute to the longitudinal continuity and integrity of muscle cells. Desmin connects adjacent myofibrils at the level of Z line.
- *Sarcoplasmic proteins.* These proteins are water-soluble proteins, composing about 30-35% of total muscle proteins. Sarcoplasmic proteins comprise a high number of different proteins, mainly metabolic enzymes (mitochondrial, lysosomal, microsomal, nuclear or cytosolic) and myoglobin. Some hemoglobin may also remain in the muscle, although most blood is drained from muscle during bleeding. Myoglobin is the protein pigment responsible for the red meat color.
- *Stromal proteins.* Collagen is the basic protein-forming part of the connective tissue that surrounds the fibers and muscles (epimysium, perimysium and endomysium). Collagen provides strength and support to the muscle structure (Toldrà, 2002).

  Several muscle proteases (cathepsins, calpains, peptidases and aminopeptidases) and lipases (lysosomal acid lipase, acid phospholipase and adipose tissue lipase) are involved in important biochemical mechanisms taking place during the processing of dry-cured meat products which are directly related to the final quality. These enzymes are affected by the conditions typically found in the processing of dry-cured meat products, being dehydration one of the most important factors (Toldrà, 2006).

2.2.3 Proteolysis

One of the most important biochemical changes that occurs during dry-curing is the intense proteolysis that takes place as a result of the action of endogenous muscle peptidases. It is assumed that the main enzymes responsible for the degradation of muscle proteins during this period are endopeptidases, mainly calpains

and cathepsins, and certain groups of exopeptidases (Toldrá, 1998b;Toldrà, 2002). The proteolysis process is summarized in the following Figure 3:

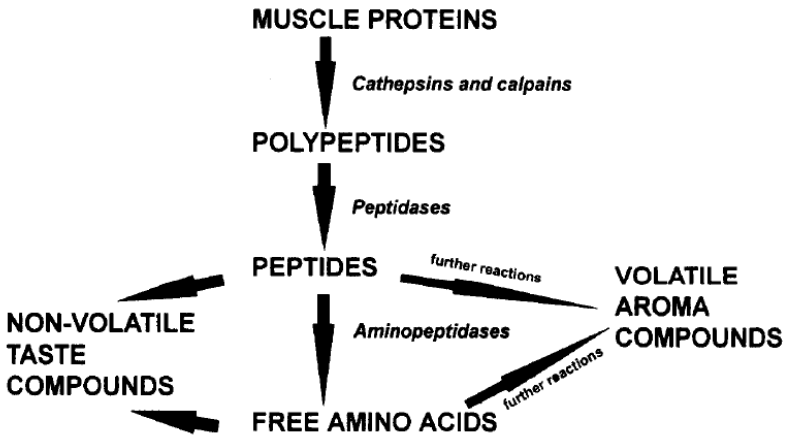

Figure 3. Flow chart showing the major steps in post-mortem proteolysis (Toldrà, 1998a).

Endopeptidases. The action of muscle endopeptidases such as cathepsins and calpains is of great importance during processing because they are directly responsible for changes in postmortem muscle texture: in fact they degrade the muscle protein structure by cleaving intact proteins, giving rise to large polypeptides which are further degraded by exopeptidases (Lametsch, 2003). Between these endopeptidases, cathepsins B, H , and L have been reported to be stable during dry-cured ham processing, showing activity even after 15 months of processing (Toldrá, 1993), whereas cathepsin D activity disappears after 6–10 months of processing (Rico, 1991). An excess of activity of cathepsins can damage the texture of the product, inducing a clear effect also on sensory quality (Virgili, 1995). Calpains can participate in the post-mortem muscle proteolysis only during the first stages of the curing processes since its stability is rather poor and the activity disappears in near 2 weeks (Toldrá, 1998b).

Exopeptidases. These proteolytic enzymes degrade the large polypeptides generated by endopeptidases, giving rise to small peptides and free amino acids. In this sense, dipeptidyl peptidases (DPP I, II, III, and IV) are a group of enzymes able to release different dipeptides from the N-terminal site of peptides. The DPP II activity is relevant only from the end of the post-salting period (50 days) up to 240 days. The contribution of DPP IV activity during dry-curing is also expected although in a lower degree, as it expresses a low percentage of activity at pH around 6.0 and it is partially inhibited by NaCl (Sentandreu, 2001). Its activity decreases to very low values at 240 days, although it remains active until the end of the process.

Aminopeptidases. These enzymes are responsible for the release of amino acids from the N-terminus of peptides and proteins. Their activities are relevant for the development of the characteristic dry-cured

flavour of dry-cured ham that is attributed to the accumulation of free amino acids. Aminopeptidase activity is involved in the later stages of protein degradation: in meat products it has been detected even after more than 12 months of processing. Curing agent, the presence of other peptides or many other factors can modulate the activity of these enzymes (Toldrá, 2000).

Exopeptidases. Carboxypeptidases and peptidyl dipeptidases are responsible for the hydrolysis of amino acids and dipeptides, respectively, from the C-terminal side of the protein fragments. Their stability during curing period remains unknown: these groups of enzymes have not been so well studied in dry-cured ham (Mora, 2013).

2.3 THE ROLE OF SODIUM CHLORIDE AS CURING AGENT

Salt is one of the most widely used additives in food industries because of its low cost and varied properties. The first capacity of sodium chloride is to reduce water activity values and so it's used for his preservative and antimicrobial effect but an excess of intake of this salt could have negative effects on human health.

2.3.1 Sodium and health implications

The trends in food industries point to salt replacement or reduction due to the negative effects of excess dietary intake of sodium, which has been linked to hypertension and, consequently, to an increased risk of cardiovascular disease (Morgan, 2011). The main source of sodium in the diet is sodium chloride and great contributions are given by meat products. The average total daily sodium intake per capita in developed countries is 4–5 g of Na (10–12 g of NaCl), which is up to 25 times greater than the minimum adult requirement (0.5 g of NaCl) (Katsiari, 2000). Therefore, the need to reduce salt intake for the serious public health implications.

2.3.2 Salt reduction in dry-cured ham

Salt reduction is not an easy task to achieve in dry-cured ham. In fact salt has a great number of advantages, inducing:

- a good microbial stability through the reduction of water activity;
- a pleasant salty taste;
- a partial solubilization and cohesiveness of myofibrillar proteins;
- an important inhibition of most muscle proteases (Armenteros, 2008);

- the development of the typical color: the pigment responsible for the stable pink-red color is the Zinc-protoporphyrin IX (Wakamatsu, 2004), generated through a substitution process of the heme iron atom by a zinc atom, a metal of which pork meat is rich. The zinc-chelatase enzyme seems to be involved in this reaction, catalyzing the substitution of iron with zinc in presence of salt and of mild temperature (Benedini, 2008);
- formation of flavor volatiles in dry-cured hams catalyzing oxidation as a pro-oxidant agent (Coutron-Gambotti, 1999).

The reduction in the total amount of salt has been found to cause excessive proteolysis and considerable softening due to the intense action of muscle endopeptidase enzymes (Virgili, 1995). Furthermore, hams cured with 6% salt have been found to be significantly drier, harder and more fibrous than hams cured with 3% salt, which (3% salt) moreover have a better salty taste (Andrés, 2004). The reduction could have a negative impact on the control of microorganisms such as Pseudomonas, Acinetobacter, Enterobacteriacee, Clostridium and Bacillus.

2.3.3 Salt reduction in dry-cured ham: strategies

In dry-cured hams, salt uptake can be managed by means of the selection of raw matter, by varying the length of the salting phase, by reducing the salt amount added to fresh ham or with the replacement of sodium with others salts (Schivazappa, 2013).

Replacement of NaCl by other salt. The replacement with alternative salts like KCl, $CaCl_2$ and $MgCl_2$ can affect the sensory quality as for instance: an excess of KCl imparts a noticeable bitterness and astringency to the ham. In a recent study, however, differences in perceived quality between dry-cured loins with 50% substitution of NaCl by KCl and controls with 100% NaCl were found to be non-significant (Armenteros, 2009).

Replacement of NaCl by flavour enhancers. To correct the salty taste it is possible to use flavor enhancers like monosodium glutamate, sodium lactate or yeast extracts. Taste enhancers work by activating receptors in the mouth and throat, which helps to compensate for the salt reduction. Potassium lactate has also been used recently as an alternative salt for restructured hams (Fulladosa, 2008). Also phosphates may be used as salt substitutes.

Optimization of the physical form of salt. Another option is based on the use of a different physical form of salt, i.e. changing from granular to flaked: the flaked salt showed greater functionality, in fact it has a faster solubility, which allows to use it in smaller amounts (Sacks, 2001).

Alternative processing techniques.

- Reduction of salt added in % of the weight of the ham: this approach implies consequent changes in the process parameters (i.e., longer period of post-salting phase or maturation time). The longer resting phase is necessary for microbiological safety reason (Simoncini, 2009).
- *Pre-rigor meat.* Meat removed from the carcass early postmortem, when it is still physiologically active (it responds to electrical stimulation; it has a high pH and available energy) and it has not entered the onset of rigor (stiffening process), is referred to as pre-rigor meat. Pre-rigor meat has higher emulsifying capacity and it shows greater extractability of salt soluble proteins (myosin, actin, tropomyosin) than post-rigor meat (Claus, 2006). The use of this approach allows to improve the extractability of myofibrillar proteins, water-binding and water-holding capacity.
- *High pressure technologies*. The texture of the products may be improved by high pressure application: this allows to obtain an improvement of protein functionality.

The combinations of any of the above mentioned approaches represent other ways to obtain a reduction in salt content in meat product.

2.4 PEPTIDES RELEASED BY PROTEOLYSIS

In literature, a great number of papers focus their attention on the evolution of peptides throughout the complete processing of high-quality dry-cured ham in order to better understand the path of proteolysis mechanisms, flavour development and texture on the final products. Proteomic techniques have been successfully applied to the identification of the generated peptides and their sequencing in order also to reveal key biomarker peptides controlling the process and establish strategies to drive and optimise enzyme reactions for the production of optimal quality products (Mora, 2013).

For example, a proteomic approach has been carried out to investigate the extensive proteolysis occurring in the processing of Serrano ham: a total of 14 peptide fragments derived from myosin light chain I and titin have been identified (Mora, 2009). Peptides like TKQEYDEAGPSIVHR, ITKQEYDEAGPSIVHRK DSGDGVTHNVPIYE and DSGDGVTHNVPIYEG originated instead from muscle actin (Sentandreu, 2007). Other 27 peptides generated during the dry-cured processing originated from Troponin T, a myofibrillar protein with important structural and regulatory functions have been founded by Mora et al. (Mora, 2010). Eleven fragments of myoglobin detected at the end of the processing of dry-cured have been

confirmed by (Mora,2012). The increase of small peptides (MW <3000Da) generated during the long process of italian-style dry-cured ham have been analyzed by Hansen-Møller et al., (Hansen-Møller, 1997) and by Sforza et al. (Sforza, 2006). In this last paper were also detected the presence of γ-glutamyl dipeptides. The characteristics of these compounds are their remarkable increase during ham aging and their function as taste-active compounds.

2.4.1 NPADs

Proteolysis is the most important phenomenon occurring during ageing of dry-cured ham, giving rise to an accumulation of free amino acids and small peptides having a fundamental high nutritional value and playing a fundamental role in flavour development.

Among the low-molecular weight nitrogen fraction of several cheeses and non-dairy products, a particular group of unusual amino acyl derivatives of non-proteolytic origin, namely γ-glutamyl-amino acids, lactoyl-amino acids and pyroglutamyl-amino acids, collectively known as Non-Proteolytic Aminoacyl Derivatives (NPADs), were found in consistent amount (Figure 4).

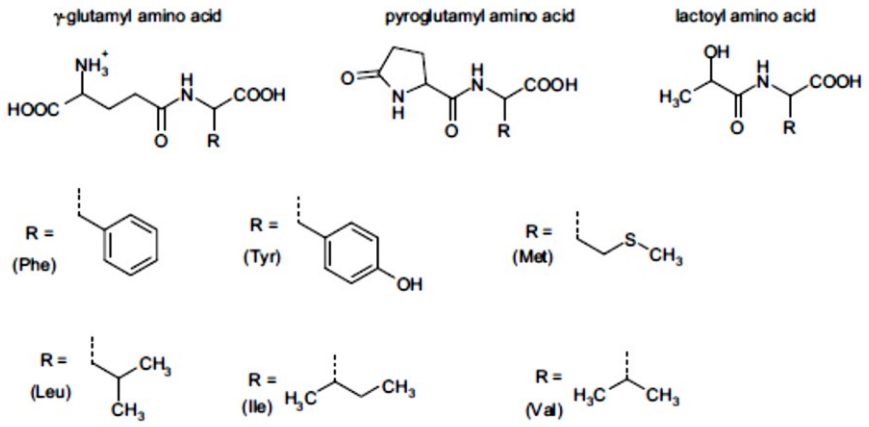

Figure 4. Chemical structures of NPADs (Sforza, 2009).

In particular, γ-glutamyl-phenylalanine (γ -Glu-Phe, brothy, salty, slighty sour), γ-glutamyl-tyrosine (γ -Glu-Tyr, sour and salty) and γ -glutamyl-leucine (γ -Glu-Leu) have been reported in Comte´ cheese (Roudot-Algaron, 1994). In addition to these three compunds, also γ -glutamyl-methionine (γ -Glu-Met), γ -glutamyl-valine (γ -Glu-Val) and γ -glutamyl-methionine (γ -Glu-Ile) were reported in Grana Padano, Parmigiano Reggiano and Asiago cheese (Sforza, 2009). γ -Glutamyl-amino acids has also been reported in

Gouda cheeses (Toelstede, 2009), thus confirming their widespread distribution in dairy products. Their total amounts vary between few mg and more than 50 mg per 100 g of cheese.

Properties of these compounds are still largely unknown, apart from their peculiar characteristic taste, which for most of them have been described as kokumi taste (Toelstede, 2009).

Indeed, γ-Glutamyl-leucine, γ-glutamyl-valine and γ-glutamyl-cysteinyl-alanine having this characteristics have been isolated by edible beans (Dunkel, 2007) as well as γ-glutamyl–valyl–glycine discovered in Vietnamese fish sauce (Kuroda, 2012). A patent to prepare these interesting flavour compounds have been registered (Dunkel, 2007).

In a recent paper (Bottesini, 2014), the conditions that favour the bioproduction and biodegradation of these compounds were investigated using pure enzymes and biological media both on NPAD and their amino acidic precursors. The data suggested that their production in cheese, and also their partial degradation, might be due to the action of peptidases and γ -glutamyl transpeptidase.

As well as the other NPADs, pyroglutamyl peptides have been reported in wheat (Higaki-Sato, 2003) and mushrooms (Altamura, 1970) and for the first time also in Parmigiano-Reggiano cheese (Sforza, 2009). The occurrence in cheese is probably related also to the widespread occurrence of the precursor pyroglutamic acid which has been reported to be present in Parmigiano-Reggiano and other hard cheeses, derived from an enzymatic reaction that catalyses the internal cyclization of glutamic acid (Mucchetti, 2002). Derivatives of pyroglutamyl peptides have been patented by Nestle´ SA as umami taste enhancers to foodstuffs (Schlichtherle-Cerny, 2003).

Lactoyl-amino acids were recently reported for the first time in cheeses which have been proved to be formed by enzymatic actions of Lactic acid bacteria in Parmigiano-Reggiano cheese starting from lactic acid and amino acids. The same activities are responsible for the formation of g-glutamyl-amino acids in cheeses.

Lastly, in a recent paper, all these compounds have been also found in fermented soy sauce: in particular, γ -glutamyl, γ -glutamyl, and pyroglutamyl dipeptides, as well as lactoyl amino acids, were identified in the acidic fraction of soy sauce (Frerot, 2013).

Again, the most studied characteristics of all these compounds is their taste enhancing properties both for kokumi and umami taste, as proved by several recent activities and patents (Winkel, 2008).

The presence of small dipeptides with the composition Glu-Phe, Glu-Ile, Glu-Leu and Glu-Tyr has already been reported in Parma ham, although at that time not recognized as γ-glutamyl derivatives: their content was found to increase during the ripening time and to be possibly linked to the pleasant aged flavour of the product (Sforza, 2006).

2.5 BIOACTIVE PEPTIDES

Food proteins have long been recognized for their nutritional and functional properties associated with their amino acid content and their physiological utilization of specific amino acids upon digestion and absorption but also for their contribution to the physico-chemical and sensory properties of foods. In the last years, a considerable amount of research are also focused on the release of bioactive peptides encrypted in food proteins. Bioactive peptides have been defined as "food derived components (genuine or generated) that, in addition to their nutritional value exert a physiological effect in the body". Bioactive peptides are usually composed of 2–20 amino acid residues in length, but some bioactive peptides have been found also with >20 amino acid residues. To exert their different physiological effects, bioactive peptides may be absorbed through the intestine and subsequently enter the circulatory system, but they also may induce local effects in the digestive tract. Food derived bioactive peptides have been shown to display a wide range of physiological functions including antihypertensive, antioxidant, opioid agonistic, immunomodulatory, antimicrobial, prebiotic, mineral binding, antithrombotic and hypocholesterolemic effects.

Meat proteins offer huge potential as novel sources of bioactive peptides displaying antihypertensive, antioxidant, antimicrobial and antiproliferative effects (Ryan, 2011).

2.5.1 Antihypertensive peptides

Hypertension affects one third of the Western worlds' population and is a known risk factor for stroke and cardiovascular disease. ACE inhibitory peptides were first discovered in snake venom and since then numerous synthetic ACE inhibitors have been produced, with Captopril being the most common. Captopril and other synthetic ACE inhibitors are known to exert various side-effects such as coughing, taste disturbances and skin rashes (Atlas, 2007): for these reasons, research efforts have been oriented towards the discovery of antihypertensive peptides derived from food, as an alternative and/or complementary way to control blood pressure.

2.5.1.1 Mechanism of ACE inhibition

ACE, or kininase II, is a dipeptidyl carboxy peptidase (a zinc metallopeptidase) found in various tissues in the body and it is integral to the moderation of blood pressure and normal heart function (Shalaby, 2006).

In the rennin-angiotensin system, ACE catalyzes the conversion of the inactive form of angiotensin I (Ang I) to the potent vasoconstrictor angiotensin II (Ang II) and at the same time it deactivates the hypotensive peptide, bradykinin, by cleaving the C-terminal dipeptide from both Ang I (His-Leu) and bradykinin (Figure 5). Ang II is a documented potent vasoconstrictor and it is also responsible for the expansion of vascular volume via sodium retention and fluid retention (Brown, 1998).

While synthetic ACE inhibitors, such as Captopril function by directly blocking the action of ACE, ACE inhibitory peptides function by reacting with ACE, thus ACE is unavailable to cleave Ang I and prevent the production of the vasoconstrictor Ang II (Iwaniak, 2014) .

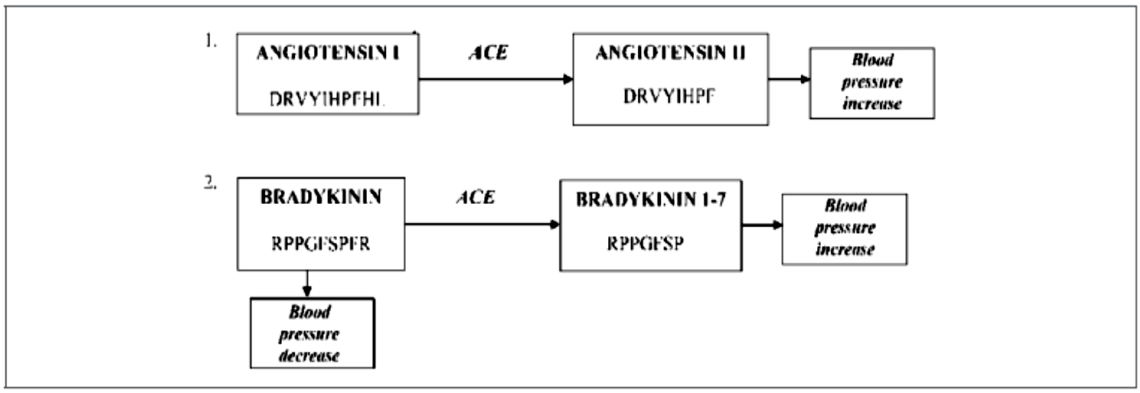

Figure 5. Action of Angiotensin-Converting-Enzyme (Iwaniak, 2014).

2.5.1.2 ACE inhibitor type peptides

The ACE inhibitory peptides can be divided into three categories:

1. "true inhibitor type" peptides: peptides that bind to the enzyme, blocking the activity of ACE without being modified, thus preventing the binding of the natural substrate (Ang I) to the active site;
2. "substrate type" peptides: they are hydrolyzed by ACE resulting in weak inhibitory activity;
3. "pro-drug type" inhibitors: the peptides in this category are converted to "true inhibitor type" peptides by ACE or proteases of the digestive tract.

In vivo studies have demonstrated that only peptides belonging to the groups of true inhibitor type or pro-drug type reduce the systolic blood pressure of spontaneously hypertensive rats (SHR).

To date, the majority of ACE inhibitory peptides found in meat can be classified as true inhibitor type peptides (Ahhmed, 2010).

2.5.1.3 Structure correlation of ACE inhibitory peptides

The mode of action of the majority of ACE inhibitory peptides is thought to be as competitive substrates for ACE. Several structure activity relationships have been hypothesized for ACE inhibitory peptides, thus characteristics positively influencing the ACE inhibitory activity are the followings:

- for tripeptides, the presence of hydrophobic amino acid residues at the C-terminal end with, at the penultimate positions, aliphatic (V, I, and A), basic (R) and aromatic (Y and F) residues; the

presence of aromatic (W, Y, and F), proline (P) and aliphatic (I, A, L and M) residues at the ultimate position of the C-terminal end of the peptide, probably on account of the interaction of these residues with the three hydrophobic sub-sites located on the active site of ACE. The positive charge associated with arginine or lysine at the C-terminus has also been associated with the ACE inhibitory activity of some peptides; the presence of hydrophobic amino acids also at the N-terminal end of the peptide (Ahhmed, 2010);

- for tetrapeptides, the most favorable amino acids (starting from C-terminus) are Tyr and Cys. Residues such as His, Trp, and Met are usually found in the 2nd position of a tetrapeptide. In the 3rd position, Ile, Leu, Val, and Met are favorable with Trp placed as the 4th residue (Iwaniak, 2014).

Hydrophilic peptides possess weak or no ACE inhibitory activity, since hydrophilic peptides are incompatible with the active sites of ACE. Indeed, inhibition of ACE is achieved by hydrophobic peptides which display high affinity to the active sub-sites of ACE (Matsui, 2006).

2.5.1.4 Ace-inhibitory peptides from meat protein

Antihypertensive peptides have been identified in meat from various sources (Jang, 2005; (Escudero, 2013a): as far as pork meat, it has been demonstrated that biopeptides may be released by meat proteins by the action of endogenous or exogenous peptidases during processing (Lafarga, 2014a). Antihypertensive peptides have also been identified and their activity has been proven both in vitro as well as on spontaneously hypertensive rats (Escudero, 2013a). Antihypertensive peptides were found after gastro-intestinal digestion of pork meat by the action of pepsin and pancreatin at simulated gut conditions: ER, KLP, and RPR with IC_{50} values of 667 μM, 500 μM, and 382 μM, respectively. The strongest ACE inhibition was observed for peptide KAPVA (IC_{50} = 46.56 μM) followed by the sequence PTPVP (IC_{50} = 256.41 μM), peptides that derive from muscle titin (Escudero, 2010).

In Spanish dry-cured hams, the peptide AAATP was found with an IC50 value of 100 μM which also showed a good in vivo activity: indeed, it decreased systolic blood pressure by −25.62±4.5 mm Hg ($p<0.05$) in spontaneous hypertensive rats after 8 h administration (Escudero, 2013a). Another paper reported that the water soluble fractions of a Spanish dry-cured ham extract, corresponding to a peptide fraction with molecular weight up to 1700 Da, determine changes in systolic blood pressure of spontaneously hypertensive rats after oral administration (decrease of 38.38 mm Hg) (Escudero, 2012).

The identification of bioactive peptides may be also supported by in-silico techniques as recently demomstrated by using software able to predict and to identify bioactive peptides (Lafarga, 2014a).

2.5.2 Antioxidant peptides

Antioxidants play a vital role in both food systems as well as in the human body to reduce oxidative processes. In foods antioxidants are useful to maintain flavor, texture, and, in some cases, the color during storage. In the human body, endogenous antioxidants help to protect tissues and organs from oxidative damage caused by reactive oxygen and reactive nitrogen species. However, in certain circumstances the endogenous defense system fails to protect the body against reactive radicals: thus, a great interest has aroused for the potential health benefits with no or little side effects of natural antioxidants from food resources (Sarmadi, 2010).

Use of dietary antioxidants has been recognized as potentially effective to promote human health by increasing the body's antioxidant load: in vitro studies using various chemical assays have indicated the potential of food-derived peptides to act as antioxidant agents to control various oxidative processes in the human body as well as in food. Antioxidant peptides can be released from different proteins of plant or animal origin during preparation of protein hydrolysates using exogenous or endogenous enzymes, food processing or during microbial fermentation, as well as during gastro-intestinal (GI) digestion of food proteins.

2.5.2.1 Mechanisms of action of antioxidant peptides

The antioxidant potential is strongly influenced by the amino acid composition, sequence, and size of peptides. The different amino acid residues and peptide sequences are responsible for the inhibition of oxidative reactions that are initiated by different types of free radicals or pro-oxidants such as metal ions, as well as in different molecular environments i.e., aqueous, lipid, or emulsion systems, or different pH conditions, or the presence of other compounds in the food matrices or biological systems, etc. (Elias, 2008). The majority of the antioxidant peptides derived from food sources have molecular weights ranging from 500 to 1800 Da (Ranathunga, 2006). In general, all 20 amino acids found in proteins can interact with free radicals: the most reactive ones include the nucleophilic sulfur-containing amino acids Cys and Met, the aromatic amino acids Trp, Tyr, and Phe, and the imidazole-containing amino acid His; others include hydrophobic amino acid residues such as Val or Leu at the N-terminus of the peptides, and Pro, His, Tyr, Trp, Met, and Cys in their sequences (Elias, 2008).

2.5.2.2 Antioxidant assay

Specific assays have not yet been developed or standardized to measure the antioxidant capacity of peptides or peptide mixtures. Therefore, assays that are commonly used for measuring antioxidant capacity of non-peptidic antioxidants have been used in the literature to measure the antioxidant capacity of peptides as well.

On the basis of the chemical reactions, the assays for measuring antioxidant capacity are classified into two groups: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET) :

- HAT assays: oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP). The HAT-based assays usually involve the use of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. Quantification is obtained from kinetic curves derived from competitive reaction kinetics (Sarmadi, 2010);
- ET assays: Trolox equivalent antioxidant capacity (TEAC), the ferric ion reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl radical-scavenging capacity (DPPH) or 2,2-Azino-bis-3-ethylbenzothyazoline-6-sulfonic acid (ABTS). These methods measure the capacity of an antioxidant to reduce a chromophoric oxidant, which changes colour when reduced: the degree of color change is correlated with the sample's antioxidant activity. These assays measure the radical-scavenging activity of an antioxidant (Di Bernardini, 2011).

More commonly used assays include measuring the inhibition of lipid peroxidation in a linoleic acid model system and the capacity to scavenge the DPPH radical in studying radical-antioxidant interactions. The DPPH, however, is a test that is more affected by the composition of the peptides, the abundance in free amino acids, the size and the molecular weight of the peptides, the effect of the solvent / solubility of the peptide due to steric hindrance that is created between the radical and the same amino acid residues. It is also a test which does not allow to evaluate the hydrophilic antioxidants because it is conducted in ethanol. The ABTS assay instead allows to study both hydrophilic and lipophilic antioxidants and it is not affected by steric hindrance (Samaranayaka, 2011).

Lipid oxidation occurs when oxygen reacts with lipids in a series of free radical chain reactions that lead to complex chemical changes. Oxidation of lipids in foods causes quality losses. In vivo, lipid oxidation may play a role in coronary heart disease, atherosclerosis, cancer, and the ageing process (Joshipura, 1996). The susceptibility of low density lipoproteins (LDL) to radical peroxidation is believed to be an important factor in the process responsible for foam-cell formation and atherosclerosis.
Many factors affect oxidation, including temperature, oxygen pressure, metal catalysts, fat composition and form, that may vary depending on the oxidation conditions used (Frankel, 1993). Antioxidants, when present in the medium, can delay or inhibit lipid oxidation. The use of iron or copper enhance the rate of oxidation, principally by homolytic cleavage of the weak O-O bond in hydroperoxides and subsequent formation of radicals (Bondet, 2000). Through reaction with acidic Fe(II)-thiocyanate, these peroxides can be colorimetrically determined by formation of the red Fe(III)-thiocyanate complex. Peroxide production as a function of time in a LA system was used to determine both antioxidant and prooxidant activities initiated

by a metal catalyst (Cu(II)) (Fukumoto, 2000). The antioxidant activity of protector compounds added to the medium is inversely proportional to the amount of Fe(III)‑thiocyanate complex formed per unit time.

Lipid peroxidation induced by transition metal ions (e.g., copper(II)) requires the occurrence of a redox cycle with Cu(II) (initiation), where AH symbolizes an antioxidant (such as ascorbic acid) and LOOH a lipid hydroperoxide:

$Cu^{2+} + AH \rightarrow A\cdot + H^{+} + Cu^{+}$ (1a)

$Cu^{2+} + LOOH \rightarrow LOO\cdot + H^{+} + Cu^{+}$ (1b)

Propagation proceeds via molecular oxygen or Fenton-type reactions:

$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-}\cdot$ (slow) (2)

$Cu^{+} + LOOH \rightarrow Cu^{2+} + LO\cdot + OH^{-}$ (fast) (3)

The lipid oxidation chain reaction propagates as these lipid radicals (LO· and LOO·) react with other lipids in their immediate vicinity or with other radicals (McClements, 2000).

2.5.2.3 Antioxidant peptides in meat products

The total antioxidant capacities of different types of raw meat (beef, chicken, pork) has been investigated as ABTS scavenging activity and they ranged between 25.9±1.0 and 51.7±1.2 mmol Trolox Eq./k: the highest value has been measured for raw chicken, followed from pork and beef (Serpen, 2012).

The antioxidant activity of peptides in a water soluble extract of Spanish dry-cured ham have been studied using the DPPH radical-scavenging assay and the reducing power assay. The strongest radical-scavenging activity was observed for the peptide SAGNPN whit an inhibition of 50% at a concentration of 1.5 mg/ml. On the other hand, the peptide GLAGA showed the higher reducing power with 0.5 units of absorbance at 700 nm at a concentration of 1 mg/ml (Escudero, 2013b). In another paper, the peptide SNAAC was identified with an IC_{50} of 75.2 μM for DPPH radical-scavenging assay and 205 μM for ferric-reducing antioxidant (Mora, 2014).

Well known antioxidant peptides are also, for example, carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-L-3-methyl-L-histidine). Carnosine can act both as a free radical scavenger as well as a metal ion chelator, and it has demonstrated both in vivo and in vitro antioxidative activity in rat skeletal muscle lipid and protein components under the conditions of oxidative stress (Nagasawa, 2001).

2.5.3 Models of gastrointestinal digestions

Simulated gastro-intestinal digestion is widely employed in many fields of food and nutritional sciences: this method typically include the oral, gastric and small intestinal phases, and occasionally large intestinal fermentation. In addition, it tries to mimic in vivo physiological conditions, taking into account the presence of digestive enzymes and their concentrations, pH, digestion time, and salt concentrations, among other factors. However, the results of in vitro digestion model are often different if compared to those found using in vivo models because of the difficulties in accurately simulating the highly complex physicochemical and physiological events occurring in animal and human digestive tracts. In vivo feeding methods, using animals or humans, usually provide the most accurate results (Boisen, 1991), but they are time consuming and costly, which explains why so much effort has been devoted to the development of in vitro procedures (Coles, 2005).

The ideal in vitro digestion method would provide accurate results in a short time (Hur, 2011).

In the literature many in vitro digestion models are reported that differ for:

- the number and type of steps included in the digestion sequence (mouth, stomach, small intestine, large intestine);
- the composition of the digestive fluids used in each step (enzymes, salts, buffers, biological polymers, and surface-active components);
- the mechanical stresses and fluid flows utilized in each step in the digestion sequence (magnitude and direction of applied stresses, flow geometries, and flow profiles). However, the majority of models reported in literature are static ones, that are useful to study the digestion of single substrates or simple meals under specific conditions (Hur, 2011).

The most frequently utilized enzymes and other biological molecules used within in vitro digestion models were pepsin, pancreatin, trypsin, chymotrypsin, peptidase, α-amylase, lipase, bile salt, and mucin. Several studies have utilized enzymes collected from human subjects, whereas others have used enzymes extracted from animal or plant sources. The types of enzyme included within an in vitro digestion model tend to reflect the major food components being investigated, e.g., lipases for lipid digestion, proteases for protein digestion, and amylases for starch digestion. It should be noted that different enzymes are usually added sequentially, rather than all together, so as to simulate the different steps of the digestive process. The enzyme composition of a particular digestive fluid can often be simulated by mixing together appropriate amounts of pure enzymes (Boisen, 1991). It should also be noted that enzymes often require additional components within the digestive fluids to operate efficiently.

For all in vitro digestion models the digestion temperature was 37 °C.

The length of the incubation times of samples in the various simulated digestive fluids should mimic the reported digestion times in humans. Important factors influencing the digestion time depends upon

individual characteristics (age, sex, health status, mental state, time of the day) and food properties (total amount, composition, particle size) (Hur, 2009).

Many paper reported the use of a single purified enzyme, also for complex biological mixture, because it facilitates the standardization of in vitro digestion models, which enables more consistent laboratory-to-laboratory comparisons (Coles, 2005). However, the digestion of one nutrient is often influenced by the digestion of other nutrients, and so it is often more realistic to use a complex mixture of enzymes rather than a single purified one (Boisen, 1991).

In order to allow in the future the comparison between different in vitro static gastrointestinal digestions models, a recent paper, within the COST Infogest network (which is an international network joined by more than 200 scientists from 32 countries working in the field of digestion), proposed a general standardized and practical static digestion method based on physiologically relevant conditions that can be applied for various endpoints, which may be amended to accommodate further specific requirements. (Minekus, 2014).

Another fundamental part of the study of peptide bioactivity is related to their ability to cross the intestinal barrier thus reaching blood circulation and allowing to exert a systemic action. Also the eventual modification taking place on account of epithelial cell enzymatic activities should be considered.

2.5.4 Peptides transport across the intestinal barrier

Cell culture models have been utilized as part of in vitro digestion models, in order to assess the bioavailability of nutrients. In particular, the Caco-2 cell culture model has been widely used as a predictive tool for the absorption of bioactive components from foods and pharmaceutical preparations.

As far as peptides are concerned, small di- and tri-peptides may be absorbed intact across the brush border membrane using H^+-coupled PepT1 transporter system (Vermeirssen, 2002). Larger water-soluble peptides can cross the intestinal barrier paracellularly via the tight junction between cells, while highly lipid-soluble peptides may diffuse via the transcellular route. Peptides may also enter the enterocytes via endocytosis, which entails membrane binding and vesiculisation of the material (Ziv, 2000). The intestinal basolateral membrane also possesses a peptide transporter, which facilitates the exit of hydrolysis-resistant small peptides from the enterocyte into the portal circulation. Further, the contribution of each route and the ability of individual peptides to cross the membrane depends upon the molecular size and other structural characteristics such as hydrophobicity, as well as their resistance to brush-border peptidases (Satake, 2002). The major pathways for intestinal absorption of peptides are display in Figure 6.

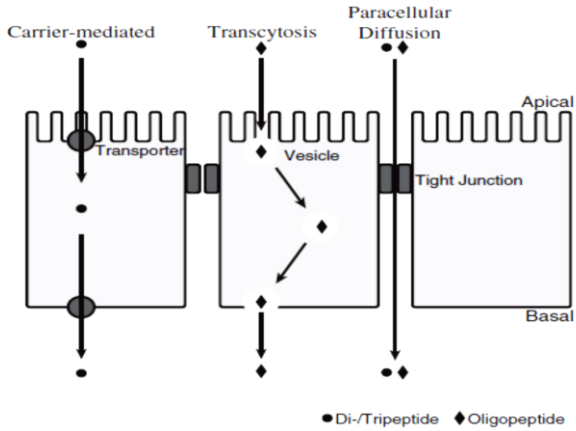

Figure 6. Major pathways for intestinal absorption of bioactive peptides. Mechanisms for absorption of bioactive peptides in the intestine are largely categorized into carrier-mediated transport, transcytosis and paracellular passive diffusion (Wadaa, 2014) .

2.5.4.1 Caco-2 cells line

Peptides may exert their actions locally at the level of the gastrointestinal tract or after intestinal absorption that allows their distribution to peripheral tissues and organs. Peptides that are resistant to proteolytic degradation and are taken up to a significant extent are good candidates to exert beneficial effects in vivo. The in vivo determination of intestinal uptake of nutrients in the intestine is technically difficult, so transport studies across human colon adenocarcinoma (Caco-2) cell monolayers have been routinely utilized as an in vitro model to mimic absorption by human intestinal epithelium. The parental cell line undergoes in culture a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of the mature enterocyte such as cell polarization, microvillous structure, carrier-mediated transport systems, functional tight junctions between adjacent cells and expression of brush border membrane associated proteases and peptidases. The intestinal absorption predicted by using Caco-2 cell monolayers is well correlated with intestinal uptake in vivo, at least from a qualitative viewpoint (Picariello, 2013). To better reproduce the steric conditions existing in the intestine in vivo, Caco-2 cells were cultured on permeable filter supports that allow free access of ions and nutrients to the two sides of the cell monolayer. Trans-epithelial electrical resistance after confluence and permeability of marker molecules have been used to monitor the integrity of the cell layer, and ultrastructure morphology, by transmission electron microscopy, has been utilized to check for morphological differentiation. The mosaic expression of brush border enzymes in confluent Caco-2 cell is present.

Culture-related conditions were shown to influence the expression of these characteristics, in part due to the intrinsic heterogeneity of the parental cell line, leading to selection of sub-populations of cells becoming

prominent in the culture. Culture-related conditions, as well as different Caco-2 cell lines utilized in different laboratories, often make it extremely difficult to compare results in the literature (Sambuy, 2005).

2.5.4.2 Ussing chamber

The Ussing chamber provides a physiological system to measure the transport of ions, nutrients, and drugs across various epithelial tissues. One of the most studied epithelia is the intestine. The method was developed over 50 years ago by the Danish biologist Hans H. Ussing as a means to understand the phenomenon of active NaCl transport (Clarke, 2009). The intestinal segment is mounted in an Ussing chamber where one side will be exposed to buffer with the compound of interest (mucosa side) and the other side to buffer without the compound of interest (serum side). This method is defined as an ex-vivo technique due to use of an intestinal tissue.

2.6 METHODS FOR THE STUDY AND THE CHARACTERIZATION OF PEPTIDES

2.6.1 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is now firmly established as the premier technique for the analysis, characterization and purification of peptides and proteins. Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity: the separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. The solute mixture is initially applied to the stationary phase in the presence of aqueous buffers, and the solutes are eluted by the addition of organic solvent to the mobile phase: elution can proceed either by isocratic conditions (concentration of organic solvent is constant) or by gradient elution (the amount of organic solvent is increased over a period of time). The solutes are, therefore, eluted in order of increasing molecular hydrophobicity.

RP-HPLC is a very powerful technique for the analysis of peptides and proteins for the following factors:

1. excellent resolution that can be achieved under a wide range of chromatographic conditions for very closely related molecules as well as structurally quite distinct molecules;
2. experimental easiness: the chromatographic selectivity can be manipulated through changes in mobile phase characteristics;
3. generally high recoveries and, hence, high productivity;

4. excellent reproducibility of repetitive separations carried out over a long period of time, which is due partly to the stability of the stationary phase materials, resistant to a wide range of mobile phase conditions;
5. extreme versatility for the isolation of peptides and proteins from a wide variety of synthetic or biological sources;
6. applicability to both analytical and preparative experiments. Analytical applications range from the assessment of purity of peptides following solid phase peptide synthesis, to the analysis of tryptic maps of proteins. In the case of synthetic peptides, RP-HPLC is generally employed both for the initial analysis and the final large-scale purification. The purification of synthetic peptides usually involves an initial separation on an analytical scale to assess the complexity of the mixture followed by large-scale purification and collection of the target product. A sample of the purified material can then be subjected to RP-HPLC analysis under the same or different elution conditions to check for purity (Aguilar, 2004).

2.6.2 Mass spectrometry

Mass spectrometry is a central analytical technique for protein research and for the study of biomolecules in general. A wide range of new mass spectrometry–based analytical platforms and experimental strategies have emerged driven by the need to identify, characterize, and quantify proteins and peptides at ever increasing sensitivity and in ever more complex samples. Mass spectrometers are used both to: to measure simply the molecular mass of a polypeptide as well to determine additional structural features including the amino acid sequence or the site of attachment and type of post-translational modifications. In the former case, single-stage mass spectrometers are used, acting essentially as balances to weigh molecules. In the latter case, after the initial mass determination, specific ions are selected and subjected to fragmentation through collision.

A triple quadrupole, able to perform MS/MS experiments, is achieved by combining three quadrupole units in series. A quadrupole consists of four cylindrical rods, set parallel to each other: it's the component of the instrument responsible for filtering sample ions, based on their mass-to-charge ratio (m/z). Ions are separated in a quadrupole based on the stability of their trajectories in the oscillating electric fields that are applied to the rods. In a triple quadrupole, as shown in Figure 7, the first and third quadrupoles act as mass filters and the middle quadrupole is employed as a collision cell.

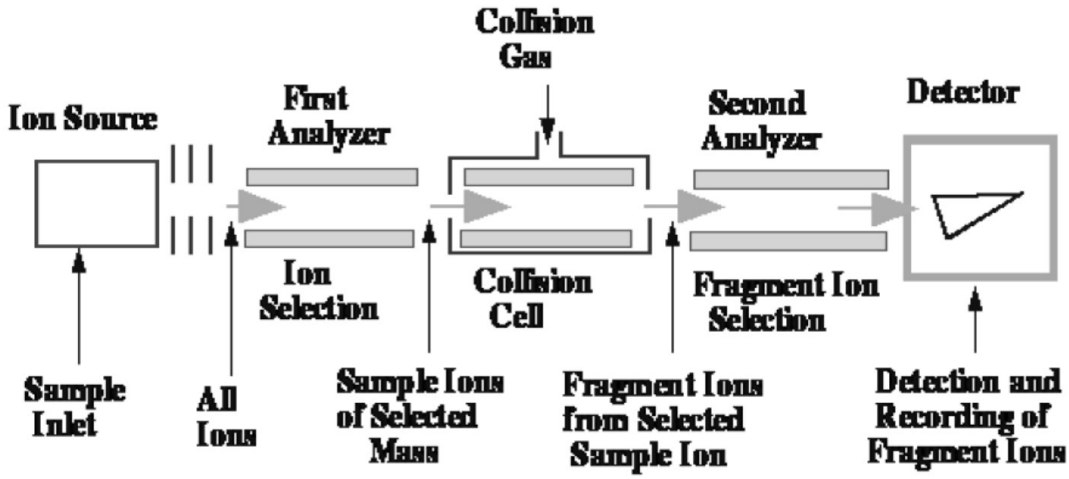

Figure 7. The Triple Quadrupole Mass Spectrometer (Scott).

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In such experiments, referred to as tandem mass spectrometry (MS/MS), detailed structural features of the peptides can be inferred from the analysis of the masses of the resulting fragments. Schematic representation of various types of tandem mass spectrometry experiments is reported in Figure 8.

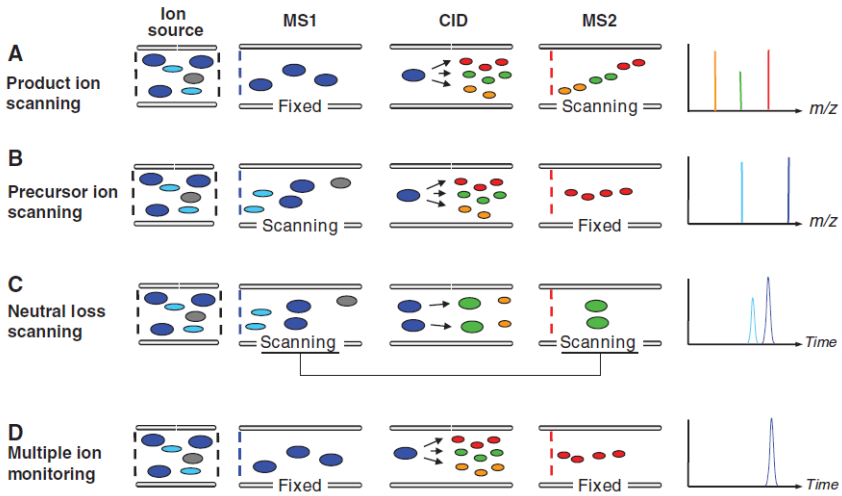

Figure 8. Schematic representation of various types of tandem mass spectrometry experiments: a) product ion scanning; precursor ion scanning; c) neutral loss scanning; d) multiple ion monitoring (Domon, 2006).

(A) Product ion scanning is the most common MS/MS experiment in proteomics: generation of fragment ion spectra for the identification of the amino acid sequence of specific peptides. In this experiment, the first analyzer (MS1) is set to a value that selects one specific precursor ion at a time. The selected ion

undergoes CID in the collision cell, and the resulting fragments are analyzed by the second analyzer (MS2). This process is repeated for different precursors.

(B) Precursor ion scanning sets the second analyzer (MS2) to transmit only one specific fragment ion to the detector. MS1 is scanned to detect all the precursor ions that generate this fragment. Typically, this method is used to detect a subset of peptides in a sample that contain a specific functional group, for instance a phosphate ester or a carbohydrate modification.

(C) Neutral loss scanning scans both analyzers in a synchronized manner, so that the mass difference of ions passing through MS1 and MS2 remains constant. The mass difference corresponds to a neutral fragment that is lost from a peptide ion in the collision cell. The neutral loss scan is therefore used to detect those peptides in a sample that contain a specific functional group, as the detection of peptides phosphorylated at serine or threonine residues via a loss of phosphoric acid.

(D) Multiple ion monitoring (MRM) consists of a series of short experiments in which one precursor ion and one specific fragment characteristic for that precursor are selected by MS1 and MS2, respectively. Typically, the instrument cycles through a series of transitions (precursor fragment pair) and records the signal as a function of time (chromatographic elution). MRM is used for the detection of a specific analyte with known fragmentation properties in complex samples. (Domon, 2006)

A typical peptide fragmentation is reported in Figure 9.

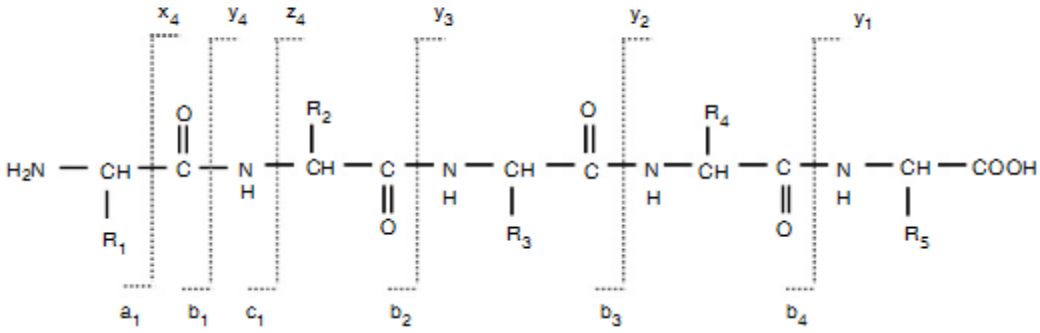

Figure 9. A typical peptide fragmentation: x, y, z, a, b, c fragments.

The fragments, obtained from MS/MS experiments, are conventionally denoted by the letters “a, b or c” if the charge is retained in the N-terminal fragment and the letters “x, y or z” if the charge is instead retained in the C-terminal fragment. The site of fragmentation more likely is the peptide bond, generating the fragments to “b and/or y”.

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3 TRADITIONAL AND REDUCED SALT DRY-CURED HAM: STUDY OF THE PEPTIDE FRACTION

3.1 INTRODUCTION

Recent innovations in the meat industry are addressed towards the production of healthier meats and processed meats. The strategies are based on either reducing the content of unhealthy substances (like less added sodium chloride, less nitrate and nitrite) or improving the content of substances with healthy benefits (i.e. natural antioxidants, omega-3 fatty acids, probiotics and bioactive peptides). These trends are due to the increased attention of consumers to well-being.

One of the unhealthy substances present in dry-cured ham is the sodium. In Western societies the intake of salt is very high, thus leading to a higher risk of hypertension, cardiovascular diseases, diabetes, and kidney disease. The reduction of salt in hams is not an easy task, as salt content affects the microbiological growth, the proteolysis, the texture of the product, the development of the aroma and flavor, the color.

In particular, proteolysis, which is the main process taking place during ham maturation and ageing, is strongly influenced by salt content, as salt affects a_w and enzyme activity.

To establish strategies for optimizing the enzyme reactions towards optimal quality of the products, and so benefit the production and economy related to this important sector, the optimization of the proteomic strategy is crucial in the identification of key peptides acting as biomarkers to control and direct the industrial processes for dry-cured ham.

The aim of this work is to study the peptide fraction of traditional hams, obtained by the normal procedure applied in the production of Parma ham, and compare it with the peptide fraction characterizing salt-reduced dry-cured hams, the latter being obtained by a modified processing aimed at reducing by 25% the sodium chloride content. In particular, the salt reduction techniques tested are the conventional technique where salt is reduced of 25% as a function of ham weight and another technique that is similar to conventional one but it considers also the amount of fat/lean tissue present in legs.

The aim of the project is to obtain healthier products that nevertheless still maintain the peculiar organoleptic properties of the traditional ones.

3.2 MATERIALS AND METHODS

3.2.1 Reagents and solvents

Bidistilled water was produced in our laboratory by a Millipore Alpha Q purification system (Waters, Billerica, MA, USA). Acetonitrile, methanol, L-phenylalanyl-L-phenylalanine (Phe-Phe), acetic acid, hydrochloric acid (0,1N), sodium hydroxide, sodium phosphate dibasic dodecahydrate, sodium phosphate monobasic, potassium phosphate monobasic, diethyl ether, trypsin, dithiothreitol, iodoacetamide, tris(hydroxymethyl)aminomethane (TIS), trifluoroacetic acid, dimethylformamide, n-butanol, methylene chloride, N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIPEA), ethyl acetate, potassium bisulfate, sodium bicarbonate, sodium sulfate anhydrous, tetrahydrofuran (THF), barium hydroxide octahydrate, m-cresol and thioanisol were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (37% v/v), potassium chloride, phosphoric acid were purchased from Carlo Erba (Milan, Italy). Formic acid was purchased from Acros Organics (Geel, Belgium). Thionyl chloride and ammonium bicarbonate was purchased from Fluka (St. Louis, MO, USA). Sodium chloride was purchased from VWR (Milan, Italy). SDS-PAGE standards Broad Range, XT Reducing Agent, XT Sample Buffer 4x, XT MES Running Buffer 20x, Comassie Brillant Blu R-250 were purchased from BIO-RAD (Hercules, CA, USA).

3.2.2 Samples

Dry-cured ham samples (n=95) were purchased from the market and from the Experimental Station for the Food Preserving Industry (Parma). Samples of Biceps Femoris muscles were analyzed at different ageing time: salting (n=19), post salting (n=30), 18 (n=35) and 24 (n=11) ripening months with low salt content (<5.4%) and "traditional" content (> 5.4%). Samples are taken from the Biceps femoris muscle, regarded as the reference muscle for evaluating most technological and nutritional parameters of dry-cured ham, such as salt content, texture, colour, etc. The muscles were triturated by a common mill (Moulinex, Milano, Italia) and stored at freezing temperature (-22 °C) until analysis.

3.2.3 Extraction of the peptide fraction (MW<10 kDa)

To 5 g of minced dry-cured ham 45 ml of a HCl 0.1N solution and 250 µl of a 1mM Phe-Phe aqueous solution were added and the mixture homogenized for 1 minute with Ultra Turrax T50 Basic (IKA Werke, Staufen, Germany) at 322g. After centrifugation at 4°C (3200g for 1 h), the supernatant is purified by a series of filtration steps using different filters (paper, 5µm type SMWP, 0.45µm type HPLV: for sample at salting and post-salting steps, it's also necessary another centrifugation at 4°C, 3200g for 15 minutes between filtration at 5µm and at 0.45µm). 4 ml of the obtained extract is evaporated to dryness under

vacuum by rotary evaporator and the residue was dissolved with 2 ml of a 0.1% formic acid aqueous solution. This solution was then filtered on a Vivaspin 2 (Sartorius) filtration system with 10 kDa cut off filters, at room temperature and 5423g for 45 minutes. The filter is washed for 3 times with 2 ml of a 0.1% formic acid aqueous solution. All filtered samples is evaporated by rotavapor and the residue dissolved in 150 µl of a 0.1% formic acid aqueous solution and is centrifuged at 4°C, at 16060 g, for 10 minutes before the UPLC-ESI-MS analysis.

3.2.4 SDS-PAGE analysis on extracted samples

Extracted samples: 5 µl of extracted samples filtered at 0,45 µm was evaporated under nitrogen flux and dissolved in 25 µl of reducing sample buffer (1x, BIO-RAD).

Reducing sample buffer is composed by 5% of XT Reduce Agent (BIO-RAD) in Sample buffer 1x (BIO-RAD). The running buffer (1x) is prepared by dilution of XT MES Running Buffer 20x (BIO-RAD) with bidistilled water. Comassie staining solution: for 1 liter of solution, 1g of Comassie Brillant Blu R-250 (BIO-RAD) was dissolved with 100 ml of acetic acid, 400 ml of methanol and 500 ml of bidistilled water. Comassie destaining solution: for 1 liter of solution, 100 ml di 100 ml of acetic acid, 400 ml of methanol and 500 ml of bidistilled water were mixed. Marker: the molecular marker SDS-PAGE standards (BIO-RAD), is in ratio of 1:20 with the reducing sample buffer. GEL: CriterionTM XT Precast gel, 12% Bis-Tris (BIO-RAD). Run time: around 50 minutes at 150 V. Volume of marker used: 5µl. Data analysis with scanner GS-800 Calibrated Densitometer controlled by software "Quantity one" (BIO-RAD).

3.2.5 In-gel digestion

The gel was destained and the reduction, alkylation and digestion of the bands of interest were carried out as described by the in-gel digestion protocol of the Biological Mass Spectrometry Laboratory (Ontario Wide Protein Identification Facility, http://www.uwo.ca/biochem/bmsl/in-gel_digestion.html#coom).

3.2.5.1 UPLC-ESI-MS analysis

UPLC/ESI-MS analyses were performed with an UPLC/ESI-MS system (UPLC Acquity Waters equipped with a single quadrupole mass spectrometer Waters Acquity Ultraperformance). Conditions were as follows: column, RP ACQUITY UPLC BEH 300 C18 (1.7 µm, 2.1 x 150 mm, Waters); gradient elution: eluent A, water with 0.1% formic acid and 0.2% acetonitrile, eluent B, acetonitrile with 0.1% formic acid; gradient: 0-7 min 100% A, 7-50 min linear from 100% A to 50% A, 50-52.6 min isocratic 50% A, 52.6-53 min linear from 50% A to 0% A, 53-58.2 min isocratic 0% A, 58.2-59 min linear from 0% A to 100% A, 59-72 min isocratic 100% A. LC parameters: flow rate, 0.2 ml/min; analysis time, 72 min; column temperature, 35°C; sample temperature, 18°C; injection volume, 4 µl for extract samples (no solvent delay). MS parameters:

Full Scan mode, acquisition time 7-58.2 min; ionization type, ESI + (positive ions); scan range, 100-2000 m/z; capillary voltage, 3.2 kV; cone voltage, 30 V; source block temperature, 150°C; desolvation temperature, 300°C; cone gas flow, 100 l/h; desolvation gas flow, 650 l/h. Data were acquired and analyzed by MassLynx 4.0 software (Waters Co., Milford, MA, USA).

3.2.6 HPLC/ESI-MS/MS analyses

HPLC/ESI-MS/MS analyses were performed using a HPLC (Mod. Alliance 2695, Waters) equipped with a triple quadrupole mass spectrometer (Mod. 4 Micro, Waters) and a RP column JUPITER 5 µm C18, 300 Å 250 x 2 mm I.D. (Phenomenex, Bologna, Italy). Gradient elution was as follows: eluent A, water with 0.1% formic acid and 0.2% acetonitrile; eluent B, acetonitrile with 0.1% formic acid; gradient: 0-12 min 100% A, 12-77 min linear from 100% A to 50% A, 77-81 min 50% A, 81-82 min linear from 50% A to 0% A, 82-90 min 0% A, 90-91 min linear from 0% A to 100% A, 91-110 min 100% A. Samples (extracts samples) were first analyzed in Full Scan mode, to identify the characteristic ions and the retention time of the unknown compounds, then in Daughters Scan modality, using a variable collision energy from 10 to 30 eV. HPLC/ESI-MS/MS parameters were as follows: flow rate, 0.2 ml/min; analysis time, 110 min; column temperature, 35°C; sample temperature, 23°C; injection volume, 10 µl for Full Scan mode and 30 µl for Daughter Scan mode; acquisition time, 90 min; ionization type, ESI + (positive ions); scan range, 100-2000 m/z; capillary voltage, 3.2 kV; cone voltage, 35 V; source block temperature, 100°C; desolvation temperature, 200°C; cone gas flow, 100 l/h; desolvation gas flow, 650 l/h. The peptide sequences were assigned on the basis of the obtained mass spectra and using the Bioinfomatics resource portal ExPasy with the tools FindPept (Swiss Institute of Bioinformatics, Switzerland) and with the web application "Proteomics Toolkit" (Institute for Systems Biology, Seattle, WA, USA).

3.2.7 LTQ-Orbitrap analysis

Analyses were performed by a LTQ-Orbitrap (Thermo Fischer Scientific, Milano, Italy) equipped with a column Jupiter Proteo 4 µm (90 Å, 150 x 0.30 mm, Phenomenex). Gradient elution was as follows: eluent A, H_2O + 0.2% HCOOH; eluent B, CH_3CN + 0.2% HCOOH. Gradient: 0-4 min, 95%A, 4-60 min, linear from 95% to 50% A, 62-72 min, isocratic 95%B, 70-72 min linear from 5% A to 95% A, plus reconditioning (3 min in 95% A). Samples (extracts) were analysed using a column Acclaim PepMap 300Å 5 µm (300 Å, 150 × 0.30mm, LC Packings, Dionex Company). Gradient elution was as follows: eluent A, H2O + 0.2% HCOOH; eluent B, CH3CN + 0.2% HCOOH. Gradient: 0–4 min, 90%A, 4–60 min, linear from 90% to 50% A, 62–72 min, isocratic 95%B, 72–74 min linear from 5% A to 90% A, plus reconditioning (16 min in 95% A). Samples were desalted by ZipTip (Millipore) before the analyses. Other parameters: flow rate, 5 µl/min; injection volume, 5 µl; MS run time: 65 min; scan event details: (1) FTMS + res = 30000; (2) ITMS + Dep MS/MS most intense ion (1); (3) ITMS + Dep MS/MS second most intense

ion from (1); (4) ITMS + Dep MS/MS third most intense ion from (1); (5) ITMS + Dep MS/MS fourth most intense ion from (1); collision energy, 35. Data-dependent setting: charge state rejection enabled; Unassigned charge states: rejected, charge state 1 rejected, charge states 2, 3 and 4 not rejected. Dynamic exclusion enabled. Repeat count: 3. Repeat duration 30.00 s. Exclusion list size: 50. Exclusion duration: 180.00 s. Exclusion mass width by mass. Exclusion mass width low and high: 1.5000.

3.2.8 Synthesis of NPADs

3.2.8.1 Synthesis of L-Phenylalanine methyl ester hydrochloride (deuterated)

1g of deuterated L-phenylalanine (d_5-L-Phe) were dissolved in 50 ml of dry-methanol and kept under continuous stirring in an ice bath; $SOCl_2$ was added to a final concentration of 1 M (3,62 ml of $SOCl_2$ plus 46,38 ml dry-methanol). The reaction closed with a valve of calcium chloride, was left to react overnight and was monitored by TLC (n-butanol: acetic acid:water 4:1:1 as eluent, UV and ninidrine detection). The reaction mixture was dried at rotavapor. Residual HCl was eliminated by adding methanol (50 ml) and redrying by rotavapor (4 times). Characterization MH^+ (ESI–MS): 185.2 L-Phenylalanine methyl ester hydrochloride (deuterated).

3.2.8.2 Synthesis of L-Lactoyl-L-Phenylalanine (d_5)

(S)-(-)-2-Acetoxypropionic acid (1.56 mmol) was diluted in CH_2Cl_2 (3 ml) together with 1.48 mmol of HBTU and the mixture was kept under continuous stirring at room temperature for 30 min, in order to activate the carboxylic function. L-Phenylalanine-methyl ester d_5 (1.56 mmol) was dissolved in 2 ml of CH_2Cl_2, together with DIPEA (4.68 mmol) and then added to the activated acetoxypropionic acid. The reaction was left to react under magnetic stirring overnight at room temperature. The reaction was monitored by TLC (ethyl acetate as eluent, UV absorbance detector). The organic solution was washed with 10 ml of saturated solutions of $KHSO_4$ (3 times) and $NaHCO_3$ (3 times) in order to remove the unreacted reagents, dried with Na_2SO_4 and filtered. The product was dried under nitrogen flux and vacuum. The methyl and acetyl protecting groups were removed by reaction in 5 ml of THF (without inhibitor) and 5 ml of water with 0.78 mmol of $Ba(OH)_2$ (which corresponded to 2 equivalents of the protected molecules, ratio between THF and H_2O is 1:1) for 20 min, on ice bath. THF was then eliminated under vacuum by rotavapor and the aqueous solution was acidified to pH 3 with HCl. Characterization MH^+ (ESI–MS): 243.2 L-Lactoyl-L-Phenylalanine (d_5).

3.2.8.3 Synthesis of L-Pyroglutamyl-L-Phenylalanine (d_5)

1.5 mmol of L-pyroglutamic acid were diluted in CH_2Cl_2 (5 ml) together with 1.42 mmol of HBTU and the mixture was kept under continuous stirring at room temperature for 30 min, in order to activate the carboxylic function. L-Phenylalanine-methyl ester d_5 (1.5 mmol) was dissolved in 2 ml of CH_2Cl_2, together

with DIPEA (4.5 mmol) and then added to the activated pyroglutamic acid. The reaction was left to react under magnetic stirring overnight at room temperature. The reaction was monitored by TLC (ethyl acetate as eluent, UV absorbance detector). In order to make the reaction complete, 0.75 mmol of L-pyroglutamic acid and 0.71 mmol of HBTU in 2 ml of CH_2Cl_2:dry DMF (1:1) plus 2.25 mmol of DIPEA were added. The organic solution was evaporated under vacuum, at 40°C, in order to eliminate the DMF and redissolved in 5 ml of CH_2Cl_2. The organic solution was washed with 10 ml of saturated solutions of $KHSO_4$ (3 times) and $NaHCO_3$ (3 times) in order to remove the unreacted reagents, dried with Na_2SO_4 and filtered. The product was dried under nitrogen flux and vacuum. The methyl protecting group was removed by dissolving the ester in 12 ml of THF (without inhibitor) and 12 ml of water with 0.9 mmol of $Ba(OH)_2$ (which corresponded to 2 equivalents of the protected molecules, ratio between THF and H_2O is 1:1) for 1h and 30 min, on ice bath. The reaction was monitored by TLC (ethyl acetate as eluent, UV detector). THF was then eliminated under vacuum by rotavapor and the aqueous solution was acidified to pH 3 with HCl. Characterization MH^+ (ESI–MS): 282.2 L-Pyroglutamyl-L-Phenylalanine (d_5).

3.2.8.4 Synthesis of γ-glutamil-L-Phenylalanine (d_5)

N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester (2.055 mmol) was dissolved in DMF (2.5ml) together with 1.955 mmol of HBTU and the mixture was stirred at room temperature for 30 minutes. L-Phenylalanine-methyl ester d_5 (2.055 mmol) was dissolved in DMF (1 ml) together with 6.125 mmol of DIPEA and added to the reaction. The mixture was left to react under magnetic stirring overnight and the reaction was monitored by TLC (ethyl acetate as eluent, UV detector). In order to react the residual phenylalanine, 1.027 mmol of N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester and 0.511 mmol of HBTU in 1.5 ml of DMF and 3.06 mmol of DIPEA were added. The organic solution was evaporated under vacuum, at 40°C, in order to eliminate the DMF and redissolved in 5 ml of CH_2Cl_2. The organic solution was washed with 10 ml of saturated solutions of $KHSO_4$ (3 times) and $NaHCO_3$ (3 times) in order to remove the unreacted reagents, dried with Na_2SO_4 and filtered. The product was dried under nitrogen flux and vacuum. The residue was dissolved in 15 ml of a trifluoroacetic/methylene chloride 1:1 solution, containing also 4% of m-cresol and 4% of thioanisol as scavengers. The mixture was stirred at room temperature for 1 h and then the solvent was evaporated under vacuum by nitrogen flux. Cold ethyl ether was added to the residue in order to precipitate the free dipeptide. The precipitate was washed several times with cold ethyl ether and the product dried by nitrogen flux and under vacuum. The methyl protecting groups was removed by dissolving the ester in 5 ml of THF (without inhibitor) and 5 ml of water with 0.5 mmol of $Ba(OH)_2$ (which corresponded to 2 equivalents of the protected molecules, ratio between THF and H_2O is 1:1) for 20 min, on ice bath. The reaction was monitored by TLC (ethyl acetate as eluent, UV detector). THF was thne eliminated under vacuum by

rotavapor and the aqueous solution was acidified to pH 3 with HCl. Characterization MH^+ (ESI–MS): 300.2 γ-glutamil-L-Phenylalanine (d_5).

For product characterization, 5 µl of all obtained NPADs were dissolved in 1 ml of H_2O+0.1% HCOOH and the solution (1 µl) was injected in UPLC-ESI-MS (the analysis conditions are the same described in paragraph number 3.2.5.1).

3.2.8.4.1 Purification of Npads: semipreprative HPLC-UV

The purification of compounds has been made with semipreparative HPLC-UV (1525 Binary HPLC Pump, Waters) using a RP column (JUPITER 5 µm C18 300 Å 250 x 10 mm, Phenomenex). Injection volume 500 µl, flow 5 ml/min. Gradient elution was as follows: eluent A, water with 0.1% formic acid; eluent B, acetonitrile with 0.1% formic acid; gradient: 0-5 min 100% A, 5-10 min linear from 100% A to 81% A (this % change in function of compound to purified), 10-20 min 81% A, 20-25 min linear to 0% A, 25-30 min linear to 100% A, 35-50 min 100%A.

3.2.8.4.2 Quantification of Npads

The concentration of purified compounds has been made with spectrophotometer UV (Jasco V-530, UV-Vis, Easton, USA) monitoring the absorbance of the amino acid phenylalanine (ε=200$M^{-1}cm^{-1}$) at 257 nm in water. The calibration curve was set up plotting the ratio between areas NPADs:FF (internal standard) vs different concentration of NPADs (γ-glutamil-L-Phenylalanine (d_5) and L-Lactoyl-L-Phenylalanine (d_5) from 25 µM to 500 µM; L-Pyroglutamyl-L-Phenylalanine (d_5) from 10 µM to 500 µM).

3.2.9 Data statistical analysis.

The peptide profiles of the extracts, obtained by the analysis of spectra from UPLC-ESI-MS, were examined by principal component analysis (PCA), T-student test and ANOVA with the software SPSS Statistics 17.0.

3.3 RESULTS AND DISCUSSION

3.3.1 Extraction method

To perform the extraction of the peptide fraction of interest a preliminary necessary step is the elimination of the protein components by denaturation with hydrochloric acid (0.1 N), centrifugation and molecular cut-off filtration (10 kDa). Moreover, although the samples from Biceps femoris muscle had been previously separated from the fatty tissue, a lipid fraction removal have been generally applied to the isolation of peptide fraction from food as this work up could be necessary in order to obtain a proper chromatographic separation. In order to test this aspect, we analyzed the results obtained with and without a diethyl ether

extraction step, comparing the obtained chromatograms and the semi-quantification values (ratio of "area of the peptide" and "area of internal standard", Phe-Phe) of some major peptides identified in the mixture (Sforza, 2001). These latter results are reported in Figure 10, in which the molecular weights of the considered peptides are reported on the x-axis and the semiquantification values are reported on the y-axis.

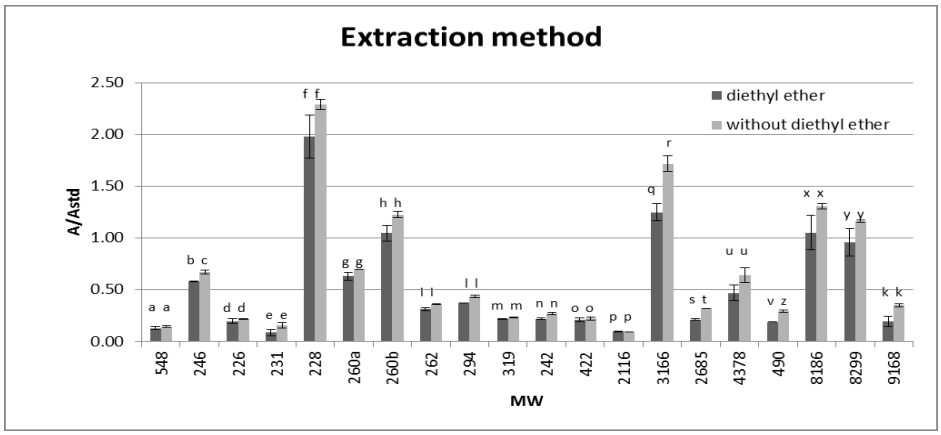

Figure 10. Semi-quantification values of some peptides: MWs of the considered peptides are reported on the x-axis and the semiquantification values (ratio of "area of the peptide" and "area of internal standard", Phe-Phe) are reported on the y-axis. T-student test, $p<0.05$.

No significant differences were found in the chromatographic separation (efficiency, resolution, etc.) and, moreover, the extraction without diethyl ether (in grey) allows a greater recovery of the main peptide fraction with lower losses of analytes. Therefore, it has been decided to proceed with the extraction of the peptide fraction without the use of diethyl ether, as reported in other papers (Mora, 2009; Sentandreu, 2007).

3.3.2 Characterization of the peptide fractions extracted from "traditional" dry-cured hams

3.3.2.1 SDS-page analysis of extracted samples as a function of the ageing time

Biochemical reactions underlying the dry-cured processing are of key relevance to understand the mechanisms of protein degradation and peptide generation. In this sense, the use of proteomic techniques contributes to an improved comprehension of the action of muscle proteolytic enzymes through the identification and sequencing of the peptides generated during the processing. The SDS-PAGE has been the method of choice for the separation of the proteins in meat and its extracts. In this sense, the structural alteration and progressive disappearance of muscular proteins during maturation and dry-cured processing have also been extensively studied. In Figure 11 a typical SDS-PAGE gel obtained from the analysis of water soluble extracts of traditional dry-cured hams at different ageing period is reported.

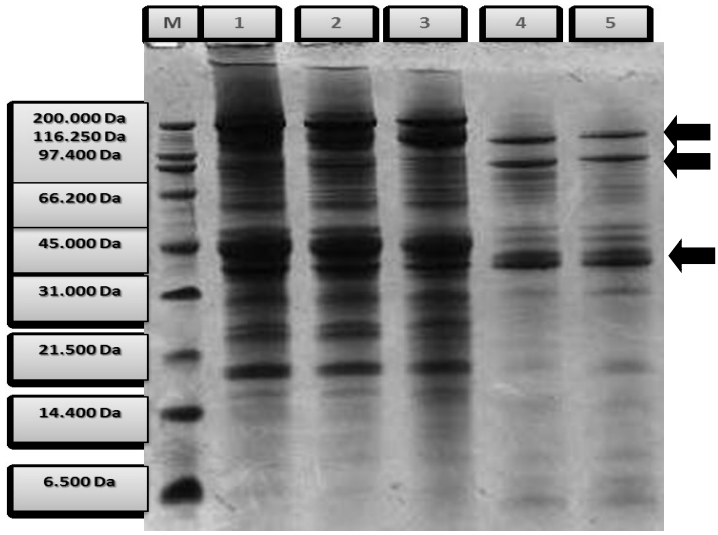

Figure 11. SDS-page of aqueous extracts of ham samples at different ageing period: salting (line 1-2), post-salting (line 3), 18 months (line 4), 24 months (line 5). M is the molecular weight marker.

Comparing an aqueous acidic extract (filtered at 0,45 μm) of an ham sampled at the salting step (line 1 and 2) with the profile of the extract at post-salting (line 3) and at different ageing times (18-24 months, line 4 and 5), large differences were observed: several bands corresponding to different muscle proteins disappear upon the maturation time. As already reported (Larrea, 2006), the most significant change in the profile of water-soluble proteins of the Biceps femoris occurs during ripening, from post-salted ham to dry-cured ham. Indeed, during this period, most proteins undergo extensive proteolysis by the muscle enzymes: there are important decreases in enolase (46.4 kDa), phosphate creatin kinase (44.3 kDa), aldolase (41.6 kDa), b-tropomyosin (35.2 kDa), myosin LC1 (24.75 kDa), troponin C (20.7 kDa), myosin heavy chain, actin. This decrease is attributable to the intense proteolysis taking place, particularly in the final stage of ripening: the long processing of dry-cured ham allows for a more intense action of muscle proteases, and results in extensive protein breakdown. Proteins still detectable in the aqueous acidic extract and, thus, more resistant to the proteolysis process were identified by LTQ-Orbitrap analysis after in gel digestion: the bands corresponding to molecular weight of 116.250 Da and 97,4 kDa are a mixture of isoforms of myosin (myosin 4, myosin 1, 2 and 7). The bands corresponding to 45 kDa MW are the myofibrillar proteins tropomyosin α-1 and tropomyosin α-3 chain and the sarcoplasmic protein L-lactate dehydrogenase.

Analysing by SDS-PAGE the peptide fraction isolated by ultrafiltration with MW cut-off filters of 10 kDa, some interesting observation can be made.

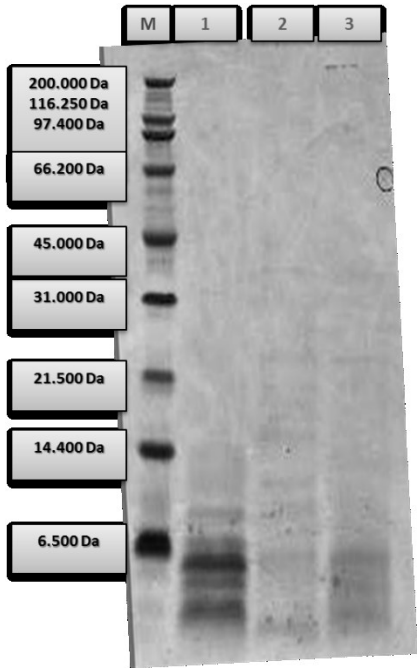

Figure 12. SDS-page of aqueous extracts (MW<10 kDa) at different ageing period: salting (line 3), post-salting (line 2), 18 months (line 1). M is the molecular weight marker.

As shown in Figure 12, if we compare sample with 18 months (a similar result was obtained for 24 months, not shown) of ripening (line 1), samples at post-salting (line 2) and salting step (line 3) we can observe a far higher intensities of the bands corresponding to low MWs (around and lower than 6500 Da): a very huge amounts of low MW peptides are produced by protein breakdown during the long maturation time.

3.3.2.2 Mass spectrometry analysis: characterization of the peptide profile in sample extracts of traditional hams.

Mass spectrometry is a powerful analytical technique for both protein and peptide analysis because it can rapidly and reliably identify the components of complex matrices. The most popular analyzers in proteomics are ion traps, triple quadrupoles, time of-flight tubes, orbitrap, and Fourier transform ion cyclotron resonance, with their specific advantages: high sensitivity and multiple-stage fragmentation for ion traps, high selectivity for triple-quadrupoles, high sensitivity and speed for time-of-flight. A complete sequence of the peptides of interest is often achieved by using tandem MS (MS/MS), by fragmentation of a selected precursor peptide ion to generate specific fragment ions for sequence elucidation (data-dependent acquisition): to identify these peptides, spectra are scanned against protein-sequence databases using search algorithms.

The water extracts of traditional dry-cured hams, already described in the paragraph 42, have been analyzed first in UPLC-ESI-MS. LC-MS analysis of the peptide pattern up to 10 kDa reveals different chromatograms profile, following the maturation process of dry-cured hams, as reported in Figure 13.

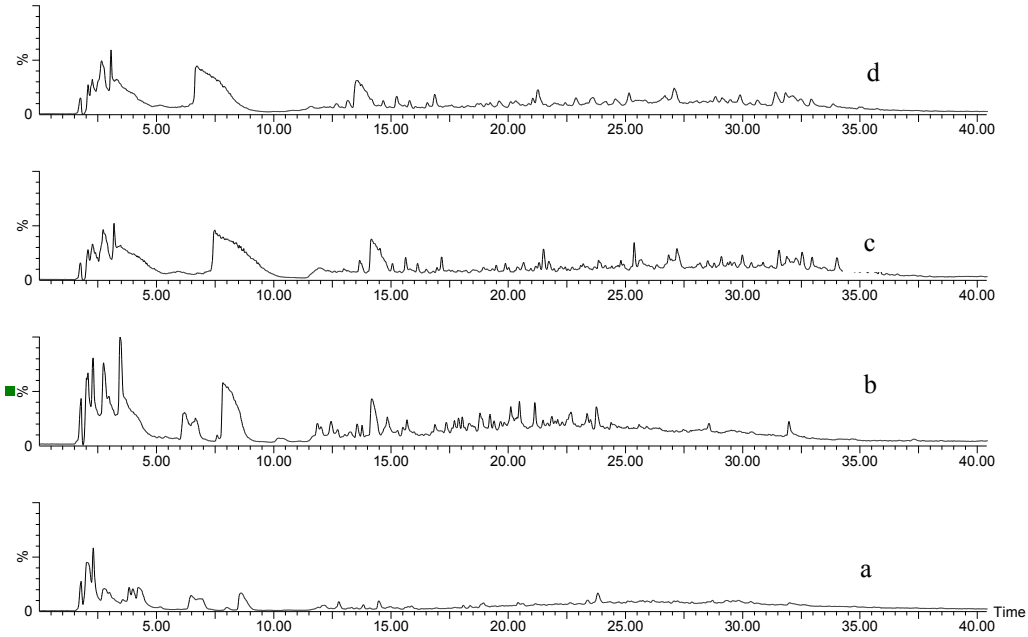

Figure 13. Total Ion Chromatograms obtained in UPLC-ESI-MS of aqueous extracts (MW<10Da) of ham samples at different ageing period: salting step (a), post salting step (b), 18 months (c) and 24 months (d).

At the salting step a very poor peptide profile was obtained, with low intensity signals (Figure 13, a); at post-salting step a sharp increase was observed, particularly in the first 25 minutes of run time (Figure 13, b), a elution window which corresponds to low molecular weight and polar peptides; the chromatograms of samples at 18 and 24 months of ripening time (Figure 13, c and d) are very similar and richer in comparison with the other two steps: the peaks of peptides are present all along the chromatograms, with a particular signal intensification also after 25 minutes of run time, corresponding to high molecular weight and more apolar peptides.

The evolution of the peptide profile is strictly determined by the action of muscle proteases and by the fact that peptides, once generated, may then undergo to further proteolytic actions. Indeed, the molecular weight range in samples at salting is approximately spans between 200 to 8500 Da, in post-salting samples approximately from 200 to 10300 Da and in samples at18-24 months approximately from 200 to 9200 Da. Short peptides ever increase during the aging time, while, in contrast, the high molecular mass peptides show a tendency to accumulate in the first part of the ageing period and to decrease in the last part, on

account of their double role of products and substrates of the proteolytic activity. Raw meat (genotype, age, sex, ante- and post-mortem treatment) and process technology have a decisive influence on the activity of muscle enzymes responsible for these transformations.

The Orbitrap analysis reveals that the main peptides present in all samples originated as expected from sarcoplasmatic and miofibrillar proteins: in particular, peptides originated myofibrillar proteins like actin, myosin, troponin T, enzymes like glygeraldehyde-3-phosphate dehydrogenase, beta-enolase, but also myoglobin and hemoglobin. All these proteins constitute relevant substrates for proteolysis that occurs during the processing of dry-cured ham: muscle endopeptidases like catepsins and calpains can degrade the myofibrillar structure giving rise to large polypeptides. These polypeptides are then degraded, in further stages, to smaller peptides and free amino acids by the action of exopeptidases such as dipeptidyl peptidases and aminopeptidases. The main enzymes responsible for proteolysis of sarcoplasmic proteins are muscle endopeptidases and certain groups of exopeptidases. The action of these enzymes during the dry-curing process has great importance because they are directly responsible for the changes in the post-mortem muscle texture and for the flavour development that occurred during the ripening of dry-cured products.

The presence of fragments of myoglobin, the most important protein of sarcoplasm, is very important because it is responsible for meat color, which is associated with the product quality. Myoglobin protein has been found to be degraded during the post-mortem storage of pig meat as well as during the processing of dry-cured ham. Fragments of myoglobin detected at the end of the processing of dry-cured ham and the intense proteolysis of myoglobin during dry curing has been confirmed (Mora, 2012).

The main peptides present in hams at step of salting and post-salting are reported below in Table 1.

Table 1. List of main peptides (Molecular Weight, MW), retention time and observed ions in ham extracted samples at the salting and post-salting step with UPLC-ESI-MS analysis.

Retention time (min)	Ions	MW
6.2	136.9+268.9	268
7.59	217	216
10.21	246.9+229.8	246a
11.86	246.9	246b
12.44	179.0+357.1	356
	401	400
12.72	219.9	218
	136.9+296.9	296
13.25	232	231
	216.9	217
	222.9+119.9	222
13.56	229	228a

Retention time (min)	Ions	MW
13.77	136.8+340.9	340
14.85	115.9+229.0	228b
15.28	261	260a
15.53	178.0+261.0	260b
15.63	231.2+132.0	230
15.69	115.9+229.0	228c
16.03	388.4+776.0	775
16.23	263.2+401.3	400
16.87	305.1	304
	336.6+672.1	671
17.32	344.3	343
17.35	383.7+511.4+766.3	1531
17.68	320.1	319a
17.75	369.9+554.2	1106
17.88	415.8+554.3+831.1	1660
	319.9	319b
18.04	408.8+510.8+680.8+1020.6	2039
18.35	513.3+769.5	1537
18.61	322.5+386.8+483.2+644.0	1929
18.79	444.2+592.0+887.5	1773a
	535.8+803.2	1604
18.9	245	244
19.22	355.7+444.2+592.0+887.5	1773b
19.39	381.3+476.3+634.9+951.8	1902
	262.1	261
19.69	444.1+517.8+621.2+776.4+1034.5	3101
19.82	355.7+426.7+533.1+710.3	2128
	622.1+1243.1	1242
	304	303
20.08	416.3+520.0+693.0+1039.2	2076
20.11	509.4+407.4	2033
20.41	499.7+624.3+832.2	2493
20.48	391.8+469.9+587.1+782.5+1173.4	2345
	525.5+656.6+875.2	2622.5
20.68	589.4+687.5+824.5+1030.5	4118
21.14	438.6+548.1+730.4+1095.1	2188
21.49	410.7+547.3	1639
21.66	487.3+973.3	972

Retention time (min)	Ions	MW
21.68	585.1	584
21.76	703.4+844.0+1055.0+1405.7	4215
21.87	516.8+645.9+860.9	2579
21.9	530.1+618.1	3703
22	441.6+529.9	2644
22.11	469.4+563.1+703.7+938.0	2811
22.29	519.8+606.2+727.4+908.9	3631.5
	469.4+586.4+781.6	2342
22.54	494.6+593.3+741.4+988.6	2962
22.67	457.8+548.9+686.2+914.2	2740
22.78	455.2+546.1+682.4	2752
22.83	275.9	275
23.36	431.6+517.8+646.9	2584
	204	203
23.51	851.0+1701.3	1700
23.77	119.9+165.9+313.0	312 (Phe-Phe)
23.99	524.6+699.0	2094
24.39	915.6+1830.4	1829
24.51	610.0+762.3+1016.0	3045
25.08	854.5+1139.3	3414
25.59	676.1+1013.6	2025
	738.4+1107.0	2212
25.9	589.0+785.0+1177.2	2352
26.14	528.6+634.3	3166
27.43	434.1	433
28.23	547.3+656.5+820.4+1093.7	3278
28.56	687.2+763.4+858.7+981.3+1144.6+1373.6	6862
28.7	789.3+902.0+1051.9+1262.3	6306
29.37	514.9+600.5+720.5	3597
29.57	643.9+715.5+804.8+919.6+1072.9+1287.3	6430.5
29.88	847.5+1271.2	2540
30.36	558.2+669.4+836.6+1114.9	3342
	730.1+875.8+1094.5	4374
	628.7+785.6	3138
31.29	643.6+771.9+964.9	3855
	574.1+860.7	1719
31.98	650.8+704.9+769.0+845.8+939.7+1057.1+1207.8+1409.3	8448
32.56	572.2+715.3+953.3	2857

About 89 peptides were identified in terms of MW in the mixture. The only compound that has been asily identified is inosine and its degradation product hypoxanthine (MW 268 Da), generated by enzymatic reactions in the post mortem metabolism of muscle that still occur at the beginning of dry-cured processing. This is the case for the ATP (adenosine triphosphate) degradation with the generation of nucleotide breakdown products and glycolysis resulting in the generation of lactic acid. The pathway of ATP catabolism, as a degradative sequence, has been widely studied and used in freshness and quality assessment in different fish species, beef, poultry and pork meat. Thus, after slaughter, ATP degradation to ADP (adenosine diphosphate) and AMP (adenosine monophosphate) takes place rapidly, with the subsequent accumulation of IMP (inosine 5′-monophosphate). The IMP is hydrolysed by autolytic enzymes (5′-nucleotidase, N) to inosine (Ino), which, in turn, is degraded to hypoxanthine (Hx) by autolytic and/or microbial action (nucleoside phosphorylase, NP). Further, the Hx will be oxidized to xanthine (X) and then to uric acid (UA) through a much slower reaction due to xanthine oxidase (XO) in case of spoilage by microorganisms. Some of the ATP degradative compounds or ratios of these (Ko, K value, K′ value, Hx ratio or H value) have been proposed as freshness indexes. The simplest is the determination of the Hx concentration that, due to its accumulation during storage, has been considered an excellent index of meat aging and quality. On the other hand, some of these compounds affect meat flavor: indeed, IMP is a flavour enhancer, which contributes to the umami taste, whereas hypoxanthine together with some amino acids and peptides may contribute to a bitter taste in meat (Hernández-Cázares, 2011).

Dry-cured hams are generally commercialized at 18-24 months, so we focused our characterization on the ham sample with these ageing times.
Peptide sequences present in 18 and 24 months were identified and characterized by UPLC-ESI-MS and HPLC-ESI-MS/MS, confirming the identification by exact mass determination with LTQ-Orbitrap, as reported in Table 2.

Table 2. Peptide sequences in 18 and 24 months samples, identified and characterized by UPLC-ESI-MS.

Retention time (min)	Ions	MW	Sequences	Proteins
11.63	189.4	188	n.i.	
11.95	247.3	246a	ID	
12.67	358.5	357	PIE	actin
	247.4	246b	LD	
13.00	220.3	219	SN	

Retention time (min)	Ions	MW	Sequences	Proteins
	227.3	226	piroglutamil-Pro	
13.67	229.4	228a	PI	
14.41	229.4	228b	PL	
	281.4	280	n.i.	
15.06	261.4	260a	γ-EI	
15.62	261.4	260b	γ -EL	
16.14	222.3	221	n.i.	
16.50	263.4	262	PF	
16.96	295.4+166.3	294	γ -EF	
17.17	190.4+144.1	189	lactoil-val	
17.6	231.4+ 214.4	230	n.i.	
	320.5	319a	DW	
18.51	243.4+197.3	242a	pir-ile	
18.8	229.4	228c	n.i.	
18.91	320.5	319b	n.i.	
18.93	128.3+156.4+174.3	173	n.i.	
18.95	245.4	244a	II/LL/IL/LI	
19.11	477.3+636.1+953.7	1905	PAPAPAPAPAPAPPKEEK	A1XQT6
19.15	341.2+511.2+1020.9	1020	ALPHAIMRL	P68137
	538.6	537	YNEL	
	552.6	551	n.i.	
19.49	243.4+197.3+132.3	242b	pir-leu	
19.61	245.5	244b	II/LL/IL/LI	
19.88	174.3+128.3+156.3+ 196.3+304.4	303	n.i.	
20.38	214.4+232.4	231b	VN	
	387.0+772.7+275.3	771	n.i.	
20.65	324.0+646.5+515.4	645	FHETL	F1RZV8
	275.3+202.3+159.3	274	n.i.	
21.31	158.4+204.4	203a	lacto-ile	
21.49	158.4+204.4	203b	lacto-leu	
21.74	301.4+451.4+901.7	901	n.i.	
22.23	498.9+598.5+747.8+99 6.7	2987	n.i.	
22.41	530.1+706.3+1059.0	2116	n.i.	
	279.5	278	n.i.	
	471.6+942.0	941	n.i.	
22.98	344.6+213.5	343a	IVL	

Retention time (min)	Ions	MW	Sequences	Proteins
23.19	238.4+192.3+120.3	237	lacto-phe	
23.85	120.3+166.4+313.4	312	Phe-Phe	Internal standard
24.11	484.2+553.3+645.2+774.2+967.2	3865	n.i.	
	422.2 +527.6+703.0	2106	n.i.	
24.61	445.7	444	VITI	actin
24.81	213.4+344.5	343b	IVL	
25.37	528.6+634.0+792.5	3166	GIITNWDDMEKIWHHTFYNELRVAP (y phos)	actin
25.67	553.6+622.7+711.4+829.9+995.8	4973	n.i.	
	608.5+695.1+810.9+972.7	4859	n.i.	
	559.6	558	**IGGSIL**	Actin
26.35	530.0+618.2+741.8	3703	n.i.	
	381.0+476.2+634.4	1900	VTTAEREIVRDIKEKL	Actin
26.67	507.6+634.2+845.3	2533a	DTYKSEIAHRFKDLGEQYFKG	PO8835 serum albumin
	660.1 + 754.3 + 879.8 + 1055.6	5273	n.i.	
	705.2+822.7+987.1	4930	n.i.	
	568.0+681.4+851.3+1134.9	3402	n.i.	
	816.8	815	n.i.	
27.06	441.5+514.9+617.6+771.7+1028.6	3083	n.i.	
27.19	507.7+634.3+845.3	2533b	DTYKSEIAHRFKDLGEQYFKG	PO8835 serum albumin
27.98	614.5+691.1+789.6+921.2+1105.2	5521	n.i.	
	698.1+1046.7	2091	n.i.	
28.17	646.6+738.7+861.6+1033.8	5164	n.i.	
28.27	569.1	1136	LAGRDLTDYL	P68137 actin
	462.7 + 693.2	1385	YQKSSVKTLAFL	Q9TV61 myosin 1
28.37	565.9+753.8+1130.5	2259	n.i.	
28.52	659.1+753.1+878.4+1053.7	5264	n.i.	
28.78	563.2 + 643.5 + 750.4 + 900.5 +1125.3	4497	n.i.	

Retention time (min)	Ions	MW	Sequences	Proteins
29.1	672.2+895.9+1343.5	2684	PKPHSDAGTAFIQTQQLHAAMADTF	swine pyruvate kinase
29.11	549.5+659.2+823.6	3291	n.i.	
29.17	486.6+972.0	971	VLIREDNI	F1RP07
29.49	502.0+602.2+752.3	3006	n.i.	
	717.3+956.0	2865	n.i.	
29.88	473.9+552.7+662.9+828.2	3310	n.i.	
29.98	626.3+730.5+876.4+1095.2	4378	n.i.	
30.37	480.3+600.1+799.7	2396	IDHLSEDKLRDKAKELWDAL	Q75NG6 troponin T
30.87	491.5	490	VL(I)L(I)F	
31.55	580.0+632.6+695.7+772.8 +869.5+993.4+1158.8	6947	n.i.	
31.88	683.0+745.0+819.4+910.4+ 1024.0+1170.1	8184	n.i.	
32.08	645.7+699.4+762.8+839.1+932.2 +1048.5+1198.1	8381	n.i.	
32.26	589.3+643.0+707.0+785.4+883.5+ 1009.6+1177.5	7060	n.i.	
32.53	689.4+751.9+827.0+918.8+1033.5 +1181.0+1377.8	8260	n.i.	
32.54	573.0+715.9+954.2	2860	n.i.	
32.96	692.5+755.2+830.7+922.9+1038.1+ 1186.3+1383.6	8297	n.i.	
33.8	665.9+887.4+760.8	5318	n.i.	
	528.1+659.8	2635	n.i.	
	681.8+1021.1	2041	n.i.	
	507.6+608.9+760.8	3039	n.i.	
	670.7+726.6+792.5+968.2+871.6 +1098.2+1245.0	8706	n.i.	
34.03	706.1+764.9+834.3+917.6+1019.4 +1146.7+1310.3+1528.5	9166	n.i.	

Retention time (min)	Ions	MW	Sequences	Proteins
	661.4+716.4+781.5+859.6+955.0+1074.2+1227.3	8586	n.i.	
34.35	591.2+709.1+886.2	3541	n.i.	
35.11	727.1+ 581.6+968.8	2904	n.i.	
35.12	604.6+755.4	3018	n.i.	
	567.7+662.4+794.5	3968	n.i.	
35.44	568.6(14)+612.4+663.4+723.6+795.8+884.1+994.6+1136.2	7948	n.i.	
35.85	667.2+833.5+1111.3	3331	n.i.	

n.i.: not identified

The identification has been performed by online bioinformatics resource portal such as "Expasy" with the tool "FindPept" and by the "Proteomics Toolkit"(MS/MS Fragment Ion calculator): the identification process is based on the comparison of the theoretical fragmentation (Proteomics Toolkit) of the possible peptide sequences obtained by in silico experiments from unspecific cleavage of proteins (FindPept) with the sequence fragmentation obtained by MS/MS experiments. An example is reported Figure 14.

Sequence: **VITI**, pI: **5.49502**

Fragment Ion Table, average masses

Seq	#	A	B	C	X	Y	Z	# (+1)
V	1	72.13045	100.14055	117.17111	-	445.57979	428.54923	4
I	2	➡ 185.28988	➡ 213.29998	230.33055	372.44145	346.44723	329.41667	3
T	3	286.39497	➡ 314.40507	331.43563	259.28201	➡ 233.28779	216.25723	2
I	4	399.55441	➡ 427.56451	-	158.17693	➡ 132.18271	115.15214	1

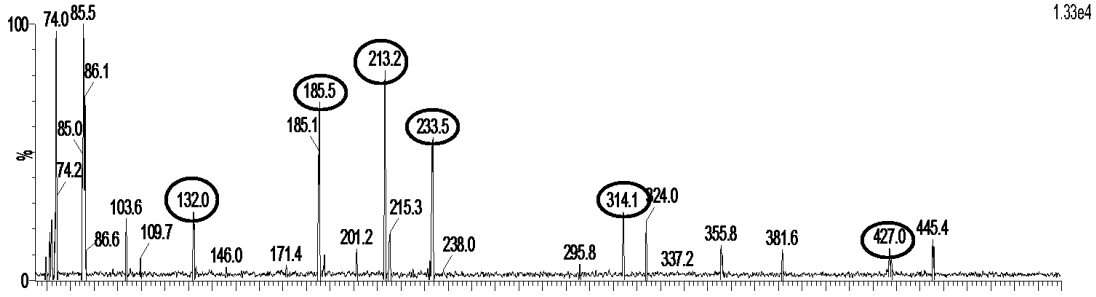

Figure 14. Identification of sequence VITI by HPLC-ESI-MS/MS analysis.

Proteolysis during the ageing time determines changes in the peptide profile in term of type of sequences and/or relative amounts. The main peptides found in samples at different ripening periods have been semiquantified using the internal standard Phe-Phe (ratio between the areas) in order to verify the possibility to discriminate the samples on the base of their maturation time. A PCA analysis was performed and the results are reported below, in Figure 15. The total explained cumulated variance is 65%, 49 % from component 1 and 15% from component 2.

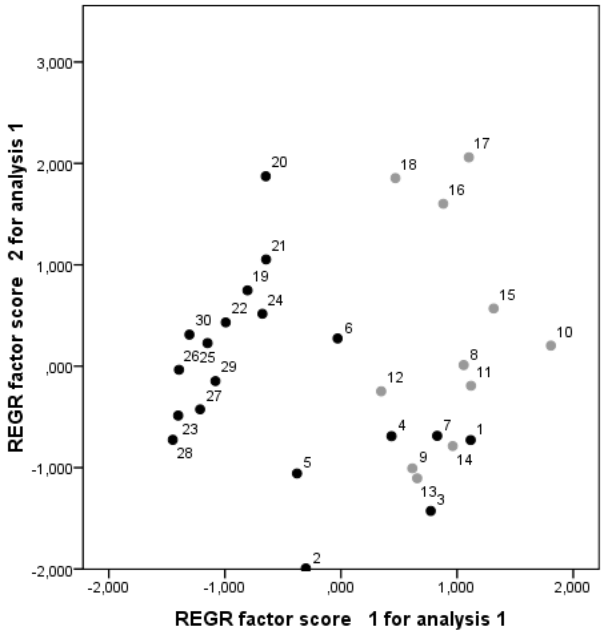

Figure 15. Principal Component Analysis of extracted samples at 18 (in black) and 24 months (in grey).

The results of statistical analysis allow to conclude that it's possible to distinguish dry-cured hams on the basis of their ageing period on the base of a different peptide profile, on account of the prolonged enzymatic proteolytic activities. Principal Component Analysis of extracted samples at 18 (in black) and 24 months (in grey). In order to verify which peptides are responsible for the observed difference, the semiquantification values of all the peptides were compared by T-student test ($p<0.05$): all the identified peptides are found in higher amounts in 24 months aged ham than in 18 months aged ones the peptides with molecular weight of 173 Da and 303 Da.

The knowledge of the protein fragments naturally generated during dry-curing would be very important for a better understanding of the texture changes and flavor development mechanisms occurring in this process. Due to the complexity of this study, in the literature a lot of papers focused on peptides generated from breakdowns of one muscle protein for time. Peptide profile of aged Parma ham has been already partially

characterized (Sforza, et al., 2001).We confirmed these previous findings and identified some other sequences among the most abundant peptides in the extract, i.e. the sequence PAPAPAPAPAPAPPKEEK (MLC1f), a fragment of the myosin light chain already identified in Serrano dry cured ham (Mora, 2009), and PKPHSDAGTAFIQTQQLHAAMADTF, a sequence originating from swine pyruvate kinase, already previously identified by Sforza et al. (Sforza, 2003). Moreover, fragments from actin were found such as GIITNWDDMEKIWHHTFYNELRVAP (Y phos), VITI, PIE confirming the extensive proteolysis of this protein during curing.

Some specific compounds have been identified in these dry-cured ham extracts, namely non-proteolytic amino acyl derivatives, NPADs. These compounds of non-proteolytic origin have been already identified in cheeses and some other food matrices (soy sauce, etc.). They are de novo synthesized by non-proteolytic enzymatic activity and are generally characterized by an "umami" taste.

3.3.2.2.1 NAPADs: non-proteolytic amino acyl derivatives

The amino acyl derivatives are peptides of non-proteolytic origin but synthesized de novo by enzymatic activities in food. In particular, gamma-glutamyl amino acids, pyroglutamyl-amino acids and lactoyl-amino acids have been already observed in dairy products and are known to contribute to the taste of the products. Gamma-glutamyl amino acids have been already found in dry cured hams (Sforza, 2006): they are formed during ageing and tend to accumulate in the product.

So, we investigated the occurrence of non-proteolytic amino acyl derivatives (NPADs) in dry cured ham.

We have been able to identify a lot of compounds, namely: γ-glutamyl-tyrosine, pyroglutamyl-proline, γ-glutamyl-isoleucine, γ-glutamyl-leucine, lactoyl-metionine, lactoyl-tyrosine, γ-glutamyl-phenilalanine, lactoyl-valine, pyroglutamyl-isoleucine, pyroglutamyl-leucine, lactoyl-isoleucine, lactoyl-leucine and lactoyl-phenialalnine.

A typical fragmentation obtained by HPLC-ESI-MS/MS analysis is reported below in Figure 16 for pyroGlu-Ile/Leu.

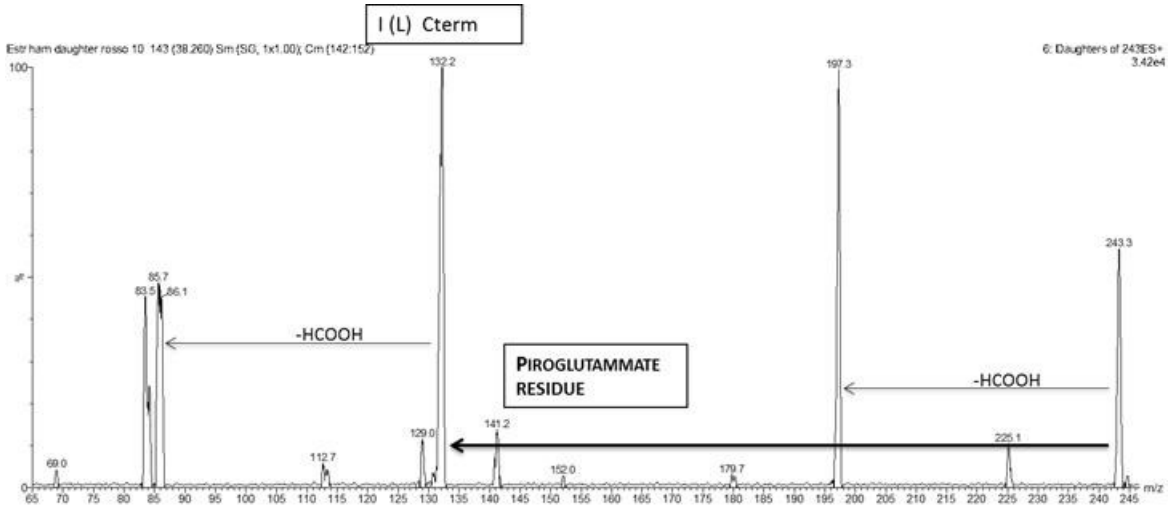

Figure 16. Spectra of piroGlu-Ile (Leu) obtained by HPLC-ESI-MS/MS analysis.

In order to quantify the identified compounds and evaluate their amount variation during ageing, we synthesized some derivatives to be used as standards for confirming the identification and performing the quantification. In particular, one NPAD for type has been synthesized: thus, γ-glutamyl-Phe, lactoyl-Phe, pyroglutamyl-Phe were prepared by chemical synthesis and used to quantify all the compounds occurring in the samples. The calibration curves were set up plotting different concentration of Npads with the ratio area NPADs/area FF (internal standard).

The results are reported in Figure 17.

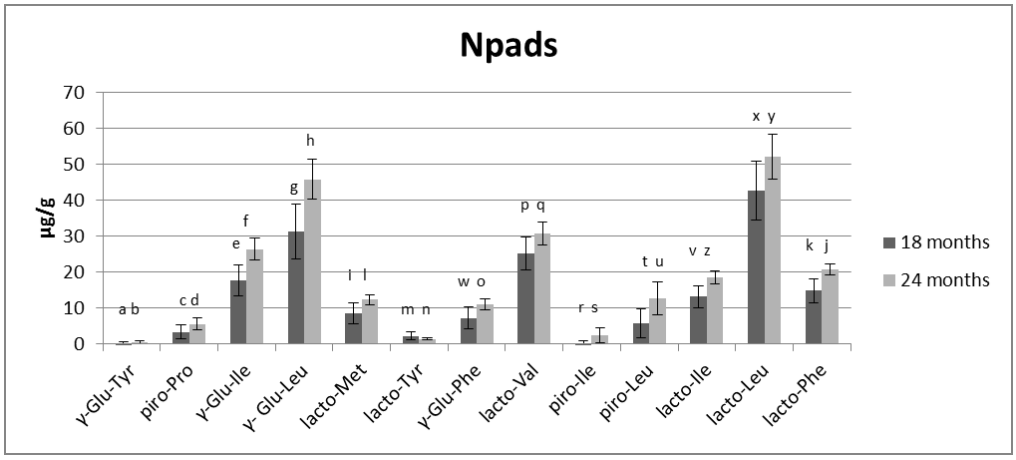

Figure 17. NPADs present in aged dry-cured hams: 18 and 24 months (T-student test: p<0.05).

Several compounds are present in the extracts and they tend to slightly increase from 18 to 24 months: this change with ageing has been already observed in literature in particular for cheeses. This is due to the fact

that these compounds, once formed, are not furtherly degraded by the proteolysis process. The two most abundant compounds found in aqueous acidic extracts of dry-cured ham are γ-glutamyl-leucine and lactoyl-leucine: they have been found in maximum amount at 24 months with 46 µg and 52 µg per gram of ham.

Only two compounds have been found in detectable amounts in the post-salting process, i.e. lactoyl-Methionine and lactoyl-Leucine, neveretheless in far lower amounts then in 18 and 24 months samples (Figure 18).

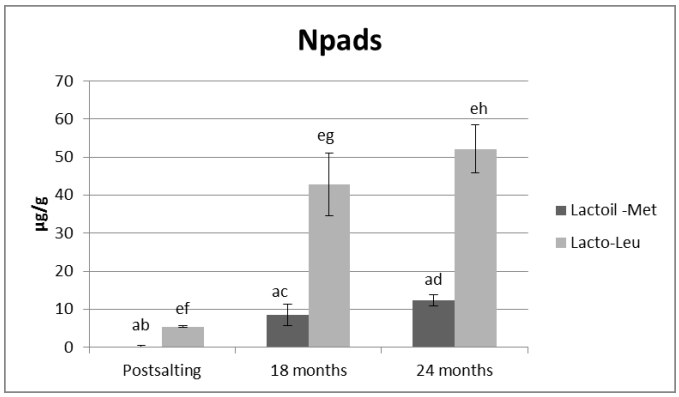

Figure 18. Variation of lactoyl-Met and lactoyl-Leu during ageing of dry-cured hams: from post-salting to 24 months of ageing time.

These compounds, already detectable in the first months of processing (post-salting period), strongly increased during the ripening time: in particular, lactoyl-leucine a 10 times increase was recorded, from 5.4 µg to 52 µg per g of product.

3.3.2.3 Characterization of the peptide profile of traditional dry-cured hams vs dry-cured hams with reduced amount of salt: similarity and differences.

Health authorities are associating high levels of sodium chloride (NaCl) intake with raised blood pressure, also known as hypertension: these data have pushed the current research and strategies to reduce sodium intake (He, 2003). In the case of low-salt meat products, the claim "with reduced salt", stating that the content in sodium or the equivalent value for salt has been reduced, may only be reported on the label if the reduction is at least 25% if compared to the sodium or salt content of similar products (Nutrition claims and conditions applying to them as listed in the annex of regulation (EC) n°1924/2006).

Biceps femoris is the muscle generally taken for the reference values of different product parameters such as salt content. Salt content in the Biceps femoris of traditional hams is about 5.3%: to have a reduction of 25% it's necessary to have 4.8% of salt in Biceps femoris at 12 months of ripening. Nevertheless, hams are normally commercialized at 18-24 months of ripening: thus, taking into account the weight loss of about 5% during the ageing period from 12 to 19 months, at the end of ripening hams with a salt content (in Biceps femoris) <5.4% are considered "at reduced salt", whereas for salt content >5.4% samples are considered "traditional" hams. The new salt-reduced products have been prepared by SSICA (Experimental Station for the Food Preserving Industry of Parma).

The extraction of peptide fraction was performed as described for the traditional hams (paragraph n° 3.2.3). The peptide database created with the main peptides present in traditional dry-cured hams has been used to compare the hams at low salt content with those at the post-salting step first and at 18-20 months of ripening time after. The peptides were semiquantified using the internal standard (Phe-Phe).

The first Principal Component Analysis (PCA) on "cold" steps (post-salting periods) is reported in Figure 19. The total cumulative variance explained is 49.5% (in which the variance of the component 1 is 31.2% while that of component 2 is 18.3%).

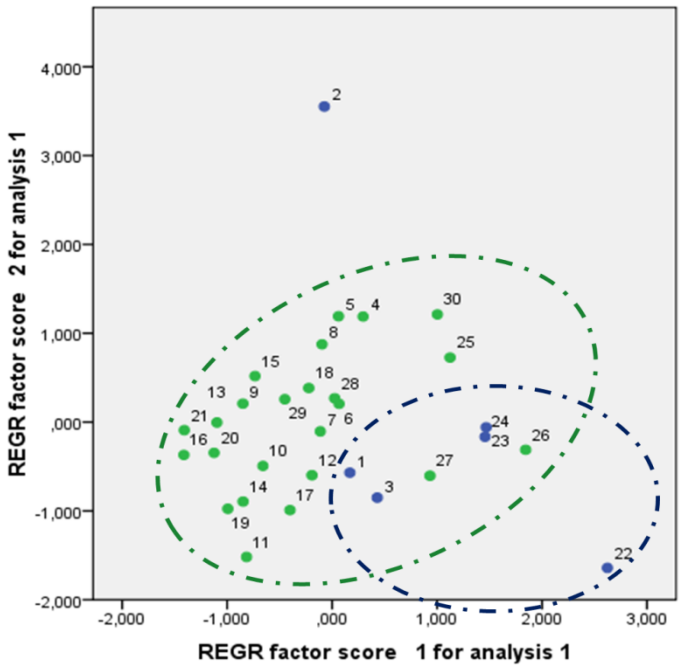

Figure 19. Principal Component Analysis of traditional (green) and reduced salt (blue) hams at the end of the "cold" step (post-salting).

The peptide profile (<10 kDa) of low salt and traditional hams at the end of resting are slightly different and it allows a partial grouping of the two types of products. A little difference can be also observed in the intensity of the different peaks in the chromatographic profile of the peptide fractions obtained in UPLC-ESI-MS, as shown in Figure 20 .

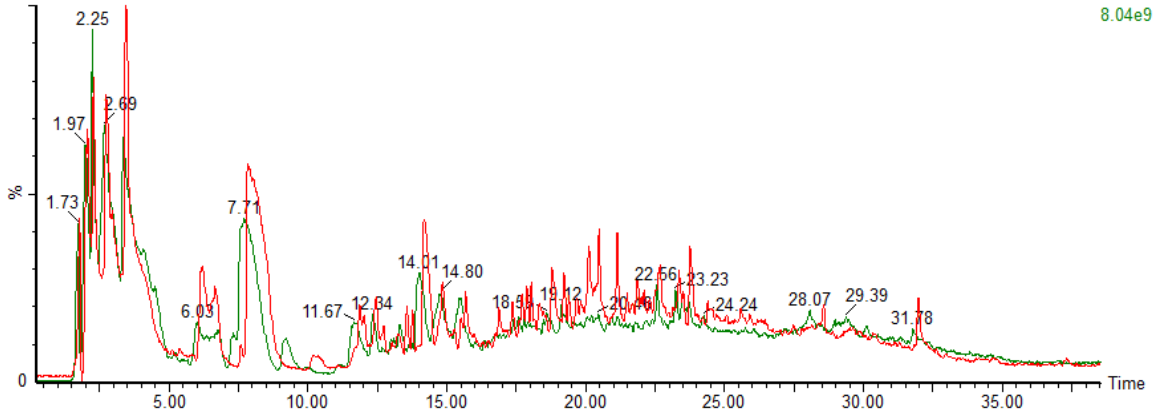

Figure 20. Chromatograms of the peptide fraction of a traditional ham (red) and of a salt reduced ham (green).

The resting phase is a critical stage in the production of cured ham. The salt, absorbed during the salting and concentrated in the superficial muscles (eg. semimembranosus muscles), during the resting phase diffuses into the internal muscles such as Biceps Femoris, due to the concentration gradient of the salt in the tissue fluids, and the moisture migrates from the inside to the outside of the ham then evaporating into the environment. During the resting phase, the diffusion of the salt and the loss of moisture lead to a reduction of water activity (a_w) in the inner parts of the ham, a necessary condition to achieve the microbiological safety before the subsequent stages at higher temperatures (14-16 °C), but these events also have an influence on and the activity of the endogenous proteolytic enzymes. Hams with low salt content show a decrease of a_w in BF as far as resting time increases: a longer resting time is necessary to reach the same values of the traditional products, which have been demonstrated to assure the microbiological safety: in this case, resting time up to 145-160 days, almost 2 months longer than the traditional production, is necessary.

This phenomenon can be explained by the fact that the salt content in the ham with reduced salt is lower than for the traditional hams and that diffusion occurs more slowly but still allows the salt to reach the inside of the ham and to concentrate.

The effect of the duration of the resting phase on the proteolysis is, really, determined by the complex combination of different events that occur simultaneously: 1) the time available for the expression of the proteolytic activity; 2) the variation of the concentration of the salt with inhibitory activity against proteolytic enzyme activity; 3) the variation of the concentration of the salt which affects protein solubility. In Biceps femoris, a higher proteolysis index was measured for the hams characterized by longer resting times (145 and 160 days): this may be the result of the lower salt content and the increased times available for enzymatic degradation of proteins. Indeed, when the concentration of NaCl in the BF is low, it exerts a lower inhibition of the enzyme activity. In particular, cathepsins, that are likely to be inhibited by the salt concentration in traditional ham, could have an higher activity on low salt ham.

In order to verify if these differences occurring at the resting step between the two type of products will be confirmed at the end of ageing time, the peptide profile of the samples at 18-20 months of ripening have been also characterized. The Principal Component Analysis (PCA) on the "hot" step is reported in Figure 21: the total cumulative variance explained is 59%, being the variance of the first component 41% and the variance of the second component 18%.

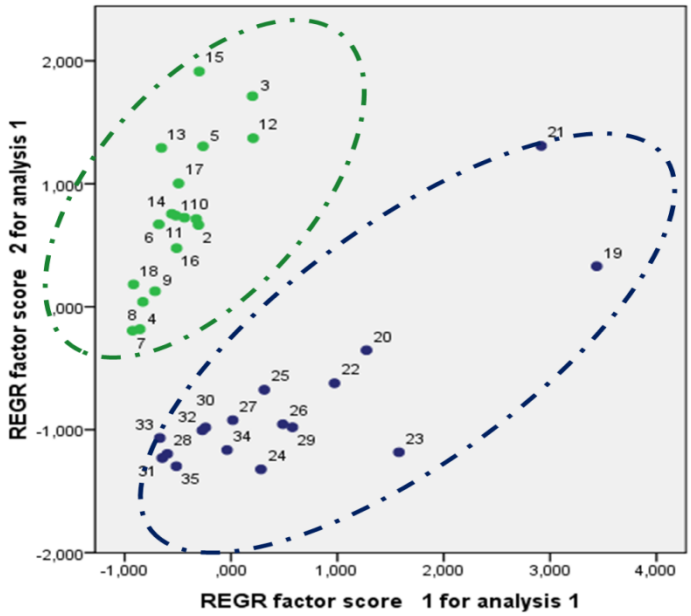

Figure 21. PCA of reduced salt (green) and tradiotional hams (blue) at 18-20 months of ageing time.

The graph of the scores show as the reduced salt samples (in green) sharply differ from the traditional ones (in blue). These differences are also particularly evident comparing the peptide profile obtained in UPLC-

ESI-MS for the two sets of samples: the comparison between the sample chromatogram of a low-salt (in green) and a traditional sample (in red) is reported Figure 22.

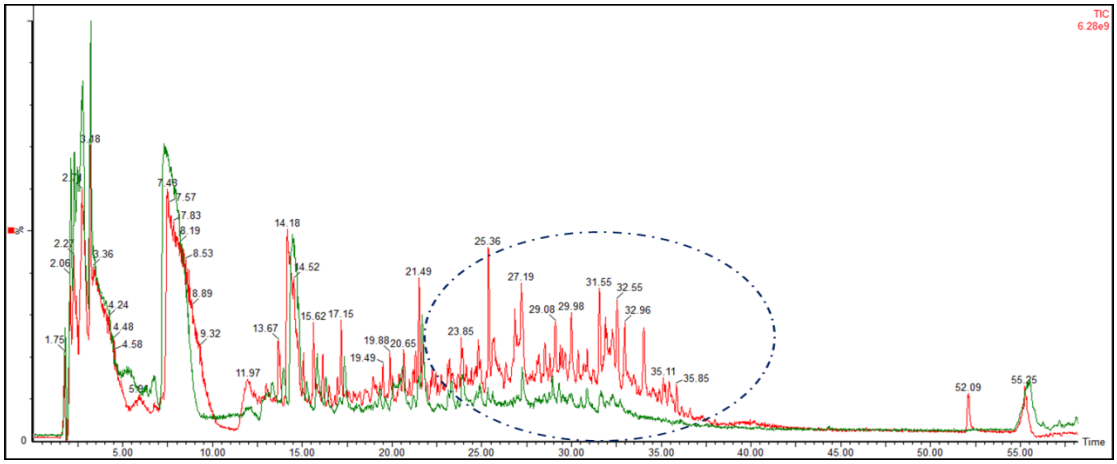

Figure 22. Comparison between the chromatograms of the peptide fraction of a sample of low-salt ham (green) and a sample of traditional ham (red).

Clear differences may be appreciated both in the first part of the chromatogram, where amino acids and small peptides generally are eluted (retention time up to 20 minutes), as well as in the second part of the chromatogram between 25 and 40 minutes, where generally medium-high MW peptides are eluted. Regarding the amino acids, they seem to be most abundant in the low salt ham samples while the medium-high MW peptides are considerably more abundant in traditional hams. The relative amounts (semiquantification data) of each peptide in low salt sample or in conventional sample were compared and T-student test was assessed in order to evaluate significant differences (at $p < 0.05$). In Table 3, the amount of each significant peptide is reported.

Table 3. T Amount of peptides (MW, molecular weight) in traditional and reduced salt hams.

MW	Average amount ± std. dev.		MW	Average amount ± std. dev.	
	Reduced salt	***Traditional***		***Reduced salt***	***Traditional***
188	***0.41±0.16***	*0.20±0.07*	3703	*0.01±0.01*	***0.05±0.02***
219	***0.26±0.04***	*0.22±0.06*	5273	*0.00±0.00*	***0.03±0.03***

MW	Average amount ± std. dev.		MW	Average amount ± std. dev.	
	Reduced salt	***Traditional***		***Reduced salt***	***Traditional***
226	***0.20±0.04***	*0.14±0.04*	4930	*0.01±0.01*	***0.04±0.03***
228b	***1.30±0.08***	*0.05±0.02*	3402	*0.01±0.01*	***0.09±0.09***
189	***1.15±0.14***	*0.93±0.20*	3083	*0.20±0.07*	***0.39±0.18***
230	***0.14±0.06***	*0.04±0.03*	5521	*0.00±0.00*	***0.02±0.03***
228c	***0.08±0.04***	*0.02±0.03*	5164	*0.01±0.01*	***0.07±0.02***
173	***0.21±0.09***	*0.15 ±0.05*	5264	*0.01±0.02*	***0.16±0.12***
1020	***0.11±0.05***	*0.04±0.04*	4497	*0.05±0.02*	***0.15±0.06***
551	***0.03±0.01***	*0.02±0.02*	3291	*0.00±0.00*	***0.06±0.09***
303	***0.60±0.20***	*0.47±0.15*	3006	*0.04±0.03*	***0.12±0.09***
231b	***0.16±0.03***	*0.13±0.03*	3310	*0.04±0.02*	***0.08±0.08***
274	***0.40±0.26***	*0.20±0.11*	4378	*0.12±0.05*	***0.28±0.16***
203b	***2.04±0.22***	*1.63±0.33*	8184	*0.50±0.17*	***1.56±0.50***
343a	***0.25±0.15***	*0.10±0.14*	8381	*0.09±0.06*	***0.15±0.1***
237	***0.61±0.11***	*0.49±0.10*	7060	*0.04±0.04*	***0.15±0.17***
343b	***0.26±0.11***	*0.15±0.15*	8260	*0.03±0.04*	***0.44±0.44***
246b	*0.08±0.01*	***0.08±0.01***	2860	*0.17±0.13*	***0.01±0.07***
357	*0.03±0.03*	***0.06±0.06***	8297	*0.16±0.057*	***0.73±0.48***
228a	*1.30±0.56*	***1.72±0.57***	5318	*0.00±0.00*	***0.06±0.06***
280	*0.11±0.06*	***0.19±0.0***	2635	*0.00±0.00*	***0.02±0.1***
319a	*0.10±0.04*	***0.15±0.05***	3039	*0.01±0.01*	***0.07±0.06***
244a	*0.10±0.03*	***0.16±0.06***	8706	*0.01±0.01*	***0.04±0.03***
1905	*0.01±0.01*	***003±0.03***	9166	*0.01±0.01*	***0.30±0.16***
244b	*0.08±0.02*	***0.12±0.04***	8586	*0.00±0.01*	***0.14±0.11***
2987	*0.03±0.04*	***0.10±0.14***	3541	*0.01±0.01*	***0.06±0.05***
2116	*0.08±0.03*	***0.17±0.14***	2904	*0.01±0.01*	***0.04±0.05***
278	*0.03±0.01*	***0.05±0.01***	3018	*0.00±0.01*	***0.03±0.04***
4973	*0.01±0.01*	***0.21±0.13***	3968	*0.00±0.00*	***0.05±0.03***
4859	*0.01±0.01*	***0.44±0.18***	3331	*0.01±0.01±*	***0.04±0.06***

As already inferred by the analysis of the chromatograms, peptides with high molecular weight (from 3000 to 8000 Da) were found to be in higher amount in the traditional ham samples. On the contrary, peptides

with a MW ranging from 200 to 300 Da were found in higher amount in ham samples with low salt content. These results can be explained by the fact that proteolytic activity is favoured in low salt ham, thus inducing the accumulation of peptides with low MW and amino acids. On the contrary, in traditional products proteolysis is less favoured on account of the higher amount of salt, thus inducing the accumulation of a greater number of high MW peptides. A lower salt content could also induce a lower extraction of myofibrillar proteins (known to be soluble in concentrated salt solutions), which are therefore less accessible to the action of proteolytic enzymes. Indeed, proteolysis index of the two type of products are significantly different: 31% in the case of traditional products and 34% for low salt hams. Also, sensorial analysis underlines the greater smell of fresh meat perceived for the low salt products. Finally, also the texture is different: the products with low salt show lower hardness values (TPA analysis, Texture Profile Analysis) than those of the traditional products while the values of adhesiveness are higher. All these results confirmed the difference between the two type of products clearly revealed by the peptide profile analysis.

To obtain a good quality dry-cured ham both the parameters controlling salt diffusion and weight loss (preserving factors) and those affecting the final flavour, such as proteolysis must be taken into account (Schivazappa, 2002). In traditional hams there is an intense dehydration process during the ripening/drying stage, reaching moisture contents around 60% in the inner part of the ham (region of the muscle Biceps femoris) and around 50% in the outer part (region of the muscle Semimembranosus). Accordingly, water activity decreases during drying and its values can be reduced to near 0.90 in the inner part and around 0.85 in the outer part of the ham. Water activity inside the dry-cured meats is important in controlling the enzyme actvity, especially when the ripening/drying progresses and a_w approaches 0.90 or even lower values. Most of the muscle enzymes (cathepsins, aminopeptidases) are strongly affected by the decrease in a_w along the processing of dry-cured meats with few exceptions. So, calpain appears to be slightly affected by a_w reduction. The length of the process is also important for the activity of the enzymes, as a more pronounced action of the enzymes can be observed, especially if the drying conditions are mild. Thus, a more intense proteolysis can be expected when the ripening/drying time is longer and the conditions are milder. Most of the muscle enzymes are very stable and exhibit activity during all the process period and even some residual activity may be found by to 2 years. This allows a prolonged enzyme action even though its activity is reduced as drying progresses due to lower water activity values. Calpain and pyroglutamylaminopeptidase constitute an exception since they exhibit reduced stability and their activities are detectable only in the initial weeks of the process. Finally, other factors like salt and other curing agents must be taken into account because they exert inhibitory or activation effects on the muscle enzymes (Toldrà, 2006). During ripening and drying a large number of variable conditions as time, temperature, relative humidity and air velocity could determine difference in the final product. In other cases, the excess of proteolysis may be due

to variations in cathepsin B: low pH favours its activity, while an increase in salt concentration and a decrease of a_w inhibits it. The inhibitory effects of sodium chloride on cathepsin B is well known. An impairment of texture, increase in bitter flavour, white surface film formation and tyrosine chalks (Virgili, 1995) are observed with an excess of proteolysis. So, salt can help in controlling some defects such as the reported soft defective Parma hams, where the activity of cathepsin B is too high.

The reduction of sodium in processed dry-cured hams requires innovations in the whole production process, since the use of conventional complementary treatments (syringing, use of substitutes of sodium chloride, and vacuum) is not allowed by PDO dry-cured ham production rules. The main technique used to reduce salt content in traditional ham has been generally the reduction of 25% of sodium chloride obtained with conventional technique (salt as a function of ham weight). Some other techniques taken into account have been the reduction of 25% of salt on the weight of legs but considering also the amount of fat tissue present in legs: to determine if the legs "fat" or "lean" the system MIS Lenz Prototype (Magnetic Induction Spectroscopy) have been used. The system equipment's operate in a non-invasive way: the former is based on carcass response to ultrasound, the latter on the different response of lean and fat tissues to a frequency dependent electromagnetic field (Simoncini, 2012). Two classes of fleshiness: hams with a content of fat or less than 65%, defined as "fat", and hams with a lean content equal to or higher than 67%, defined as "lean". This last technique has not particular advantages in comparison to the first one.
The last techniques experimented has been the "satured" salt addition (where an excess salt cover all the leg and the absorbed salt depends on the duration of the salting step), but with this approach a very high influence of the raw material is observed on salt adsorption: the high variability between different legs (especially in terms of lean and fat tissue percentage) determine a great variability in salt content that it's difficult to control. For this reason, this technique has been discarded.

Some process parameters should be further improved (temperature, humidity and time duration of different phases) as well as a strict control of the characteristics of the raw material should be implemented in order to obtain products with characteristics similar to those of the traditional ones (Benedini, 2012).

3.3.3 Conclusions

The study of peptide fraction of traditional dry-cured hams is fundamental to realize innovative hams. The database of peptides created to compare reduced salt hams with the traditional ones has allowed to evidentiate large difference between the products, mainly based on the different extent of the proteolysis process that takes place in hams with low amount of salt: the lower value of salt increases the activity of proteolytic enzymes, decreases the extraction of myofibrillar proteins, determines a necessary increase of

period of resting in order to obtain the microbiological safety. The obtained products are characterized by an higher proteolysis index, a more "gummy" texture (due to the higher humidity), with a peptide profile richer in low MW peptides. As previously stated, to obtain a ham with the characteristics more similar to the traditional products, it will be necessary to change the process parameters (as temperature, water activity, humidity, etc) in addition to a strict control of the characteristics of the raw material.

3.4 ACKNOWLEDGEMENTS

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4 BIOACTIVE PEPTIDES RELEASED BY THE GASTROINTESTINAL-DIGESTION OF DRY-CURED HAMS

4.1 INTRODUCTION

Dry-cured hams are meat products mostly appreciated by consumers for their organoleptic properties; they also represent an important example of traditional methods to preserve, and eventually improve, the nutritional properties of meat. In fact, while the excessive consumption of meat products is indicated as a risk factor for certain diseases, it is equally true that proteolysis is able not only to positively influence the digestibility of the products, but also to generate a pool of peptides that might be characterized by interesting biological activities, which may potentially counteract these negative effects. The bioactive peptides can be naturally present in the product or may be released during the gastro-intestinal digestion step. Several studies have reported health related activities of biopeptides derived from food protein sources of animal origin, in particular from dairy proteins (Phelan, 2009). These bioactive peptides have been defined as "food components that may influence biological processes and thus have an impact on body functions or conditions and finally on health" (Roberts, 1994). Their activity depends on the composition of the amino acid sequence and on its length, and many peptides have shown multifunctional properties (on the cardiovascular, digestive, immune and nervous system). In this contest, a number of research investigations are currently focusing on the identification of functional biopeptides from meat proteins as meat is widely used as a nutritional source of high quality proteins (Ryan, 2011).

Potentially antioxidant (Samaranayaka, 2011) and antihypertensive peptides have been identified in meat from various sources (Lafarga, 2014a): as far as pork meat, it has been demonstrated that biopeptides may be released by meat proteins by the action of endogenous or exogenous peptidases during processing. Peptides exhibiting in vitro antioxidant activities have been characterized in water soluble fraction from dry cured Spanish hams (Mora, 2014). Antihypertensive peptides have also been identified and their activity has been proven both in vitro as well as on spontaneously hypertensive rats (Escudero, 2010).

It is worth noting that biopeptides may exert their physiological actions both in the intestinal lumen as well as upon crossing intestinal epithelia and reaching blood circulation: thus, their modifications by the gastrointestinal digestion process, the transport mechanism across the intestinal epithelium and the enzymatic modifications taking place there, all have to be considered.

Thus, the application of *in vitro* digestion models are a necessary step to study the release of potentially bioactive and functional peptides from food proteins and to identify the most interesting sequences

(Ferranti, 2014), taking also in consideration the eventual effect of processing or maturation time of food products on the general digestibility and the release of these compounds.

4.2 MATERIALS AND METHODS

4.2.1 Samples

Dry-cured ham samples (n=10) with 18 and 24 ripening months, were provided by the Experimental Station for Food Preserving Industry (Parma). Samples of Biceps femoris muscle were minced by a common mill (Moulinex, Milano, Italia) and stored at freezing temperature (-22 °C) until analysis.

4.2.2 Reagents and solvents

Bidistilled water was produced in our laboratory by a Millipore Alpha Q purification system (Waters, Billerica, MA, USA). Pepsin from porcine gastric mucosa, trypsin from porcine pancreas, α-chymotrypsin from bovine pancreas, α-amylase from barley malt (type VIIIa), uric acid, mucin from porcine stomach (type III), glucose, glucuronic acid, glucosamine hydrochloride, bovine serum albumin, pancreatin from porcine pancreas, lipase from porcine pancreas (type II), bovine and ovine bile, sodium dihydrogen phosphate, potassium chloride, sodium phosphate dibasic dodecahydrate, monobasic potassium phosphate, acetonitrile, methanol, urea, L-phenylalanyl-L-phenylalanine (Phe-Phe), acetic acid, hydrochloric acid (0.1 N), potassium persulfate, sodium hydroxide, trypsin, dithiothreitol, iodoacetamide, tris(hydroxymethyl)aminomethane (TIS), trifluoroacetic acid, sodium phosphate dibasic dodecahydrate, potassium phosphate monobasic, dimethylformamide (DMF), n-butanol, methylene chloride, N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIPEA), ethyl acetate, potassium bisulfate, sodium bicarbonate, sodium sulfate anhydrous, tetrahydrofuran (THF), barium hydroxide octahydrate, m-cresol, thioanisol, sodium phosphate monobasic, methanol, ammonium thyocynate, ferrous chloride, sodium phosphate monobasic, cupric nitrate trihydrate, Krebs-ringer bicarbonate buffer, glycerol, Angiotensin-Converting-Enzyme (ACE) 0.1U and 0.5U, captopril, **2**-(Cyclohexylamino)ethanesulfonic acid (CHES), Ethylenedinitrilotetraacetic acid (EDTA), N-Hippuryl-His-Leu hydrate (HHL), L-piroglutamic acid and (S)-(-)-2-Acetoxypropionic acid were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (37% v/v), sodium hydroxide, sodium hydrogen carbonate, calcium chloride, ammonium chloride, potassium thiocyanate, potassium hydroxide, potassium chloride, phosphoric acid were purchased from Carlo Erba (Milan, Italy). Formic acid was purchased from

Acros Organics (Geel, Belgium). Sodium chloride was purchased from AnalaR Normapur (Milan, Italy). Sodium sulfate was purchased from–Riedel de Haen (Seelze, Germany). Thionyl chloride, ammonium bicarbonate, 2,2-Azino-bis-3-ethylbenzothyazoline-6-sulfonic acid (ABTS), boric acid and magnesium chloride hexahydrate was purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). SDS-PAGE standards Broad Range, XT Reducing Agent, XT Sample Buffer 4x, XT MES Running Buffer 20x, Comassie Brillant Blu R-250 were purchased from BIO-RAD (Hercules, CA, USA). Sodium chloride was purchased from VWR (Milan, Italy).

Wang-resin, L- phenylalanine deuterated and N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester were purchased from Novabiochem (Billerica, MA, USA). Dulbecco's modified Eagle's minimal essential medium (DMEM with glucose 4500 mg/L), inactivated fetal calf serum (FCS), non-essential amino acids (NEAA), Hank's balanced salt solutions (HBSS), and penicillin/streptomycin (PS) were purchased from Gibco (Fisher Scientific). Cell culture inserts with polycarbonate membranes (pore sizes, 0.4 μm; effective area, 4.2 cm^2) were obtained from Millipore (Saint-Quentin-en-Yvelines, France). Plastic dishes were purchased from Dutscher (Brumath, France).

4.2.3 Extraction of the peptide fraction (MW<10 kDa)

To 5 g of minced dry-cured ham 45 ml of a HCl 0.1N solution and 250 μl of a 1mM Phe-Phe aqueous solution were added and the mixture homogenized for 1 minute with Ultra Turrax T50 Basic (IKAWerke, Staufen, Germany) at 322 g. After centrifugation at 4°C (3200 g for 1 h), the supernatant was purified by a series of filtration steps using different filters (paper, 5 μm type SMWP, 0.45 μm type HPLV). 4 ml of the obtained extract were evaporated to dryness under vacuum by rotary evaporator and the residue was dissolved with 2 ml of a 0.1% formic acid aqueous solution. This solution was then filtered on a Vivaspin 2 (Sartorius) filtration system equipped with 10 kDa cut off filters, at room temperature and 5423 g for 45 minutes. The filter was washed for 3 times with 2 ml of a 0.1% formic acid aqueous solution. The filtered sample was evaporated to dryness under vacuum by rotary evaporator and the residue dissolved in 150 μl of a 0.1% formic acid aqueous solution and centrifuged at 4°C, at 16060 g, for 10 minutes before the UPLC-ESI-MS analysis.

4.2.4 Simulated in vitro gastro-intestinal digestion

The simulated gastro-intestinal digestion and the preparation of the relative artificial digestive juices (simulated salivary juice, simulated gastric juice, simulated duodenal juice, and bile) were performed according to the protocol of Versantvoort (Versantvoort, 2005).

Briefly, the digestion procedure was as follows: minced Biceps femoris muscle (2 g) was dispersed in 3 ml of simulated salivary juice (pH = 6.8±0.2) and incubated at 37°C under agitation with Vortex and a horizontal shaker (230 rpm) for 5 min. After this first step, 6 ml of the simulated gastric juice (pH = 1.3±0.2) was added and the mixture was left to react for 2 h under the same conditions. The last step was the addition of bicarbonate (1 ml, 1 M), simulated duodenal juice (6 ml, pH = 8.1±0.2) and bile (3 ml, pH = 8.2±0.2) and the mixture was left to react at 37°C for other 2 h under agitation on a horizontal shaker. The enzyme reaction was stopped heating at 95°C for 15 min and the mixture was centrifuged at 3200 g for 20 min to separate the supernatant (chyme) and the pellet (residual matrix). The supernatant was subjected to a final clean-up step by a Sep-Pak C18 cartridge (Waters Co., Milford, MA, USA): after conditioning the cartridge with methanol (2 ml) and bidistilled water (3 ml), chyme was applied (2 ml) and after discarding the first eluate, peptides were eluted with 2 ml of a solution of H_2O:CH_3OH (50:50, v/v). The eluate (1 ml) was evaporated to dryness under nitrogen flow in an Eppendorf tube and the residue was resuspended with 1 ml of aqueous formic acid (0.1%), centrifugated and, then, analyzed in UPLC-ESI-MS (conditions reported below). Peptides were semiquantified comparing their peak area with that of the internal standard Phe-Phe: in particular, 4 µl of internal standard (1 mM aqueous solution) were added to 196 µl of digested sample (after clean-up) prior to the analysis by UPLC-ESI-MS.

4.2.4.1 SemiprepLC-UV fractionation of digested samples

10 ml of digested samples were fractionated by semipreparative HPLC-UV (□=214), collecting a fraction per minute. The digested samples were separated by a RP column (JUPITER 5 µm C18 300 Å 250 x 10 mm, Phenomenex) using a semipreparative HPLC-UV (1525 Binary HPLC Pump, Waters). The following gradient conditions were applied: eluent A, water with 0.1% formic acid and 0.2% acetonitrile, eluent B, acetonitrile with 0.1% formic acid; gradient: 0-12 min 100% A, 12-77 min linear from 100% A to 50% A, 77-81 min 50% A, 81-82 min linear from 50% A to 0% A, 82-90 min 0% A, 90-91 min from 0% A to 100% A, 91-110 min 100% A. Other parameters were as follows: flow rate, 5 ml/min, analysis time, 110 min; column temperature, r.t.; sample temperature, r.t.; injection volume, 2 ml; fractions collected every minute for a total of 52 fractions. UV detector (2998 Photodiode Array, Waters) set at λ = 214 nm.

4.2.5 SDS-PAGE analyses

Extracted samples: 5 µl of extracted filtered at 0,45 µm were evaporated to dryness under nitrogen flux and dissolved in 25 µl of reducing sample buffer (1x, BIO-RAD). Digested samples (after clean-up) : 500 µl of digested sample was evaporated under nitrogen flux and dissolved in 25 µl of reducing sample buffer (1x, BIO-RAD).

Reducing sample buffer was composed by 5% of XT Reduce Agent (BIO-RAD) in Sample buffer 1x (BIO-RAD). The running buffer (1x) is prepared by dilution of XT MES Running Buffer 20x (BIO-RAD) with

bidistilled water. Comassie staining solution: for 1 liter of solution, 1g of Comassie Brillant Blue R-250 (BIO-RAD) was dissolved with with 100 ml of acetic acid, 400 ml of methanol and 500 ml of bidistilled water. Comassie destaining solution: for 1 liter of solution, 100 ml of acetic acid, 400 ml of methanol and 500 ml of bidistilled water were mixed. Marker: the molecular marker (SDS-PAGE standards, BIO-RAD) is in the ratio of 1:20 with the reducing sample buffer. Gel: CriterionTM XT Precast gel, 12% Bis-Tris (BIO-RAD). Run time: 50 minutes ca. at 150 V. Volume of marker used: 5µl. Data analysis with scanner GS-800 Calibrated Densitometer controlled by the software "Quantity one" (BIO-RAD).

4.2.5.1 In-gel digestion

The gel was destained and the reduction, alkylation and digestion of the bands of interest were carried out as described by the in-gel digestion protocol of the Biological Mass Spectrometry Laboratory (Ontario Wide Protein Identification Facility, http://www.uwo.ca/biochem/bmsl/in-gel_digestion.html#coom).

4.2.6 Peptide transport studies

4.2.6.1 Co-culture Caco2-HT29-MTX

The Caco-2 TC7 cells obtained by the laboratory of Thécla Lesuffleur (INSERM) were cultured in DMEM supplemented with 20% heat-inactivated FCS, 1% PS, and 1% NEAA. They were incubated at 37°C in a humidified atmosphere with 10% CO_2. The mucus cells HT29-MTX were cultured in DMEM supplemented with 10% heat-inactivated FCS, 1% PS. They were incubated at 37°C in a humidified atmosphere with 10% CO_2. The co-culture has been prepared and were seeded in cell culture inserts with polycarbonate membranes at a cell density of 250000 cell/ml for inserts of 4.1cm^2, in ratio 90:10 Caco2 TC7 / HT29-MTX. The cells used in the studies had a number of passage of 44. Measurements of digesta transport with co-culture Caco-2/ HT29-MTX cell monolayer were performed in triplicate by transepithelial electrical resistance (TEER). The cells were incubated at 37°C, in a humidified atmosphere containing 10% CO_2, and the medium was changed daily. The confluent monolayer was used between 21 and 23 days after seeding. The Caco-2 cells monolayer was rinsed three times with 1 mL of HBSS. The cells were incubated with HBSS for 30 min in the apical compartment and in the basolateral compartment. After incubation, the solutions in both compartments were removed, and the digesta solution was added as follows: 2 mL with samples (digesta at 18, 24 months and blank (matrix without ham)) prepared in HBSS (dilution 1:23 to have 2mg/2ml of proteins and 0.5mM bile salts) and 2 mL of HBSS in the basolateral compartment. Aliquots were taken from the apical and basolateral compartments after 30, 60, and 120 min of incubation time (two inserts for each samples tested were incubated for one time determined). The aliquots were boiled at 95°C for 15 minutes to stop any enzymatic reaction. At the end of the experiments, the cells were washed three

times with 1 mL of HBSS, and fresh culture medium was added to the apical and basolateral compartments in order to measure the TEER and the aliquots were boiled at 95°C for 15 minutes.

The Measurement of Transepithelial Electrical Resistance (TEER) was then measured to determine the integrity of the Caco-2 cell monolayer: the measure of TEER with a Millicell-ERS Ohm Mete (Millipore, Billerica, MA) just before, immediately after, and 2, 4, and 20 h after the peptide transport studies. An increase to 350 Ω cm^2 of the TEER indicated a confluent cell monolayer with tight junctions.

4.2.6.2 Ussing Chamber

The experiment must be performed in one hour after the animal death. Take the intestine, the proximal jejunum, 20-25 cm after the stomach (after the duodenum and the angle of Treitz). Wash the intestine in a Krebs –ringer bicarbonate buffer. The intestine undergoes stripping in order to facilitate assembly and oxygenation (elimination of the muscular outer side of the intestine). Put the intestine in the chamber in the right direction to properly identify mucosal compartments (donor) and serous (receiver). Place a sheet of parafilm on top of the room to separate the two compartments (with bubbling, the Krebs ringer foams and can pass into the serosa compartment). Screw the two half-chambers flat on the bench and put them on the carrier thermostat. The choice of the temperature is a function of the animal: for the pork, 40 °C. Put simultaneously on both sides of the tissue 4 ml of Krebs ringer with bubbling of 95% O_2 5% CO_2. Allow to equilibrate for 20 min. Then, fill 2 half-empty compartments at the same time with digested solutions (dilution 1:11 in Krebs-ringer in order to have 8mg/4ml of protein): 2 chambers was charged with Krebs-ringer as system blank, 2 with sample at 18 months, 2 with samples at 24 months and 2 with the digestion blank. Aliquots of 1 ml (and substituted by 1 ml of Krebs ringer buffer) have been taking after 30, 60 and 120 minutes of incubation times. The aliquots were boiled at 95°C for 15 minutes to stop any enzymatic reaction.

4.2.7 UPLC-ESI-MS analysis

UPLC/ESI-MS analyses were performed with an UPLC/ESI-MS system (UPLC Acquity Waters equipped with a single quadrupole mass spectrometer Waters Acquity Ultraperformance). Conditions were as follows: column, RP ACQUITY UPLC BEH 300 C18 (1.7 µm, 2.1 x 150 mm, Waters); gradient elution: eluent A, water with 0.1% formic acid and 0.2% acetonitrile, eluent B, acetonitrile with 0.1% formic acid; gradient: 0-7 min 100% A, 7-50 min linear from 100% A to 50% A, 50-52.6 min isocratic 50% A, 52.6-53 min linear from 50% A to 0% A, 53-58.2 min isocratic 0% A, 58.2-59 min linear from 0% A to 100% A, 59-72 min isocratic 100% A. LC parameters: flow rate, 0.2 ml/min; analysis time, 72 min; column temperature, 35°C; sample temperature, 18°C; injection volume, 2 µl for digested samples (with 7 minutes of solvent delay), 4 µl for extracted samples (with no solvent delay), 10 µl for samples of Caco-2/HT29-MTX and Ussing

chamber. MS parameters: Full Scan mode, acquisition time 7-58.2 min; ionization type, ESI + (positive ions); scan range, 100-2000 m/z; capillary voltage, 3.2 kV; cone voltage, 30 V; source block temperature, 150°C; desolvation temperature, 300°C; cone gas flow, 100 l/h; desolvation gas flow, 650 l/h. Data were acquired and analyzed by MassLynx 4.0 software (Waters Co., Milford, MA, USA).

4.2.7.1 Data statistical analysis

Peptide profiles of the digesta (as a function of the ageing time), obtained by the analysis of spectra from UPLC-ESI-MS, were examined by Principal Component Analysis (PCA) and T-student test with the software SPSS Statistics 17.0.

4.2.8 HPLC/ESI-MS/MS analyses

HPLC/ESI-MS/MS analyses were performed using a HPLC (Mod. Alliance 2695, Waters) equipped with a triple quadrupole mass spectrometer (Mod. 4 Micro, Waters) and a RP column JUPITER 5 µm C18, 300 Å 250 x 2 mm I.D. (Phenomenex, Bologna, Italy). Gradient elution was as follows: eluent A, water with 0.1% formic acid and 0.2% acetonitrile; eluent B, acetonitrile with 0.1% formic acid; gradient: 0-12 min 100% A, 12-77 min linear from 100% A to 50% A, 77-81 min 50% A, 81-82 min linear from 50% A to 0% A, 82-90 min 0% A, 90-91 min linear from 0% A to 100% A, 91-110 min 100% A. Samples (extracted and digested samples) were first analyzed in Full Scan mode, to identify the characteristic ions and the retention time of the unknown compounds, then in Daughters Scan modality, using a variable collision energy from 10 to 30 eV. HPLC/ESI-MS/MS parameters were as follows: flow rate, 0.2 ml/min; analysis time, 110 min; column temperature, 35°C; sample temperature, 23°C; injection volume, 10 µl for Full Scan mode and 30 µl for Daughter Scan mode; acquisition time, 90 min; ionization type, ESI + (positive ions); scan range, 100-2000 m/z; capillary voltage, 3.2 kV; cone voltage, 35 V; source block temperature, 100°C; desolvation temperature, 200°C; cone gas flow, 100 l/h; desolvation gas flow, 650 l/h. The peptide sequences were assigned on the basis of the obtained mass spectra and using the Bioinfomatics resource portal ExPasy with the tools FindPept (Swiss Institute of Bioinformatics, Switzerland) and with the web application "Proteomics Toolkit" (Institute for Systems Biology, Seattle, WA, USA).

4.2.9 LTQ-Orbitrap analyses

Analyses were performed by a LTQ-Orbitrap (Thermo Fischer Scientific, Milano, Italy) equipped with a column Jupiter Proteo 4 µm (90 Å, 150 x 0.30 mm, Phenomenex). Gradient elution was as follows: eluent A, H_2O + 0.2% HCOOH; eluent B, CH_3CN + 0.2% HCOOH. Gradient: 0-4 min, 95%A, 4-60 min, linear from 95% to 50% A, 62-72 min, isocratic 95%B, 70-72 min linear from 5% A to 95% A, plus

reconditioning (3 min in 95% A). Samples (digesta) were desalted by ZipTip (Millipore) before the analyses. Other parameters: flow rate, 5 µl/min; injection volume, 5 □l; MS run time: 75 min; scan event details: (1) FTMS + res = 30000; (2) ITMS + Dep MS/MS most intense ion (1); (3) ITMS + Dep MS/MS second most intense ion from (1); (4) ITMS + Dep MS/MS third most intense ion from (1); (5) ITMS + Dep MS/MS third most intense ion from (1); collision energy, 35. Data-dependent setting: charge state rejection enabled; Unassigned charge states: rejected, charge state 1 rejected, charge states 2, 3 and 4 not rejected. Dynamic exclusion enabled. Repeat count: 3. Repeat duration 30.00s. Exclusion list size: 50. Exclusion duration: 180.00s. Exclusion mass width by mass. Exclusion mass width low and high: 1.5000.

4.2.10 Solid phase peptide synthesis

The peptides NSIM, GVVPL, LGL, SFVTT, ALM were synthesized on solid phase according to Fmoc/t-butyl strategy using a Syro I Fully Automated Peptide Synthesizer (Biotage, Uppsala, Sweden). The peptides were cleaved from the Wang-resin (Wang, Calbiochem-Novabiochem, Läufelfingen, Switzerland) using a TFA:TIS:H_2O:DTT (94:1:2.5:2.5) solution and purified using a C18 cartridge Sep-pak (Waters) and semipreparative RP-HPLC-UV (λ=214 nm). Characterization MH^+ (ESI–MS): 464.2 NSIM, 484.3 GVVPL, 302.2 LGL; 554.3 SFVTT, 334.2 ALM.

4.2.10.1.1 Purification of peptides: semipreprative HPLC-UV

The purification of compounds has been made with semipreparative HPLC-UV (1525 Binary HPLC Pump, Waters) using a RP column (JUPITER 5 µm C18 300 Å 250 x 10 mm, Phenomenex). Injection volume 500µl, flow 5ml/min. Gradient elution was as follows: eluent A, water with 0.1% formic acid; eluent B, acetonitrile with 0.1% formic acid; gradient: 0-5 min 100% A, 5-10 min from 100% A to 85% A (this % change in function of compound to purified), 10-20 min linear in 85% A, 20-25min 0%A, 25-30min 100A%, 35-50 min 100%A.

4.2.11 Synthesis of NPADS

4.2.11.1 Synthesis of L-Phenylalanine methyl ester hydrochloride (deuterated)

1g of deuterated L-phenylalanine (d_5-L-Phe) were dissolved in 50 ml of dry-methanol and kept under continuous stirring in an ice bath; $SOCl_2$ was added to a final concentration of 1 M (3,62 ml of $SOCl_2$ plus 46,38 ml dry-methanol). The reaction closed with a valve of calcium chloride, was left to react overnight and was monitored by TLC (n-butanol: acetic acid:water 4:1:1 as eluent, UV and ninidrine detection). The reaction mixture was dried at rotavapor. Residual HCl was eliminated by adding methanol (50 ml) and redrying by rotavapor (4 times). Characterization MH^+ (ESI–MS): 185.2 L-Phenylalanine methyl ester hydrochloride (deuterated).

4.2.11.2 Synthesis of L-Lactoyl-L-Phenylalanine (d_5)

(S)-(-)-2-Acetoxypropionic acid (1.56 mmol) was diluted in CH_2Cl_2 (3 ml) together with 1.48 mmol of HBTU and the mixture was kept under continuous stirring at room temperature for 30 min, in order to activate the carboxylic function. L-Phenylalanine-methyl ester d_5 (1.56 mmol) was dissolved in 2 ml of CH_2Cl_2, together with DIPEA (4.68 mmol) and then added to the activated acetoxypropionic acid. The reaction was left to react under magnetic stirring overnight at room temperature. The reaction was monitored by TLC (ethyl acetate as eluent, UV absorbance detector). The organic solution was washed with the 10 ml of saturated solutions of $KHSO_4$ (3 times) and $NaHCO_3$ (3 times) in order to remove the unreacted reagents, dried with Na_2SO_4 and filtered. The product was dried under nitrogen flux and vacuum. The methyl and acetyl protecting groups were removed by reaction in 5 ml of THF (without inhibitor) and 5 ml of water with 0.78 mmol of $Ba(OH)_2$ (which corresponded to 2 equivalents of the protected molecules, ratio between THF and H_2O is 1:1) for 20 min, on ice bath. THF was then eliminated under vacuum by rotavapor and the aqueous solution was acidified to pH 3 with HCl. Characterization MH^+ (ESI–MS): 243.2 L-Lactoyl-L-Phenylalanine (d_5).

4.2.11.3 Synthesis of L-Pyroglutamyl-L-Phenylalanine (d_5)

1.5 mmol of L-pyroglutamic acid were diluted in CH_2Cl_2 (5 ml) together with 1.42 mmol of HBTU and the mixture was kept under continuous stirring at room temperature for 30 min, in order to activate the carboxylic function. L-Phenylalanine-methyl ester d_5 (1.5 mmol) was dissolved in 2 ml of CH_2Cl_2, together with DIPEA (4.5 mmol) and then added to the activated pyroglutamic acid. The reaction was left to react under magnetic stirring overnight at room temperature. The reaction was monitored by TLC (ethyl acetate as eluent, UV absorbance detector). In order to make the reaction complete, 0.75 mmol of L-pyroglutamic acid and 0.71 mmol of HBTU in 2 ml of CH_2Cl_2:dry DMF (1:1) plus 2.25 mmol of DIPEA were added. The organic solution was evaporated under vacuum, at 40°C, in order to eliminate the DMF and redissolved in 5 ml of CH_2Cl_2. The organic solution was washed with 10 ml of saturated solutions of $KHSO_4$ (3 times) and $NaHCO_3$ (3 times) in order to remove the unreacted reagents, dried with Na_2SO_4 and filtered. The product was dried under nitrogen flux and vacuum. The methyl protecting group was removed by dissolving the ester in 12 ml of THF (without inhibitor) and 12 ml of water with 0.9 mmol of $Ba(OH)_2$ (which corresponded to 2 equivalents of the protected molecules, ratio between THF and H_2O is 1:1) for 1h and 30 min, on ice bath. The reaction was monitored by TLC (ethyl acetate as eluent, UV detector). THF was then eliminated under vacuum by rotavapor and the aqueous solution was acidified to pH 3 with HCl. Characterization MH^+ (ESI–MS): 282.2 L-Pyroglutamyl-L-Phenylalanine (d_5).

4.2.11.4 Synthesis of γ-glutamil-L-Phenylalanine (d_5)

N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester (2.055 mmol) was dissolved in DMF (2.5ml) together with 1.955 mmol of HBTU and the mixture was stirred at room temperature for 30 minutes. L-Phenylalanine-methyl ester d_5 (2.055 mmol) was dissolved in DMF (1 ml) together with 6.125 mmol of DIPEA and added to the reaction. The mixture was left to react under magnetic stirring overnight and the reaction was monitored by TLC (ethyl acetate as eluent, UV detector). In order to react the residual phenylalanine, 1.027 mmol of N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester and 0.511 mmol of HBTU in 1.5 ml of DMF and 3.06 mmol of DIPEA were added. The organic solution was evaporated under vacuum, at 40°C, in order to eliminate the DMF and redissolved in 5 ml of CH_2Cl_2. The organic solution was washed with 10 ml of saturated solutions of $KHSO_4$ (3 times) and $NaHCO_3$ (3 times) in order to remove the unreacted reagents, dried with Na_2SO_4 and filtered. The product was dried under nitrogen flux and vacuum. The residue was dissolved in 15 ml of a trifluoroacetic/methylene chloride 1:1 solution, containing also 4% of m-cresol and 4% of thioanisol as scavengers. The mixture was stirred at room temperature for 1 h and then the solvent was evaporated under vacuum by nitrogen flux. Cold ethyl ether was added to the residue in order to precipitate the free dipeptide. The precipitate was washed several times with cold ethyl ether and the product dried by nitrogen flux and under vacuum. The methyl protecting groups was removed by dissolving the ester in 5 ml of THF (without inhibitor) and 5 ml of water with 0.5 mmol of $Ba(OH)_2$ (which corresponded to 2 equivalents of the protected molecules, ratio between THF and H_2O is 1:1) for 20 min, on ice bath. The reaction was monitored by TLC (ethyl acetate as eluent, UV detector). THF was thne eliminated under vacuum by rotavapor and the aqueous solution was acidified to pH 3 with HCl. Characterization MH^+ (ESI–MS): 300.2 γ-glutamil-L-Phenylalanine (d_5).

For product characterization, 5 µl of all obtained NPADs were dissolved in 1 ml of H_2O+0.1% HCOOH and the solution (1 µl) was injected in UPLC-ESI-MS (the analysis conditions are the same described in paragraph number 3.2.5.1).

4.2.11.4.1 Purification of Npads: semipreprative HPLC-UV

The purification of compounds has been made with semipreparative HPLC-UV (1525 Binary HPLC Pump, Waters) using a RP column (JUPITER 5 µm C18 300 Å 250 x 10 mm, Phenomenex). Injection volume 500 µl, flow 5 ml/min. Gradient elution was as follows: eluent A, water with 0.1% formic acid; eluent B, acetonitrile with 0.1% formic acid; gradient: 0-5 min 100% A, 5-10 min linear from 100% A to 81% A (this % change in function of compound to purified), 10-20 min 81% A, 20-25 min linear to 0%A, 25-30 min linear to 100 A%, 35-50 min 100%A.

4.2.11.4.2 Quantification of Npads

The concentration of purified compounds has been made with spectrophotometer UV (Jasco V-530, UV-Vis, Easton, USA) monitoring the absorbance of the amino acid phenylalanine (ε=200$M^{-1}cm^{-1}$) at 257 nm in water. The calibration curve was set up plotting the ratio between areas NPADs:FF (internal standard) vs different concentration of NPADs (γ-glutamil-L-Phenylalanine (d_5) and L-Lactoyl-L-Phenylalanine (d_5) from 1 μM to 20 μM; L-Pyroglutamyl-L-Phenylalanine (d_5) from 10 μM to 150 μM).

4.2.12 Ace-inhibitory activity

4.2.12.1 Ace-inhibitory activities: fractions, purified peptides, Npads

Ace-inhibitory activity was determined by the methods of Cushman et al (Chushman, 1971) and Nakamura et al (Nakamura, 1995), with some modification. The following solutions were prepared: sodium borate buffer (0,1 M, NaBB) with NaCl (300 mM), pH = 8.3; potassium phosphate buffer (0,01 M, KPB) with NaCl (500 mM), pH = 7; 5 mM HHL (Hippuryl-Histidyl-Leucine) in NaBB buffer. The experiment is carried out at 37°C determining the following parameters: maximum activity of ACE (MAX) = 200 μl HHL + 80 μl NaBB + 20 μl ACE; control blank of ACE (Bmax) = 200 μl HHL + 80 μl NaBB + 20 μl KPB; minimum activity of ACE (MIN) = 200 μl HHL + 80 μl sample/digestion blank at different concentration + 20 μl ACE; control blank of sample/digestion blank (Bmin) = 200 μl HHL+ 80 μl sample/digestion blank at different concentration + 20 μl KPB. After 60 minutes of incubation the reaction was quenched with 250 μL HCl (1N). The analysis was performed by HPLC-UV (Alliance 2695 separation, Waters with Dual λ Absorbance 2487, Waters) with the following conditions: column, RP JUPITER C18, 5 μm, 300 Å 250 x 2 mm I.D.; gradient elution: eluent A, water with 0.1% formic acid and 0.2% acetonitrile, eluent B, acetonitrile with 0.1% formic acid; gradient, 0-10 min 100% A, 10-22.50 min linear from 100% A to 50% A, 22.50-23.50 min 50% A, 23.50-30 min linear from 50% A to 100% A; column temperature, 35°C; sample temperature, r.t.; injection volume, 10 μl; acquisition time, 30 min. UV detector: λ = 228, aufs 2. Data analysis was performed with Empower software.

4.2.12.2 ACE-inhibitory activity: samples from co-culture Caco-2/ H29-MTX and from Ussing chamber

Ace-inhibitory activity was determined by the methods of Cushman et al (Chushman, 1971) with some modifications. Prepare the following solution: CHES 50mM buffer containing 300mM of NaCl, pH 8.3; HHL 5,2mM in CHES 50mM buffer (as prepare before); ACE 0,025U/ml is solubilized in CHES buffer 50Mm (as prepared before) with 5% glycerol; stop solution composed by captopril 15μM, EDTA 3mM and 0.2% TFA. The ace-inhibitory activity was performed as follow: 65μl HHL 5.2mM + 25 μl samples from co-culture/HBSS or 12 μl samples from Ussing chamber/Krebs-ringer+ 10μl ACE (0,025U/ml) at 37°C.

After 1 hour of incubation time, the reaction is inactivated by 37 µl stop solution. The analysis was performed in UHPLC-UV (Shimadzu, Kyoto, Japan), injection volume 30 µl, samples temperature 4°C, Column temperature 29°C, flow 0,25ml/min, eluent A: water and 0,1%TFA, eluent B: ACN and 0,1%TFA, Column Kinetex C18 2.6µ XB 100Å 150*2.10mm (Phenomenex), Gradient: 0-7min from 87% A to 50%A, 7-8 min from 50% A to 1%A, 8-17 min 1inear 1%A, 17-18 min 1% to 87%A, 18-35 min linear 87% A. Detector UV, PDA (Shimadzu, Kyoto, Japan), λ=228nm. Data were elaborated by software LabSolutions (Shimadzu).

4.2.12.3 ACE: in silico model

Molecular modeling. The models for both C- and N-domains of ACE were derived from the Protein Data Bank (http://www.rcsb.org) structures having PDB codes 4APH and 4BZS, respectively (Masuyer et al., 2012; Kramer et al., 2014). Protein structures and ligands were processed by using the software Sybyl, version 8.1 (www.tripos.com).

Docking simulations and rescoring procedure. The coupling of GOLD (version 5.1), to perform docking simulations, and HINT software, as re-scoring function, has been already proved to be effectively able to evaluate the bioactivity of small molecules including peptides (Dellafiora, 2012-2014).

4.2.13 Antioxidant activity.

4.2.13.1 ABTS assay

Antioxidant activity was measured using the method of Re R. et al (Re, 1999) with same modifications. For the analysis, digested samples were diluted 1:100 in PBS, whereas no dilution was applied to the fractionated samples. The test parameters were determined as follows: MAX = 0.2 ml PBS + 1.8 ml ABTS work solution; MIN = 0.2 ml sample + 1.8 ml ABTS work solution; B_MIN = 0.2 ml sample + 1.8 ml ABTS work solution. Each prepared solution was analysed after 60 minutes with a UV-VIS spectrophotometer (LAMBDA BIO 20, Perkin Elmer) at 734 nm. Data analysis was performed with the LambdaBio software.

4.2.13.2 Acid linoleic assay

The method of Beker et al. (Beker, 2011) was used with some modification.

All solutions were prepared daily. Aqueous solutions of ethanol (75%), ammonium thiocyanate (NH_4SCN) 30%, and ferrous chloride (0.02M in 3.5% of HCl), sodium phosphate monobasic buffer 0.2M (up to pH 7 with NaOH), cupric nitrate trihydrate 0.05 M were used in the determination of lipid hydroperoxides with the Fe(III)-SCN reagent. A stock linoleic acid emulsion was prepared as followed linoleic acid: tween 20 1:1 in 0.2M of phosphate buffer and homogenized for 1 minute with UltraTurrax T18 Basic (Ika).

Solutions to test are composed as report below:
-blank: 3.125 mL of emulsion (already described before) + 2.5 mL phosphate buffer (0.2M) + 0.125 mL cupric nitrate (0.05M) + 0.500 mL ethanol absolute;
-blank of digestion: 3.125 mL of emulsion (already described before) + 2.5 mL phosphate buffer (0.2M) + 0.125 mL cupric nitrate (0.05M) + 0.125 mL sample blank of digestion + 0.375 ml ethanol absolute;
- digested samples: 3.125 mL of emulsion (already described before) + 2.5 mL phosphate buffer (0.2M) + 0.125 mL cupric nitrate (0.05M) + 0.125 mL digested samples + 0.375 ml ethanol absolute;

The solutions, in contact with air, are placed in a water bath thermostated at 37 ◦C, in continuous magnetic stirring. After 20 minutes of beginning to the test and after every hour for 24 hours, take aliquots of solutions and test them for lipidic oxidation with the ferric thiocyanate method (Lea, 1952; Mihaljevic et al., 1996): the degree of oxidation was measured by sequentially adding ethanol (2,350 mL, 75%), ammonium thiocyanate (0.05 mL, 30%), sample solution (0.05 mL), and ferrous chloride (0.05mL of 0.02Min 3.5% HCl). After 3 minutes of incubation time of the mixture, the peroxide concentration was spectrophotometrically determined by reading the absorbance at 500nm against a reagent blank containing identical components without the sample solution (substituted by phosphate buffer) (Yen and Hsieh, 1998). Data analysis has been made with software Lambda 20 Bio.

4.3 RESULTS AND DISCUSSION

4.3.1 Simulated in vitro gastro-intestinal digestion

The gastrointestinal digestion process has a major influence on the release of peptide sequences encrypted in food proteins. In this contest, although simplified approaches based on the use of gastrointestinal enzymes may be used to evaluate protein digestibility, digestion model better simulating the real environment of the human gastrointestinal tract are more effective to assess the nutritional value and the biological activity of the released peptides.
Thus, in order to understand the effect of the digestion process, dry-cured ham samples have been subjected to a simulated gastro-intestinal digestion. Samples are taken from the Biceps femoris muscle, regarded as the reference muscle for evaluating most technological and nutritional parameters of dry-cured ham, such as salt content, texture, colour, etc. The digestion procedure was adapted from (Versantvoort, 2005): the method mimics the subsequent steps of the digestion process in terms of composition of the juices in the

different compartments (simulation of salivary juice, gastric juices, duodenal juice and bile) as well as the relative residence times.

4.3.1.1 Sample clean-up

The chyme at the end of the digestion process is characterized by an high amount of and an high salt concentration, which may impair the chromatographic analysis by UPLC-ESI-MS. Thus, we have developed a properly clean-up procedure of the samples..

In Figure 23 the UPLC-ESI-MS chromatograms obtained for the same sample subjected both to no clean-up (a) or to clean-up with Sep-Pak C18 cartridge (b) are reported.

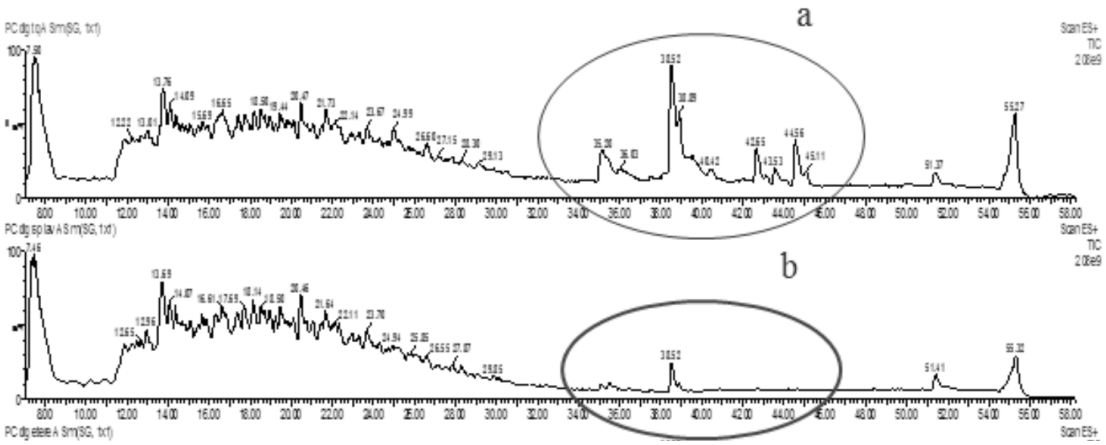

Figure 23. Chromatograms of a sample of digested ham sample not subjected to clean-up (a) or subjected to clean-up by Sep-Pak C18 cartridges (b).

Clean-up on Sep-Pak C18 cartridges allows to eliminate very efficiently bile salts, thus we have developed the clean-up method testing different solvent mixtures to elute the peptides from the cartridge. The tested eluents are:

- H_2O: CH_3OH 30:70 v / v;
- H_2O: CH_3CN 70:30 v / v;
- H_2O: CH_3CN 50:50 v / v;
- H_2O: CH_3OH 50:50 v / v;

The UPLC-ESI-MS chromatograms obtained using the different mixtures are reported in Figure 24.

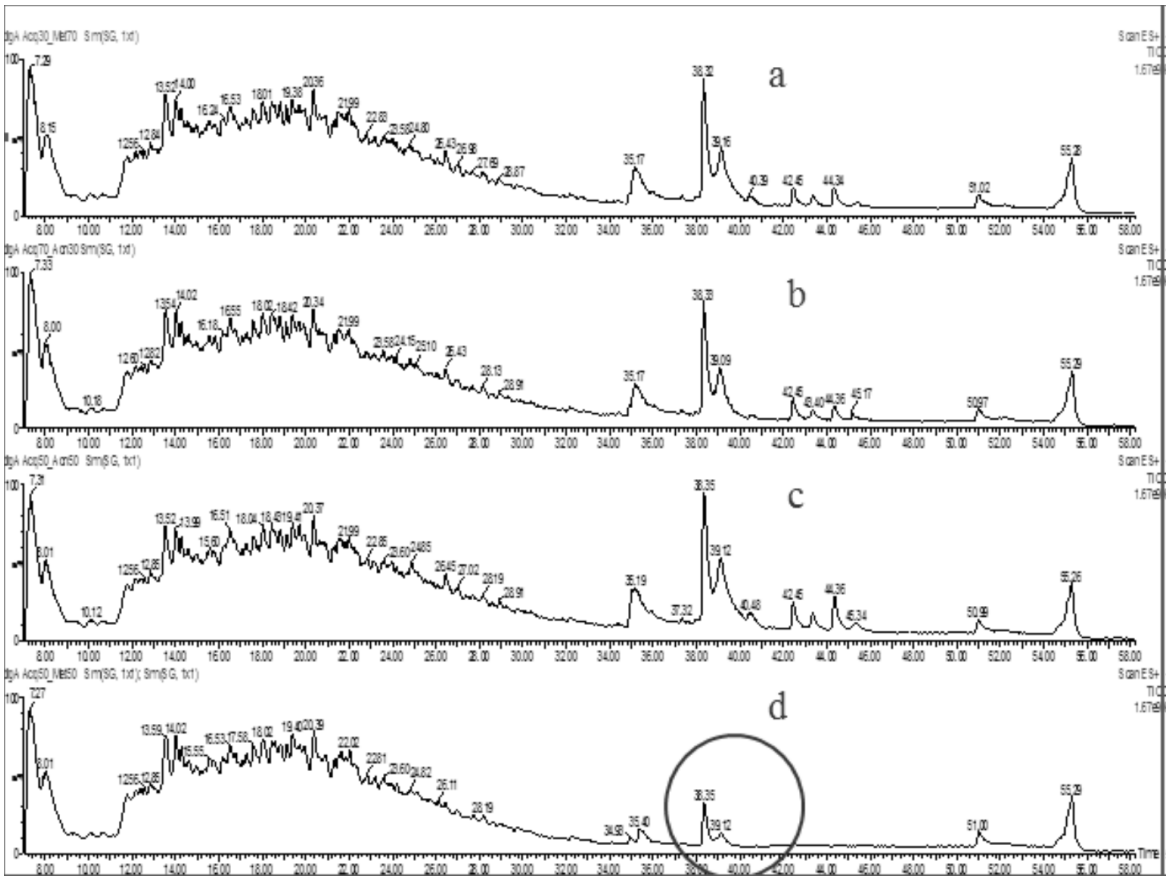

Figure 24. Chromatograms of a sample of raw ham after clean-up on Sep-Pak cartridge using as eluents: H_2O: CH_3OH 30:70 v / v (a), H_2O: CH_3CN 70:30 v / v (b), H_2O: CH_3CN 50:50 v / v (c), H_2O: CH_3OH 50:50 v / v (d).

From the analysis it can be concluded that no significant differences were observed for the peptide profile, whereas the best solvent mixture to efficiently eliminate the maximum amount of bile salts is the mixture H_2O: CH_3OH 50:50 (v / v) (chromatogram d). Thus, the clean-up method using Sep-Pak C18 cartridge with the eluent H_2O: CH_3OH 50:50 was applied to the analysis of all the digested ham samples.

4.3.2 Efficiency of digestion process

The efficiency of the digestion process was evaluated comparing the protein and peptide profile of dry-cured ham samples before and after the digestion process.

In particular, comparing an aqueous acidic extract of the ham with the profile of the digesta by monodimensional gel electrophoresis large differences were observed: several bands of muscle proteins and

protein fragments were found in the extracts, whereas these bands completely disappear upon digestion with the concurrent accumulation of low molecular weight compounds (Figure 25).

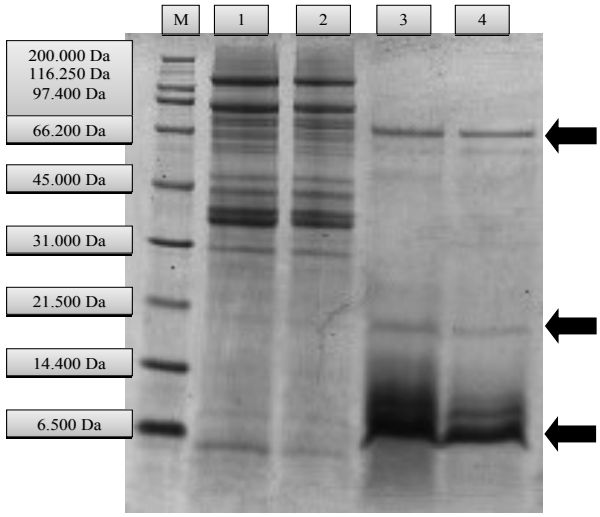

Figure 25. Sds-page of extracted and digested samples: 1, extract at 18 months; 2, extract at 24 months; 3, 18 months digesta; 4, 24 months digesta, M marker.

The only protein bands present in digested samples are those of serum albumin (with molecular weight around 66200 Da), troponin C (the band slightly below 21500 Da) and fragment probably derived by superoxide dismutase (around band 6500Da). It appears evident that the digestion process was very effective and we focused our attention to the characterization of the peptide pattern by liquid chromatography-mass spectrometry analysis.

4.3.3 Chromatographic profile of ham samples before and after the digestion process

First, we performed an UPLC-ESI-MS analysis of the peptide fraction before and after the digestion process, focussing on peptides with a MW up to 10 kDa.

A typical UPLC-MS peptide chromatographic profile of a digested sample compared with that of the corresponding extract as well as of the digestion blank is reported in Figure 26.

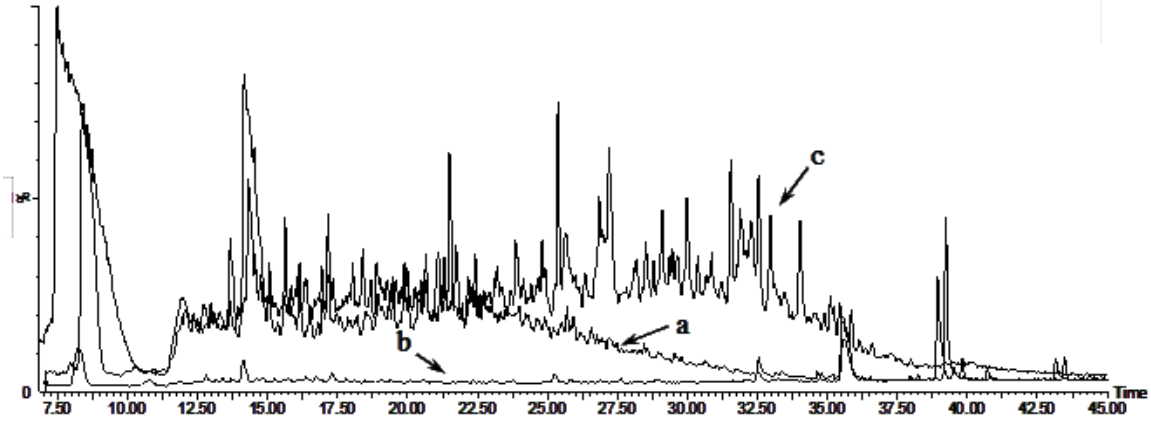

Figure 26. Total Ion Chromatogram in UPLC-ESI-MS of: a) digesta sample; b) blank digesta (matrix without ham); c) extract sample.

The peptide profile of digesta from dry-cured ham (a) is completely different from that of the extract before digestion (c) and absolutely richer than that obtained for the digestion blank (matrix-without ham) (b), which contains only some endogenous peptides in far lower amounts than those observed in the case of ham.

Some common peaks are related to the free amino acids (phenylanine and tryptophan) and to the bile salts of the digestive juices (the last eluting peaks between 35 and 45 min). In particular, as already underlined, the peptide profile of the extract before digestion (c) is characterized by a higher amounts of high MW peptides and a lower amounts of low MW peptides in comparison with the profile of the digested sample.

Indeed, LC-MS analysis reveals large differences in terms of molecular weights: in the extract before digestion we observed a very complex mixture of peptides with a range of MWs between 188 and 9166 Da: 54% of these peptides have MWs > 1000 Da and 15% have MWs > 5000 Da. In the digesta, MWs span between 200 and 1600 Da, with 80% of the peptides having a MW < 1000 Da.

Peptide sequences were identified and characterized by HPLC-ESI-MS/MS, confirming the identification by exact mass determination with LTQ-Orbitrap. The main peptides occurring in ham extract before digestion are not found in the digesta: degradation by the digestion process leads to a completely different pattern, which has been characterized as reported below.

4.3.4 Characterization of the peptide profile of the digested samples

The cleavage of peptide bonds by digestive proteases results into the release of peptides of various length and free amino acids. The peptide profile of digested ham samples was analyzed in order to identify the peptides regarded as the most significant (obtained by UPLC-ESI-MS): on account of the large number of peptides and the great complexity of the chromatogram, the identified peptides are those corresponding to

the most intense signals. The identification was carried out matching the results obtained by HPLC-ESI-MS/MS and LTQ-Orbitrap analyses. As stated before, most peptides found in the profile of digested samples have MWs between 200 and 1700 Da: these dimensions are typical of most known bioactive peptides and also of peptides able to easily cross the gut lumen, thus potentially able to exert a nutritional or systemic bioactive function. The length of the peptide fragments are between 2 and 14 amino acids, in agreement with other studies of peptide profile obtained upon a digestion process (Escudero, 2010; Bauchart, 2007).

The most important proteins of origin with reference to the amount of the peptides found in the mixture were identified by LTQ-Orbitrap analysis (Table 4).

Table 4. Main proteins of origin of the peptides present in the digested samples.

Protein Code (UniProtKB-Swiss Prot-ExPASy)	Score	Protein Description
Q9TV61	411.40	Myosin-1 OS=Sus scrofa GN=MYH1 PE=2 SV=1 - [MYH1_PIG]
Q9TV62	361.68	Myosin-4 OS=Sus scrofa GN=MYH4 PE=2 SV=1 - [MYH4_PIG]
Q9TV63	246.12	Myosin-2 OS=Sus scrofa GN=MYH2 PE=2 SV=1 - [MYH2_PIG]
P79293	218.49	Myosin-7 OS=Sus scrofa GN=MYH7 PE=2 SV=2 - [MYH7_PIG]
F1RZC8	176.06	Uncharacterized protein OS=Sus scrofa GN=TTN PE=4 SV=1 - [F1RZC8_PIG]
K7GMH0	174.08	Uncharacterized protein (Fragment) OS=Sus scrofa GN=MYH6 PE=4 SV=1 - [K7GMH0_PIG]
P68137	128.36	Actin, alpha skeletal muscle OS=Sus scrofa GN=ACTA1 PE=3 SV=1 - [ACTS_PIG]
F1SHL9	101.93	Pyruvate kinase (Fragment) OS=Sus scrofa GN=PKM PE=2 SV=2 - [F1SHL9_PIG]
F1SS61	93.60	Uncharacterized protein OS=Sus scrofa GN=MYHC PE=4 SV=1 - [F1SS61_PIG]
F2Z5B6	76.92	Tropomyosin alpha-1 chain OS=Sus scrofa GN=TPM1 PE=3 SV=1 - [F2Z5B6_PIG]
F1SG00	72.09	Uncharacterized protein OS=Sus scrofa GN=TPM2 PE=3 SV=1 - [F1SG00_PIG]
A1XQT6	58.04	MLC1f OS=Sus scrofa GN=MYL1 PE=2 SV=1 - [A1XQT6_PIG]
Q5XLD3	42.86	Creatine kinase M-type OS=Sus scrofa GN=CKM PE=2 SV=1 - [KCRM_PIG]
F1RFY2	42.73	Beta-enolase OS=Sus scrofa GN=ENO3 PE=3 SV=1 - [F1RFY2_PIG]
F1RJ25	42.66	Fructose-bisphosphate aldolase OS=Sus scrofa GN=ALDOC PE=2 SV=2 - [F1RJ25_PIG]

The identification has been performed by online bioinformatics resource portal such as “Expasy” with the tool “FindPept” and by the “Proteomics Toolkit”(MS/MS Fragment Ion calculator): the identification

process is based on the comparison of the theoretical fragmentation (Proteomics Toolkit) of the possible peptide sequences obtained by in silico experiments from unspecific cleavage of proteins (FindPept) with the sequence fragmentation obtained by MS/MS experiments. An example is reported in Figure 27.

Fragment Ion Table, average masses

Seq	#	A	B	C	X	Y	Z	# (+1)
D	1	88.08649	116.09659	133.12715	-	933.98795	916.95738	8
I	2	201.24593	229.25602	246.28659	844.89357	818.89935	801.86879	7
D	3	316.33453	344.34463	361.37519	731.73412	705.73991	688.70935	6
D	4	431.42312	459.43323	476.46378	616.64553	590.65131	573.62075	5
L	5	544.58256	572.59266	589.62322	501.55693	475.56271	458.53215	4
E	6	673.69804	701.70814	718.73870	388.39749	362.40326	345.37271	3
L	7	786.85748	814.86758	831.89814	259.28201	233.28779	216.25723	2
T	8	887.96256	915.97266	-	146.12257	120.12835	103.09779	1

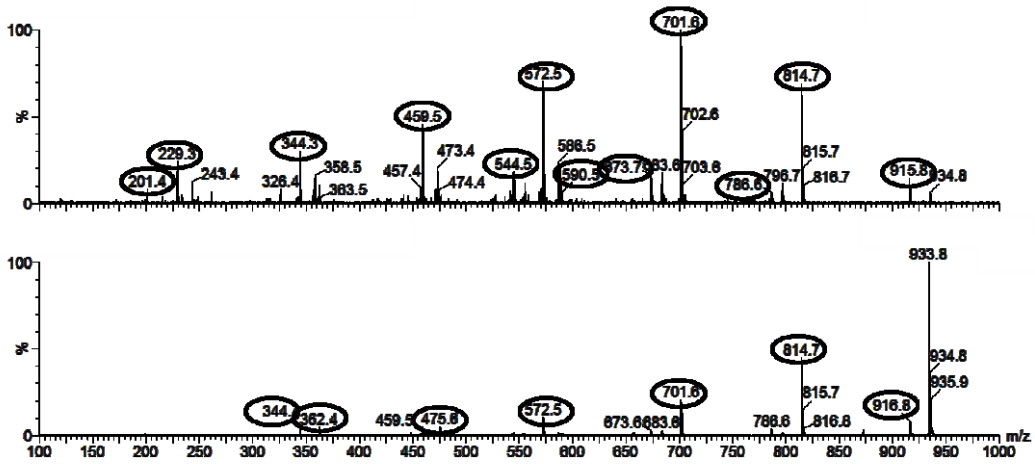

Figure 27. Peptide identification example: comparison of the theoretical fragmentation with the sequence fragmentation obtained by MS/MS experiments.

The sequences of the peptides identified in the digesta are reported in Table 5, along with their main putative proteins of origin.

Table 5. Identified peptide sequences in digesta of dry-cured hams analysed at 18 and at 24 months of ageing.

MW	Sequences	Parent Protein	Average amount ± std. dev. *	
			18 months	24 months
202	AI(L)		**5.80±0.95**	2.16±0.53
248a	VM		**0.98±0.18**	0.32±0.08
932	n.i.		**0.22±0.09**	0.09±0.06
232	TI(L)		**1.50±0.17**	0.65±0.15

188	GI(L)		**1.39±0.14**	0.60±0.15
242a	SH		**2.31±0.30**	0.90±0.20
248b	MV		**0.81±0.07**	0.26±0.05
230a	I(L)V		**3.39±0.41**	0.82±0.15
230b	VI(L)		**2.10±0.32**	0.72±0.13
331a	n.i.		**0.82±0.18**	0.22±0.05
230c	L(I)V		**2.56±0.32**	0.81±0.15
1035	RVAPEEHPT	actin B	**2.62±0.56**	1.32±0.30
879	VAPEEHPT	actin	**1.05±0.21**	0.30±0.09
487	LAPST	actin	**0.46±0.08**	0.11±0.04
477	FQPS	actin	**1.19±0.17**	0.44±0.09
639	APLNPK	actin	0.10±0.03	0.12±0.10
256	n.i.		0.01±0.02	0.01±0.01
501	n.i.		**0.38±0.05**	0.11±0.04
262a	IM		**1.60±0.24**	0.53±0.11
230d	VI(L)		**3.06±0.34**	1.17±0.22
331b	SIL/SLI	myosin 1	**0.84±0.12**	0.54±0.12
471	PGIAD	actin	**0.95±0.16**	0.09±0.02
802	KLEGDLK	myosin 1	**0.58±0.11**	0.29±0.11
262b	LM		**0.27±0.04**	0.09±0.02
659	n.i.		**0.79±0.13**	0.25±0.07
688	n.i.		**0.21±0.07**	0.21±0.06
264	FV		**1.19±0.15**	0.44±0.09
244a	II/LL/IL/LI		**0.97±0.16**	0.25±0.04
672	YPIEH methylated	actin	**0.84±0.13**	0.25±0.07
869	TEAPLNPK	actin	**2.26±0.43**	0.72±0.29
455	IVAPG	Fructose aldolase biphosphate	**0.88±0.17**	0.14±0.04
662	MDLER	myosin 1	0.19±0.08	**0.23±0.09**
262c	MI(L)		**0.07±0.1**	0.04±0.01
359	ELV	myosin 1	**0.25±0.04**	0.16±0.03
333	ALM	myosin 1	**0.33±0.06**	0.28±0.06
244b	II/LL/IL/LI		**1.31±0.15**	0.53±0.08
463	NSIM	actin	**1.10±0.15**	0.29±0.07
244c	II/LL/IL/LI		**6.18±0.73**	1.91±0.33

795	IIAPPER	actin	**0.61±0.12**	0.44±0.09
402	DIR	actin	**0.93±0.22**	0.55±0.14
445	IGGSI	β -actin	**0.73±0.17**	0.09±0.02
1002	n.i.		**1.05±0.15**	0.36±0.06
506	MDLE	myosin 1	**0.80±0.13**	0.23±0.06
988	n.i.		0.08±0.03	**0.22±0.06**
244d	II/LL/IL/LI		**2.02±0.42**	0.99±0.18
830	LQDLVDK	myosin 1	**1.38±0.28**	1.02±0.22
982	LTEAPLNPK	actin	**3.25±0.80**	1.37±0.33
415	PSIV	actin	**1.64±0.40**	0.26±0.07
553	SFVTT	actin	**0.40±0.08**	0.35±0.10
329	DPV	pyruvate kinase	**1.15±0.16**	0.42±0.09
625	DLTDY	actin	0.45±0.14	**0.54±0.14**
937	NWDDMEK	actin	**1.18±0.22**	0.38±0.07
441	NVPI	actin	**5.67±0.75**	1.69±0.34
1066	KMEGDLNEM	myosin 1	**0.41±0.08**	0.11±0.02
278a	FI(L)		**0.93±0.13**	0.32±0.06
345	LTL	myosin 1	**0.24±0.05**	0.04±0.01
430	LEGI	myosin 1	**0.65±0.12**	0.24±0.05
908	SYELPDGQ	actin	**0.30±0.06**	0.10±0.02
331c	SIL/SLI	myosin 1	**0.40±0.06**	0.08±0.02
444	PTVE	β-enolase	**0.93±0.22**	0.21±0.06
301	LGL		**2.66±0.35**	0.93±0.2
278b	I(L)F		**1.11±0.22**	0.57±0.10
801	IKAKSALA	myosin 1	**0.84±0.14**	0.40±0.12
278c	FI(L)		**0.24±0.07**	0.14±0.03
372	L(I)KL(I)/L(I)QL(I)		**0.81±0.13**	0.20±0.04
1349	PEILPDGDHDLK	aldolase A fructose biphosphate	**1.50±0.32**	0.28±0.15
635	n.i.		**0.42±0.08**	0.22±0.05
474	IDDL/LDDL	myosin 1	**1.01±0.22**	0.09±0.02
649	SLSTEL	myosin 1	**0.71±0.14**	0.45±0.12
343	LVL	myosin 1	**0.91±0.15**	0.29±0.06
1577	VEPEILPDGDHDLK	aldolase A fructose biphosphate	**0.98±0.35**	0.28±0.07

335	AVF / GLF / LGF	actin / myosin1/ myosin1	**0.12±0.03**	0.04±0.01
776	TSLINTK	myosin 1	**0.23±0.05**	0.18±0.06
317	VTV	myosin 1	**0.25±0.08**	0.14±0.03
1285	LKGADPEDVITGA	myosin light chain 2	**0.65±0.24**	0.21±0.06
1084	DIDSPPITAR	pyruvate kinase	0.03±0.02	**0.27±0.09**
483	GVVPL	aldolase A fructose biphosphate	**0.76±0.14**	0.16±0.04
558	n.i	n.i	**1.14±0.18**	0.40±0.10
1220	PEILPDGDHDL	aldolase A fructose biphosphate	**0.32±0.09**	0.02±0.02
970	DQIISANPL	myosin 1	**0.22±0.14**	0.07±0.04
1097	LLASIDIDHT	myosin 2	**0.37±0.11**	0.07±0.02
1109	TVKDLQHRL	pyruvate kinase	0.15±0.08	**0.29±0.14**
1314	IQLVEEELDRA	tropomyosin a-1	0.25±0.09	**0.40±0.10**
933	DIDDLELT	myosin 1	**0.22±0.05**	0.09±0.02
1015	LFDKPVSPL	creatin chinase	**0.27±0.06**	0.11±0.03
1393	RMKKNMEQTVK	myosin 1	0.25±0.11	**0.41±0.09**
571	RVAPE	actin	**0.30±0.07**	0.18±0.04
1048	INTTLETKQ	myosin 7	**0.21±0.04**	0.10±0.03
1653	NAYEESLDQLETLK	myosin 1	0.09±0.05	**0.22±0.07**
1160	n.i.		**0.21±0.05**	0.05±0.02

n.i.: not identified

* semiquantification values (expressed as ratio between "Area peptide" and "area Phe-Phe" as internal standard). When reported peptides are significantly different between 24 or 18 months of ageing time ($p < 0.05$, Student's t-test), the higher one is reported in bold character.

As already underlined and also observed by other authors, the identified sequences in digested samples show that most peptides originated from myofibrillar proteins (actin and myosin) and from sarcoplasmic proteins (such as creatine kinase, pyruvate kinase, fructose bisphosphate aldolase, etc.). In comparison to what observed in the literature (Ferranti, 2014), in our case a higher amount of short sequences were found in the digesta: indeed, up to 21 dipeptides and 11 tripeptides were found among the 81 identified sequences.

As far as the identified sequences, peptides originated by the sequential activity of pepsin and pancreatin are present and many N- or C-terminal amino acid residue correspond to the known cleavage site of these enzymes. Nevertheless, it's not possible to certainly assign one particular sequence to a specific enzyme activity due to the combined and complex action of the enzyme pool.

Some of the identified peptides are already known in the literature. In particular, the peptide sequences IIAPPER, PEILPDGDHDL, LTEAPLNPK have been identified in *in vitro* digestion of pork meat

(Escudero, 2010), whereas DIDSPPITAR and SYELPDGQ share partial homology with DIDSPPIT, YELPDGQ, as well as VTV, AVF, LGF with VTVNPY, AGDDAPRAVF, LGFNPPDL. The sequences RVAPEEHPT, VAPEEHPT, IGGSI, LFDKPVSPL were also found in pork meat digesta (Escudero, 2010). A partial homology occurs between DLTDY and DLAGRDLTDYL, identified in the *in vivo* proteolytic digestion of beef meat and trout flesh in pigs (Bauchart, 2007).

Many identified sequences are encrypted in those found in a recent work on Bresaola (Ferranti, 2014) like LAPST, PGIAD, IVAPG, PSIV, SFVTT, DLTDY, NWDDMEK, NVPI, SYELPDGQ, IDDL, LKGADPEDVITGA, TVKDLQHRL, but also in a study on Spanish dry-cured ham (Mora, 2014).Peptide sequences like LTEAPLNPK, PTVEVDLH, LFDPVSPL, NAYEELSDQLETLK also occur.

As a general remark, most sequences found in our work are shorter than those reported in other studies, and/or are parts of the longer ones identified in the above mentioned works: it is indeed worth noting that in these papers the minimum length of identified peptides is around 8 amino acid residues, whereas in our samples lots of dipeptides and tripeptides have been found. These findings could be ascribed to a number of factors such as a more efficient digestion model, but also to the advanced proteolytic process in long aged hams, which are characterized by a high percentage of low MW oligopeptides more easily prone to further degradation during digestion. In order to evaluate the latter effect we have compared the results obtained with hams of 18 and 24 months of maturation.

4.3.5 NPADS

Npads are peptides of non-proteolytic origin. They are present in dry-cured hams as already described in chapter 3.3.2.2.1. In the literature it is already known that these compounds are resistant to gastro-intestinal digestion (Bottesini, 2014). We have checked the digested samples for the occurrence of these compounds and their eventual quantification. The results are reported in Figure 28.

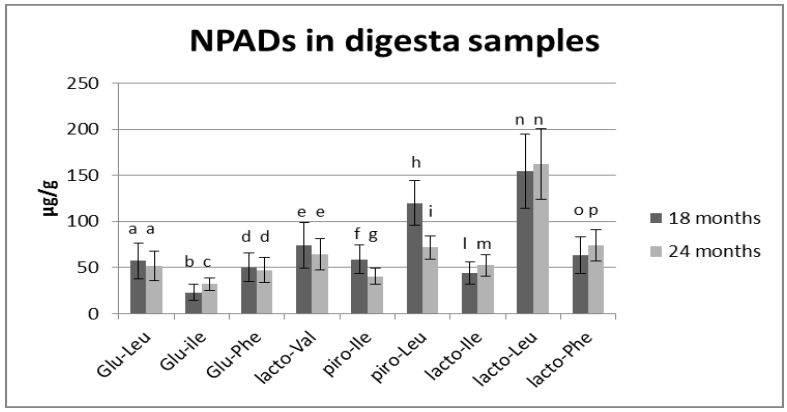

Figure 28 NPADs in digesta of dry-cured hams of 18-24 months of ripening (statistical analysis:T-student test, p<0.05).

The NPADs detected in dry-cured ham extracts such as γ-Glu-Tyr, pyro-Pro, lacto-Met, lacto-Tyr and lacto-Phe are not found in the digested samples. NPADs in extracted samples are present in maximum amount of 50µg/g, instead in digested samples the maximum amount is about 150 µg/g. As these compounds may not be originated by digestive enzyme (Bottesini, 2014), their higher amount in digesta may be due to a more efficient extraction on account of the degradation of the food matrix. Anyway, both in the extracted as well as in the digested samples, Lactoyl-Leu is the amino acyl derivative present in highest amount.

4.3.6 Peptide profile of the digesta as a function of the ageing time

Ham samples of 18 and 24 months were subjected to the simulated gastrointestinal digestion process, the peptide profile was characterized and the identified peptides were semiquantified using a suitable internal standard (Phe-Phe, 1mM), by measuring the ratio between the peptide area and the area of the internal standard. Data were statistically evaluated by principal component analysis in order to highlight the possible differences occurring between samples: indeed, dry-cured hams can be grouped as a function of ageing time (18-24 months) after the gastro-intestinal digestion step (Figure 29).

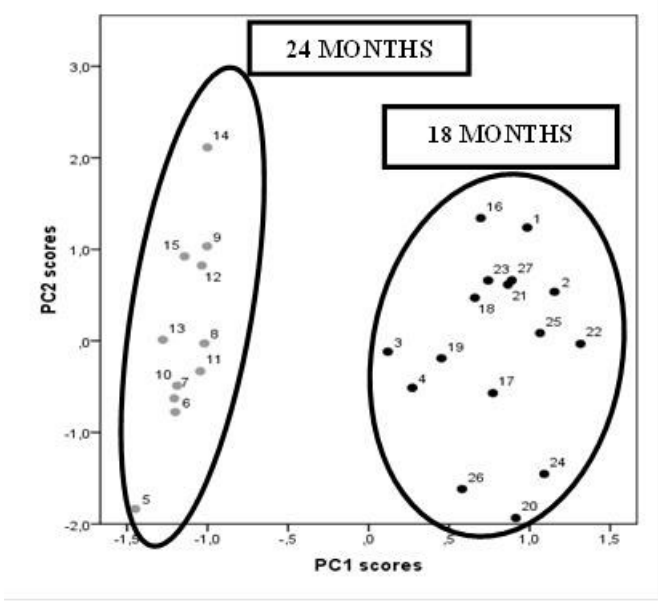

Figure 29. PCA digesta samples at different ageing time.

The cumulative variance explained is 86%, of which 76% determined by component 1.

In order to verify which peptides are responsible for the observed difference, the semiquantification values of all the peptides were compared by T-student test: results are reported in Table 2.

Most peptides are found in higher amounts in 18 aged hams than in 24 aged ones: this result could be determined by an higher digestibility and bioaccessibility of the nitrogen fraction in more aged samples on account of the proteolytic process during maturation.

Although somehow expected, these findings underline that the proteolysis process taking place during the ageing period strongly influences the peptide profile of the digested sample, and, as a consequence, the nutritional value and possible biological activity of the peptides encrypted in meat proteins.

4.3.7 Study of peptides transport across the intestinal barrier

In order to exert their functional bioactivity peptides have to be able to cross the intestinal barrier and enter the blood circulation in an intact form. In order to study this ability, the digested samples at 18 and 24 months (with also the digestion blank as control) have been tested on model of intestinal barrier systems like Caco-2 and Ussing chamber. The first method is a simplified model that mimics the intestinal barrier (as a good and valuable model for assessing the physiological response of enterocytes) re-creating the two compartments: the apical one represents the intestinal lumen, the basolateral compartment represents the

blood circulation. Caco-2 cells are able to mimic the intestinal barrier: they express a number of carrier-mediated transport systems and enzymes (as the enterocytes) (Behrens, 2003) and numerous brush border membrane peptidases similar to those of human intestinal epithelium (Xie, 2014). To obtain a better physiological model, it's possible to create a co-culture of Caco-2 with HT29-MTX that are mucus cells (Picariello, 2013). The second method (Ussing chamber) is an ex-vivo method because the two compartments (mucosa = apical, serum = basolateral) are created using a real intestinal epithelium of a pig, excited immediately after the slaughter of the animal. This second model is more complex but more similar to the real physiological environment.

In the literature these methods have been applied to test the ability of few purified peptides with bioactive-function to cross the barrier of the intestinal epithelium (Cakir-Kiefer, 2011; Bejjani, 2013) or to evaluate fractions of protein of different source subjected to similar gastrointestinal digestion and Caco-2 cells permeation (Samaranayaka, 2010) and by (Cristina Megías, 2009).

The aim of our work is to apply these systems to study the behavior of a complex matrix such as the digesta of dry-cured ham in order to verify the resistance of the peptides to brush border peptidases and their ability to cross intestinal barrier.

4.3.7.1 Caco2/HT29-MTX

Briefly, the two different types of cells (Caco2 and HT29-MTX) are first cultivated separately and then combined together in ratio 90:10 for 21 days in specific inserts and medium. Then, the medium is substituted by the digested sample/ HBSS (as a control of the system) in the apical compartment and only HBSS in the basolateral compartment, like in Figure 30.

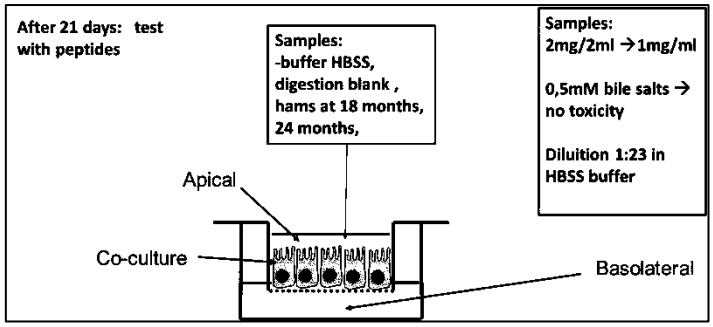

Figure 30. Scheme of co-culture experiment.

Sampling was performed at 30, 60 and 120 minutes. Some preliminary experiments were made in order to verify the proper sample dilution in order to obtain a non-toxic bile salt concentration for the cells (Cakir-

Kiefer, 2011,): the Measurement of Transepithelial Electrical Resistance (TEER) allows to assess integrity of the Caco-2 cell monolayer, as reported in Figure 31.

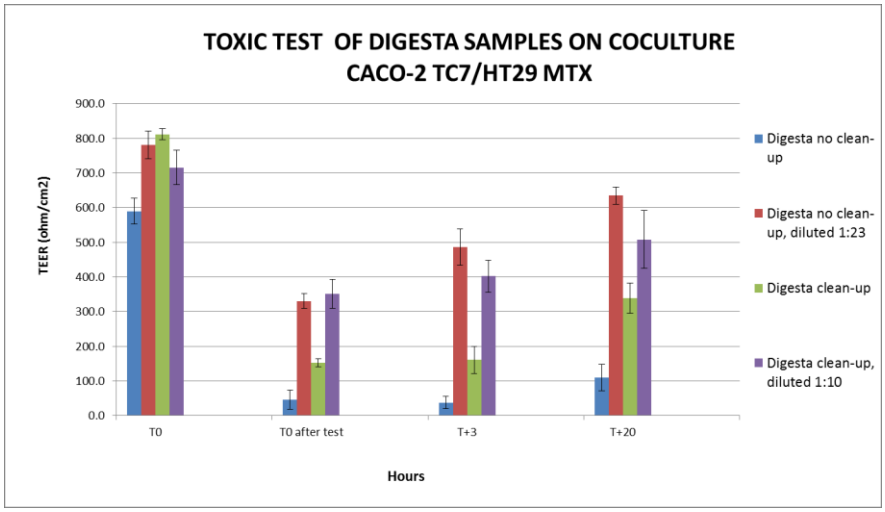

Figure 31. Toxic test of samples on co-culture Caco-2/HT29-MTX: digested samples without clean-up (blue); digested sample diluted 1:23 without clean-up (red); digested sample with clean-up on Sep-Pak C18 cartridges; digested sample with clean-up on Sep-Pak C18 cartridges diluted 1:10.

An increase to 350 Ω cm^2 of the TEER indicated a confluent cell monolayer with tight junctions. As the graph show, the TEER of samples with and without clean-up collapses immediately after the test and at 20 hours of the end of experiments its value is very low: these results indicated that the monolayer has been damaged. Instead, the same digested sample but with an opportune dilution in HBSS allows to maintain a confluent monolayer. For these reason has been choice the samples diluted 1:23 were chosen because the samples cleaned-up on Sep-Pak cartridges does not provide additional benefits.

The concentration of digested samples (only the supernatant) has been determined with the Kjeldhal method.

The samples obtained at 30, 60 and 120 minutes of test on co-culture have been analyzed by UPLC-ESI-MS in order to evaluate their resistance to peptidases and their ability to cross the intestinal barrier in their native form or after modification by intestinal enzymes. An example of the total-ion-chromatogram (TIC, full scan mode) obtained from tested samples of HBSS, blank digestion, 18 and 24 months ham digesta (60 minutes, apical compartment) is reported in Figure 32.

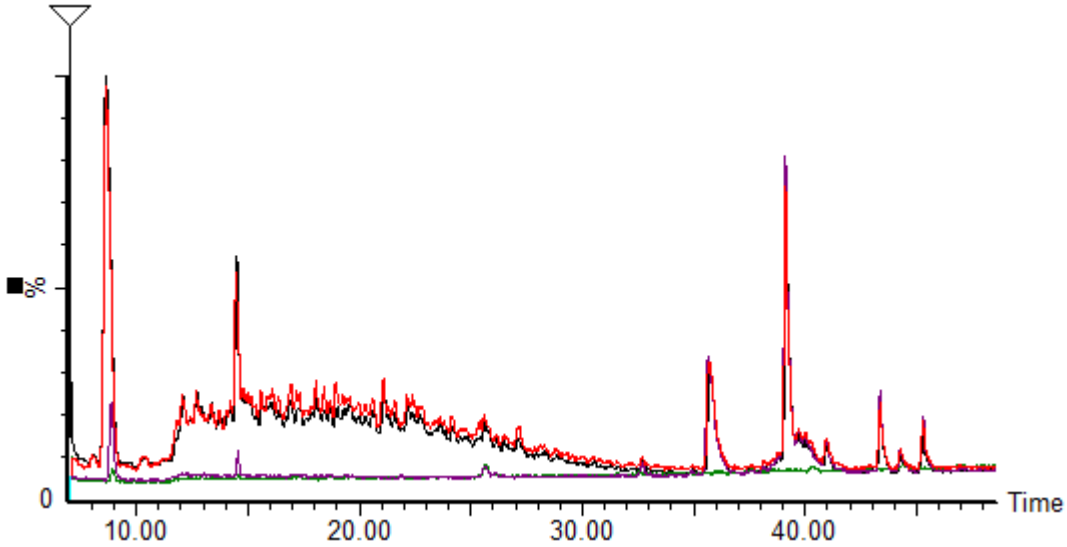

Figure 32. Superimposed chromatograms obtained by the analysis of the apical compartment of Caco2 system: 24 months ham digesta at the top (time zero and 120 min), digestion blank and HBSS at the bottom.

Between 35 and 45 minutes the bile salts are eluted. The chromatograms are very similar to the chromatograms obtained from digested samples before the test on co-culture Caco-2/HT29-MTX: the profile of peptides is very rich and so it's difficult to identify new peptides or peptides with some modifications due to enzymatic actions. In order to evaluate the hydrolysis of peptides by brush border peptidases, the peptides already identified in chapter 4.3.4 (in Table 4) have been searched.

Evaluating the variation of these peptides, it's possible to note that generally most of the peptides seem to decrease during time, from zero to 120 minutes. An example of this decrease is show in Figure 33: the main four highest peaks corresponds to peptides II/LL/IL/LI and their decrease during time in the apical compartment is evident.

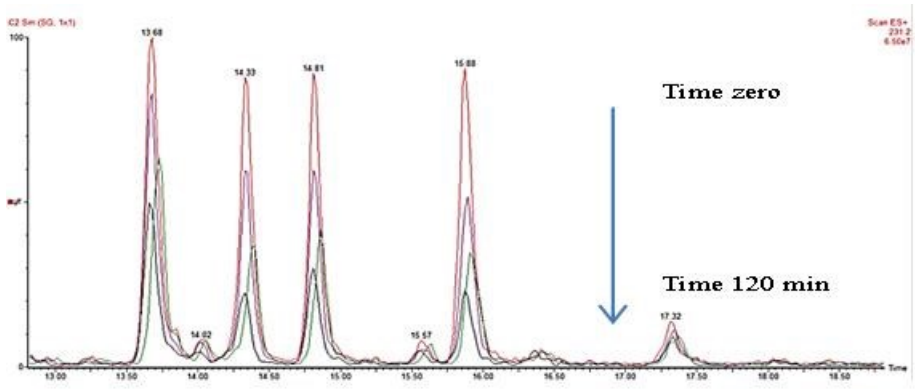

Figure 33. Peptides decrease from time zero to 120 minutes of test on Caco-2/HT29-MTX: apical compartment.

The decrease of peptides may be due to the actions of hydrolytic enzyme present in the apical compartment released from Caco-2 cells. There are some peptides that seem to be resistant: for example peptides like VTV, ELV, PTVE seem to be constant during all time of experiment. This decrease may be also due to the crossing of the Caco2 layer.

In order to evaluate the ability of peptides to cross into the basolateral compartment, the total ion chromatogram (TIC, obtained in full scan mode) have been analyzed. An example of chromatogram obtained from the sample taken at 60 minutes of experiment is reported below, in Figure 34.

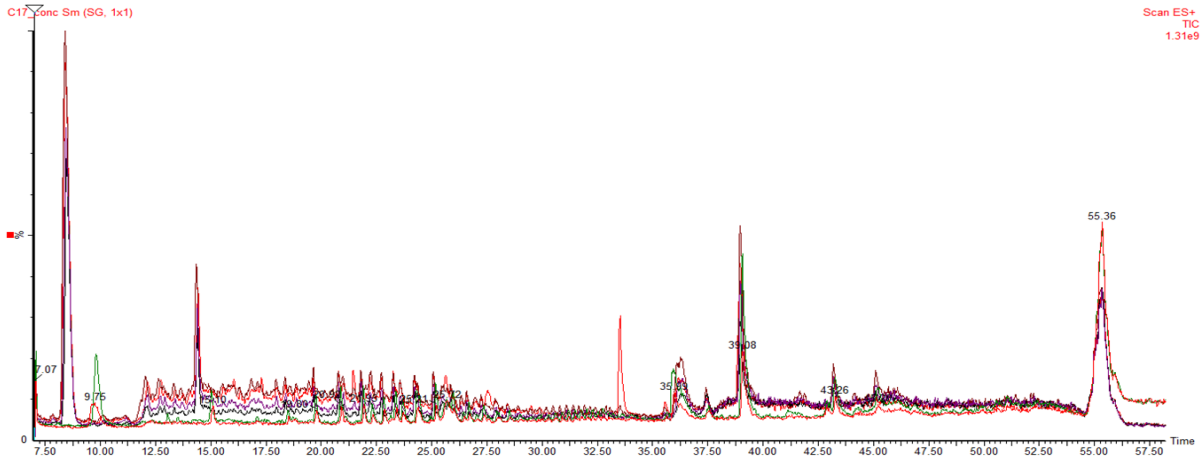

Figure 34. Chromatograms of digested samples in systems like co-culture Caco-2/HT29-MTX: basal compartment. TIC are of HBSS, blank of digestion, 18 and 24 months. Bile salts are present from 35 to 45 minutes.

The peaks between 35 and 45 min correspond to the bile salts. Chromatograms are very complex and rich of several signals that are also present in the TIC of HBSS, thus, it is very difficult to identify eventual peaks related to the peptides of the digesta. Thus, we decided to test for the presence of peptides already identified in the digesta in order to verify if they are able to cross the intestinal barrier, although in low amounts.

By applying this approach, surprisingly we found most of the peptides identified in the profile of the digested ham: indeed, we were able to identify 55 sequences out of the 80 previously identified in the digesta, although most of them are present in very low amount. Considering the type of peptides found and their MW, probably different transport mechanisms are involved: carrier-mediated transport system as PepT1 for di-tripeptides, transcytosis (an intracellular vesicle mediated transport) for small oligopeptides and paracellular diffusion for larger oligopeptides as well as di- and tripeptide. The amount of most peptides in the basolateral compartment seems to increase during time, as shown in Figure 35.

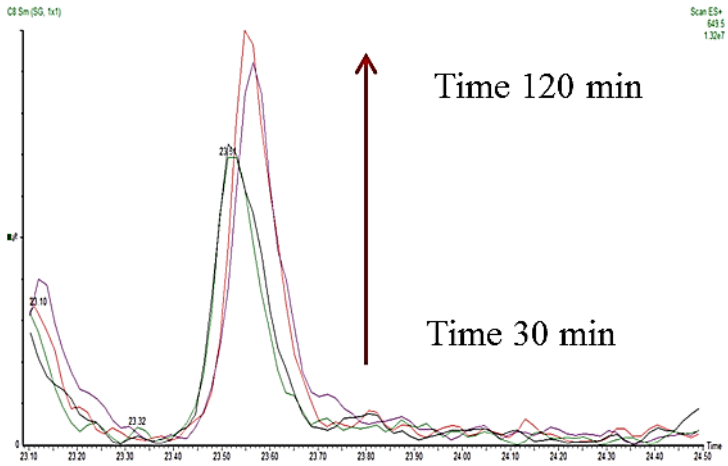

Figure 35. Peptides increase during time in basolateral compartment in systems like Caco-2/HT29-MTX.

More reliable indications may be obtained a simpler mixture of peptides, nevertheless these results are very interesting as they clearly demonstrate the ability of several peptides of the digesta to cross the intestinal barrier without being modified or degraded, thus being potentially able to exert a functional bioactivity in vivo.

4.3.7.1.1 NPADS in basolateral compartment of co-culture

Also NPADs have been searched in the basolateral compartment: some of them seem to be able to cross the intestinal barrier, probably on account of their low molecular weight and their resistance to the proteolytic activities of blood serum (Bottesini, 2014). More studies will be needed to outline their ability to be absorbed in the gastrointestinal tract and their eventual biological and nutritional significance.

4.3.7.2 Ussing chamber

The Ussing chamber provides a physiological system to measure the transport of ions, nutrients, and drugs across various epithelial tissues. One of the most studied epithelia is the intestine. Ussing chamber is a particular chamber where the two compartments are created by the insertion of a real piece of pig intestine. The Ussing chamber is shown in Figure 36.

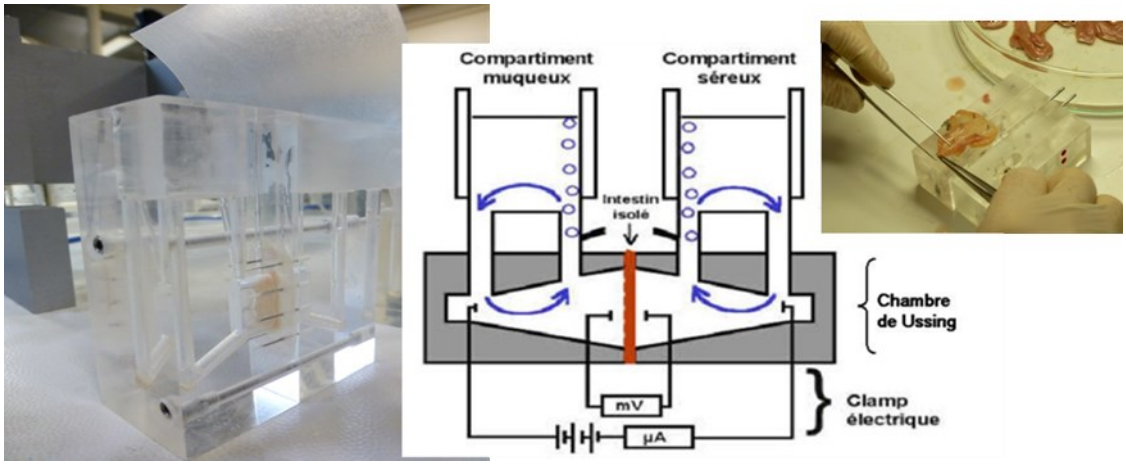

Figure 36. Ussing chamber.

The samples analyzed are 18 and 24 months hams, digestion blank and Krebs solution as control of the system. The analyzed samples have been diluted (11 times) in Krebs ringer: in this case bile salts at the concentration present in the digesta are not toxic for the intestine, thus it's possible to use a more concentrated sample in comparison with the experiment on Caco-2. The sampling time is always 0, 30, 60, 120 minutes for the mucosa compartment whereas for serum compartment only one sampling at 120 minutes was performed: it must be taken into account that the subsequent sampling steps of the mucosa compartment causes a dilution of the components, as it is necessary to replace the sample volume with the same volume of Krebs-Ringer.

All samples have been analyzed in UPLC-ESI-MS. The chromatograms for the mucosa compartment are shown in Figure 37.

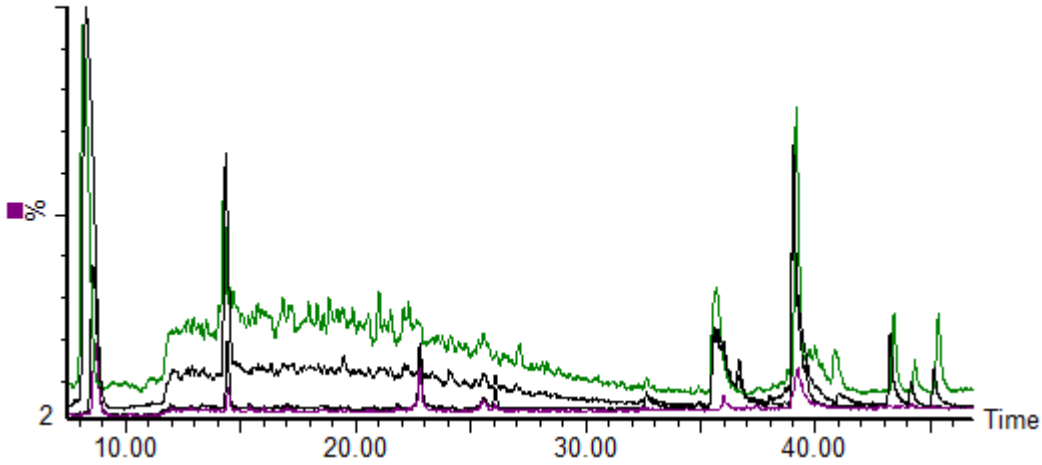

Figure 37. Chromatograms obtained by the analysis of the mucosa compartment in the Ussing chamber in the presence and in the absence of the digested samples: 24 months digested hams (time zero in green and time 120 min. in black) at the top, Krebs buffer and digestion blank at the bottom.

As already observed in the experiments with Caco-2, the chromatographic profile is very complex. Nevertheless, also in this case it was possible to identify all the peptides of the digesta and to verify their strong decrease during time as shown in Figure 38 in the case of VAPEERHPT.

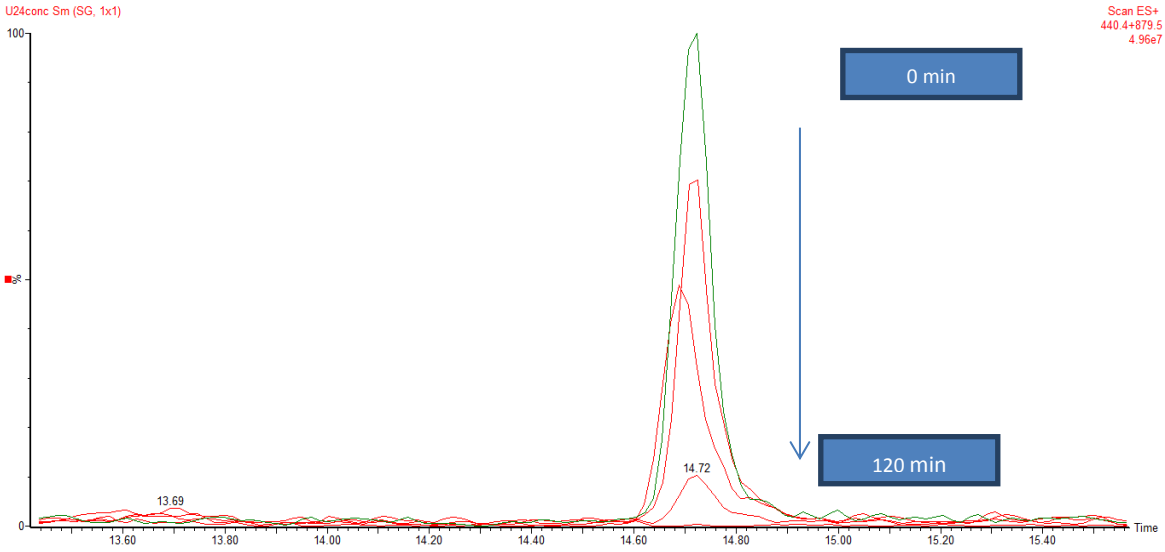

Figure 38. Peptides in Ussing chambers: variation of the peptide VAPEERHPT with time.

The decrease is stronger than that observed in the case of Caco-2. The chromatograms obtained from the analysis of the serum compartment are much more complex as outlined in Figure 39:

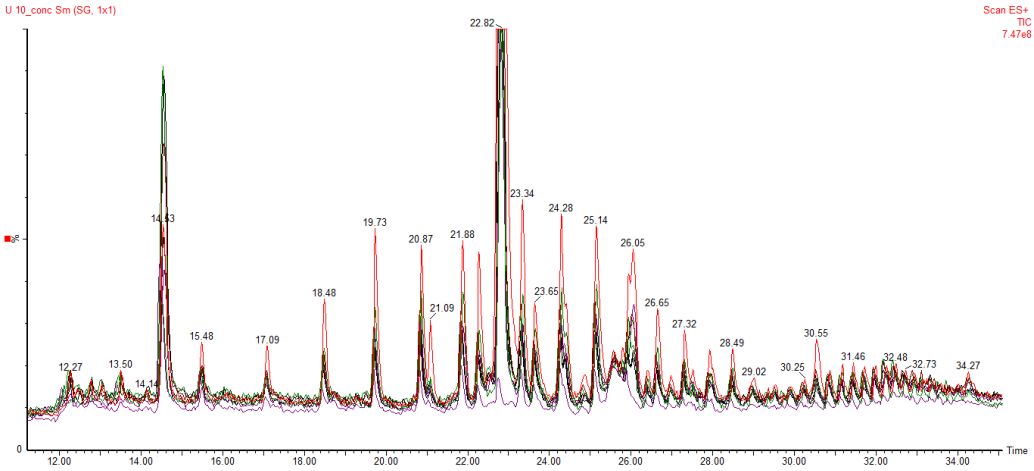

Figure 39. Chromatograms obtained by the analysis of the serum compartment of the Ussing chamber in the presence and in the absence of digested ham samples (18, 24 months ham, digestion blank and Krebs ringer).

We tried to verify, as already done in the case of Caco2, if some peptides of the digesta were able to cross the intestinal barrier: unfortunately, in this case we didn't succeed in identifying any of the peptides occurring in the digesta. As a plausible explanation of this negative result, it is worth noting that there are several interfering substances coming from the complex composition of the Krebs-ringer and, as already evidenced in the case of the Caco2, the amount of peptides that crosses the epithelial barrier is very low.

4.3.8 ACE-inhibitor activity

Angiotensin I converting enzyme (ACE) inhibitor peptides are in the last years the most studied owing to their potential beneficial effects on hypertension: there is a growing interest in using these bioactive peptides as efficient agents in treating and preventing hypertension. Many peptides from enzymatic hydrolysis of proteins of meat product have been identified showing the ability to inhibit ACE and/or to lower blood pressure in vivo (in spontaneously hypertensive rats, SHR). Among meat products, attention has been devoted also to dry-cured hams as important and appreciated high value products: researches on this topic are continuously growing. To exert a systemic function, bioactive peptides must be able to cross the intestinal barrier and enter the blood stream in an intact form. The first phase in which structure modification takes place is during the gastrointestinal digestion of food and for these reasons ham have been subjected to simulated gastrointestinal digestion and the analysis and bioactivity tests have been performed on digesta. Among the identified sequences, some short sequences have been found that are already known

and tested in the literature for their ACE inhibitor activity, such as AI(L), GI(L), I(L)F, LVL (BIOPEP online database). Some sequences were identified that are known not to have an inhibitor activity, such as IGGSI. Most of the other identified sequences have never been tested, such as RVAPEEHPT, VAPEEHPT, and LFDKPVSPL.

Thus, the first step was to evaluate the ACE inhibitor activity of digesta and the eventual possibility for peptides to cross the intestinal barrier as well as the bioactivity could be transferred from the gut lumen to the blood stream, also using the previously described system like co-culture Caco-2/HT29-MTX and Ussing chambers.

The antihypertensive activity of the samples was measured by monitoring the release of hippuric acid originated from the substrate hippuryl-histidyl-leucine (HHL) by the action of ACE (Figure 41): most inhibitory peptides are able to inhibit the enzyme activity thus leading to a lower release of hippuric acid.

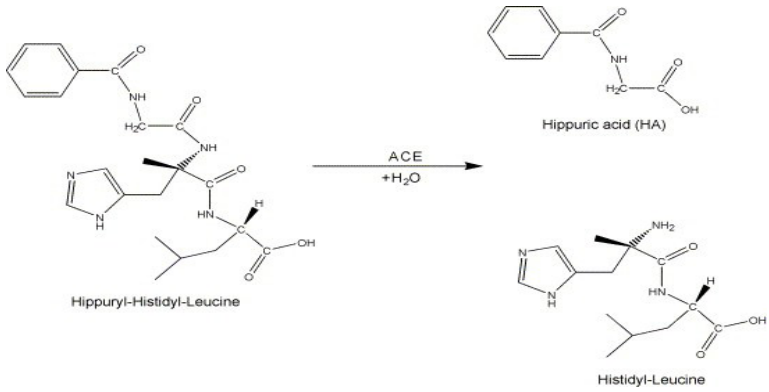

Figure 40. Release of hippuric acid from Hippuryl-Histidyl-Leucine by enzyme action of Angiotensin-Converting-Enzyme (ACE).

Generally the result are expressed as IC_{50}, that is the amount of peptide mixture required to inhibit of 50% the activity of the enzyme ACE or as the percentage of inhibition generated taking in account the maximum of activity of ACE without inhibitor (ACEmax) and the minimum value generated by the presence of inhibitor (Ace min), calculed as follow:

$$I\% = \frac{(ACEmax - Bmax) - (ACEmin - Bmin)}{(ACEmax - Bmax)} * 100$$

where Bmax is the maximum value of absorbance of the solution of the substrate (HHL), to take into account the eventual release of hyppuric acid by the auto-hydrolysis of HHL; Bmin is the solution of the

inhibitor with HHL in the absence of the enzyme again to verify the eventual release of hyppuric acid from the auto-hydrolysis of HHL or the contribution of some interfering substances; ACEmax is the maximum absorbance value in the presence of the enzyme (maximum release of hyppuric acid) and ACEmin is the minimum absorbance value obtained in the presence of the inhibitor.

4.3.8.1 Caco-27/HT29-MTX

We have tested for ACE inhibitor activity the solution coming from the apical and basolateral compartments in Caco2 experiments at the different sampling time already tested for the presence of peptides. Thus, the samples collected at 30, 60 and 120 minutes in apical and basolateral compartment were tested for ACE-inhibitory activity. The results are expressed as % of inhibition and are shown in Figure 41.

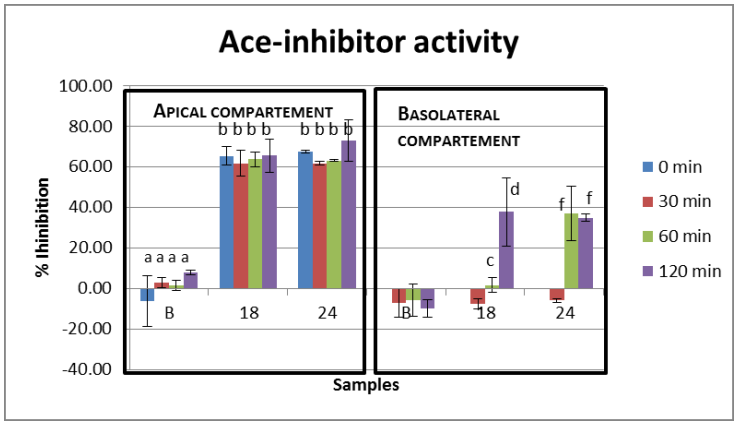

Figure 41. Ace-inhibitor activity of digested samples collected at different time (minutes) in the two compartments of a co-culture of Caco-2/HT29-MTX (statistical analysis Anova test, LSD post-hoc test, $p<0.05$).

Digesta samples at 18 and 24 months showed high ACE-inhibitory activity, with an inhibition % around 60%. Monitoring the apical compartment at 30, 60 and 120 minutes no significant changes have been measured. The most important results are about the activity in the basolateral compartment that represent the potential transfer of the activity into the blood serum. ACE inhibitor activity appears at time 60 minutes for samples aged 24 months and at 120 min for samples aged 18 months. These results indicate that peptides with ace-inhibitory activities are able to cross the Caco2 cell layer.

4.3.8.2 USSING CHAMBER

In the case of Ussing Chamber's experiments, results are much more difficult to explain, as already seen also for the evaluation of occurrence of peptides. The samples collected at 30, 60 and 120 minutes in mucosal compartment and only at 120 minutes in the serum were tested for ace-inhibitory activity. The results are expressed as % of inhibition and they are shown in Figure 42.

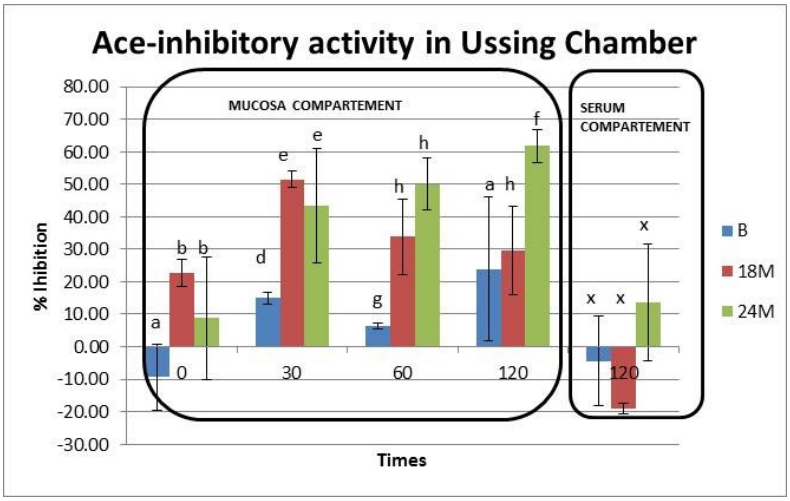

Figure 42- Ace-inhibitory activity in Ussing Chamber of digesta samples (18 and 24 months) during 0, 30, 60 and 120 minutes of test: mucosa and serum compartment (statistical analysis Anova test, LSD post-hoc test, $p<0.05$).

An ACE inhibitor activity has been measured in the mucosa compartment, but with high variability. It's possible to note that for 18 months aged sample % inhibition seems to decrease with time, whereas it seems to increase for sample at 24 months. These variations are probably complex results of uptake from the intestinal epithelia and by the enzymatic activity released by the cells.

Results in serum compartment are too much affected by a very high variability and it's not possible to draw a conclusion.

In this case, probably the use of a very complex peptide mixture didn't allow to obtain clearly understandable results.

4.3.8.3 Ace-inhibitor activity: integration in vitro and in silico approach

In order to identify new peptide sequences with ACE inhibitory activity in the dry-cured ham digesta, the in vitro experimental approach has been coupled to an in silico evaluation of the ability of peptides to interact with the active site of ACE.

4.3.8.3.1 Experimental assay

First of all, in order to identify the peptide sequences responsible for the ACE inhibitor activity found in the digested ham samples, a fractionation of the digesta by semipreparative HPLC-UV has been performed, collecting 1 fraction for each minute (starting from the minute 1) for a total of 51 fractions. All fractions have been then tested for antihypertensive activity.

The results of ACE-inhibitory test, expressed as % inhibition, are reported below in Figure 43 for 24 and 18 months, respectively.

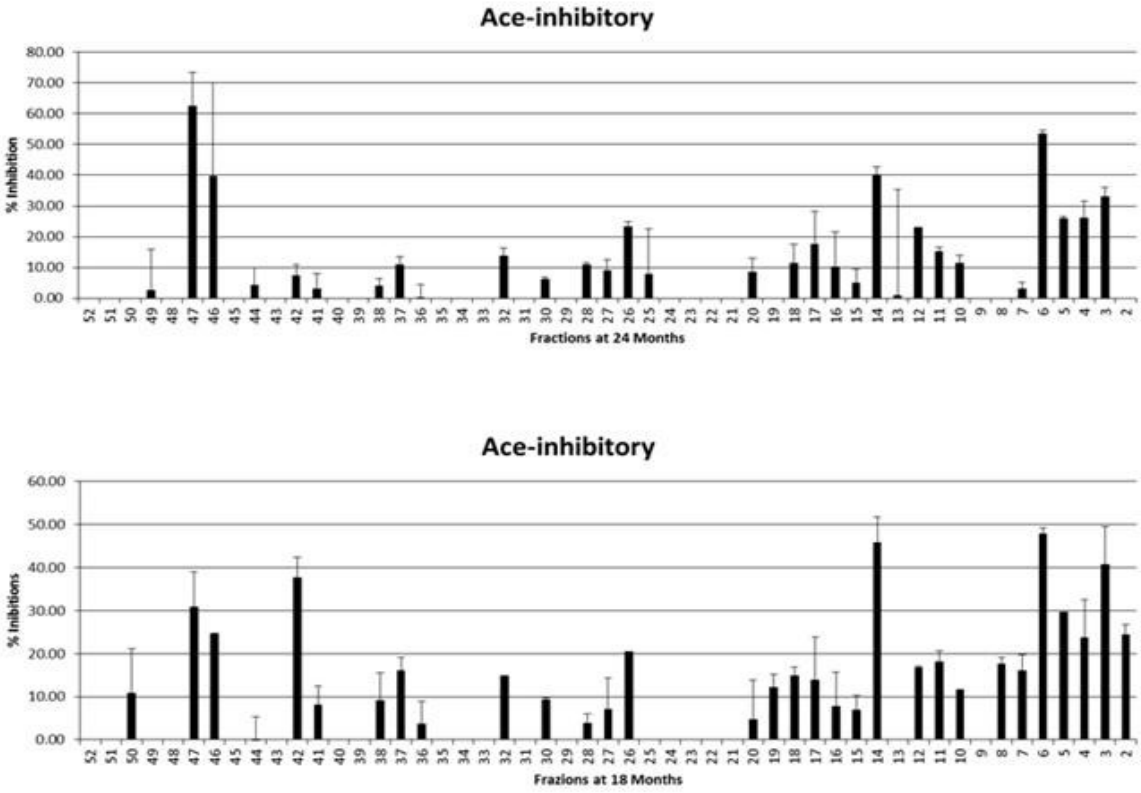

Figure 43. Ace-inhibitory activity of fractions of digested dry-cured hams at 24 and 18 months.

Several fractions have shown significant ACE inhibitor activity, the most significant being fractions number 6, 14, 46 and 47 for samples at 24 months to which also the fraction number 42 may be added for samples at 18 months. In order to identify the peptides present in these fractions, LC-MS analysis have been performed. Mostly amino acids like tyrosine, phenylalanine and some dipeptides occurred in fractions number 6 and 14. In the fractions 47, 46 and 42 peptides like RVAPE, IQLVEEELDRA, DIDDLELT, DIDSPPITAR, LKGADPEDVITGA, and GVVPL were found, as reported in Figure 44.

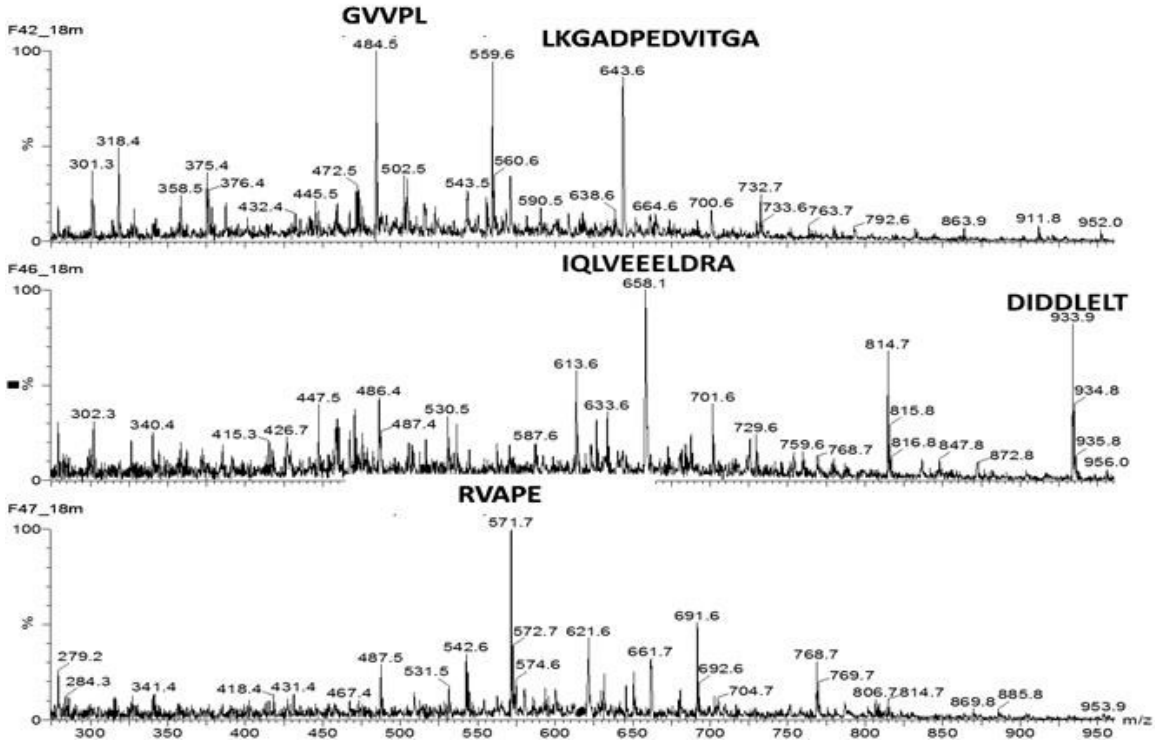

Figure 44. Mass spectrum of fractions number 42, 46 and 47 of samples at 18 months of ageing time.

4.3.8.3.2 In silico docking simulations

The computational model involves the sequential docking simulation of peptides within both the two domains of human enzyme ACE (C-domain an N-domain). It has been hypothesized that if a given molecules is predicted to be able to interact with at least one domain, it might be able to exert inhibitory effects. Therefore, the coupling of docking simulations and rescoring procedures by using the HINT scoring function– whose correlation with the free energy of binding was previously reported (Cozzini, 2002)– can be effectively used to predict the inhibitory activity. In silico analysis involved docking simulations and rescoring procedures by using the empirical scoring function HINT. HINT score provides the evaluation of thermodynamic benefits of protein-ligand interaction (Cozzini, 2002). In general, low and negative scores indicate the instability of protein-ligand complex. Therefore molecules with low and negative scores should be considered unable to interact with the target under investigation. At this stage of model development, a quantitative evaluation cannot be obtained; rather, a qualitative cut-off is provided to identify compounds virtually able to interact with the catalytic binding sites.

The simulation of the interactions between peptides and ACE has been performed using the entire peptide profile previously identified after the *in vitro* gastrointestinal digestion of 18-24 months aged dry-cured ham: solely the unambiguous sequences were considered for the analysis. Moreover, since most dipeptides were already known as active like I(L)F, GI(L), AI(L) (BIOPEP database http://www.uwm.edu.pl/biochemia/index.php/pl/biopep), they were excluded throughout. Overall, a total of 54 sequences were analyzed. The results are reported in Table 6.

Table 6. HINT scores and expected activity of peptides.

Peptide sequence	C-domain	N-domain	Expected Interaction
RVAPEEHPT	-5202	-3442	Negative
VAPEEHPT	-3113	-5273	Negative
LAPST	1046	416	Positive
FQPS	-494	308	Positive
APLNPK	-396	-4126	Negative
PGIAD	195	728	Positive
KLEGDLK	-211	-3021	Negative
YPIEH methylated	-1238	-616	Negative
TEAPLNPK	< -10000	-1859	Negative
IVAPG	267	-228	Positive
MDLER	1434	-2354	Positive
ELV	503	581	Positive
ALM *	1885	1200	Positive
NSIM *	1001	848	Positive
IIAPPER	-2734	-787	Negative
DIR	-677	-1314	Negative
IGGSI	846	283	Positive
MDLE	729	365	Positive
LQDLVDK	-1342	-2949	Negative
LTEAPLNPK	-5231	< - 10000	Negative
PSIV	688	1558	Positive
SFVTT *	1481	542	Positive
DPV	1398	1463	Positive
DLTDY	-5	-2265	Negative
NWDDMEK	-2130	-410	Negative
NVPI	400	-227	Positive
KMEGDLNEM	-3471	-2962	Negative
LTL	1030	1432	Positive
LEGI	169	847	Positive
SYELPDGQ	-2173	-2000	Negative

Peptide sequence	C-domain	N-domain	Expected Interaction
PTVE	428	1242	Positive
LGL *	2503	2285	Positive
IKAKSALA	251	-852	Positive
PEILPDGDHDLK	< -10000	< -10000	Negative
SLSTEL	-1359	492	Positive
LVL	1473	1641	Positive
VEPEILPDGDHDLK	< -10000	< -10000	Negative
TSLINTK	1094	-284	Positive
VTV	1930	1180	Positive
LKGADPEDVITGA	< -10000	-7295	Negative
DIDSPPITAR	-3137	-3436	Negative
GVVPL *	1075	-1129	Positive
INAEL	-92	101	Positive
PEILPDGDHDL	< -10000	-6684	Negative
DQIISANPL	-3516	-2260	Negative
LLASIDIDHT	< - 10000	-3236	Negative
TVKDLQHRL	-5802	< -10000	Negative
IQLVEEELDRA	< -10000	< -10000	Negative
DIDDLELT	-3807	-1487	Negative
LFDKPVSPL	-7900	-1585	Negative
RMKKNMEQTVK	< -10000	< -10000	Negative
RVAPE	-735	-1897	Negative
INTTLETKQ	-3525	-789	Negative
NAYEESLDQLETLK	< -10000	< -10000	Negative
***Note* → * indicates peptides selected for experimental trials**			

A total of 25 peptides were predicted as active and, with the exception of 3 peptides (i.e. IKAKSALA, SLSTEL and TSLINTK), most of them ranged from 3 to 5 amino acids.

4.3.8.3.3 Experimental and in silico assay: potential bioactive peptides

Among the putatively active peptides, 5 were selected for the *in vitro* evaluation of ACE inhibition, namely ALM, NSIM, SFVTT, LGL and GVVPL. The inclusion criteria were based (i) on the finding that short peptides may be easily adsorbed trough the intestinal epithelium, thus giving a greater physiological significance; (ii) on the relative abundance within the most active fraction of digested mixture; and (iii) on the computational results, with the aim to cover a reasonable high range of scores. Specifically, LGL showed highest scores for both ACE domains, GVVPL – whose relevant abundance was detected in the most active fraction – showed a putative interaction only with the C-domain, and ALM, NSIM and SFVTT

were arbitrarily selected among the remaining peptides with high scores. With respect to this, albeit a correlation between HINT scores and inhibitory activity cannot be sustained yet, since HINT scores have proved to be correlated with the free energy of binding (as aforementioned) it is reasonable to think that the selection of high scoring peptides may increase the chances to discover truly active peptides.

ALM, NSIM, SFVTT, LGL are peptides that have been find in fractions number 32, 31, 33 and 37 respectively. NSIM and SFVTT are present in fractions without any activity. ALM and LGL are present in fractions with low ACE-inhibitor activity. Only GVVPL is present in one of the most ACE-inhibitory fraction (number 42). All five peptides, after synthesis and purification, have been tested for Ace-inhibitory activity in order to verify if they actives and second to determine the $IC_{50,}$ the concentration of peptide required to inhibit 50% the ACE activity.

All peptides results actives: the in vitro experiment about ACE-inhibitory activity show the silico model adopted is useful to discover potential bioactive peptides. The results are reported in Table 7 and an example of IC_{50} curve is show in Figure 45.

Table 7. IC_{50} (μM) of peptides.

	IC_{50} (μM)
LGL	145
ALM	>1100
SFVTT	395
GVVPL	956
NSIM	>1100

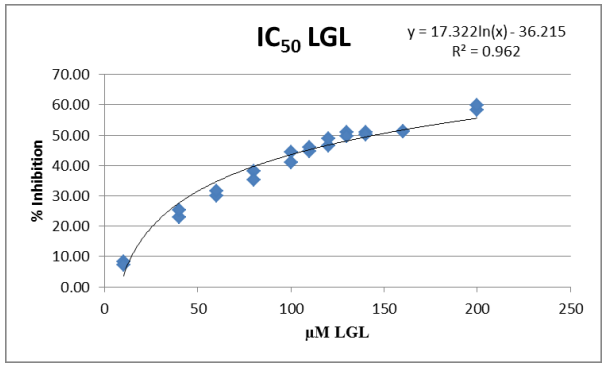

Figure 45. Curve inhibition LGL: determination of IC_{50}.

The peptide with the highest activity is LGL with an IC_{50} of 145 µM. The structure activity relationships of ACE inhibitory peptides in the literature is not clear but as reported by different papers it seem that the presence at the penultimate positions at the C-terminal end of aliphatic residues as I present in peptide our NSIM or of L and M residues at the ultimate position like peptides LGL, NSIM, ALM and GVVPL are preferred.

In the literature GL is known as an ACE-inhibitor peptide (BIOPEP database): thus, in order to verify if LGL is a true-inhibitor, the mixture LGL-ACE-HHL has been analyzed in UPLC-ESI-MS. The Extracted-ion chromatogram (XIC) are reported below, Figure 46.

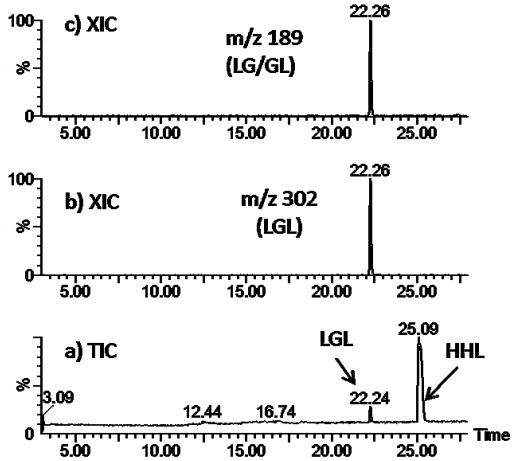

Figure 46. a) TIC of LGL in presence of ACE and HHL; b) XIC of LGL; XIC of LG/GL.

The peptide LGL is a true inhibitor as peptides like ALM, SFVTT, GVVPL but with high ace-inhibitory activity. Instead, NSIM instead seem to be a substrate due to release of dipeptide IM as shown in Figure 47.

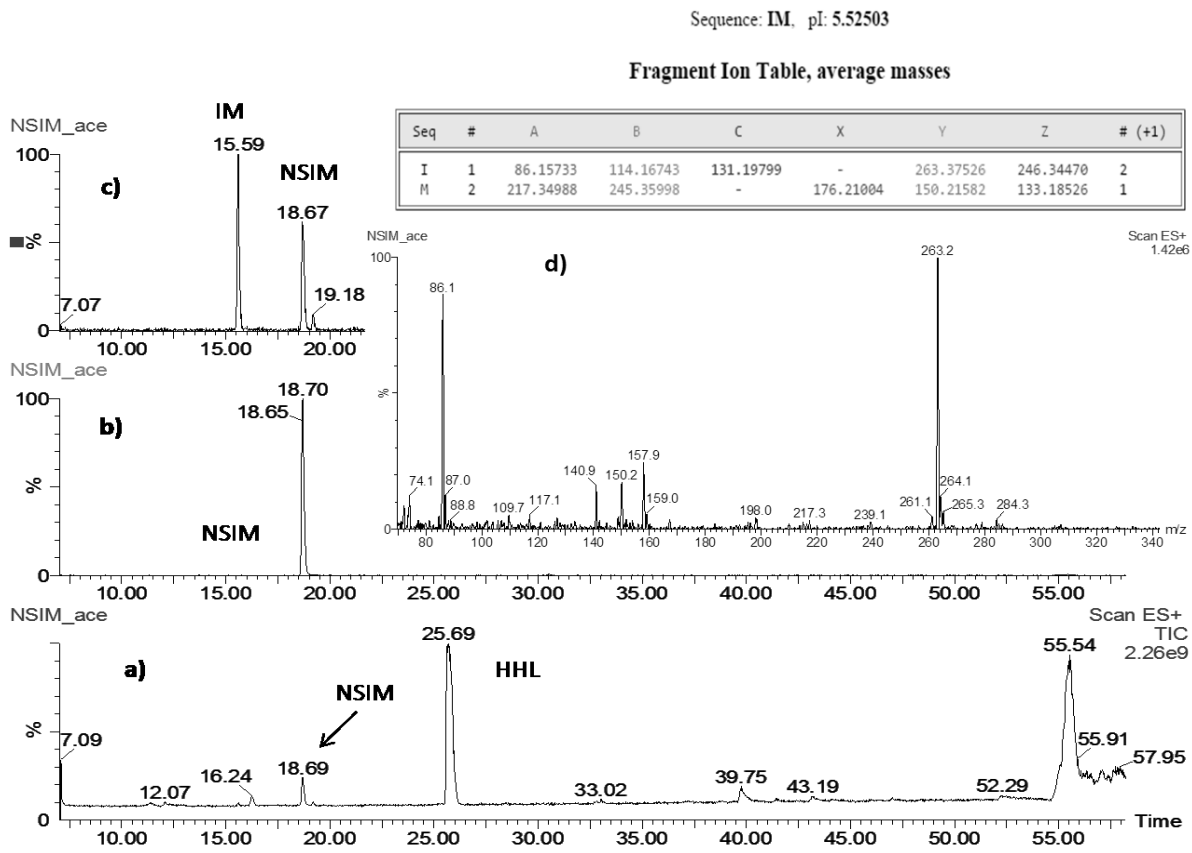

Figure 47. a) TIC of mixture NSIM, ACE, HHL; b) XIC of NSIM; c) XIC of IM; d) fragmentation in source of IM and theoretical fragmentation.

Further analysis are necessary to confirm these results and to confirm the eventually activity of IM.

It is worth noting that all these peptides have been found to be able to cross the intestinal barrier in the model Caco-2/HT29-MTX co-culture.

LGL is the peptide with the highest ace-inhibitory activity between the five peptides tested in vitro. The IC_{50} of this peptide allows to consider it a good potential bioactive peptide. In order to verify its amount in the digesta of dry-cured ham, a calibration curve of ratio LGL:PhePhe (internal standard) vs concentration from 1 to 125 µM of LGL has been done.

In digested samples at 24 and 18 months of ageing time, the LGL peptide's amount is 61,65 µg/g and 112,04 µg/g, respectively. Being the IC_{50} = 145 µM for LGL obtained by 829,255 µg of peptide, a slice of ham (about 15 g) is enough to reach this value: 13,56 g of ham at 24 months and only 7,4 g of ham at 18 months contain this amount.

4.3.8.4 NPADs ace-inhibitory activity

Also NPADs were tested for ace-inhibitory activity. No bioactivity studies have been performed until now on these compounds, apart from their well-recognized contribution to the umami taste. These compounds are interesting because they have been shown to be resistant to gastro-intestinal digestion and to proteases in blood samples and they are dipeptides that can easily cross the intestinal barrier. First of all they have evaluated by the described docking simulation.

The results are reported below, in Table 8.

Table 8. HINT scores and expected activity of peptides.

SEQUENCE	AVERAGE C-domain	AVERAGE N-domain
γGlu-Ile	-2035	382
γGlu-Leu	-2126	304
γGlu-Met	-2127	450
γGlu-Phe	-1927	484
γGlu-Trp	-1875	618
γGlu-Tyr	-1186	961
γGlu-Val	-2069	270
Lactoyl-Met	964	704
Lactoyl-Tyr	2054	1291
Lactoyl-Val	819	853
Pyroglutamil-Ile	221	353
Pyroglutamil-Pro	217	36
Pyroglutamil-Leu	326	70
Lactoyl-Ile	929	948
Lactoyl-Leu	689	852
Lactoyl-Phe	1360	566
Pyroglutamil-Phe	603	-593

All Npads have a positive response on one domain, so they could be considered potentially active, althouhgh their scores are not very high. The predict activity is tested on the three standards already synthetized and purified: γ-Glu-Phe (d5), Lactoyl-Phe (d5) and Pyro-Phe (d5).

Results in terms of IC_{50} are reported in Table 9.

Table 9. Npads IC_{50}

Npads	IC_{50} (μM)
Lacto-Phe	>1100
Pyro-Phe	Inactive
γ-Glu-Phe	>1100

The results show that Lactoyl-Phe and γ-Glu-Phe are active compounds but that their IC_{50} is >1100μM, whereas Pyro-Phe seems to be totally inactive (up to 1100 μM). These findings indicate that NPADs aren't good candidate for ace-inhibitory activity.

4.3.9 Antioxidant activity

Among the several bioactivities potentially exerted by peptides, the antioxidant is one of the most studied and a lot of paper deals with this topic in the literature. Several methods exist to study the antioxidant activity of a molecule: most methods have been developed to study the antioxidant activity of polyphenols. Owing to the fact that these compounds mainly behave as radical scavenging compounds, thus counteracting the autooxidation process of food lipids, many assays have been developed based on the evaluation of the ability of a particular molecule to quench active radicals. Thus, the famous ABTS assay or DPPH assays have been developed, which are spectrophotometric methods based on the measurement of the ability of a particular molecule to quench a chromophoric radical. Antioxidant capacity is then ranked against a proper standard antioxidant. Also, some methods have been developed which are closer to the physiological conditions in which an antioxidant molecule could encounter in vivo, such as for the protection of the peroxidation of phospholipids of cell membrane, i.e. the study of peroxidation of linoleic acid in an emulsion system that mimic the cellular membranes.

4.3.9.1 ABTS assay

The digested samples after gastro-intestinal digestion have been evaluated for their capacity to act as radical scavengers by ABTS assay. The samples tested are in particular the same obtained from co-culture and Ussing chambers. In this way, we evaluate the potential antioxidant exerted by a digested sample and its ability to be transferred into the bloodstream crossing the intestinal barrier where antioxidant activity against free radicals could be exerted.

The results expressed as % of inhibition, obtained in the apical compartment of co-culture Caco-2/HT29-MTX are reported in Figure 48.

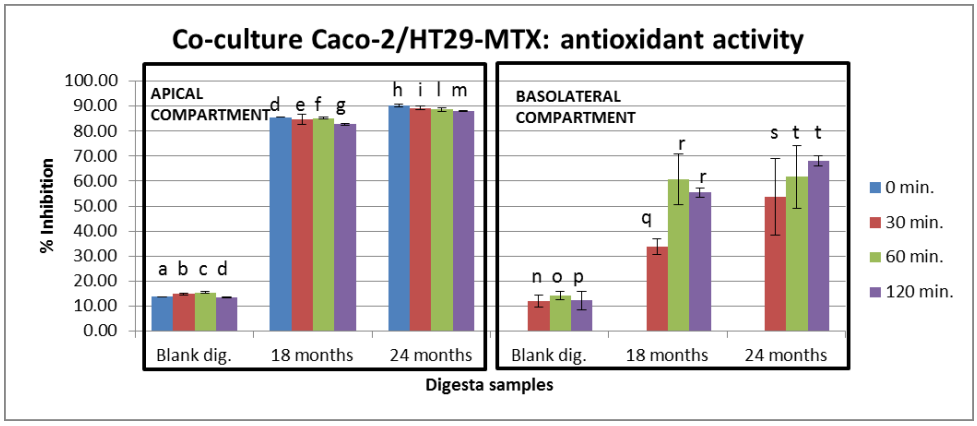

Figure 48. Antioxidant activity: ABTS assay of samples in the apical and basolateral compartments of the co-culture Caco-2/HT29-MTX (statistical analysis Anova test, LSD post-hoc test, $p<0.05$).

From the graphs, it is possible to note that the potential antioxidant of dry-cured hams after gastro-intestinal digestion is very high in the apical compartment and those samples of 18 and 24 months of ripening are characterized by similar values which did not change during the experiment. Noteworthy is the fact that antioxidant peptides are able to cross the intestinal barrier: indeed, after only 30 minutes a sharp increase of the antioxidant power in the basolateral compartment is observed, which tends to increase with time from 60 to 120 min.

Also samples obtained from Ussing Chamber have been tested with ABTS assay. The results are reported below in Figure 49.

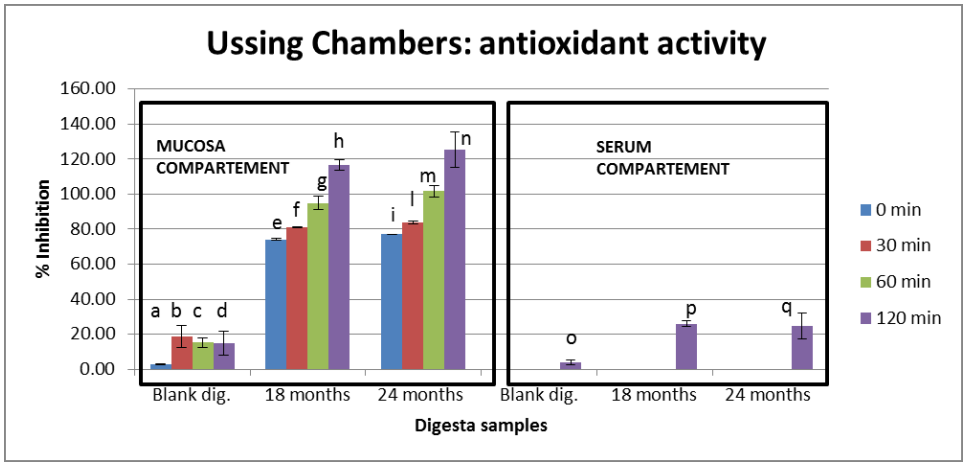

Figure 49. Antioxidant activity: ABTS assay of samples in the mucosa and in the serum compartments of Ussing (statistical analysis Anova test, LSD post-hoc test, $p<0.05$).

Again, results of these experiments are not so clearly explainable as in mucosa compartment an increase of antioxidant activity is observed, and this could be due to the activity of the living intestine cells which can release antioxidant compounds in the medium or exert some enzymatic activity which can have an influence on the recorded antioxidant values. Moreover, in the serum compartment a low increase of antioxidant activity has been measured at 120 min incubation: it could be inferred that this activity could have been generated by antioxidant peptides able to cross the pig intestinal barrier, but evidently other experiments should be performed in order to better clarify these results.

As already done in the case of the ACE experiments, the digested samples have been fractioned by semipreparative HPLC-UV, collecting 51 fractions, one for minutes in order to find out the most interesting fractions and eventually to identify the peptides able to exert this antioxidant activity.

The results expressed as % of inhibition (I%) for samples at 24 and 18 months are reported below respectively in Figure 50.

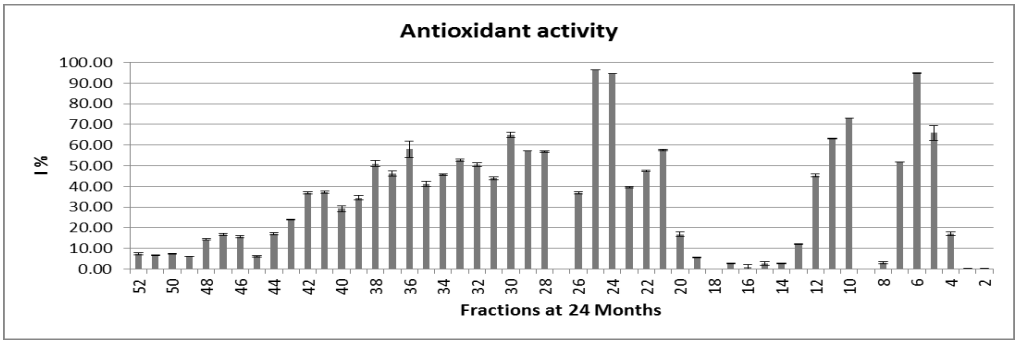

Figure 50. Antioxidant activity of LC-fractions of digested dry-cured hams at 24 months.

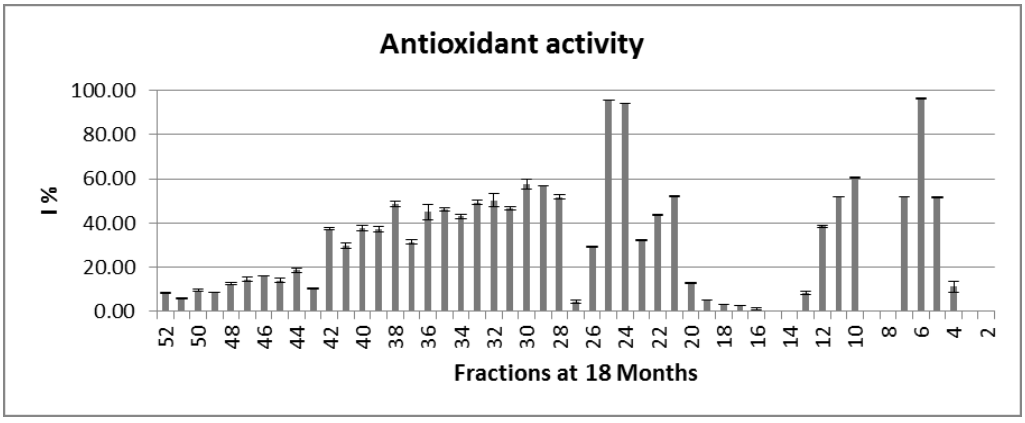

Figure 51. Antioxidant activity of LC-fractions of digested dry-cured hams at 18 months.

Most of the fractions are characterized by an antioxidant activity: in particular the most antioxidant fractions are the 6, 24, 25 both at 24 and 18 months of ripening. The antioxidant activity of these fractions seems to be mainly related to the presence of the antioxidant amino acids Histidine, Tryptophan, Tyrosine, which have been identified by LC-ESI-MS analysis of the specific fraction and which are well known antioxidant molecules (Bottesini, 2013).

4.3.10 Linoleic acid assay

The linoleic acid assay was set up according to Beker et al. (Beker, 2011). The test is aimed at evaluating the ability of antioxidant molecules to inhibit linoleic acid peroxidation within an emulsion system, which mimicks the conditions encountered in lipid cellular membranes, rich in unsaturated fatty acids such as linoleic and arachidonic acids, highly prone to peroxidation reactions.

Therefore, to simulate the lipid membrane an emulsion of linoleic acid and tween20 is prepared; then, the oxidation of linoleic acid is induced maintaining the emulsion in contact with oxygen, under constant agitation, in the presence of copper (II) (the LA/Cu(II) molar ratio = 10 with [LA]: 0.01M and [Cu(II)]:

0.001 M have themost strongest prooxidant effect) and incubated at 37° C (physiological temperature). The digested samples obtained from ham of different ageing times are then added to the emulsion in order to test the ability of the peptide mixture to counteract lipid peroxidation. Taking aliquots at regular intervals of time and testing it by the iron thiocyanate method, it's possible to quantify the increase in peroxides with time.

The determination of peroxides is performed by the iron thiocyanate colorimetric assay, which is based on the formation of red-rust complex of iron (III) thiocyanate in acidic environment, which originate from the reaction with oxygen: absorbance measurement is done at 500 nm.

The presence of peptides with antioxidant activity causes a prolongation of the lag phase and / or a decrease of the maximum speed and / or a lower accumulation of oxidized compounds.

The change in absorbance has been recorded for 27 hours: results for digested samples obtained from hames aged 18 and 24 months are shown in the graphs below, Figure 52.

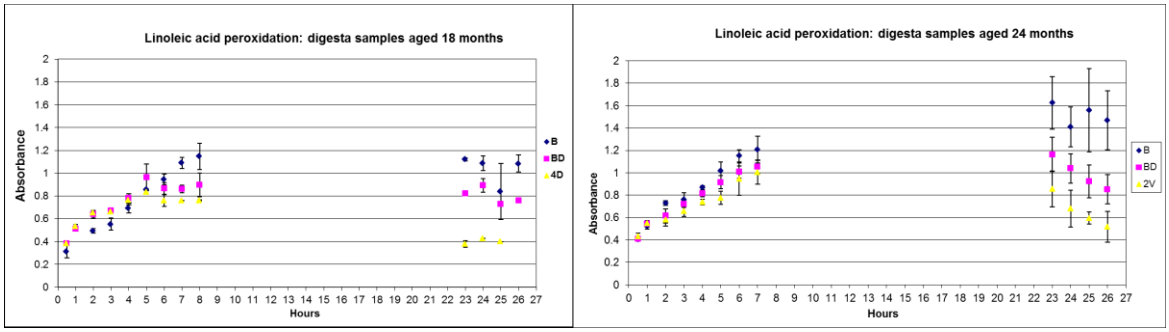

Figure 52. Antioxidant activity of digested samples at different ageing time by the linoelic acid assay: B is the blank sample (maximum of peroxidation); BD is the blank of digestion; 4D and 2V are digested dry-cured ham, aged 18 months and 24 months, respectively.

In the first eight hours there are no significant differences between samples and the blank control (blue, maximum of oxidation) and the blank of digestion (pink). Only in the second part of the test, at 24 hours, the samples seem to exert an inhibitory action against peroxide formation, thus underlying a possible antioxidant effect of the peptide mixture also in lipophilic systems. Nevertheless, the measured activity is probably low on account of the relative water solubility of most of the considered peptides.

4.4 CONCLUSIONS

The simulated gastrointestinal digestion of ham using a physiological digestion model was performed for the first time and gave rise to a complex mixtures of peptides derived mainly from myofibrillar and sarcoplasmic proteins. MWs spans from 200 to 1700 Da, with high numbers of di- and tripeptides. The peptide pattern is influenced by the proteolysis process occurring in ham during ageing, thus leading to a

different profile in 18 and 24 months aged products. Among the identified sequences, many are known to be bioactive or precursors of potential bioactive sequences, which may be qualitatively and quantitatively different in raw meat or in meat products matured for more or less time.

The bioactive substances have to be absorbed in an active form in order to exhibit physiological activity in human body. The use of models like co-culture Caco-2/HT29-MTX or Ussing chambers are useful to evaluate the ability of peptides to cross the intestinal barrier an t to evaluate their resistance to brush border peptidases. Between the peptides able to join the "bloodstream", with the help of in silico model, some peptides have been evaluated for their potential as ace-inhibitor peptides. The peptide LGL, in particular, has been characterized by a high ace-inhibitor activity: its amount in digesta is compatible to a physiologically relevant concentration to exert a bioactive function. In vivo tests are of course necessary to evaluate its real potential as antihypertensive compound.

4.5 ACKNOWLEDGEMENTS

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5 GENERAL CONCLUSIONS and PERSPECTIVES

Dry-cured ham is one of the most important traditional products in the Mediterranean area, with a strong popularity among consumers, on account of its peculiar organoleptic properties. Ham production plays a key role in the meat industry, with high market value. Ham is characterized by high content of protein with high biological value, it has a moderate caloric value and an interesting lipid profile, being also a good source of vitamins and minerals. Nevertheless, some aspects its composition could impair its nutritional value such as the high sodium content and the saturated fatty acid and cholesterol content. Consumers are increasingly aware of the aspects of health and well-being. Therefore, a new strong trend in the food industry is to take various measures at the level both of livestock husbandry as well as during production and processing to face the problems aroused by compounds with health implications, yet without compromising the quality of the finished product. On the other side, new scientific investigations are increasing about functional and bioactive components naturally occurring in foods or generated during processing with positive effects on human health. One of the most negative aspects of processed meat product is the salt content which, on the other side, is a fundamental ingredient to obtain the specific texture, color, and taste, to control the enzymatic activities (proteolysis) and to assure the microbiological safety. Generally, the reduction of salt is obtained by partial or total substitution of sodium chloride with other salts like KCl or with flavor enhancers that maintain the taste and the texture of the final product. AT present, this substitution is not allowed in high quality POD products as dry-cured hams. An alternative approach is to lower the amount of salt used by applying different salting techniques and varying the duration of the different processing steps opportunely. The reduction of 25% of sodium chloride in high quality dry-cured hams have been studied in chapter 3 , in particular taking into account the proteolysis process and the changes occurring in the peptide fraction. The results confirm that salt-reduced hams are different from the traditional ones: the long resting step necessary to obtain the microbiological safety and the low amount of salt added gave rise to several differences in terms of proteolysis and, thus, peptide fraction as well as in texture, color, organoleptic properties. Changes in the process parameters and a strict control of raw materials are probably necessary in order to obtain products more similar to the traditional ones.

In order to check for the release of bioactive peptides during ham ageing and/or human digestion, an in vitro physiological digestion model has been applied in order to characterize the released peptides, to test their ability to cross the intestinal barrier and to evaluate their bioactivity (chapter 0). The characterization with mass spectrometry of digested samples has allowed to determinate the sequences of several new peptides, generated from the digestion model. Then, following these peptides in systems like co-culture Caco-2/HT29-MTX or Ussing chamber, it has been possible to

discover the most peptides are strongly affected by brush border enzymes (peptidases) but at the same time most of them are able to cross the Caco-2 monolayer as a model of the intestinal barrier. In more complex system, such as the living excited pig intestine in Ussing chamber, the experiments must be improved in order to determine the peptides able to cross the intestinal barrier. Several bio-peptides with antioxidant and antihypertensive activity were found in the digesta: biopeptides are able to transfer these bioactivities into the bloodstream. Among the several bioactive sequences identified, the peptide LGL has been found to be characterized by a significant ace-inhibitory activity (IC_{50}=145 μM): its quantification in samples have shown its occurrence at a physiologically relevant concentration already in a slice of ham. In vivo tests are nevertheless needed in order to definitely prove the bioactivity of these compounds.

6 SUMMARY

Among the different types of dry-cured meat products, dry-cured hams are the most appreciated by consumers. Dry-cured ham is produced by pork legs properly worked and salted during a relatively complex process and a long maturation and ageing period. The most important changes during ageing are consequences of biochemical (enzymatic) and chemical reactions: in particular, proteolysis and lipolysis, as the effects of the action of endogenous enzymes characteristic of the muscle such as proteases and lipases on proteins and lipids are the most relevant (Toldrá, 1998a). The speed of these reactions depend largely on the process conditions (temperature, relative humidity and time) (and, especially, on the salting process (amount and type of added salt (Toldrà, 2006). Proteolysis generates a considerable amount of small peptides and free amino acids, accumulating during ageing, through the breaking of myofibrillar proteins (such as myosin, actin, troponin, tropomyosin) by endoproteases (calpains and cathepsins) and, subsequently, by exoprotease (peptidase and aminopeptidase). Recent innovations in the meat industry are addressed towards the production of healthier meats and processed meats. Recent innovations in the meat industry are addressed towards the production of healthier meats and processed meats. The strategies are based on either reducing the content or substitute unhealthy substances like salt. Indeed, sodium chloride has been linked to hypertension and to an increased risk of cardiovascular disease (Morgan, 2011): the salt intake has serious public health implications nowadays. The reduction of salt in hams is not easy task. Salt content affects the microbiological growth, the proteolysis, the texture of the product, the development of the aroma and flavor, the color. To establish strategies for optimizing the enzyme reactions towards optimal quality of the products, and so to benefit the production and economy related to this important sector, the optimization of the proteomic strategy is crucial in the identification of key peptides acting as biomarkers to control and direct the industrial processes for dry-cured ham. The peptide fraction of traditional ham has been investigated in order to understand the differences between traditional hams and salt-reduced dry-cured hams obtained reducing sodium chloride by 25% (but without use of substitutes), with the final aim of identifying the changes in the production processing necessary to obtain healthier products but similar to the traditional ones in terms of other qualitative characteristics. The main peptides characterizing all phases of production (from salting to ripening step) of hams have been identified by mass spectrometry coupled to chromatographic and electrophoretic separations. Among the identified peptides there are 13 compounds that are aminoacyl derivatives of non proteolityc origins (Npads): 3 NPADs have been synthesized, purified and quantified.

The results obtained from principal component analysis, from the elaboration of the chromatograms, show that reduced-salt and traditional hams are different: the lower value of salt could increase the activity of proteolytic enzymes, could decrease the extraction of myofibrillar proteins. The longer period of rest,

necessary to obtain the microbiological safety, generated hams with an increased proteolysis index, more "gummy" texture, with a peptide profile richer in peptide with low molecular weight. To obtain a ham with the characteristics more similar to the traditional ones, it will be necessary to change the process parameters (as temperature, activity waters, humidity, etc) and to strictly control the raw materials.

If the excessive consumption of meat products is indicated as a risk factor for certain diseases, it is equally true that proteolysis is able not only to positively influence the digestibility of the products, but also to generate a pool of peptides that might be characterized by interesting biological activities, which may potentially counteract these negative effects. The bioactive peptides can be naturally present in the product or may be released during the gastro-intestinal digestion step but they can exert their biological functions if they are able to join the bloodstream (chapter 0). For this reason, first an in vitro physiological digestion model has been applied in order to characterize the peptides released upon digestion of traditional hams of different ageing time, thus elucidating the effect of proteolysis during maturation on the peptide profile generated by digestion. The sequences characterized with mass spectrometry are numerous and most of them are not present in the literature: the in vitro physiological digestion method is never been applied to dry-cured hams. Secondly, the peptide transport across the intestinal barrier and their resistance to intestinal peptidases have been studied: to this purpose models such as co-culture of Caco-2/HT29-MTX and an ex-vivo technique such as Ussing Chambers have been applied. These models are very popular for studies of absorption of nutrients, bioactive diet components, and drugs. The results obtained show that the majority of peptides are strongly hydrolyzed by brush border peptidases, in particular in the more physiological system as Ussing chamber, but also that they are able to cross the Caco-2 cell monolayer. These good results allowed to investigate the digested samples for their potential to exert antioxidant and ace-inhibitory activities, the bio-functions more studied in the last years. The results show that traditional hams possess bioactive peptides able to join intact the blood circulation. With the help of fractioning experiments and in silico model, it has been found that aminoacids like tryptophan, tyrosine and fenilalaniline are the main responsible of antioxidant activity. Instead, for ace-inhibitory activity several peptide sequences were identified. 5 peptides (LGL, NSIM, ALM, SFVTT, GVVPL) have been synthesized, purified and tested in order to determine their IC_{50} value: the peptide LGL have been found to possess a significant ace-inhibitory activity (IC_{50}=145μM), to be a true-inhibitor of Angiotensin-Converting-enzyme (Ace) and its quantification allowed to determine that its amount in a slice of ham may be sufficient to reach a physiologically relevant concentration. Also three Npads have been tested for the first time for this activity but, unfortunately, one is completely inactive (pyro-Phe (D5)) and the others two (Glu-Phe (D5), Lacto-Phe) have an $IC_{50,}$ although they were good candidate as bioactive peptides due to their resistance to the digestion process and someone also maybe to brush border peptidases. Among the identified sequences present there are RVAPEEHPT, VAPEEHPT, and LFDKPVSPL have not been tested in the literature for Ace-inhibitor

activity. Instead, IGGSI have not shown any ace-inhibitory activity. Peptides like AI(L), GI(L), I(L)F, LVL, instead, are ace-inhibitor sequences known in the literature (BIOPEP online database). Finally, the digested samples have shown also their potential to exert an antioxidant activity as radical scavengers and against peroxides.

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7 CURRICULUM VITAE

PERSONAL INFORMATION Sara Paolella

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Sex Female | Date of birth 03/1986 | Nationality Italian

POSITION **Research fellow - Food Science (CHIM/10)**

WORK EXPERIENCE

01/04/2014–Present **Research fellow- Food Science (CHIM/10)**

Parma University, Parma (Italy)

Study of the evolution of the proteolytic phenomenon in dry-cured hams as a function of the technological process (with particular emphasis on the possible technological solution aimed at reducing salt content) and on the evaluation of the functional properties linked to the occurrence and possible formation of bioactive peptides (in particular, antioxidant and antihypertensive activity).

01/01/2012–31/12/2014 **PhD student in Food Science and Technology**

Parma University, Parma (Italy)

Proteolysis in dry-cured hams: salt reduction and bioactive peptides.

Study of the evolution of the proteolytic phenomenon in dry-cured hams as a function of the technological process (with particular emphasis on the possible technological solution aimed at reducing salt content) and on the evaluation of the functional properties linked to the occurrence and possible formation of bioactive peptides (in particular, antioxidant and antihypertensive activity).

May-July 2014: the study of peptides trasfers has been performed abroad in **France**, in the laboratory of URAFPA , Unité de Recherche «Animal & Fonctionnalités des Produits Animaux» Équipe «Protéolyse & Biofonctionnalités des Protéines et des Peptides», Université de Lorraine, Vandroeuvre-les-Nancy, France

01/12/2011–31/05/2012 **Scholarship holder- Parma university**

ISMAC-CNR, Biella (Italy)

Identification and quantification of different species in animal fibres by LC/ESI-MS analysis of keratin-derived proteolytic peptides.

27/01/2011–26/04/2011 **Scholarship holder**

Department of Organic and Industrial Chemistry (University of Parma), Parma (Italy)

Determination of proteolytic and non-proteolytic peptides in Parmigiano-Reggiano. Determination of amino acids in protein hydrolysates originating from poultry products

by HPLC-FLD analysis. Identification of peptides for the discrimination of animal fibers by UPLC-ESI-MS.

08/2004 **Internship**

Technical office Local Health Unit
11100 Aosta (Italy)

Creation archive documents.

07/2000 **Forest sector: internship**

Valle D'Aosta
11100 Aosta (Italy)

Protection forest land.

EDUCATION AND TRAINING

01/01/2012–31/12/2014 **PhD student in Food Science and Technology**

Department of Food Science- Parma University, Parma (Italy)

- Study of peptides, proteins in dry-cured hams
- Nitrogen fraction characterization using Kjeldahl, chromatographic techniques (LC), UV, fluorescence, mass spectrometry, MS/MS
- Bottom-up proteomic: mono- and bidimensional electrophoresis, in-gel digestion, MS/MS
- Simulated gastrointestinal digestion of dry-cured and characterization of the peptides generated
- Solid phase and liquid peptide synthesis of peptides of interest in food
- Study of transport of peptides in systems like co-culture Caco-2/H29-MTX and Ussing Chamber
- Bioactive peptides: antioxidant and ace-inhibitory peptides

2008–2010 **Master Degree in Food Science and Technology (106/110)**

Faculty of Agriculture, Parma (Italy)

Master thesis degree of six months: "Characterization of the Parmigiano-Reggiano soluble fraction and study of bioaccessibility using simulated gastrointestinal digestion" in the Department of Organic and Industrial Chemistry - University of Parma.

- Chemistry, biochemistry and nutrition applied to the food sector
- Food microbiology
- Food businesses management
- Food products and processes design

2005–2008 **Bachelor Degree in Food Science and Technology (102/110)**

Faculty of Agriculture, Parma (Italy)

Bachelor thesis of three months: "Determination of free amino acids in complex food matrices: comparison of extraction, chromatographic and detection methods" at the Department of Organic and Industrial Chemistry, University of Parma. .

- Food chemistry,
- Food microbiology
- Food technologies
- Food nutrition

2000–2005 **High School diploma: scientific Lyceum (80/100)**

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- Chemical-biological science
- Physics, mathematics
- History, philosophy, latin
- Italian, French, English

PERSONAL SKILLS

Mother tongue(s): Italian

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
French	B1	B2	B1	B1	B1
English	B1	B1	A2	A2	A2

Levels: A1/A2: Basic user - B1/B2: Independent user - C1/C2: Proficient user
Common European Framework of Reference for Languages

Communication skills

Team spirit gained from scouting and football activities.

Good ability to adapt to multicultural environments gained university experience.

Laboratory of food chemistry for the bachelor degree in Food Science and Technology

Organisational / managerial skills

Laboratory activity correlator of master degree and bachelor thesis in food science and technology.

Job-related skills

- Liquid chromatography techniques
- Mass spectrometry and MS/MS, UV, FLD

- Protein electrophoresis
- Antioxidant activity
- Ace-inhibitory activity
- Peptides and peptide-derivatives synthesis
- UV-Vis spectrophotometry

Computer skills

- Good command of Microsoft Office™ (Word™, Excel™ e PowerPoint™);
- Software for chromatographic data acquisition (MassLynx, Empower) and spectrophotometric data acquisition (LambdaBio)

Driving licence B

ADDITIONAL INFORMATION

Publications

- Sara Paolella, Falavigna Claudia, Faccini Andrea, Virgili Roberta, Sforza Stefano, Dall'Asta Chiara, Arnaldo Dossena, Galaverna Gianni (2014). Effect of dry-cured ham maturation time on simulated gastrointestinal digestion: Characterization of the released peptide fraction, Food Research International, 2014
- Claudia Vineis, Cinzia Tonetti, Sara Paolella, PierDavide Pozzo and Stefano Sforza,A UPLC/ESI–MS method for identifying wool, cashmere and yak fibres, Textile Research Journal, 2013
- Chiara Bottesini, Sara Paolella, Francesca Lambertini, Gianni Galaverna, Tullia Tedeschi, Arnaldo Dossena,Rosangela Marchelli, and Stefano Sforza,Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese, Int J Food Sci Nutr, 2013
- Sara Paolella, Mariangela Bencivenni, Francesca Lambertini, Barbara Prandi, Andrea Faccini, Cinzia Tonetti, Claudia Vineis and Stefano Sforza,Identification and quantification of different species in animal fibres by LC/ESI-MS analysis of keratin-derived proteolytic peptides, J. Mass Spectrom. 2013, 48, 919–926
- Claudia Alessandri, Stefano Sforza, Paola Palazzo, Francesca Lambertini, Sara Paolella, Danila Zennaro, Chiara Rafaiani, Rosetta Ferrara, Maria Livia Bernardi, Mario Santoro, Sara Zuzzi,Ivana Giangrieco, Arnaldo Dossena, Adriano Mari,Tolerability of a Fully Maturated Cheese in Cow's Milk Allergic Children: Biochemical, Immunochemical, and Clinical Aspects, PLoS ONE,July 2012, Volume 7, Issue 7, e40945.

Conferences

- Sara Paolella, Characterization of bioactive peptides in dry-cured ham , PhD worshop 2014, 19th Workshop on the Developments in the Italian PhD research on Food Science and Technology and Biotechnology, 24-26 Settembre 2014, Bari.
- Gianni Galaverna, Sara Paolella, Claudia Falavigna, Roberta Virgili, Chiara Dall'Asta, Arnaldo Dossena,BIOACTIVE PEPTIDES GENERATED BY IN VITRO GASTROINTESTINAL DIGESTION OF DRY-CURED HAM, 6th International Symposium on Recent Advances in Food Analysis, 45, November 5–8, 2013, Prague, Czech Republic.

- Sara Paolella, Characterization and bioactivity of the peptide fraction of dry-cured ham as a function of production technologies, PhD workshop 2013, XVIII Workshop on the Developments in the Italian PhD research on Food Science and Technology and Biotechnology, 25-27 Settembre 2013, Conegliano.
- Sara Paolella,Claudia Falavigna, Iulia Pantea, Arnaldo Dossena, Roberta Virgili, Gianni Galaverna,BIOACTIVE PEPTIDES GENERATED BY IN VITRO DIGESTION OF DRY-CURED HAM: EFFECT OF MATURATION TIME AND SALT CONTENT, EuroFoodChem XVII, Book of Abstracts,May 07-10,2013, Istanbul (Turkey).
- S. Sforza, C. Bottesini, S. Paolella, F. Lambertini, G. Galaverna, M. Nocetti, A. Dossena, R. Marchelli, ANTIOXIDANT ACTIVITY OF PARMIGIANO-REGGIANO CHEESES AT DIFFERENTAGEING TIMES, Summilk, IDF World Dairy Summit, Parma, 15-19 ottobre 2011, Final Programme, Nutrition and Health: Dairy products, a healthy choice, p58.
- S. Sforza, F. Lambertini, M. Bencivenni, S. Paolella, A. Dossena, SHEEP CHEESES AND CASHMERE PULLOVERS: MS-CERTIFIED AUTHENTICITY THROUGH PEPTIDE ANALYSIS,XXIV Congresso Nazionale della Società Chimica Italiana, Lecce, 11-16 settembre 2011, DSM-OR-03, p1170.
- C. Bottesini, S. Paolella, F. Lambertini, G. Galaverna, A. Dossena, R. Marchelli, S. Sforza ANTIOXIDANT ACTIVITY OF THE PEPTIDE FRACTION FROM PARMIGIANO-REGGIANO AT DIFFERENT AGEING TIMES, EuroFoodChem XVI Polish Journal of Food and Nutrition Sciences,Conference Proceeding, Gdansk 6-8 luglio 2011.
- P. Palazzo, S. Sforza, C. Alessandri, F. Lambertini, S. Paolella, M. Liso, D. Zennaro, A. Dossena, A.Mari, MILK ALLERGEN DETECTION IN ORIGINAL PARMIGIANO-REGGIANO CHEESE USING ISAC INHIBITION ASSAY (SPHIAa), 30th Congress of the European Academy og allergy and Clinical Immunology, Instanbul, Turkey, 11-15 giugno 2011.

Honours and awards

Winner of the third award of Trophelia 2008: award for food innovation in Italy and Europe with the project "Edible container of surprises for chocolate eggs." Award obtained with the team FIAMS STA, Faculty of Agriculture, University of Parma

Competition organized by Trophelia-Federalimentare, May 8, 2008