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Expression of genes involved in meat tenderization of bovine and caprine muscles

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“... fatti non foste a viver come bruti, ma per seguir virtute e canoscenza …"

(Dante Alighieri, Divina Commedia, Inferno, Canto XXVI)

Abstract

From a technological and commercial perspective, the scope of meat quality takes into consideration several aspects including colour, pH, water losses, tenderness, chemical composition, and hygienic conditions. Among the sensory aspects, meat tenderness is the most important eating factor that influences consumer's preference.

Meat tenderness is related to many animal characteristics, such as breed, age, gender, feeding and management, but also to specific properties of the muscle, such as the collagen and fat content, the metabolic and fibre type, the myofibrillar structure, the pH, etc. The myofibrillar structure of the muscle and the destructuration that occurs during meat ageing, seem to be the most important factors that affect meat tenderization. The degradation of the muscle is the result of the actions of many enzymatic and protein systems of the muscle cell, e.g. calpains/calpastatin, cathepsins, proteasome, caspases and heat shock proteins.

Calpain-1 and 2 are calcium-dependent proteases and calpastatin is their competitive inhibitor. Calpain/calpastatin system is considered the most important enzymatic system involved in degradation of myofibrillar structure of the muscle and the calpain/calpastatin ratio is considered as the best predictor of meat tenderness.

More recently, apoptosis has been recognized to cause the death of muscle cell during the slaughter of the animal. Caspases are the effectors of apoptosis, and in particular, caspase 3 and 9 are involved in the intrinsic pathway of apoptosis that is the most important in the muscle cell. Caspase 3 is an effector caspase, which can degrade many proteins in the sarcoplasm, while caspase 9 is an initiator enzyme, which can activate caspase 3. Based on these roles, caspases are investigated in relation to their possible involvement in meat tenderization process, as apoptosis effectors and/or as proteolytic enzymes.

Heat shock proteins (Hsp) comprise factors that are constitutively expressed and are involved in protein assembly and transport, folding and unfolding, and in the refolding of damaged proteins. Hsp include also resistance factors that are expressed in cells subjected to various types of stress. For their protective function on myofibrillar proteins and their anti-apoptotic properties, Hsp are also taken into account for a possible role in meat tenderization. AlphaB-crystallin and Hsp27 are small Hsp that can inhibit the apoptosis pathways, acting on various actors of the death chains.

Furthermore, αB-crystallin is involved in the protection of myofibrillar proteins, like desmin and titin, acting against their denaturation and degradation. Hsp27 is also involved in the stabilization of myofibrillar proteins and in particular, it regulates polymerization and protect actin filaments, which constitute the base of cell muscle structure. Hsp40 is a co-chaperon of the large Hsp70 and it assists the latter in the folding and compartmentalization of nascent proteins. Moreover, the complex Hsp70-40 have been identified to directly inhibit the intrinsic and extrinsic apoptotic pathways and Hsp70 is known to have a cellular role in resistance against heat stress.

In the present thesis, we made an attempt at investigating the role of calpain/calpastatin system, some caspases and Hsp proteins in meat tenderization process in different experimental conditions: two different muscle types in the bovine species, the same muscle (*Longissimus dorsi*, LD), in two different bovine species and the same muscle (LD) in animals of different ages, in caprine species.

In general, we confirmed the primary role of the calpain/calpastatin system in meat tenderization process, showing its positive relatedness to meat tenderness in the experiments conducted. In particular, in mature animals, higher expression levels (both at mRNA and protein level) and activity of calpain-1 and lower levels of calpastatin mRNA are associated with higher levels of tenderness. Still before, the calpain system confirmed its involvement in the turnover of the muscle proteins, with a degradative action, being the calpain-1 and 2 less expressed, at least at mRNA level, in the phase of higher muscle growth in young animals. This degradative function is the basis of the role that this enzymatic system has at the *post-mortem*, during the muscle to meat transformation.

Regarding caspases, our results confirmed the action of these proteases in early *post-mortem*, as characteristic of the apoptotic process, but seems to negate a fundamental importance of these enzymes in meat tenderization. In fact, even though we confirmed the expression of caspases 3/9 mRNAs and the activity of caspase 3 in *post-mortem* period, it seems these facts were not in relation with the different levels of tenderness in muscles. Moreover, we observed that caspase 3 is active only in the early *post-mortem* (the first few hours after slaughter) and at lower levels compared to calpain-1, which confirms its primary role in meat tenderization. However, similarly to calpains, during the phase of great muscle growth in young animals, caspase 3 mRNA seems to be espressed at a lower level probably because of the necessity of a low degradation of myofibrillar proteins in this phase of life.

With respect to Hsp, we hightlighted a correlation between αB-crystallin, Hsp27 and Hsp70 and meat tenderization; instead, on the contrary of some other studies, Hsp40 seems to be not related to the process. The expression (at least at mRNA level) of Hsp27, Hsp70 and αB-crystallin are in relation with meat tenderness, showing that higher expression levels of these Hsp correspond to higher levels of toughness of meat. Basing on their functions, high grades of protection of myofibrillar proteins and/or high levels of inhibition of apoptosis lead to a slowing-down of the maturation process of the meat, producing tougher meat. Furthermore, in mature animals, this process of myofibrillar proteins protection seems to overhang the degradative potential of calpains. We observed a growing level of Hsp with age, which could be due to a progressive adaptation of animal to environmental stresses, probably in addition to a physiological hormonal change. Hsp70 has also confirmed its importance in thermoprotection of muscle cells.

In conclusion, in this thesis, we confirmed the importance of calpain/calpastatin system in meat tenderization process, underlining the lesser importance of caspases. Regarding αB-crystallin, Hsp27 and 70, we pointed out the relationship of their higher presence with lower tenderness of meat, suggesting their possible utilization as new, negative markers of tenderness.

Table of contents

General introduction

Meat is the principal source of proteins in the diet of the developed countries and the tenderness is the most important factor that influence consumer satisfaction for meat palatability (Miller *et al*., 2001).

In this thesis, I wanted to address the argument of meat tenderness, considering some factors that can condition this aspect of meat quality. In particular, I focalized the study on the expression of some genes which can influence meat tenderness.

In this introduction, I considered first the structure and ultrastructure of the muscle and its metabolism, and then the enzymatic systems and the proteins that can be involved in meat tenderization process.

After this introduction, I presented the experimental part of the thesis, with some general conclusions.

Muscle composition

Muscle cells are among the most highly organized cells in the animal body and perform a varied array of mechanical functions. The organization, structure and metabolism of the muscle are key to its function and to the maintenance of its integrity, both during contraction and during the early *post-mortem* period. Ultimately, these *post-mortem* changes will influence the suitability of meat for further processing.

Essentially, meat is composed of water, proteins, lipids, minerals and carbohydrates. Mammalian muscle tissue contains approximately 75% water, 18.5% protein, 3% lipid, 1% carbohydrate, 1.7% non-protein nitrogenous substances and 0.85% other non-protein substances (minerals, vitamins, etc.; U.S. Department of Agriculture, 2008).

Muscle proteins

Proteins constitute the major compounds in the muscle, approximately 16-22%, and have important roles for the structure, normal function and integrity of the muscle.

There are three main groups of proteins in the muscle: myofibrillar proteins, sarcoplasmic proteins and connective tissue proteins.

Myofibrillar proteins are primarily associated with the contractile organelles, the myofibrils. This class of proteins includes both the proteins directly involved in movement (contractile

proteins) and proteins that regulate the interactions between the contractile proteins (regulatory proteins). **Sarcoplasmic proteins** are soluble proteins that include proteins involved in cellular signaling processes and enzymes important in metabolism and in protein degradation/cellular remodeling. **Connective tissue proteins** constitute the main stromal proteins in connective tissue.

Proteins undergo important changes during the conversion of muscle to meat, that mainly affect tenderness; additional changes occur during further processing, through the generation of peptides and free amino acids as a result of the proteolytic enzymatic chain.

Myofibrillar proteins

Myofibrillar proteins make up approximately 50-60% of the total extractable muscle proteins. These proteins are responsible for the basic myofibrillar structure, and thus they contribute to the continuity and strength of the muscle fibres. **Myosin** and **actin** are by far the most abundant myofibrillar proteins and they form part of the structural backbone of the myofibril. **Tropomyosin** and **troponins** C, T, and I are considered as regulatory proteins because they play an important role in muscle contraction and relaxation (Pearson, 1987). There are many proteins in the Z line region of the sarcomere (see below), although in a low percentage, that serve as bridges between the thin filaments of adjacent sarcomeres. **Titin** and **nebulin** are two very large proteins, present in a significant proportion, that are located in the void space between the filaments and the Z-line and contribute to the integrity of the muscle cells (Robson *et al*., 1997). **Desmin** is located on the external area of the Z-line and connects adjacent myofibrils.

Sarcoplasmic proteins

These are water-soluble proteins, comprising about 30–35% of the total protein in muscle. Sarcoplasmic proteins contain a high diversity of proteins, mainly metabolic enzymes (mitochondrial, lysosomal, microsomal, nuclear, or free in the cytosol) and myoglobin. Some of these enzymes play a very important role in *post-mortem* meat and during further processing. Minor amounts of hemoglobin may be found in the muscle if blood has not been drained properly during slaughter. **Myoglobin** is the main sarcoplasmic protein, responsible for the red meat colour of meat as well as the typical pink colour of drippings. The amount of myoglobin depends on many factors. Red fibres contain higher amounts of myoglobin than white fibres (see below). The species is very important, and thus beef and lamb contain more

myoglobin than pork and poultry. For a given species, the myoglobin content in the muscle increases with the age of the animal (Lawrie & Ledward, 2006).

Connective tissue proteins

Collagen, reticulin, and elastin constitute the main stromal proteins in connective tissue. The total **collagen** content in bovine muscles varies from 1% to 15% of dry weight, while elastin is a smaller component, varying from 0.6% to 3.7% (Bendall, 1967). **Reticulin** is secreted by reticular cells; it forms fibres that crosslink to form a fine network that acts as a supporting in tissue. **Elastin** is found in lower amounts, usually in capillaries, tendons, nerves and ligaments; it allows the tissue to resume the shape after stretching or contracting.

Collagen, the most abundant mammalian protein, is a connective tissue constituent that is present in all tissues. There are several types of collagen (I, III, IV, V, VI, XII, and XIV) containing different polypeptide chains (Listrat *et al.*, 1999, 2000; Nishimura *et al.*, 1997). The major types of collagen in skeletal muscle are type I and III (Bailey & Light, 1989). Type I collagen is the major component of the epimysium and perimysium that surround the muscles (see below). Types III, IV, and V collagen are found in the endomysium, which provides support to the muscle fibres (Eskin, 1990). There is a high number of cross-linkages in the collagen fibres, that increase with age and this is why meat is tougher in older animals (Purslow, 2005).

Muscle structure

The structure is important for the properties of the muscle and its changes during *post-mortem* events influence the quality properties of the meat. The muscle structure of meat is similar in all species. Although meat includes a number of different tissues, the majority is skeletal muscle tissue.

The muscle mass is elongated and covered by a connective tissue termed the **epimysium**, which binds both individual fibre bundles and groups of muscle bundles together (Hulting, 1976) (Figure 1). It tapers into a tendon that connects the muscle to the skeletal structure. The epimysium is often thick, tough and resistant to both shear and solubilization. However, it is easily (and usually) separated from cuts of meat and is generally not considered a factor in meat quality. The muscle is subdivided into bundles or groupings of muscle cells. The three-dimensional collagen network that surrounds large and small bundles of muscle fibre and

contains intramuscular lipid deposits and vasculature is the **perimysium**. The layer of connective tissue encircling each muscle fibre (cell) and overlying the basement membrane is the **endomysium**.

The intramuscular connective tissue is, thus, the combined peri- and endomysium depots, but the vast bulk of it, about 90%, is perimysium. The perimysium is thought to play the major role in determining meat texture differences that are related to connective tissue (Light *et al*., 1985). The role of endomysium in meat texture is less well understood.

The high tensile strength of collagen as well as its mechanical stability are conferred by the amount and type of covalent **crosslinks** linking individual collagen molecules and fibrils together. With maturation, the number of crosslinks increases (Kovanen & Suominen, 1989). Mature crosslinks and collagen concentration have an additive effect on the toughening of meat and textural differences are due largely to increase in mature crosslinking (McCormick, 1999).

The **sarcolemma** (the cell membrane of the muscular cell) retains the **sarcoplasmic fluid**, which bathe each muscle fibre and contains sarcoplasmatic proteins. In any muscular cell there are around 1000 **myofibrils** (they vary in number with type of muscle, from few hundreds to over a million per cell). Each muscle fibre is a single large multinucleate cell. The cytoplasm in these cells contains myofibrils, 2-3 μm thick, that can extend over the full length of the muscle fibre.

The streak of the muscle fibres is characteristic of skeletal muscle. It results from the regular arrangement of molecules of different density (Figure 1). The repeating contractile units, the **sarcomeres** (1.5-2.5 μm in length, depending on the state of muscle contraction) are bounded by **Z lines**/disks from which thin filaments of F-actin extend on each side. In the dark **A bands** ("anisotropic bands", with a low index of refraction) there are also thick parallel filaments of myosin. The **H bands**, in the middle of the A bands, contain only myosin, while only actin is found on each size of the Z lines, forming the light **I-bands** ("isotropic bands", with a consistent index of refraction). At the center of H bands we can found the **M-lines**.

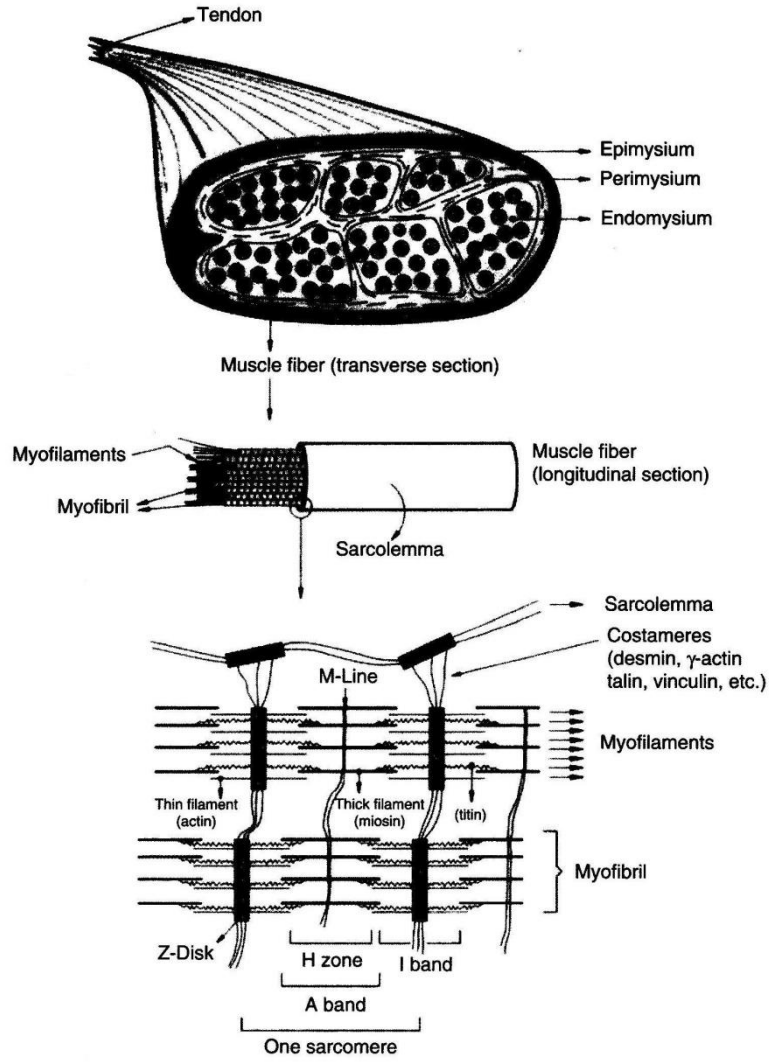

Figure 1. Schematic representation of the skeletal muscle structure and ultrastructure. (From "Handbook of Muscle Food Analysis", L.M.L. Nolet, F. Toldrá Eds., 2009. I.P. Munuera *et al.*, "Microstructure of Muscle Foods").

All the proteins that composing filaments in sarcomere are known as **myofibrillar proteins.** Adjacent myofibrils are attached to each other at the Z-line by proteinaceous filaments, known as **intermediate filaments**. Outermost myofibrils are attached to the cell membrane (sarcolemma) by intermediate filaments that interact not only with the Z-line, but also with structures at the sarcolemma known as **costameres** (Robson *et al.*, 2004). The protein constituents of the costameres (desmin, actinin, vinculin, talin, vimentin, titin, etc.) extend into the muscle cell where they encircle the myofibrils at the Z-disk and run from myofibril to myofibril and from myofibril to sarcolemma (Lluch *et al.*, 2001). Another smaller pool of actin, organized as mono-stranded microfilaments, contributes to the transversal connection of myofibrils to the membrane anchorage structures (Bard & Franzini-Armstrong, 1991).

Myosin is the most abundant myofibrillar protein in skeletal muscle, making up approximately 50% of the total protein in this organelle. It is the largest component of the **thick filament** in the sarcomere. Myosin is a large protein (approximately 500 kDa), shaped like a golf club, that contains six polypeptides. It consists of an alpha helical tail region, that forms the backbone of the thick filament, and a globular head region, that extends from the thick filament and interacts with actin in the thin filament. The head region of myosin also has **ATPase activity**, which is important in the regulation of contraction. Each myosin molecule contains two heavy chains (**MHC**; approximately 220 kDa each), that are required for enzymatic activity, and two regulatory light chains (**MLC**; 14-20 kDa). Each of the two heavy chains has a globular "head" at its amino end (N terminus) which extends into a "tail" (towards the C terminus) about 150 nm long, in which the two chains are intertwined to form a superhelix. The small subunits are attached in the head area.

Actin is the second-most abundant protein in the myofibrils, accounting for approximately 20% of the total protein. The backbone of the **thin filaments** is made up primarily of actin. It is a globular protein (**G-actin**, 42 kDa on average) that polymerizes to form filaments (**F-actin**). F-actin is also an important component of the cytoskeleton. There are approximately 400 actin molecules per thin filament. The thin filaments (F-actin polymers) are 1 μm in length and are anchored in the Z-line. Actin is in association with nebulin, and it is also present in intermediate transversal cytoskeletal filaments, where it serves to connect myofibrils to the sarcolemma, this link extending further to the extracellular matrix.

For contraction to occur, the thick and thin filaments interact via the head region of myosin, forming the complex referred as **acto-myosin**. The globular head of myosin also has enzymatic activity: it can hydrolyze ATP and liberate energy. In living muscle during contraction, the ATPase activity of myosin provides energy for myosin bound to actin to swivel and ultimately pull the thin filaments toward the center of the sarcomere. This produces contraction by shortening the myofibril, the muscle cell, and eventually, the muscle. The myosin and actin can disassociate when a new molecule of ATP is bound to the myosin head (Goll *et al*., 1984). In *post-rigor* muscle, the supply of ATP is depleted, resulting in the acto-myosin bonds becoming essentially permanent. These bonds are the genesis of the stiffness that develops in *post-mortem* muscle (*rigor mortis*).

Tropomyosin and troponin are two other proteins that are important in muscle contraction and are associated with the thin filaments. **Tropomyosin** is the second -most abundant protein in the thin filaments and it is made up of two polypeptide chains (alpha, 34kDa; beta, 36 kDa).

These two chains interact with each other to form a helix. The native tropomyosin molecule interacts with the troponin molecule to regulate contraction.

Troponin is a complex that consists of three subunits. These are termed **troponin I** (23 kDa), **troponin C** (18 kDa), and **troponin T** (37 kDa). Troponin I can inhibit the interaction between actin and myosin; troponin C has the ability to bind calcium released from the sarcoplasmic reticulum and troponin T binds very strongly to tropomyosin. The cooperative action of troponin and tropomyosin, in response to calcium increases in the sarcoplasm, regulates the interaction between actin and myosin and thus is a major regulator of **contraction**. In particular, calcium that is released from the sarcoplasmic reticulum is bound to the troponin complex and the resulting conformational changes within troponin cause tropomyosin to move away from sites on actin to which myosin binds and allows myosin and actin to interact.

Titin (aka connectin) is a mega-protein that is approximately 3 mega-Daltons in size. In striated muscle, titin thus spans fully half of a sarcomere with its C-terminal end localizing in the M-line, and the N-terminal forming an integral part of the Z-line. In particular, titin integrates the Z-line and the thick filaments, thus maintaining the location of the thick filaments (A-band) in the center of the sarcomere. Titin aids in maintaining sarcomere alignment of the myofibril during contraction.

Nebulin is another mega-protein (600–900 kDa) that is part of the filament system in muscle. Nebulin extends from the Z-line (with its C-terminal) to the pointed ends of the thin filament (Lukoyanova *et al.*, 2002), so it may aid in anchoring the thin filament to the Z-line (Komiyama *et al.*, 1992; Wang & Wright, 1988). Nebulin has also been shown to be capable of linking actin and myosin. It has been hypothesized that nebulin may also have a regulatory function in skeletal muscle contraction (Bang *et al.*, 2006; Root & Wang, 1994, 1994a).

Desmin is a member of intermediate filament proteins (O'Shea *et al.*, 1979; Robson, 1989). Intermediate filaments connect adjacent myofibrils at the level of their Z-lines and the myofibrils to other cellular structures, including the sarcolemma (Richardson *et al.*, 1981; Robson *et al.*, 1995; Robson, 1989) and thus may be important in maintaining the structural integrity of muscle cells. It is known that desmin play also a role in the development of tenderness (Boehm *et al.*, 1998; Huff-Lonergan *et al.*, 1996; Melody *et al.*, 2004; Taylor *et al.*, 1995).

Filamin is a large actin-binding protein. In skeletal and cardiac muscle, filamin is localized at the periphery of the myofibrillar Z-disk and it may be associated with intermediate filaments (Loo *et al.*, 1998; Thompson *et al.*, 2000; Van der Flier *et al.*, 2002). Thus, *post-mortem*

degradation of filamin probably could disrupt key linkages that serve to help hold myofibrils in lateral register. Degradation of filamin may also alter linkages connecting the peripheral layer of myofibrils in muscle cells to the sarcolemma (Robson *et al.*, 1995).

Muscle metabolism

In an actively exercising animal, muscle can account for as much as 90% of the oxygen consumption in the body. Muscular activity is dependent on large supplies of ATP within the muscle. Muscle can use energy precursors stored in the muscle cell, such as glucose, lactate, glycogen, lipids (free fatty acids, triglycerides) and phosphagens (phosphocreatine, ATP), and it can use energy sources recruited from the blood stream (blood glucose and circulating lipids). In general, **glycogen** is the preferred substrate for the generation of ATP. Lipids are a very energy-dense storage system and are very efficient with respect to the high amount of ATP that can be generated per unit of substrate. However, the rate of synthesis of ATP is much slower than when glycogen is used.

Which of these reserves (intracellular or circulating) the muscle cell uses depends on the activity the muscle is undergoing. When the activity is of lower intensity, the muscle utilizes a higher proportion of energy sources from the blood stream and lipid stored in the muscle cell. These are metabolized to produce ATP using **aerobic pathways** (oxidative phosphorylation) in the mitochondria. Obviously, ample oxygen availability is required for this process to proceed. During high intensity activity, when ATP is used very rapidly, the muscle uses intracellular stores of phosphagens or glycogen. These two sources, however, are utilized very quickly and their depletion leads to fatigue. Glucose and glycogen can be utilized also **anaerobically** (**anaerobic glycolysis**) in the sarcoplasm.

Concentration of ATP in skeletal muscle is critical; the relaxation of contraction is also dependent on ATP, and the removal of calcium from the sarcoplasm is an ATP-dependent process too (Hargreaves & Thompson 1999).

Aerobic metabolism, the most efficient energy system, requires **oxygen** to operate, and that oxygen is supplied by the blood to the muscle and by the oxygen transporter, myoglobin. Under conditions of extreme hypoxia (as found in *post-mortem* muscle), oxygen supplies are depleted because blood flow is not sufficient (or does not exist), and myoglobin oxygen reserves are depleted if this state continues long enough. After exsanguination, the muscle cell must turn solely to anaerobic pathways for energy production.

Phosphocreatine in living, rested muscle is available in moderate abundance compared with ATP, but very low abundance compared with glycogen. Phosphocreatine can easily transfer a phosphate group to ADP, generating ATP, in a reaction catalyzed by creatine kinase enzyme. This reaction is easily reversible and phosphocreatine supplies can be readily restored when ATP demand is low. In living muscle, when activity is intense, this system can be advantageous as it consumes H+ and thus can reduce the muscle cell acidosis that is associated with anaerobic glycolysis. However, this system is not a major contributor to *post-mortem* metabolism, as the supplies are depleted fairly rapidly. The anaerobic pathway, while comparatively less efficient is much better at producing ATP at a higher rate. Early *post-mortem* muscle obviously uses the anaerobic pathway, as oxygen supplies are rapidly depleted. This situation results in the buildup of the end-product lactate (**lactic acid**), leading to pH decline.

Metabolism in *post-mortem* muscle

A great number of chemical and biochemical reactions take place in living muscle. Some of these reactions continue, while others are altered, during the early *post-mortem* time, due to changes in pH, the presence of inhibitory compounds, the release of ions into the sarcoplasm and so on. In a few hours, these reactions are responsible for the conversion of muscle to meat; this process consists of the following steps: once the animal is slaughtered, the blood circulation is stopped, and the importation of nutrients and the removal of metabolites to the muscle ceases (Figure 2).

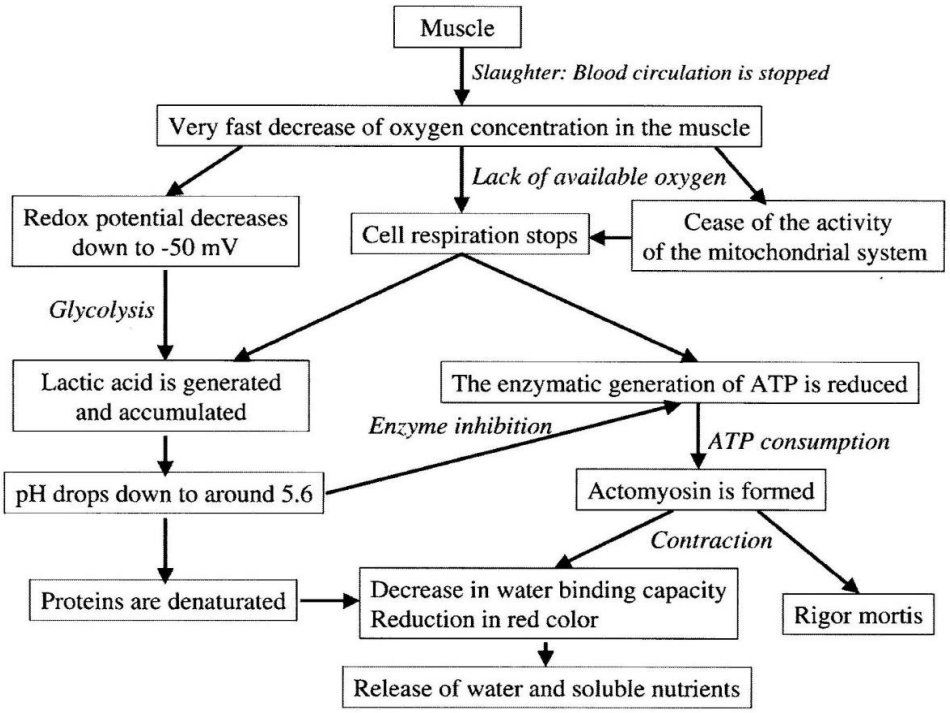

Figure 2. Summary of main changes occurring in muscle after slaughter. (From "Food Biochemistry and Food Processing", Y.H. Hui Ed., 2006. F. Toldrá, M. Reig, "Biochemistry of Raw Meat and Poultry").

This fact has very important and drastic consequences. The first consequence is the reduction of the oxygen concentration within the muscle cell because the oxygen supply has stopped. An immediate consequence is a reduction in mitochondrial activity and cell respiration (Pearson, 1987). Under normal aerobic values, the muscle is able to produce 12 moles of ATP per mole of glucose (Greaser, 1986). Instead, only 2 moles of ATP are produced per mole of glucose under anaerobic conditions (Eskin, 1990). The extent of anaerobic glycolysis depends on the reserves of glycogen in the muscle (Greaser, 1986). Glycogen is converted to dextrines, maltose, and finally, glucose through a phosphorolytic pathway; glucose is then converted into **lactic acid.**

Additionally, the enzyme creatin kinase may generate some additional ATP from ADP and **phosphocreatine** at very early *post-mortem* times, but only while phosphocreatine remains. The generation of ATP is strictly necessary in the muscle to supply the required energy for muscle contraction and relaxation and to drive the Na/K pump of the membranes and the calcium pump in the sarcoplasmic reticulum. Once phosphocreatine and glycogen are exhausted, ATP drops within a few hours to negligible values by conversion into ADP, AMP, and other derived compounds. Once the ATP concentration is exhausted, the muscle remains

contracted, as no more energy is available for relaxation. The muscle develops a rigid condition known as ***rigor mortis***, in which the cross-bridge of myosin and actin remains locked, forming **acto-myosin** (Greaser, 1986).

The lactic acid is accumulated in the muscle *post-mortem* because muscle waste substances cannot be eliminated due to the absence of blood circulation. This lactic acid accumulation produces a relatively rapid (in a few hours) pH drop to values of about **5.6–5.8 or low**. The pH drop rate depends on the glucose concentration, the temperature of the muscle and the metabolic status of the animal previous to slaughter. For example, the dark cutting beef meat are produced when the carbohydrates in the animal are exhausted before slaughter, and thus almost no lactic acid can be generated during early *post-mortem* due to the lack of the substrate. Very low or almost negligible glycolysis occurs and the pH remains high in these meats, which constitutes a risk from the microbiological point of view and a negative fact for tenderization (see below).

The fall of pH also inhibits the sarcoplasmic ATPase leading to release of Ca^{2+} from the sarcoplasmic reticulum and mitochondrial membrane into cytosol. The increased cytosol calcium ions not only regulates processes of activating calpain system (a major enzymatic degradation system in the cell; see below), but it also regulates ubiquitin-proteasome pathway (another degradation system in the cell), and it besides modulate various transcription factors leading to death of the cell (Hasselgren *et al*., 2005).

Muscle fibres types

Fibre type varies significantly between species, muscle types and regions within the same muscle. It is the variability and adaptability of fibre type that allows muscle to be a dynamic organ that responds to the needs of the animal. Several classification systems are in common used and led to understanding of the role of fibres in muscle metabolism and meat quality.

White and red muscle fibres (type I, IIA and IIB)

According to this classification, muscles contain three types of fibres, the proportions of which vary from one type of muscle to another; they can be classified as red, white or intermediate (Moody & Cassens, 1968) (Table 1). **Red** fibres (or **type I)**, **slow-contracting**, are characterized by a higher content of lipids and myoglobin, higher numbers of capillaries and mitochondria (Essén-Gustavsson *et a*l., 1992; Howald *et al*., 1985) and low glycogen

content. They exhibit **oxidative** metabolism that depends on an adequate supply of O_2, so they provide for their ATP requirements mainly (but not exclusively) from **fatty acids**, which are broken down via β-oxidation, the tricarboxylic acid cycle and the respiratory chain. The red colour in these fibres is due to the myoglobin, which they use as an O_2 reserve. They are suitable for prolonged effort (Kayar *et al*., 1988; Krenacs *et al*., 1989; Taylor & Bachman, 1999), such as endurance activities and maintaining posture. Their myosin ATPase activity is **low** and they are the smallest in diameter.

White fibres (or **type II B**) are better suited for fast, strong contractions (**fast-contracting**), such as sprinting or weight lifting. These fibres have low vascularization and low amounts of myoglobin, lipids and mitochondria and they have high content of **glycogen** (Vollenstad *et al*., 1984), exhibiting a **glycolytic** metabolism, so they are able to form sufficient ATP even when there is little O_2 available. They have immediate availability of phosphocreatine and degradation of local glycogen. They have **high** myosin ATPase activity and are the largest fibres.

Type IIA are intermediate type, **fast-contracting**, with **intermediate metabolism** (between oxidative and glycolytic). They have **high** myosin ATPase activity and are rich in mitochondria and myoglobin. They are suitable for rapid activity but also fatigue slowly. Furthers studies led to the delineation of more than one slow fibre type (I and IC; Askanas & Engel, 1975).

Fibre type	*Property*	*Role in meat quality*
I	Red, oxidative, slow contraction, slow myosin type, more myoglobin, rich in mitochondria	High fat content, small fibre diameter, red colour, low glycolytic potential
IIA	Red, intermediate, fast contraction, anaerobic	Intermediate, faster to rigor than type I
IIB	White, glycolytic, fast contraction, rich in glycogen, anaerobic, fewer mitochondria	Largest fibres so toughest, pale colour, change size with exercise regimes and age, high glycolytic potential so low pH

Table 1. Red and white myofibre types. (From "Encyclopedia of Meat Science", W.K. Jensen, C. Devine, M. Dikeman Eds., 2004. R.G. Taylor, "Myofibre types and meat quality").

Metabolic enzymes typing of fibres

Another conventional muscle fibre typing method uses histochemical staining for mitochondrial enzyme, succinate dehydrogenase (**SDH**) and reduced nicotinamide adenine dinucleotide-tetrazolium reductase (**NADH-TR**; Baker & Santer, 1990; Rahelic & Puac, 1981) (Table 2). Type I fibres, which are rich in mitochondria, stain darkly for **SDH**; IIB stain

lightly and IIA are intermediate (Gauthier, 1969). Lefaucheur (2010) reported the distinction between **red (R, SDH+)** or **white (W, SDH-)** myofibres type.

Stain	*Type I*	*Type IIA*	*Type IIB*
ATPase pH 9.4	+	+++	+++
ATPase pH 4.6	+++	–	++
ATPase pH 4.3	+++	–	–
NADH dehydrogenase	+++	++	+
Succinic dehydrogenase	+++	++	+
MHC-I	++	–	–
MHC-II	–	++	++

Table 2. Staining properties of different fibre types. (From "Encyclopedia of Meat Science", W.K. Jensen, C. Devine, M. Dikeman Eds., 2004. R.G. Taylor, "Myofibre types and meat quality").

Glucose content fibre typing

The amount of **glucose** in fibres is related to their metabolism and can be used to determine type. PAS staining method (periodic acid-Schiff reaction) distinguishes between low, intermediate and high amounts of glucose, corresponding to types I, IIA and IIB respectively. However, in some species and muscles, type IIA may richer in glucose than type IIB, so other methods must be used to validate the results of this method.

Myosin ATPase activity of fibres

One of the early **histochemistry** method used to classify fibres was based on myosin ATPase activity. This activity is correlated with muscle contraction velocity (Bárány, 1967). The myosin ATPase activity has a different sensitivity under the different pH conditions. Based on this principle, Brooke and Kaiser (1970) developed a method with a range of pH pre-incubations and so three main fibre types have been conventionally distinguished in adult skeletal muscle, i.e. types **I, IIA and IIB** fibres (Table 2). At **acid pH**, type I fibres are black, IIA are grey and IIB are white. At pH 4.3 a minor fibre type, IIC, can be distinguished by its intense staining. Pre-incubation under **alkaline conditions** has the inverse effect.

Myosin isoforms typing of fibres

Among all proteins whose expression varies from one fibre type to another, myosin isoforms are generally considered as the molecular marker of the fibre type. Myosin is the major motor protein of the muscle and it is also the determinant of the histochemical ATPase reaction after

alkali and acid pre-incubation, historically the first fibre type classification. Each myosin type is associated with specific kinetics of acto-myosin interaction and ATP hydrolysis: these properties determine the mechanical properties of the muscle fibres such as speed of actin filament sliding, mechanical power output and ATP consumption rate (Schiaffino & Reggiani, 1996).

Immunohistochemistry using monoclonal antibodies to a specific isoform of myosin can be used to stain different fibre types because one isoform always predominates (Billeter *et al*., 1980; Danieli-Betto *et al*., 1986). We can distinguish **MHC-I or MHC-fast** (**type I**), **MHC-IIa** (**type IIa**) and **MHC-IIb** (**type IIb**; Billeter *et al*., 1981; Dalla Libera *et al*., 1980), **or MHC-low,** for both of the II types (Table 2). Subsequently an additional fibre type has been identified: MHC-IIx fibres (type IIx/d; Gorza, 1990; Schiaffino *et al*., 1989). The speed of contraction increases in the following order: I < IIa < IIx < IIb (Schiaffino & Reggiani, 1996).

Combined methods to classify fibres

For a more complete understanding of the situation, multiple staining methods could be performed on the same sample. The most commonly used procedure has been to combine acidic ATPase activity with SDH, or NADH-TR, to label fibres as **slow-oxidative** (SO), **fast-oxidative-glycolytic** (FOG), or **fast-glycolytic** (FG) (Barnard *et al*., 1971; Jurie *et al.*, 1995; Pearson & Young 1989; Peter *et al*., 1972; Picard *et al.*, 2003). However, it should be pointed out that the classification systems based on stains for enzymes involved in oxidative metabolism (Peter *et al*., 1972) and ATPase activity (Brooke & Kaiser, 1970) appear to be not ever compatible. The SO fibres correspond to type I, but FG and FOG fibres do not fully match fibre types IIA, IIB or IIC (Pette & Staron, 1990).

Another common method combines ATPase with SDH to give types **β-red, α-red and α-white** for I, IIA and IIB respectively (Ashmore & Doerr, 1971).

Lefaucheur (2010) had well summarized the combination of several characteristics in different fibre types: individual fibre types exhibit different contractile, metabolic, physiological, chemical and morphological characteristics, as presented in Table 3.

	I	IIa	IIx	IIb
Contraction speed	+	+++	++++	+++++
Myofibrillar ATPase	+	+++	++++	+++++
Oxidative metabolism	+++++	++++, +++++	+, ++	+
Glycolytic metabolism	+	++++	++++	+++++
Hexokinase	+++++	+++	+	+
GLUT-4	+++++	+++	+	+
Phosphocreatine	+	+++++	+++++	+++++
Glycogen	+	+++++	++++	+++++
Triglycérides	+++++	++	+	+
Vascularization	+++++	+++	+, ++	+
Myoglobine	+++++	++++	++	+
Buffering capacity	+	++++	+++++	+++++
Diameter	++	+, ++	++++	+++++
Fatigue resistance	+++++	++++	++	+

[a] +, very low; ++, low; +++, medium; ++++, high; +++++, very high.

Table 3. Biological characteristics of individual fibre types. (From L. Lefaucheur, 2010. Meat Science, 84, 257).

Fibre type and meat quality

The interest in fibre type composition of muscles in livestocks is related to the assumption that it may play a part in determining meat quality and tenderness.

Two main opposite theories were established during years: one of this argues that the predominance of fast-glycolytic fibres lead to a high toughness of the muscle and that slow-oxidative fibres lead to more tender meat. Conversely, other authors sustain the opposite theory. Several examples in support of both theories are presented below.

Maltin *et al.* (1998; 2001) showed a positive correlation between the **tenderness** and the proportion of **SO** (slow-oxidative) fibres in a muscle and, on the contrary, a negative correlation with the proportion of fibres FG (fast-glycolytic). These authors bring the presence of a higher turnover of proteins in muscle oxidative for explaining a greater myofibrillar degradation and therefore a more pronounced tenderness. A study of Dransfield *et al.* (2003) also showed that the tender meat has a higher proportion of SO fibres and lower of FG fibres. Similarly, a study of Strydom *et al.* (2000) showed a positive correlation between the myofibrillar fragmentation (an index of muscle degradation process) and the presence of red fibres SO and intermediate FOG fibres, and a negative correlation with the presence of white fibres FG. Moreover, several authors found that cattle muscles with characteristics of high **tenderness** contained more proteins involved in **oxidative** metabolism (Succinate

dehydrogenase, SDH; Malate dehydrogenase, MDH) or **slow-contractile** properties, and less proteins associated with fast-glycolytic metabolism (Lactate dehydrogenase, LDH; Enolase; Phosphoglucomutase; Triosephosphate isomerase) or fast-contractile properties (Chang *et al.*, 2003; Hocquette *et al.*, 2007; Morzel *et al.*, 2008).

These results, reporting higher degradation of myofibrils in oxidative fibres, are in **contradiction** with the study of Ouali and Talmant (1990) which showed, in different muscles, that the maturation is more important in **glycolytic** than oxidative fibres. Similarly, Seideman *et al.* (1986) reported that the increase of **fast-twitch** glycolytic fibres has beneficial effects on *post-mortem* ageing and tenderness in cattle. Totland *et al.* (1988) also sustained that, in *post-mortem* ageing of meat, the rate of ageing is faster in fast-twitch muscles than in slow-twitch oxidative muscles. Xiong (2004) declared the same for the early *post-mortem* proteolytic degradation. Other studies on beef report positive associations between proportions of fast-twitch fibres and tenderness (Koch *et al.*, 1995) and between oxidative status and toughness (Zamora *et al.*, 1996). A study of transcriptomic (Kee *et al.*, 2008) showed a positive correlation between the **shear force** required to cut the muscle and the **glycolytic** metabolism of the muscle, supporting the results of Ouali and Talmant (1990).

Studies of Monin and Ouali (1991), Renand et al. (2001), Xiong et al. (2007) and Lee et al. (2010) moreover supported this second theory.

Another aspect that sustain this second hypothesis is the importance of the **ultimate pH** (pHu), that can influence enzymatic activity in the muscle and meat quality. The glycolytic potential of muscular fibres is an important criterion for the evolution of pH after the death of the animal. Increasing the proportion of fast-twitch glycolytic fibres in porcine *longissimus* muscle has been shown to increase the rate and extent of *post-mortem* **pH decline** (Choi *et al.*, 2007; Kim *et al.*, 2013; Ryu & Kim, 2006; Ryu *et al.*, 2006), that is positive for meat ageing. More generally, if fast-twitch glycolytic fibres are predominant in muscle, rapid glycolysis is induced, resulting in a rapid pH decline in the muscle (Choe *et al.*, 2008). Conversely, increasing the proportion of type I/oxidative fibres in muscle decreases the rate and extent of *post-mortem* pH decline (Choi *et al.*, 2007).

After all these considerations, we can conclude that the exact association between muscle fibre type characteristics and meat tenderness is **controversial** and still unclear.

Moreover, the contradictions in the results of different experiments can be attributed to the production system examined, the species, breed and sex of the animals, such as the specific muscle considered.

Meat tenderization process - conversion of muscle to meat

Multiple factors including palatability, water-holding capacity, colour, nutritional value, and safety determine meat quality. Flavor, juiciness, and tenderness influence the palatability of meat. Among these traits, tenderness is the most important during the consumption of the meat (Brewer & Novakofski, 2008; Maltin *et al*., 2003; Miller *et al*., 2001).

We can say that there are three aspects of meat tenderness: background toughness, the toughening phase and the tenderization phase. Variation in the background toughness is due to the **connective tissue** component of muscle. The toughening phase is caused by the sarcomere shortening during ***rigor mortis*** development, produced by irreversible linkages between the actin and myosin proteins (Koohmaraie *et al*., 1996; Wheeler & Koohmaraie, 1994). For beef, this process usually occurs within the first 24 h *post-mortem* (Wheeler & Koohmaraie, 1999). Finally, the **tenderization phase** is produced by protein degradation during ageing and by the ultrastructural changes that occur in the muscle tissue. This phase is also influenced by non-enzymatic aspects of the muscle, such as temperature, pH, calcium concentration, sarcomere length, collagen and lipid content and fibre type. However, many authors concluded that these muscle characteristics could explain only up to 30% of the variability in tenderness (Chriki *et al.*, 2013; Renand *et al*., 2001). It appears that the main determinant of ultimate tenderness is the extent of **proteolysis** of myofibrillar and myofibrillar-associated proteins within muscle fibres (Hopkins & Taylor, 2002; Koohmaraie & Geesink, 2006; Koohmaraie *et al.*, 2002; Maltin *et al.*, 2003; Renand *et al*., 2001; Taylor *et al.*, 1995).

The network connective of **collagen** contained in the muscle is little affected by the proteolytic systems. The connective affects positively the tenderness of the meat due to its solubility and negatively according to its quantity. During the cooking of meat, between 58 and 65°C, the collagene is transformed into an insoluble gelatin (Guillemin *et al*., 2009).

The **proteolytic systems** of muscle break the bonds intra- and inter-myofibrils, those between myofibrils and sarcolemma and the adhesion between the muscle cells and the basal lamina (Nishimura *et al.*, 1996; Taylor *et al*., 1997). In the sarcomere the most consistently reported ultrastructural change associated with tenderization is breaks at the junction of the **I band** and **Z-disk** (Ho *et al*., 1996; Taylor *et al*., 1995). Other three major cytoskeletal structures are degraded when meat is tender: Z- to Z-line attachments by **intermediate filaments (desmin)**, Z- and M-line attachments to the sarcolemma by **costameric proteins**, and the elastic filament protein **titin** (Taylor *et al*., 1995) (Figure 3).

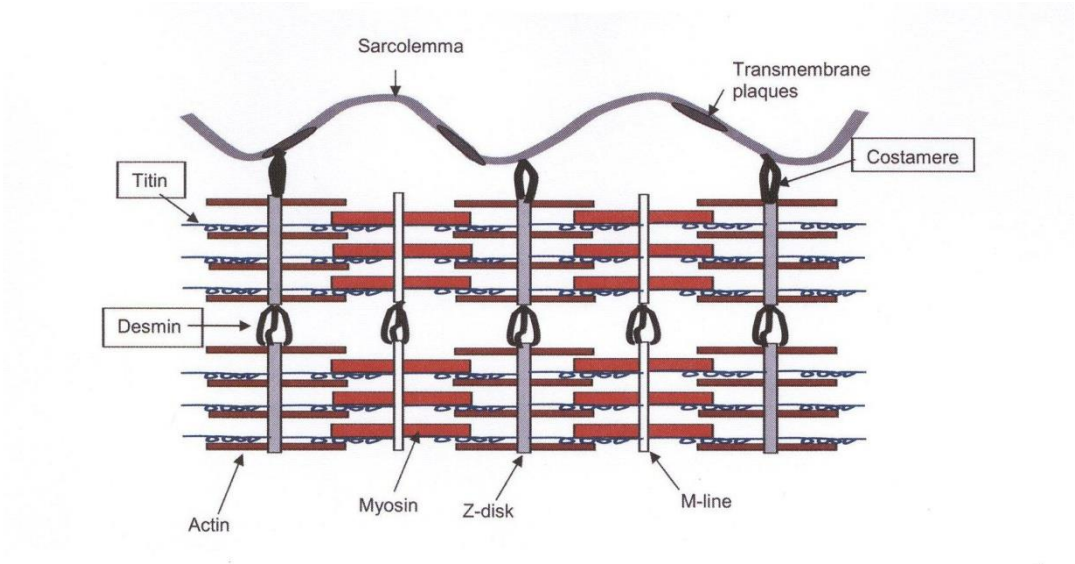

Figure 3. Schematic representation of muscle myofibrillar proteins showing the major components of the sarcomere. (From C.M. Kemp *et al.*, 2010. Meat Science 84, 248).

In addition to desmin and titin, other proteins are degraded during myofiber deterioration, for example **troponin-I, troponin-T, vinculin, meta-vinculin, dystrophin** and **nebulin**, with some cleavage of the major myofibrillar proteins such as **actin** and **myosin** (Becila *et al*., 2010; Du *et al.*, 2004; Goll *et al*., 1992; Ho *et al*., 1996; Hopkins & Thompson, 2002; Huff-Lonergan *et al*., 1996, 2010; Koohmaraie & Geesink, 2006; Lametsch *et al*., 2002, 2003, 2004; Robson *et al*., 1997; Taylor *et al*., 1995; Yang *et al*., 1998).

The question what **proteases** are primarily responsible for *post-mortem* tenderization has been a matter of debate for decades (Koohmaraie & Geesink, 2006; Ouali *et al.*, 2006). The protease systems (and their inhibitors) that have been studied in relation to *post-mortem* tenderization include the calpain system, the cathepsins, the multicatalytic proteinase complex (proteasome), the metallopeptidases and the serine-peptidases (Ouali *et al.*, 2006).

Regarding the main families of proteases involved in meat tenderization, my attention in the present thesis was focused on enzymes most involved in meat tenderization (**calpains**) and on enzymes/molecules that more recently are studied for their involvement on meat tenderization process, **caspases** and **heat shock proteins**, through the mechanism of apoptosis.

Calpains-calpastatin system

Calpains

Calpains are probably the most extensively researched proteases family with regard to meat science and it is widely accepted that proteolytic calpain activity does contribute to meat

tenderization (Koohmaraie & Geesink, 2006; Sentandreu *et al*., 2002; Veiseth & Koohmaraie, 2005). The first evidence supporting this assumption was the rapid loss of **Z-disks** and degradation of **M-lines** in calpain-treated skeletal muscle myofibrils, a change often associated with meat tenderness (Busch *et al.*, 1972; Taylor *et al.*, 1995).

Calpains are a complex family of **calcium-activated** proteases (cysteine endopeptidases; Sorimachi *et al*., 1997) with an optimum activity at **neutral pH**, also called calcium-dependent proteases, because of the need of calcium for their activity. Currently, at least 15 different calpains have been identified in mammals. Some calpains are ubiquitously expressed (calpain-1, 2, 4, 5, 7, and 10), whereas others are found in specific tissues: calpain-3 (skeletal muscle), calpain-6 (placenta), calpain-8 (smooth muscle), calpain-9 (stomach), calpain-11 (testes), calpain-12 (skin) and calpain-13 (testes and lung) (Dear *et al*., 1997; Goll *et al*., 2003; Smith & Schnellmann, 2012; Suzuki *et al*., 2004).

Calpain-1 and 2

Calpain-1 (micro-calpain/**μ-calpain**), **calpain-2** (milli-calpain/**m-calpain**) and **calpain-3** (p94) were first calpains to be detected at the protein level in mammalian skeletal muscle (Huang & Wang, 2001; Murachi *et al*., 1981; Sorimachi & Suzuki, 2001; Sorimachi *et al*., 1989, 1990; Spencer *et al*., 1995).

Some findings indicating that calpain-1 is concentrated on the N1 and N2 line region of **titin** (Z-lines/M-lines), bound to its inhibitor calpastatin (see below) and, in this position, it might constitute a reserve for the cell (Allen & Goll, 2003; Fernandez *et al*., 2005). This localization suggests a special regulation of the release of calpain-1 from its binding sites and the existence of a finely regulated equilibrium between the amounts of free and bound enzyme.

In mammalian skeletal muscle, there is also an inhibitor of calpain-1 and 2, **calpastatin**: its helical sequence prevent calpains from binding to the targets (Mellgren *et al*., 1989; Wendt *et al*., 2004).

Calpain-1 and 2 differ in their Ca^{2+} requirement for activation: from 3-10 μM to 50 μM of Ca^{2+} for calpain-1, and from 0.3 to 0.8 (1) mM for calpain-2 (Boehm *et al*., 1998; Etherington, 1984; Goll *et al*., 2003; Perrin & Huttenlocher, 2002). The terms μ-calpain and m-calpain derive from this peculiarity of the two enzymes.

Calpain-1 and 2 are inactive **heterodimers** that dissociate in the presence of Ca^{2+} into a small regulatory **28 kDa** subunit, that is identical in both isoforms (Carafoli & Molinari, 1998; Goll *et al*., 2003), and into a large catalytic subunit of **80 kDa** (Dayton *et al.*, 1976, 1976a, 1981; Dutt *et al.*, 2002; Emori *et al.*, 1986). It seems that calpain small subunit is able to modulate

protease activities of calpain-1 and 2 (Mellgren & Lane, 1988), but not that of calpain-3 (Sorimachi *et al*., 1995). Anyway, the small subunit stabilizes the enzymes, enabling them to keep the proteolytic activity (Goll *et al*., 2003; Nakagawa *et al*., 2001; Pal *et al*., 2001).

The 80 kDa subunits of calpain-1 and 2 are similar, but they are encoded by different genes (Suzuki, 1990). The 80 kDa subunits of both proteins are composed of four domains. **Domain I**, the N-terminal domain, has no sequence homology to any known polypeptide. **Domain II** is the catalytic domain. This domain contains a cysteine residue as well as a histidine residue that are in relative position that is conserved in all cysteine proteases (Suzuki, 1990). **Domain III** is not homologous to any other known protein, but has sequences that can be Ca^{2+}-binding sites (Goll *et al*., 2003). **Domain IV** is a calmodulin-like domain and it has sequences that can be Ca^{2+}-binding sites.

An important characteristic of calpain-1 and 2 is that they undergo **autolysis** (of the intact 80 kDa large subunit to a 78 kDa molecule) in the presence of calcium (that happens, for example, with the accumulation of intracellular calcium in *post-mortem* muscle). This process leads to an increase in calcium affinity and hence a rise of their activity (Goll *et al.*, 1985, 2003; Jeacocke, 1993; Koohmaraie, 1992; Suzuki, 1987). However, further autolysis of calpain-1 and 2 leads to lower molecular weight fragments of the large subunit and loss of activity (Dayton, 1982; Edmunds *et al*., 1991; Maddock *et al*., 2005; Nagainis *et al*., 1983; Suzuki *et al*., 1981).

The optimal condition for calpain activity is **pH 7.5**, at 25°C, and their activity decreases very quickly when pH decrease to 6.0 (Camou *et al*., 2007; Carlin *et al*., 2006; Geesink & Koohmaraie, 2000; Maddock *et al*., 2005), reaching the inactivity at pH 5.5 (Etherington, 1984). However, some author argues that a certain calpain activity is still detected at pH 5 (Zeece *et al*., 1986) and Koohmaraie *et al.* (1986) suggested that calpain-1 retained 24–28% of its activity at a pH of 5.5–5.8 and 5°C.

Calpains and meat tenderization

Calpain-1 is activated in early *post-mortem* (within 3 days of slaughter), when the *post-mortem* proteolysis of key myofibrillar proteins is known to take place (Taylor *et al*., 1995). Dransfield (1992, 1993) and Dransfield *et al.* (1992) examined extracts of beef muscle *in vitro*; they postulated that the low levels of free **Ca^{2+}** ions in the immediate *post-mortem* period would be insufficient to activate calpain-1. However, when the pH had fallen to *ca*. 6.1, the Ca^{2+} level would have become high enough to activate this enzyme (calpain-2 would act similarly, but at a greater Ca^{2+} level). At this pH, calpains are bound to their inhibitor

calpastatin, but the inhibitory action falls as the pH drops further from 6 to 5.5, and the activated calpains proteolyzed the calpastatin. Activity of calpains ceases as the calpains self-destruct by autolysis (Dransfield *et al.*, 1992; Dransfield, 1993).

If the role of calpain-1 in meat tenderization is certain, the role of **calpain-2** in meat tenderization is not so sure. In bovine, ovine and pig *post-mortem* muscle, the activity of extractable calpain-1 declines but the activity of calpain-2 is remarkably stable (Ducastaing *et al*., 1985; Geesink & Koohmaraie, 1999; Kretchmar *et al*., 1990; Sensky *et al*., 1996; Veiseth *et al*., 2001), suggesting that calpain-2 is not activated in early *post-mortem*. Ilian *et al*. (2001) also found, both in bovine and in ovine muscle, that calpain-1 and calpastatin activities decline significantly in 24 h *post-mortem*, contrary to calpain-2, that remains inactive and so not autolyzed. Koohmaraie *et al*. (1987) also reported the calpain-2 as more stable, just 2–3 weeks before losing its activity. The hypothesis that calpain-1 is the major responsible for *post-mortem* proteolysis was confirmed using calpain-1 **knockout mice** (Geesink *et al*., 2006): the results of this study clearly showed that *post-mortem* proteolysis was largely inhibited in calpain-1 knockout mice.

The contribution of calpain-1 and 2 in meat tenderization have been the topic of much debate in the literature (Geesink & Veiseth, 2009; Goll *et al*., 2003; Prates, 2002). The prevailing belief is that calpain-1 is the essential and predominant enzyme responsible for *post-mortem* proteolysis, and that combined calpain-1 and 2 activity may be responsible for up to **85%** of *post-mortem* meat tenderization (Delgado *et al*., 2001, 2001a; Geesink & Koohmaraie, 1999; 1999a; Geesink *et al*., 2000; 2006; Huff-Lonergan *et al*., 1996; Koohmaraie *et al*., 1986; 1988; 1990; 1991; 1991a; 1995; Koohmaraie, 1990; 1996; Shackelford *et al*., 1991; Wheeler *et al*., 1992; Whipple *et al*., 1990).

A large number of studies have shown good ability of calpains to degrade important myofibrillar proteins, such as **filamin, desmin, vinculin, α-actinin, tropomyosin, M-line protein, troponins T** (Nishimura *et al*., 1996; Zeece *et al*., 1986) and **I, titin, nebulin** (Delgado, 2001; Etherington, 1984; Huff-Lonergan *et al*., 1996, 1999; Mestre-Prates *et al*., 2002) and **integrin** (Lawson, 2004). On the other hand, they not seem active against myosin, actin and troponin C (Dayton *et al*., 1976a; Goll *et al*., 1983, 1999; Koohmaraie, 1994).

Calpain-3/p94, unique to skeletal muscle, is a protein of 94 kDa with sequence homology to the large subunits of calpain-1 and 2 (Sorimachi *et al*., 1989). It was originally of interest to meat scientists because it binds to the giant myofibrillar protein titin, at the N_2-line (Sorimachi *et al*., 1995), a site where proteolysis has been linked to meat tenderization (Taylor *et al*., 1995). Calpain-3 **knockout mice** was used to examine the effects of the absence of

calpain-3 on *post-mortem* muscle (Geesink *et al*., 2005). *Post-mortem* proteolysis occurred in a similar fashion in calpain-3 knockout mice in comparison to control wild type mice, with no differences detected in desmin, nebulin, troponin-T or vinculin degradation, suggesting that calpain-3 is not involved in meat tenderization.

Calpastatin and its relation with meat tenderness

Calpastatin is a polymorphic protein (between 50 and 172 kDa) acting as an endogenous reversible and competitive **inhibitor** of calpain-1 and 2 in the living muscle (Goll *et al.*, 2003; Maki *et al*., 1988; Parkes, 1986). It has most commonly been described as a structure of four repeats of homologous sequences at an interval of about 140 amino acids (**domains I, II, III, and IV**) and an alkaline N-terminal domain (**domain L or XL**) of variable size (Goll *et al*., 1999; Lee *et al.*, 1992; Maki *et al.*, 1990). The intact calpastatin molecule is capable of inhibiting multiple molecules of calpain (Otsuka & Goll, 1987), due to the multiple inhibitor domains I–IV. In particular, three regions (A, B, C) within domains I–IV are responsible for binding to calpains and inhibiting their activity.

Several **isoforms** of calpastatin are believed to result from various cellular events, including alternative splicing (De Tullio *et al.*, 1998; Geesink *et al.*, 1998; Lee *et al.*, 1992; Maki *et al.*, 1991; Meyers & Beever, 2008), differing start sites of translation/transcription (Cong *et al.*, 1998; Imajoh *et al.*, 1987; Parr *et al*., 2000, 2001, 2004; Raynaud *et al*., 2005, 2005a; Wang *et al.*, 1994) and differing states of phosphorylation (Averna *et al.*, 2001; Pontremoli *et al.*, 1991, 1992). The heterogeneity of calpastatin in different cells and tissues may determine its intracellular localization and its physiological role.

Calpastatin requires **calcium** to bind and inhibit calpains (Cottin *et al.*, 1981; Imajoh & Suzuki, 1985), with a concentration that are reported to be close to or below those that are required to activate calpains (Goll *et al.*, 2003). Calpastatin is also a substrate for the calpains and can be degraded in the presence of calcium (Doumit & Koohmaraie, 1999; Geesink *et al.*, 2000; Mellgren *et al.*, 1986). Anyway, degradation of calpastatin does not lead to complete loss of inhibitory activity, and even after extensive proteolysis, some inhibitory activity of this protein remains (DeMartino *et al.*, 1988; Goll *et al.*, 2003; Nakamura *et al.*, 1989).

A consistent observation of the calpain system's involvement in **tenderness** is that high levels of calpastatin are associated with poor quality meat. The model being that high levels of calpastatin reduce the activity of calpain thereby reducing the proteolysis required for tender meat. For example, some studies on a random selection of commercially slaughtered pigs have shown that high levels of calpastatin (both activity and protein levels), in the first few

hours after slaughter, are associated with an increased incidence of **toughness** (Parr *et al.*, 1999; Sensky *et al.*, 1998). Moreover, has been demonstrated that the muscle hypertrophy found in callipyge lambs is associated with the high levels of calpastatin, greatly reduced *post-mortem* proteolysis and a significant decrease in meat tenderness (Geesink & Koohmaraie, 1999). Furthermore, over-expression of calpastatin in transgenic mice resulted in reduction of *post-mortem* proteolysis of desmin and troponin T (Kent *et al*., 2004).

More specifically, most studies demonstrated that the **calpain/calpastatin** concentration ratio and, though to a lesser extent, the muscle concentration of calpains, are good predictors of the ultimate tenderness of beef (Kemp *et al.*, 2010; Ouali & Talmant, 1990; Shackelford *et al.*, 1991a; Thomson *et al.*, 1999).

Current enzymatic model of *post-mortem* proteolysis and ageing tenderization

Post-mortem improvement in meat tenderness results from a softening of the myofibrillar structure by **endogenous peptidases** (Ouali, 1992; Sentandreu *et al*., 2002). For a protease system to be considered to be involved in *post-mortem* proteolysis and meat tenderization it must meet a certain basic criteria as defined by Koohmaraie (1988). Firstly, the protease must be endogenous to skeletal muscle cells; secondly, it must be able to mimic *post-mortem* changes in myofibrils *in vitro* under *optimum* conditions, and finally it must have access to myofibrils in tissue. Of the major enzyme systems investigated by many authors, only **calpains** meet all these criteria. The location of cathepsins, another important enzymatic system of the cell, in lysosomes is thought to restrict their access on substrates. Thus, the prevailing theory is that the **calpain/calpastatin** system is the predominant driver of *post-mortem* proteolysis and ageing tenderization. However, while it is widely accepted that proteolysis of key myofibrillar proteins by the calpain enzyme system is primarily responsible for increased tenderness during *post-mortem* storage (Goll *et al*., 2003; Koohmaraie & Geesink, 2006; Koohmaraie *et al*., 1991; Uytterhaegen *et al*., 1994), it can be argued that calpains alone are **not sufficient** to fully explain *post-mortem* proteolysis and meat tenderization.

Questions regarding the role of calpains in *post-mortem* tenderization initially centered on the observation that calpain activity substantially diminishes early *post-mortem* and the fact that many of the conclusions regarding the calpain role in tenderization are based on indirect

evidence. Within the pH range of 7.5 to 5.8, both calpain-1 and 2 retain enzymatic activity, but as muscle pH drops, more autolysis of calpain-1 occurs and proteolytic activity diminishes (Koohmaraie, 1992). Even though Koohmaraie (1996) demonstrated that calpain-1 in muscle retains from 5% to 10% of its original activity even after 14 days storage, the question still remains whether this level of activity is sufficient to explain the protein degradation observed in muscle beyond 24 to 48 hours *post-mortem*.

Therefore, according to a possible hypothesis, initial meat tenderization appears to be due to calpain-1, which is optimally active at physiological pH. As pH declines in *post-mortem* muscle and calpain-1 activity becomes limited, cathepsins become increasingly active and hydrolyze myofibrillar proteins associated with the tenderization of meat *post-rigor* (Lomiwes *et al.,* 2014). In a study in primates, Yamashima *et al.* (1998) proposed "**calpain–cathepsin hypothesis**" that calpain mediated lysosomal disruption resulting in release of cathepsins. Moreover, Sentandreu *et al*. (2002) sustain that the tenderization of meat results very likely from the **synergistic action** of endogenous peptidases including mainly cathepsins, calpains and proteasome.

Many recent studies on meat tenderness have confirmed the importance of the calpain system but have further indicated that ageing tenderization is a highly complex process in which multiple enzymes and interdependent muscle factors may be necessary to fully explain *post-mortem* proteolysis and its link to tenderization.

More recently, it was hypothesized that also the **apoptotic** cellular system can be involved in the transformation of the muscle in meat, through the involvement of **caspases**, the major actors of apoptosis in the cell, and **heat shock proteins** (Hsp), a class of proteins able to inhibit apoptosis and to protect myofibrillar proteins (Ouali *et al.*, 2006).

Apoptosis theory of ageing tenderization

In 2002, Sentandreu *et al*. hypothesized that after slaughter skeletal muscle will engage in cell death, with apoptosis rather than necrosis being the most likely candidate. The hypothesis that muscle engages in a form of cell death *post-mortem* was further elucidated in 2006 by many authors (Herrera-Mendez *et al*.; Kiang *et al.*; Ouali *et al*.). These authors suggested that the **hypoxic/ischemic** conditions**,** that are induced through the process of slaughter and exsanguination, could activate **caspases** and **apoptosis** in the skeletal muscle *post-mortem*, in a similar way to that observed during neuronal or cardiac ischemia. After, in two studies, Cao

et al. (2010) and Becila *et al.* (2010) provided evidence that cell death in *post-mortem* muscle occurs via apoptosis not necrosis. In particular, Becila *et al.* (2010) clearly demonstrate a rapid phosphatidylserine externalization (an index of apoptosis) and cell shrinkage extending during the first 24 h post-exsanguination. It was therefore concluded that, in *post-mortem* muscle, cells commit suicide soon after animal bleeding through apoptosis.

Apoptosis is classified as highly organized **programmed** cell death. It is an evolutionary highly conserved process from monocellular organisms to mammals (Driscoll, 1996; Yuan, 1996). Such systematic cell clearance process is necessary for both the normal development of a multicellular organism during embryogenesis and the maintenance of tissue homeostasis in adults (Dirks & Leeuwenburgh, 2005; Meier *et al*., 2000). Indeed, this mechanism is critical in the elimination of non-functional, damaged cells that are potentially harmful (Thompson, 1995). One of the major physiognomies of apoptosis is the maintenance of the **plasma membrane** during the process, thus preventing cellular component discharge and damage to adjacent cells (Fidzianska *et al*., 1991; Green, 2011; Kerr *et al*., 1972). In fact, apoptosis occurs without inducing an inflammatory response (Wyllie *et al*., 1980). After animal slaughter, apoptosis is thought to ensue within **few minutes to 1 h** (Becila *et al*., 2010; Green, 2005; Ouali *et al*., 2006).

The apoptosis process proceeds through the following **phases** (Bratton *et al*., 1997; Majino & Jons, 1995; Martin *et al*., 1995; Matsura *et al*., 2005; Ouali *et al*., 2007; Pederson, 1999; Prochazkova *et al*., 2003; Youle & Karbowski, 2005) (Figure 4):

- first of all, cells in apoptosis are **isolated** by loss of contacts with neighboring cells;
- an important **condensation** of the nucleus and of the cytoplasm induces a significant reduction in cellular volume;
- **mitochondria** of the apoptotic cell undergo several major modifications: release of cytochrome *c* in the cytoplasm, reduction in the membrane potential and deterioration of the membrane permeability, with opening of specialized pores (by perforin action) and diffusion of diverse **pro-apoptotic proteins**;
- after condensation of the nucleus, **chromatin** is cleaved in regular fragments of approximately 180 pairs of bases (at the end the DNA results in single-stranded fragments and not in double-stranded fragments that are observed in necrosis);
- sometimes, the plasmic membrane buds and forms **apoptotic bodies**, containing some of the cell cytoplasm;

- in order to facilitate recognition of the apoptotic bodies by phagocytes, the cell signals its apoptotic state by a change of localization of **phosphatidylserines** molecules, from a cytoplasmic orientation to an extracellular orientation.

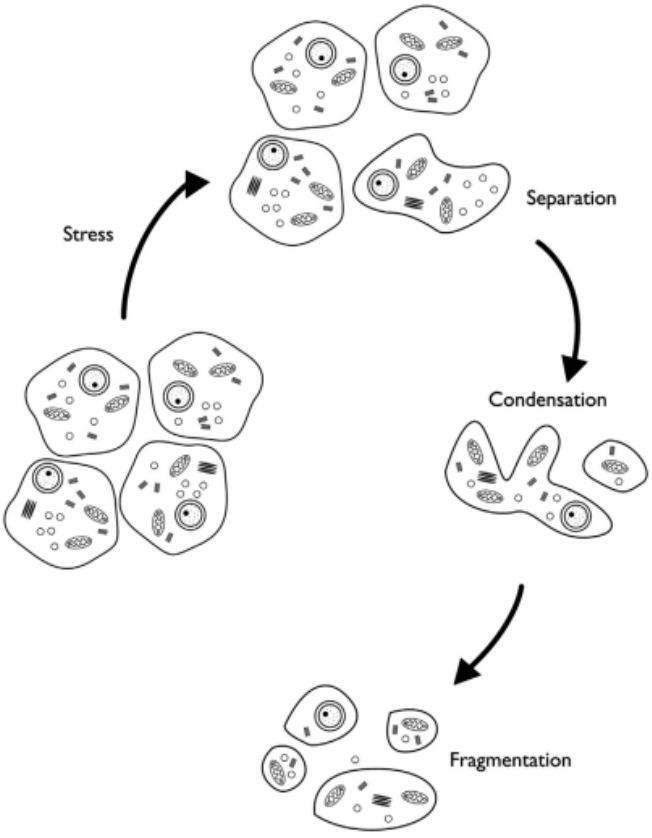

Figure 4. An illustration describing the process of apoptosis.
(From D. Lomines *et al*., 2014. Meat Science, 96, 26).

As a result of the slaughter process, the muscle tissue is deprived of oxygen and nutrients, due to the loss of the blood supply. Under these anoxic conditions, the muscle cells have no alternative but to initiate apoptosis. It seems that initiation of apoptosis occurs **immediately after death**, and therefore the conversion of muscle into meat starts by the onset of the apoptotic process a few minutes *post-mortem*. Thus, the traditional model of the conversion of muscle to meat would include a phase corresponding to the initiation of cell death through apoptosis, in addition to the phases of *rigor mortis* development and ageing tenderization (Ouali *et al*., 2006).

Caspases are the primary peptidases responsible for apoptosis (Adrain & Martin, 2001; Earnshaw *et al.*, 1999; Hengartner, 2000), so, if we reconsider meat tenderization through introduction of programmed cell death, the first active peptidases after animal bleeding would be undoubtedly caspases**.** The activation of the caspase system would induce a series of

biochemical and structural changes important in the **tenderization** process. Because capases are specialized in cell destructuration, they probably first degrade the key proteins involved in the complex spatial organization of myofibrils within muscle cells. Then, the hydrolysis of cellular components and organelles go on with the probable contribution of other proteolytic systems including very likely cathepsins, calpains, proteasome and else.

The list of proteins that are cleaved by caspases, either *in vivo* or *in vitro,* is ever growing. Cleavage of cytoskeletal proteins such as **gelsolin, foldrin, laminin, actin, and kinectin** has been found in apoptotic cells (Yamazaki *et al*., 2000). Actin degradation has been often associated to apoptosis, considering that its early degradation is ensured by caspases. Moreover, actin fragments have been proposed to be good markers of apoptosis development (Yang *et al*., 1998).

Caspases

Caspases are among the most specific **cysteine**-proteases/peptidases requiring an **aspartic acid** residue at the C-terminal side of the cleavage point of the substrate (Earnshaw *et al*., 1999). In the nomenclature suggested by Alnemri *et al*. (1996), all apoptosis-generating peptidases are designated caspase. The first letter of the name (C) stands for the cysteine of the active site; "asp" defines the strict specificity of cleavage after an aspartic acid residue and "ase" is the suffix common to all enzymes. Fourteen members of the caspases protease family have been identified and are expressed in a number of different tissues and cell types (Fuentes-Prior & Salvesen, 2004; Nakagawa & Yuan, 2000). However, some seem to be species-specific, for example with caspase 13 appearing to be present in bovine only (Barrett *et al*., 1998; Koenig *et al*., 2001).

All caspases have a highly conserved structure including:

- an N-terminal **pro-domain**, varying in size and having a prominent role in protein–protein interactions, in particular with regulators of apoptosis;
- a second domain which will become, after cleavage, the **large subunit**, carrying the active site of the enzyme with a cysteine and a histidine residue;
- a third domain, which will become, after cleavage, the **small subunit** with a conformational role.

All caspases are synthesized in the cytosol of cells as **inactive** zymogens. Precursor enzymes undergo maturation to the active form by N-terminal pro-domain elimination and subsequent

dimerization of the large and small chains, which associate to form an enzyme molecule. Two molecules join to form a dimer having two active sites in head-to-tail position (Figure 5). Catalytic sites can become active only when a dimer is formed (Fuentes-Prior & Salvesen, 2004).

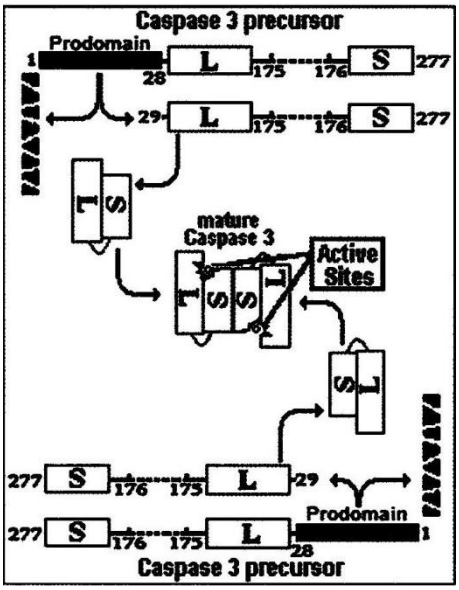

Figure 5. Maturation of the caspase 3 precursor into active caspase 3. (From C.H. Herrera-Mendez *et al*., 2006. Trends in Food Science & Technology, 17, 394).

Upon activation, caspases cleave proteins at specific aspartic acid residues (Fuentes-Prior & Salvesen 2004; Herrera-Mendez *et al*. 2006; Sentandreu *et al*. 2002).

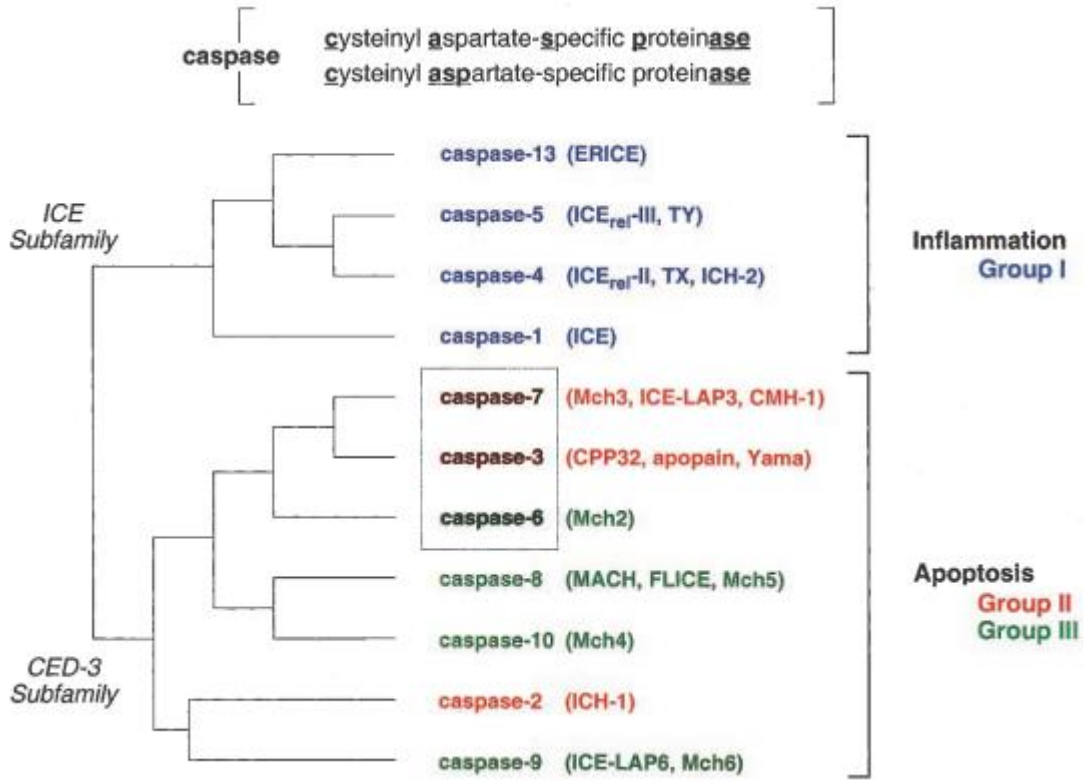

Figure 6. The human caspase gene family. (From D.W. Nicholson, 1999. Cell Death and Differentiation, 6, 1028).

Phylogenetic analysis of caspases indicates that there are two main subfamilies: caspases 1, 4, 5, 11 and 13 appear to be predominantly involved in the **inflammatory** response system, whilst caspases 3, 6, 7, 8, 9, 10 and 12 are involved in **apoptosis** (Figure 6).

The apoptotic caspases can be further subdivided, depending on their position in the cell death pathway, into **initiator** caspases such as **8, 9, 10** and **12**, that can autolytically activate themselves, and **effector** caspases such as **3, 6** and **7**, that are sequentially be activated by the initiator caspases (Danial & Korsmeyer, 2004; Earnshaw *et al*., 1999; Fuentes-Prior & Salvesen, 2004; Herrera-Mendez *et al*., 2006). Executioner caspases are activated by the initiator caspases through cleavage and subsequent dimerization; this simple proteolysis of executioner caspases is sufficient to gain maximal activity (Denault & Salvesen, 2008).

Caspases **3** and **7** have high homology, and substrates and inhibitors-specificity makes distinguishing between them virtually impossible. However, caspases 3 and 7 have differences in their pro-domains, which are thought to be involved in their subcellular targeting (Fuentes-Prior & Salvesen, 2004). Caspase **6** is also classified as an effector caspase due to its short pro-domain, however its target substrates are completely different to those of

caspases 3 and 7, suggesting that the functions of caspases 3, 7 and 6 do not overlap (Boatright & Salvesen, 2003). Skeletal muscle being one of the tissues where caspase **12** has been shown to be expressed at high levels, and some works suggest that it may play a physiological role in muscle during development (Nakanishi *et al*., 2005).

Initiator caspases are characterized by a **large** pro-domain, that often contains essential areas for their interactions with other proteins. They have homotypic **caspase recruitment domain** (CRD o CARD) or **death effector domain** (DED) at their N-termini. CRD domains direct pro-caspases to oligomeric activation assemblies in the cell and they are responsible for the interaction of caspases with a large variety of regulating molecules (activators or inhibitors) through CRD–CRD interactions. Similarly, DED domains allow binding of caspases to regulatory molecules carrying similar domains, through DED–DED interactions. For example, the pro-domains of caspases 8 and 10 contain the DED domain, instead other caspases (1, 2, 4, 5 and 9) have a CRD.

The **effector** caspases generally have **small** pro-domains and are responsible for disrupting the cellular proteins, which is a major cause of cellular death after initiation of apoptosis. The effector caspases are recognizable by the absence of recognizable homotypic recruitment domains.

The structural components of the cytoskeleton and nuclear scaffold require disassembly during apoptosis and effector caspases play a key proteolytic role in these steps (Nicholson, 1999). Similar to calpains, caspases have been shown to degrade a large number (some hundreds) of muscle (myofibrillar and cytoskeletal) proteins (Earnshaw *et al*., 1999; Fischer *et al*., 2003; Nicholson, 1999), such as **spectrin, actin** and **gelsolin**. In particular, caspase 3 has been demonstrated to cleave myofibrillar proteins in muscle during catabolic conditions (Du *et al*., 2004). Caspases can also degrade **desmin** and **vimentin** of muscle fibres (Chen *et al.*, 2003; Nakanishi *et al.*, 2001) and the products of this degradation also constitute a signal for inhibiting the synthesis of intermediate filaments, anchoring of myofibrils in the muscle structure. Moreover, caspase can target caspase-activated **DNase** (CAD). In normal cells, activity of CAD is suppressed by binding to its inhibitor ICAD. Catalytically active caspase 3 cleaves ICAD, permitting CAD to enter the nucleus and cause the degradation of chromosomal DNA into nucleosomal units, characteristic of apoptosis (Enari *et al.* 1998; Sakahira *et al.* 1998).

Apoptotic pathways and caspases

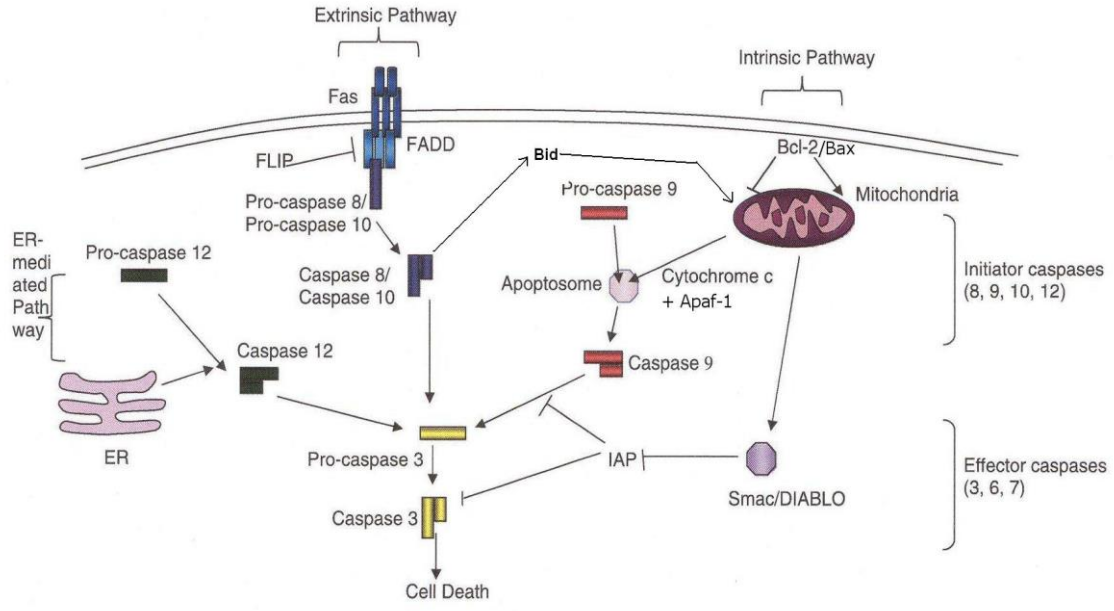

Figure 7. Diagram of apoptosis pathways and the caspases associated with each one. (From C.M. Kemp, T. Parr, 2012. Meat Science, 92, 252. Modified).

Activation of apoptosis by caspases constitutes a minimal **two-step** signaling pathway. The apical (initiator) caspases are activated in response to apoptotic stimuli; once activated, these caspases directly activate the executioner (effector) caspases downstream by limited proteolytic cleavage (Earnshaw *et al*., 1999). In caspase mediated apoptosis there are three main pathways of activation, which are presented below (Figure 7).

Intrinsic pathway

Intrinsic pathway corresponds to particular situations of environmental **stress**, such as hypoxia and ischemia, where the cell has no other solution but suicide (intrinsic stimulus). In this pathway, **mitochondria** are the central elements of the process (Kroemer *et al*., 1998; Mishra & Kumar, 2005; Mohamad *et al*., 2005; Petit *et al*., 1997). The intrinsic process implies the binding of a complex containing **Bax-type pro-apoptotic** proteins on the mitochondrial membrane, leading to the membrane deterioration itself. Then, mitochondrial membrane potential is lost and its external membrane becomes permeable, causing the release of **cytochrome *c*** (pro-apoptotic factor) and other pro-apoptotic proteins in the cytosol (Green & Reed, 1998; Yang *et al.*, 1997). In this pathway, permeability of the outer mitochondrial membrane is regulated via the opposing activities of the pro-apoptotic and anti-apoptotic

members of the **Bcl-2 family** (Adams & Cory, 1998). The proteins that promote apoptosis are **Bax, Bak, Bad, Bid** and **Bcl-xs,** whereas **Bcl-2** and **Bcl-xl** down-regulate it (Goldsby *et al*., 2003; Gross *et al*., 1999).

Free cytochrome *c* then forms a complex called **apoptosome**, composed, among others, of a protein called **Apaf-1** (Apoptotic protease activating factor-1) and **caspase 9** (Boatright & Salvesen, 2003; Fuentes-Prior & Salvesen, 2004; Zou *et al.*, 1999). Caspase 9 is activated within the apoptosome and can itself activate the effector's caspases (caspases **3** and **7**; Chen *et al.*, 2001; Earnshaw *et al*., 1999; Fadeel & Orrenius, 2005; Gao & Dou, 2000; Li *et al.*, 1997; Ouali *et al.*, 2006; Wolf & Green, 1999), that cleave specific target substrates including spectrin, actin, PARP (Poly ADP-ribose Polymerase), calpastatin and laminin. Caspase 3 can in turn activate the DNase **CAD** (Caspase Activated DNase) that degrades DNA and initiates cellular degradation (Green & Reed, 1998; Jeong & Seol, 2008).

At this level of the pathway, the activation of caspase 9 can be blocked by members of the **IAP family** (Inhibitors of Apoptosis Proteins). In the apoptosome, pro-caspase 9 is recruited and oligomerizes by homophilic CARD-domain interactions.

In the intrinsic pathway of apoptosis, the **mitochondria** and the **calcium ions** have a primary role. After slaughter, when there is cessation of aerobic conditions in the cell, the cell uses anaerobic glycolytic pathway for its survival. This results in accumulation of lactic acid leading to decrease in pH, which is detrimental to organelle membranes, causing the release of calcium ions from sarcoplasmic reticulum and mitochondria into the cytoplasm (Hwang *et al.*, 2004; Vignon *et al.*, 1989). Calcium is a crucial effector for triggering and controlling apoptosis (Orrenius *et al*., 2003; Szabadkai & Rizzuto, 2004), through its involvement in swelling and alteration of mitochondrial membrane, resulting in the release of the **cytochrome *c*** together with other pro-apoptotic proteins. Many of the key molecules involved in apoptosis are located within or attached to the mitochondrion. These molecules include, in addiction to cytochrome *c*, **AIF** (Apoptosis Inducing Factor), **Smac/Diablo** and **pro-caspase 3** (Du *et al.*, 2000; Liu *et al.*, 1996; Lorenzo *et al.*, 1999; Samali *et al.*, 1999; Verhagen *et al.*, 2000), and when released into the cytosol they function in both the initiation and execution of the apoptotic program.

Cytochrome *c* may also act as an intracellular intermediate that directly deregulates the sarcoplasmatic reticulum Ca^{2+} flux and handling, which result in caspase 7 and calpain activation.

Extrinsic pathway

The term “extrinsic apoptosis” or “death receptor pathway”, has been extensively used to indicate apoptotic cell death that are induced by extracellular stress signals that are sensed and propagated by specific **transmembrane receptors** (Wajant, 2002). Extrinsic apoptosis can be initiated by the binding of **lethal ligands** (or **death ligands)**, such as FAS ligand, CD95 ligand, TNFa (Tumor Necrosis Factor a) and TNFSF10/TRAIL (TNF ligand superfamily member 10/TNF-related apoptosis inducing ligand). These ligands interact with various cell surface **death receptors** (i.e. FAS/CD95/Apo-1, TNFa receptor 1, TRAIL receptor) (Wajant, 2002; Wang, 2000). Ligand binding induces a conformational change in the receptor that allows for the assembly of a **dynamic multiprotein complex** at the cytosolic tail of the receptor itself. This assembly occurs owing to the death domain (DD/DED) that is shared by all death receptors (and by the initiator caspases, as seen before) (Boldin *et al.*, 1995; Schulze-Osthoff *et al.*, 1998). Then, at the level of DD domain, many proteins are recruited, both pro-apoptotic factors than anti-apoptotic ones. The resulting supramolecular complex, which has been named **‘death-inducing signaling complex’** (DISC; Ashkenazi & Dixit, 1998; Nijhawan *et al.*, 2000), constitutes a platform that regulates the activation of **caspase 8** (or 10; Kischkel *et al.*, 1995).

The presence in this complex of pro-apoptotic and anti-apoptotic factors outlines the fact that the outcome of the activation of death receptors depends on which of the two parts prevails over the other. This pathway includes a mechanism of recruitment/activation of caspases through DED domain, indeed caspases activation following the recruitment of multiple homologous pro-enzymes to a common site of activation.

The active caspase 8 directly catalyzes the proteolytic maturation of **caspase 3** (and 7), thereby triggering the executioner phase of caspase-dependent apoptosis and cell disruption in a mitochondrion-independent manner (Denault & Salvesen, 2008).

To control this pathway, cells synthesize proteins containing DEDs domains, which act as **competitive inhibitors** of the activator complex for caspases binding. Excess of such proteins divert the caspases from their activator complex. Active caspase 8 or 10 can be inactivated also by **IAPs** (Inhibitors of Apoptosis Proteins), which are protein inhibitors binding close to the caspase active sites and blocking therefore its access to protein substrates.

There is to be noted the possibility of a **cross-talk** between the intrinsic and extrinsic pathways of apoptosis, that involve the mitochondrion as a control point. In some cell types, **caspase 8** mediates the proteolytic cleavage of the **Bid** factor, leading to the generation of a mitochondrion-permeabilizing fragment (tBid; Li *et al.*, 1998; Luo *et al.*, 1998). Thus, these

cells succumb from the activation of death receptors but through the involvement of the mitochondria and the caspase 9.

Third pathway

The third pathway involves **caspase 12**, which is a unique initiator caspase, as its activation does not require the assembly of a molecular platform like caspases 8, 9 and 10. Nakagawa *et al*. (2000; 2000a) demonstrated that caspase 12 is involved in cell death, initiating the apoptotic cascade via stress directly upon the **endoplasmic reticulum** (ER). Caspase 12 can also cleaves the loop region of the anti-apoptotic protein **Bcl-xl**, which associates with the mitochondrial membrane, transforming it into a pro-apoptotic molecule, and thus could potentially contribute to the induction of the intrinsic apoptotic pathway (Nakagawa & Yuan, 2000).

Caspases and *post-mortem* proteolysis of the muscle

Pulford *et al*. (2009) identified significantly higher caspase 9 activity than caspase 8 activity in *post-mortem* beef muscle samples. Furthermore, caspase 9 activity across the *post-mortem* conditioning period has been found to positively correlate to activity of effector caspases 3/7 (Kemp *et al*., 2006, 2009). Collectively, these data indicate that the predominant apoptotic pathway active in skeletal muscle *post-mortem* is the **intrinsic** pathway, involving caspase 9. Thus, supporting the hypothesis of Ouali *et al*. (2006), the process of death and exsanguination would induce hypoxia and ischemia within muscle cells, which in turn would activate the intrinsic apoptotic pathway.

Authors reported different indications about the **time of activation** of caspases in *post-mortem* muscle. Cao *et al*. (2010) and Zhang *et al*. (2013), although using different methods, found that the highest level of caspase 3 activity was at about 1 day *post-mortem*. Other authors showed that the highest caspase activity was in the early *post-mortem* phase (less than 4 - 32 hours) and then less than 6% of the caspase activity remains at 192 h (Kemp *et al*., 2006, 2009; Pulford *et al*., 2009).

Despite the activation of apoptosis in *post-mortem* muscle, caspase involvement in meat tenderization **is still debated**.

An association between caspase activity and **meat quality** was detected in **callipyge sheep** (Kemp *et al*., 2009), that are characterized by heavy muscling in the pelvic limb and loin

region but not in the thoracic region. Kemp *et al*. (2009) detected higher caspases 3/7 activity and caspase 9 activity across the *post-mortem* conditioning period, in normal sheep than in callipyge lambs, in the *Longissimus dorsi* and *Semimembranosus* muscles, but not in the *Infraspinatus* muscle, which is not affect by the callipyge phenotype. In a study of Kemp *et al*. (2006), the change in caspases 3/7 and caspase 9 activity during the early phase of conditioning has been identified to have a negative relationship with **shear force** in porcine *Longissimus dorsi* muscle. This led to the conclusion that the changes in caspase activity and caspase-mediated cleavage of muscle proteins observed during *post-mortem* ageing may be associated with meat tenderization. Ultimate pH (pHu) has been widely used as an indicator of potential meat tenderness (Young *et al.*, 2004). *Longissimus thoracis* beef muscle samples exhibiting a **low pHu** and consistently greater levels of caspases 3/7 activity than those with intermediate or high pHu, suggesting that a more vigorous rate of apoptosis was initiated in the low pHu muscles (Pulford *et al.*, 2009).

To support the hypothesis of an involvement of the caspases in meat tenderization process, from many studies derive the indication of **myofibrillar degradation** by the action of caspases. Some of these studies are reported below. Kemp and Wheeler (2011) have demonstrated, in myofibrils isolated from *Longissimus dorsi* and *Supraspinatus* muscles from normal and callipyge sheep, that some apoptotic inducers lead to the activation of the intrinsic apoptotic pathway via caspase 9, and that a recombinant caspase 3 increased proteolysis of myofibrillar proteins including **desmin** and **troponin T**, whilst a caspase specific inhibitor prevented this degradation. Chen *et al*. (2011) analyzed the effects of apoptotic inducers on caspase 3 activity and myofibrillar disruption during *post-mortem* ageing in chickens. All apoptotic inducing treatments examined in this study resulted in a significant increase in caspase 3 activity, and lead to the appearance of a proteolytic product assumed to originate from **troponin T** degradation, and to the generation of the caspase specific **alpha II spectrin** degradation product. Zhang *et al*. (2013) showed that caspases could produce the degradation patterns of **titin, nebulin, desmin and troponin-T** during the *post-mortem* degradation of geese meat.

Collectively these findings seem to indicate that during the conditioning period caspases are both active and capable of targeting and cleaving their specific substrates, adding to the evidence that apoptosis is the mode of cell death in skeletal muscle *post-mortem* and caspases have a role in meat tenderization.

By contrast, some research contend that caspases are not likely to play a major role in the *post-mortem* proteolysis associated with meat tenderization. Underwood *et al*. (2008) observed in beef muscle that caspase 3 activity is present immediately after slaughter, but that it decreases with time *post-mortem*. In this study, pro-caspase 3 was not activated during *post-mortem* storage and caspase 3 activity was not correlated with Warner-Bratzler **shear force** in beef *Longissimus* muscle. Moreover, Mohrhauser *et al.* (2011) observed no such degradation patterns in caspase 3 treated bovine *Semitendinosus* myofibrils in comparison to the control or calpain-1 treated samples. These observations led the authors to conclude that calpain-1, not caspase 3, is responsible for the degradation of myofibrillar proteins during beef ageing. Huang *et al.* (2012) examined the effects of a caspase 6 specific inhibitor on myofibrillar protein degradation. Marginal retardation of proteolysis of proteins nebulin, desmin and troponin T was detected in inhibitor incubated samples in comparison to control, suggesting that caspase 6 has **minimal role, if any**, in *post-mortem* degradation and meat tenderization. These authors support the belief that caspases do not significantly contribute to meat tenderness by means of proteolytic degradation. Therefore, some authors, considering the critical role of caspases in apoptotic cell death, proposed the involvement of caspases in meat tenderization in their ability to mediate apoptosis rather than myofibrillar protein hydrolysis.

Interactions between caspases and calpains

Numerous studies have observed cross-talk between the calpain and caspase systems. In particular, calpain activity is indirectly up-regulated by caspase enzymes (caspases 3 and 7) through the cleavage of **calpastatin** (Barnoy & Kosower, 2003; Kato *et al*., 2000; Neumar *et al*., 2003; Porn-Ares *et al*., 1998; Vaisid *et al*., 2005; Wang *et al*., 1998), thus make calpains available for activation by calcium ions (Goll *et al.*, 2003). Caspases could therefore modulate *post-mortem* proteolysis and meat tenderization via their actions on calpastatin.

On the other hand, calpains have been shown to impact caspase activity during **apoptosis** (Chua *et al*., 2000; Nakagawa & Yuan, 2000; Neumar *et al*., 2003). Drawing analogy from the studies of apoptosis in human neuronal cells, human platelets and other cell types, including atrophied muscle as a model, it can be inferred that calpains regulate apoptosis by releasing cytochrome *c* and AIF (Apoptosis Inducing Factor) from mitochondria. Moreover, calpain-2 has been reported to cleave pro-caspase 12 to generate an active caspase that could induce the intrinsic apoptotic pathway (Nakagawa & Yuan, 2000). Calpains can also act as negative

regulators of apoptosis, cleaving caspases 3, 7, 8, 9 (and other proteins that regulate progression of apoptosis) at distinctive sites to generate inactive isoforms (Chua *et al*., 2000).
Kemp *et al*. (2013) highlighted an **interaction** between calpains and caspase in calpain-1 knockout mice. Results from this study detected significantly higher caspases 3/7 activity in knockout mice in comparison to wild type, and this increased activity was particularly apparent in the younger mice when the growth is rapid. This data suggests that caspases were up-regulated to compensate for the lack of calpain-1 and to ensure normal skeletal muscle growth and development, as no differences in skeletal muscle mass were detected. This study further elucidates the complexities of the interactions between these two protease systems.
Some findings suggest that also **cathepsins**, another important group of endopeptidases working in the cell, may contribute to the apoptosis process either directly (Pennacchio *et al.*, 1998) or indirectly through the activation of caspases (Ishisaka *et al.*, 1998).
From the available literature, it can be inferred that apoptosis is a very complex process and the enzymes that mediate it appear to function via different pathways.

Heat shock proteins

It is well recognized that **stress** of animals impairs the meat ageing process, leading generally to tougher meat. At the cellular level, if the stress is particularly intense (e.g. oxidative stress) the cells receive apoptosis-inducing signals via the receptors of cellular death. If the stress is not as severe, cells prepare their defense as quickly as possible. In this sense, the most described reaction is the synthesis of various protective proteins known as **heat shock proteins (Hsp)**. These proteins help in the protection of intracellular components and structures against hazards associated with loss of their biological functions. In relation to the event of programmed cellular death, it is believed that Hsp have an **anti-apoptotic** activity (Beere, 2004). They consequently could slow down the process of cellular death and could constitute an obstacle to good meat ageing.

Structure and general functions

Hsp were originally named because of their rapid induction in response to elevated temperatures (Tissieres *et al.*, 1974). However, it has been shown that a wide variety of different physical, chemical and biological stimuli are also capable of inducing Hsp including oxidative stress, heavy metals, osmotic stress and metabolic poisons (Lindquist & Craig,

1988). Some Hsp are constitutively expressed and increase in response to stress, while the expression of others is only induced following exposure of cells to environmental and physiological stresses to preserve cellular proteins against **denaturation** and possible loss of function (Kultz, 2003). In unstressed cells, constitutively expressed Hsp are essential for maintaining cell homeostasis, functioning as molecular **chaperones** during protein assembly and transport (Haslbeck *et al.*, 2005), during protein **folding** and **unfolding** (Zietkiewicz *et al.*, 2004), and in the **refolding** of damaged proteins (Marques *et al*., 2006).

Hsp are categorized into five classes according to their monomeric molecular size. Four of these classes are **large Hsp** and they have molecular masses of about 60, 70, 90 and 100 kDa, and so they are termed **Hsp60, Hsp70, Hsp90** and **Hsp100**, respectively (Fink, 1999). As an example of large Hsp, Hsp60 and Hsp70 are recognised for their role in facilitating the correct assembly and folding of newly synthesized proteins. They are also involved in preventing the irreversible aggregation of proteins by assisting in proper reassembly of aberrantly folded and denatured proteins in an **ATP-dependent** process, and assist in the translocation of proteins to their correct cellular compartment.

Small Hsp (sHsp; **alphaB-crystallin, Hsp20, Hsp27** and **Hsp40**) are the fifth class of heat shock proteins. Small Hsp are the smallest and the most variable in size of the Hsp family, having molecular masses ranging from 12 to 43 kDa (Haslbeck *et al*., 2005; Lindquist & Craig, 1988). Small Hps are ubiquitously expressed and have homeostatic function in which they stabilize unfolded proteins, assist the refold of denatured proteins and prevent protein aggregation (Liu & Steinacker, 2001; Sun & MacRae, 2005), helping in this functions the class of large Hsp. For example, Vos *et al.* (2008) suggested that the Hsp40 proteins would assist Hsp70 in the folding and compartmentalization of nascent proteins. Moreover, it seems that Hsp27 is functionally associated with Hsp20 and alphaB-crystallin in muscle (Fontaine *et al*., 2005; Gusev *et al*., 2002; Sugiyama *et al*., 2000) to refold denatured proteins.

Unlike larger members of the heat shock protein family, sHsp function in an **ATP-independent** manner (Jakob *et al*., 1993). Small Hsp have a conserved **core region**, termed the α-crystallin domain, comprising 80-100 amino acids, which is followed by a short more freely flexible **C-terminal** extension (tail). The C-terminal of sHsp is responsible for the molecular chaperone function of this family (Muchowski *et al.*, 1997). In contrast, the **N-terminus** of sHsp is much more variable both in sequence and in length (De Jong *et al.*, 1998), and it is involved in oligomerization of the protein (Bova *et al.*, 2000). Certain sites in the α-crystallin domain are involved in the formation of β-pleated sheets that interact to form

dimers and **oligomers** (Gusev *et al*., 2002). These oligomers can either be homo- or hetero-oligomers, composed of a single or multiple sHsp type, respectively.

Functions in stressed cell

During the stress response of the cell to environmental insults, the intracellular levels of many Hsp rapidly increase, due to the increased concentration of unfolded proteins that occurs. The induced expression of these Hsp is seen to be cytoprotective, protecting cells from toxic insult and preventing their demise (Samali & Orrenius, 1998). Such inducible Hsp bind to damaged and misfolded polypeptides and mediate their **refolding** or **degradation**, thus protecting cells from potential deleterious effects and promoting cell recovery (Parsell & Lindquist, 1993). In particular, hyperthermia, ischemia and oxidative stress in mammalian cells are known to activate the constitutively expressed transcriptional factors known as **heat shock factors**. Upon activation, heat shock factors are translocated to the cell nucleus where they form trimers and undergo phosphorylation. This enables them to bind to the so-called **heat shock elements** of the heat shock protein gene**,** resulting in the synthesis of heat shock proteins (Chi & Karliner, 2004; Santoro, 2000; Shamovsky & Nudler, 2008) (Figure 8).

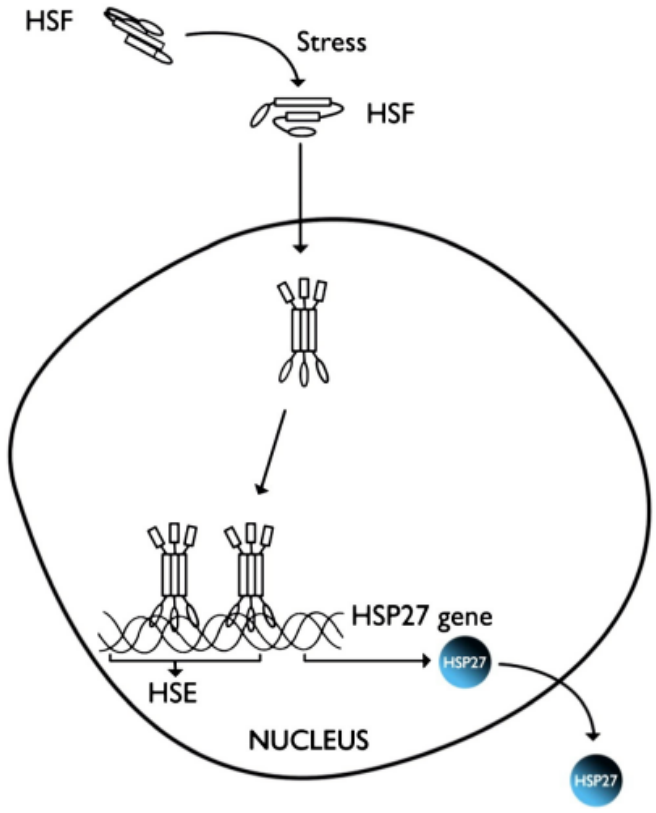

Figure 8. The transcriptional up-regulation of Hsp27 in response to stress. (From D. Lomines *et al*., 2014. Meat Science, 96, 26).

During periods of stress, sHsp already present in the cell can undergo post-translational modifications resulting in protein **phosphorylation** at defined serine residues by protein kinases. The phosphorylation promotes the **dissociation** of oligomeric complexes of sHsp into smaller oligomers, dimers and monomers (Lavoie *et al.*, 1995; Zantema *et al.*, 1992). It seems that sHsp **monomeric** structure has a high binding affinity to substrate; in contrast, oligomers with **dimeric** substructures have minimal binding to denatured proteins and little chaperone activity. Therefore, the balance of monomeric and dimeric sHsp substructures regulates the chaperone activity of sHsp oligomers (Aquilina *et al.*, 2004; Benesch *et al.*, 2008).

Although sHsp-denatured protein complexes are relatively stable, small Hsp are unable to renature damaged protein substrates. Rather, sHsp cooperatively **assist** the ATP-dependent heat shock proteins Hsp70 and Hsp60 in refolding denatured proteins back to their original functional forms (Narberhaus, 2002; Sun & MacRae, 2005; Van Montfort *et al.*, 2001). In particular, **ATP** have been shown to alter the conformation of sHsp, thus releasing their denatured client proteins and allowing other molecular chaperones, such as Hsp70, to renature the proteins (Wang & Spector, 2001).

Several studies have also shown that sHsp, especially Hsp27 and αB-crystallin, can protect the cell against **ROS** (Reactive Oxygen Species) generated through oxidative stress (Mehlen *et al*., 1995). The expression of sHsp can acts as a buffering system to prevent the oxidation of proteins such as is normally seen when intracellular levels of ROS increase.

Role in apoptosis

The events of cell stress and cell death are linked and Hsp are released in response to stress and appear to function at key regulatory points in the control of apoptosis (Arrigo *et al.*, 2002; Arrigo, 2005; Beere, 2001, 2004, 2005; Flower *et al.*, 2005; Garrido *et al.*, 2001). In the apoptotic process, Hsp may have many **anti-apoptotic actions,** which can be summarized as follow:

- formation of a **complex** with active caspases (initiators or effectors) thus hindering their function;
- **protection** of target proteins (substrates) to effector caspases, preventing or delaying their degradation by these enzymes;
- **re-establishing** the initial and active structure of proteins having undergone structural damage, following either the stress itself or the initiation of apoptosis.

However, if the stress levels become unsustainable, cellular death by the mitochondrial pathway can be induced (D'Alessandro *et al.*, 2012; Ouali *et al*., 2006).

A rich literature exists regarding the inhibition of apoptosis by Hsp. Some important points are presented below (Figure 9).

In general, Hsp27 and 70 were identified to inhibit directly both the **intrinsic** and the **extrinsic** apoptotic pathways (Creagh *et al.*, 2000; Gotoh *et al.*, 2004; Mehlen *et al.*, 1996; Mosser & Martin, 1992; Paul *et al.*, 2002; Samali & Cotter, 1996).

Concerning the **intrinsic** pathway of apoptosis, over-expression of **Hsp27** and **Hsp20** prevents the release of cytochrome *c* and the activation of **caspase 9** (Fan *et al.*, 2005; Garrido *et al.*, 1999; Samali *et al.*, 2001). In particular, Hsp27 interacts with cytochrome *c*, thus hindering the correct formation/function of the apoptosome complex (Bruey *et al.*, 2000, Concannon *et al.*, 2001; Saleh *et al.*, 2000).

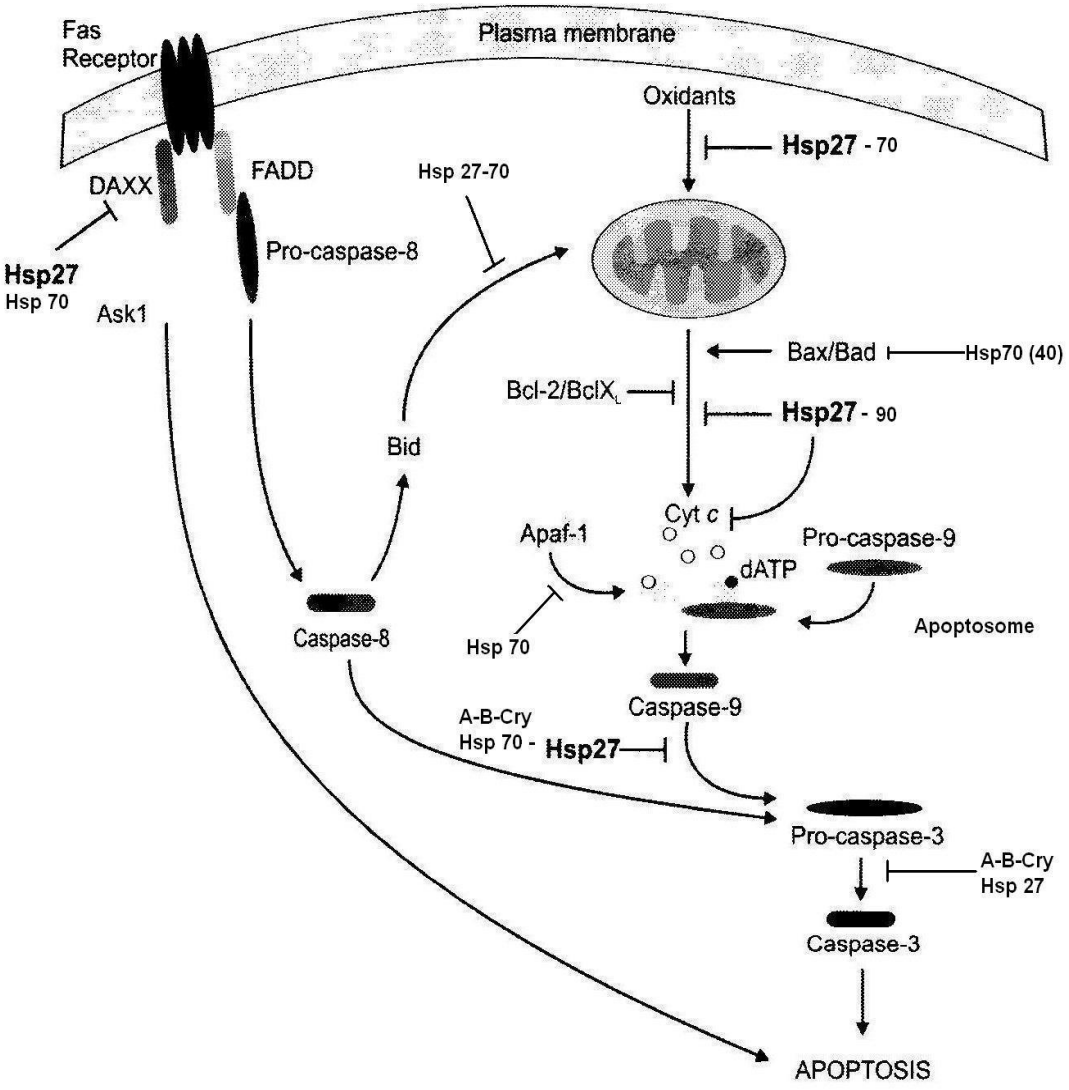

Figure 9. Inhibition of various apoptotic steps by Hsp. (From C.G. Concannon *et al.*, 2003. Apoptosis, 8, 61. Modified).

Furthermore, it has been shown that Hsp27 can inhibit **caspase 3** activation by interacting with the pro-caspase 3 molecule (Concannon *et al.,* 2001; Pandey *et al.,* 2000). An interaction with pro-caspase 3 has been demonstrated also for the **αB-crystallin** (Kamradt *et al.,* 2001). Moreover, Sun and MacRae (2005) and Fan *et al.* (2005) found that Hsp20, Hsp27 and αB-crystallin constitute a dynamic complex that is implicated in the regulation of the apoptotic pathway by sequestration of **Bcl-xs** and **Bax,** which led to the restriction of cytochrome *c* release and thus, the repression of caspase 3 activation.

Hsp27 is also associated with inhibition of **extrinsic** pathway of apoptosis initiated by the binding of **death ligands** to cell surface receptors, such as Fas (Mehlen *et al*., 1996). It has been shown that Hsp27 can interact with Daxx (Charette *et al.,* 2000), a protein that is known as a mediator of Fas induced apoptosis (Yang *et al.,* 1997a).

Both Hsp27 and **Hsp70** can modulate Bid-dependent apoptosis by suppressing caspase 8 mediated cleavage and activation of **Bid**. Hsp27 can also prevent the translocation of Bid to the mitochondria (Gabai *et al.,* 2002).

Hsp70, similarly to sHsp, can inhibit apoptosis by sequestering pro-apoptotic factors such as **Bax** (Beere & Green, 2001; Concannon *et al*., 2003). In particular, **Hsp40** is a co-chaperone of Hsp70 and the complex Hsp70-40 prevents the translocation of **Bax** to the mitochondrial membrane (Gotoh *et al.,* 2004; Ouali *et al.* 2006). *In vitro* studies conducted by Li *et al.* (2000) revealed that Hsp70 is a strong suppressor of intrinsic pathway of apoptosis, acting downstream of cytochrome *c* release and upstream of **caspase 3** activation. Hsp70 has been also reported to interact with **Apaf-1** in the apoptosome, thereby preventing the docking of pro-caspase 9 and its subsequent activation (Beere *et al.,* 2000).

Finally, **Hsp90** negatively regulates caspase activity by inhibiting the cytochrome *c*-mediated oligomerization of **Apaf-1** (Pandey *et al.,* 2000a).

Role in myofibrillar protection

In muscle, sHsp phosphorylation after the onset of stress is also associated with the translocation of sHsp from the sarcoplasm to the **myofibrils** (Golenhofen *et al.,* 1998; Paulsen *et al.,* 2007). In addition, there appears to be some specificity in the regions of the myofibrils that sHsp bind. For instance, **Hsp27** and **αB-crystallin** have been referred to translocate to the **I-band** and **Z-disc** of muscle cells following various stress insults (Bullard *et al*., 2004; Djabali *et al*., 1997; Golenhofen *et al*., 1998; Koh & Escobedo, 2004).

AlphaB-crystallin is an important chaperone in preserving the infrastructure of various cells. In skeletal and cardiac muscles, αB-crystallin associates with myofibrillar proteins in both the

Z-disc and I band, by interacting with exposed hydrophobic residues of myofibrillar structural proteins like **desmin** and **titin** (Bennardini *et al.*, 1992; Golenhofen *et al.*, 2004; Raman & Rao, 1994). Since the Z-disc region is susceptible to mechanical and enzymatic damage (Feasson *et al*., 2002; Raynaud *et al.*, 2005b), the presence of sHsp in this region may serve to stabilize myofibrillar proteins undergoing denaturation. In fact, calpain-1 has been located within the Z-disc region of the myofibrils, making this region susceptible to proteolytic degradation (Raynaud *et al*., 2005). Lomiwes *et al.* (2014) found that αB-crystallin associated with desmin and calpain-1. This observation suggests that αB-crystallin may act as an alternative substrate and may therefore be a **competitive inhibitor** of calpain-1 induced proteolysis of myofibrils.

Hsp27 has been recognized as a potent regulator of cytoskeletal dynamics, in particular of the **actin** microfilaments (Lomiwes *et al.*, 2013; Paul *et al.*, 2002). This sHsp is localized to the Z-lines and the I-bands in muscle (Sugiyama *et al*., 2000). Hsp27 is also involved in folding and assembly of **myosin** in striated muscle (Srikakulam & Winkelmann, 2004). Several studies have shown that over-expression of Hsp27 increases the stability of F-actin microfilaments during exposure to such stresses as hyperthermia and oxidants (Lavoie *et al.*, 1993; Huot *et al.*, 1995). Instead, the down-regulation of Hsp27 can accelerate actin disorganization or degradation, a process that is related to meat tenderization (Bernard *et al*., 2007; Kim *et al*., 2008; Morzel *et al*., 2008). Moreover, it was observed that muscle contraction and oxidative stress induce rapid phosphorylation of Hsp27 and the phosphorylated form of Hsp27 associates with actin and stabilizes thin myofilaments (Huot *et al.*, 1996; Liu & Steinacker, 2001; Somara & Bitar, 2004; Thompson *et al*., 2003). Actin microfilaments are not the only components of the cytoskeleton that have been reported to interact with Hsp27. Indeed, an interaction of Hsp27 and αB-crystallin with **various intermediate filaments** has been shown (Perng *et al.*, 1999). In particular, it was found that Hsp27 protects **desmin** from calpain proteolysis (Blunt *et al.*, 2007).

Role in meat tenderization

Due to their **anti-apoptotic** functions and to the **protective** action against myofibrillar degradation, heat shock proteins are increasingly being investigated as potential factors influencing the conversion of muscle to meat and meat quality. In particular, it was speculated that Hsp expression could be stimulated after slaughter in response to the muscle cell **stress** and that they may influence *post-mortem* proteolysis (Aberle *et al*., 2001; Herrera-Mendez *et al*., 2006; Ouali *et al*., 2006).

The literature is rich in references relating to the possible linkage between Hsp and meat tenderization, and the conclusions do not always agree each other. Some examples are presented below.

Due to the chaperone function of sHsp in maintaining protein integrity, the down-regulation of **Hsp27** and **αB-crystallin** was suggested to favour the proteolytic degradation of actin and myosin leading to a more tender meat (Bernard *et al*., 2007; Kim *et al*., 2008). Moreover, many studies have linked the up-regulation of Hsp27 and αB-crystallin with higher **shear force** needed to cut the meat (Hwang *et al*., 2005; Kim *et al*., 2008). Hocquette *et al.* (2007), analyzing the transcriptome of the muscle cell as a function of shear force values of meat, found that the genes for **Hsp40** and **Hsp27** are more expressed in tougher meat. These results fit well with the positive correlation, shown by Kim *et al*. (2008), between Hsp27 protein levels and shear force value in meat of Korean cattle. Kim *et al*. (2011) have also found that gene HSPB1 (encoding for Hsp27) and its regulator genes were positively correlated with a high shear force and hence low tenderness. In another experiment (Bouley *et al.*, 2004), lower levels of Hsp27 were found in a groups of Charolais young bulls with tender meat. Also in pork (Hwang *et al*., 2005), proteomic analysis have revealed a significant positive correlation between Hsp27 concentration and toughness. Furthermore, in a study comparing microarrays between high and low quality meat groups from beef (Charolais) *Longissimus* muscles, the down-regulation of αB-crystallin and Hsp27 in muscle samples was associated with improved tenderness, juiciness and flavor (Bernard *et al*., 2007).

Bjarnadottir *et al*. (2011) determined a lower abundance of **Hsp70** in electrically stimulated muscles (a known tenderization technique) in comparison to non-stimulated muscles. The authors speculated that electrical stimulation could indirectly accelerate apoptosis and *post-mortem* tenderization via its actions on Hsp70. More in detail, Guillemin *et al*. (2012) hypothesized that tenderization efficiency is dependent on the **Hsp20/Hsp70** ratio, where Hsp20 mainly assures their function of chaperone against structural proteins aggregates, while Hsp70 acts in apoptosis inhibition (by Bax sequestration).

Finally, in a study of Bernard-Capel *et al.* (2009) the expression of DNAJA1 gene, which encodes for **Hsp40**, was identified to be down-regulated in muscle samples with high meat quality. From these results, it was suggested that the anti-apoptotic activity of this gene could slow cellular death during the conversion of muscle to meat leading to a lower meat tenderization. The high expression of DNAJA1 gene would therefore be considered as a marker of toughness.

Collectively, these data suggest that the down-regulation of αB-crystallin, Hsp20, Hsp27, Hsp40 and Hsp70 could facilitate cell death and caspase activity during the *post-mortem* conditioning period and subsequently could increase meat tenderization.

However, the results are **not always univocal**. In a study of Hocquette *et al*. (2012), depending on the bovine group considered (young bulls or steers slaughtered at 1 year of age), the expression of DNAJA1, DNAJB9, DNAJC3 and DNJAC10 genes, which encodes for members of the Hsp40 family, were negatively correlated to tenderness scores, supporting the role of members of the Hsp40 family in tenderness. In this research, also Hsp27, Hsp70 and αB-crystallin were negatively correlated with tenderness in steers. However, the authors specify that the precise genes of the Hsp40 family which are individually negatively correlated to tenderness scores depends on the animal group and hence on its characteristics (**breed, management, etc.**). Similarly, a study with French breeds shown that correlation of Hsp27 level with sensory tenderness may be positive or negative depending on the **breed** considered (Picard *et al*., 2011).

Recently, Picard *et al*. (2014) highlighted that the **contractile** and **metabolic properties** of muscle play a major role in the elaboration of tenderness (Figure 10). Considering *Longissimus thoracis* and *Semidendinous* bovine muscles, that differ by their contractile type (being *Longissimus* a fast-oxido-glycolytic muscle and *Semitendinous* a fast-glycolytic muscle), proteins from the sHsp family (Hsp20, Hsp27, αB-crystallin) are inversely associated with tenderness depending on the muscle. This could demonstrate that the functions of sHsp depend on the contractile and metabolic properties of the muscle.

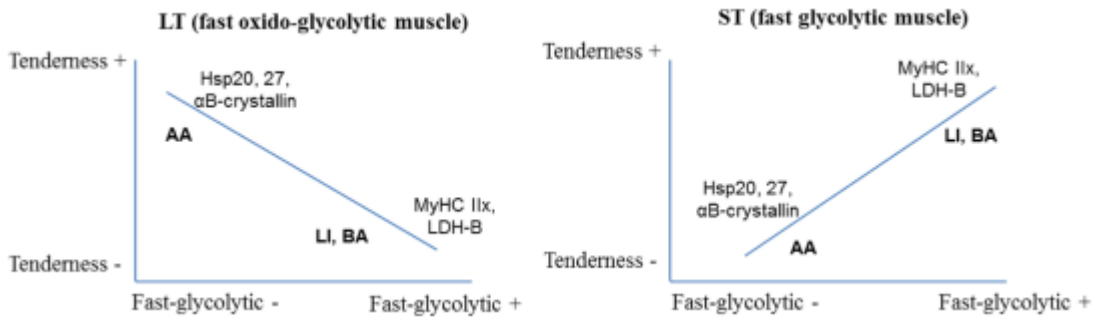

Figure 10. Schematic illustration of the relationships between muscle metabolic and contractile properties and tenderness depending on the muscle type. (From B. Picard *et al*., 2014. *Journal of Agricultural and Food Chemistry*, 62, 9808).

In the same study, Picard *et al.* (2014) pointed out the importance of the **Hsp70** family: the study suggests that Hsp70 could be a relatively general biomarker of tenderness in different muscles and breeds compared to small Hsp, which appear to be quite strongly muscle- or breed-specific.

Contrary to many studies, part of which have been illustred above, Morzel *et al.* (2008) found a positive correlation between Hsp27 levels in meat and tenderness. In this study, Blonde d'Aquitaine muscle levels of Hsp27 explained up to 91% of variation in sensory scores of tenderness. As known, Hsp27 prevents aggregation of unfolded or denatured proteins (Welch, 1992). Given the fact that formation of protein aggregates has been proposed to be a factor limiting *post-mortem* proteolysis (Morzel *et al.,* 2006), these authors explained their results with the fact that higher levels of Hsp27, associated to limited protein aggregation, could **facilitate** the action of proteolytic enzymes during meat ageing. Also Melkani *et al*. (2006) explained that the Hsp20 proteins inhibit the protein aggregates formation and maintain **protein accessibility** to enzymes, so they could enhance structural proteins proteolysis and so meat tenderness.

Finally, we can **conclude** that further data are needed to determine if Hsp levels in *post-mortem* muscle are merely indicators of *post-mortem* proteolysis and meat tenderization or if they play a mechanistic role in the ageing process.

Meat quality and some physical traits of the muscle

Meat quality refers to the compositional quality and the palatability of meat. The major parameters considered in the assessment of meat quality by a consumer are appearance, juiciness, tenderness and flavour (Lawrie & Ledward, 2006). Meat should have a desirable colour that it must be uniform throughout the entire cut. Meat should have also marbling (intramuscular fat) throughout the cut. Marbling increases juiciness, tenderness and flavour of the meat. Water-holding capacity is a factor that also determines the juiciness of meat.

Several biochemical processes that affect beef eating quality are at play during the transportation of cattle to the slaughterhouse, the immediate pre-slaughter period, the slaughtering process and meat handling after slaughter. Before these final phases, other factors affect meat quality. For example, **feeding management** and **nutritional status** (Andersen *et al*., 2005; Sañudo *et al*., 2004; Wheeler *et al*., 1996) affect glycogen depletion and other meat quality parameters, such as **ultimate pH (pHu), colour, cooking losses** and

tenderness. Meat characteristics such as tenderness, shelf life, water-holding capacity (WHC) and cooking losses can be predicted based on the knowledge of pH soon after slaughter. Meat quality traits may depending also on **breed** considered (King *et al*., 2006; Muchenje *et al*., 2008).

pH and meat quality

Several authors have reported relationships among meat quality traits such as tenderness, pHu and meat colour (Byrne *et al*., 2000; Strydom *et al.*, 2000; Vestergaard *et al.*, 2000). Stress prior to slaughter is said to be one of the most important influences on pHu and ultimate meat tenderness. It may result from transportation, rough handling, inclement temperatures or anything that can cause the animal to draw on its glycogen reserves before slaughter. The consumption of glycogen due to the impossibility of forming lactic acid in sufficient quantity to lower the pH *post-mortem*. A sufficiently low pH is necessary for the microbiological safety of meat and a proper ageing. A muscle with high pH *post-mortem* lead generally to a less tender and darker meat.

Grass-fed animals are generally reported to have darker meat than those grain-fed ones (Muir *et al*., 1998). This is caused by the higher pH values found in beef from grass-fed compared to grain-fed cattle. Muir *et al*. (1998) hypothesized that grass-fed steers are more susceptible to pre-slaughter stress and associated pre-slaughter glycogen depletion than the grain-fed steers, as the latter would be better accustomed to penning and handling.

Colour and meat quality

Meat colour is the most important factor that affect consumer purchasing of meat products. Colour measurements are generally done using the Commission International De I'Eclairage (CIE) colour system (Commission International De I'Eclairage, 1976). The three fundamental colour coordinates are L*, a* and b*. The L* measures the **lightness** and is a measure of the light reflected (100 = white; 0 = black); a* (**redness index**) measures positive red, negative green and b* (**yellowness index**) measures positive yellow, negative blue. Meat colour may be influenced by many factors, such as enzymes, diet and age of the animal and even the activity undertaken by the animal.

The myoglobin, the protein responsible for the majority of the red colour in meat, does not circulate in the blood but is fixed in the tissue cells and is purplish in colour. When it is mixed with oxygen, it becomes oxymyoglobin, and produces a bright red colour which is measured objectively by **a*** coordinates (Priolo *et al.*, 2001). The remaining red colour comes from the

hemoglobin, which occurs mainly in the circulating blood, but a small amount can be found in the tissues after slaughter.

When the muscle glycogen has been used up rapidly during the handling, transport and pre-slaughter period, after slaughter there is little lactic acid production which results in DFD (dark firm dry) meat, and this condition is measured by **L*** coordinates. This DFD meat is of inferior quality as the less pronounced taste and the dark colour are less acceptable to the consumer; moreover, this meat has a shorter shelf life, due to the abnormally high pH value, which is conducive to bacterial growth (Priolo *et al.*, 2001). Zhang *et al.* (2005) found that high pH meat had lower L* (lightness), a* (redness), b* (yellowness), hue angle (degrees) and chroma (saturation) values than normal pH meat.

Animals fed on pasture have a yellow fat colour because of the high levels of beta-carotene contained by grass. This yellow fat colour is measured objectively by **b*** coordinates. Grass-fed cattle could be more stressed than grain-fed cattle, due to differences in human exposure practice (Andersen *et al.*, 2005) and grazing animals exhibit more myoglobin than to confined animals, due to differences in physical activity (Shorthose & Harris, 1991), and this leads to differences in meat colour. Forage-based rations, as well as different forage and seasonal changes, allow the carcasses to have a darker lean appearance (low L* values), that may be attributed to increased myoglobin, decreased muscle glycogen, or both, and a yellow fat (Priolo *et al*., 2001).

Although there are contrasting reports on **breed** effects on meat colour, differences in meat colour have been associated with variations in intramuscular **fat** and **moisture** contents, **age**-dependent changes in muscle myoglobin content (Lawrie & Ledward, 2006) and the **pHu** of the muscle (Hector *et al.*, 1992), with higher pHu being associated with dark cuts. Some authors (Chambaz *et al.*, 2003; Muir *et al.*, 2000; Revilla & Vivar-Quintana, 2006) reported no breed effects on colour. On the contrary, O'Neill *et al. (*2006) and Muchenje *et al.* (2008) reported that Nguni steers (a *Bos taurus africanus* breed) produced darker meat than did the improved breeds. Although the causes of the differences in meat colour were not fully understood, O'Neill *et al.* (2006) observed that Nguni cattle released more catecholamines than did exotic breeds raised on a feedlot, during the pre-slaughter period, causing the depletion of glycogen.

Water-holding capacity and drip loss

Water-holding capacity (WHC) is defined as the ability of meat to retain its water during application of external forces, such as cutting, heating, grinding or pressing (Zhang *et al.*,

2005). In detailed studies of myofibrils, Offer and Trinick (1983) presented evidence that most of the water in muscle is held by capillary forces between the thick and thin filaments of the myofibrillar structure of the muscle. Water-holding capacity of meat is greatly affected by **pH** (Offer & Knight, 1988).

WHC is important to meat processing in that, as proteins are able to hold more water, they become more soluble. In meat, WHC is at a minimum at the iso-electric point (**pI**) of proteins (Zhang *et al.*, 2005). At this point, equal positive and negative charges on the amino acid side chains result in a maximum number of salt bridges between peptide chains and a net charge is zero. The pI of meat is in the pH range of 5.0–5.5, which is also the pH of meat after it has gone through *rigor mortis*. The exposure of proteins to a low pH (5.0 – 5.5) at high temperatures causes less water to be retained between actin and myosin filaments, thus increasing exudates (drip loss). In contrast, increasing or decreasing the pH away from the pI will result in increased water-holding capacity by creating a charge imbalance. A charge imbalance is a predominance of either positive or negative charges, which will lead to a repulsion of charged protein groups of the same charge. This repulsion results in increased capacity for water retention and leads to a juicy meat.

Drip loss is the loss of fluid from beef cuts from the shrinkage of muscle proteins (actin and myosin) in the form of drip (Yu *et al.*, 2005). Aldai *et al.* (2006) and Uytterhaegen *et al.* (1994a) reported **breed** effects on drip loss with double-muscled animals, showing increased drip loss in these beef. This effect could be the result of several factors, including, higher glycolytic **metabolism** in muscle of double-muscled animals (Gagniére *et al.*, 1997; Oliván *et al.*, 2004), differences in **collagen** structure (Uytterhaegen *et al.*, 1994a), or the lower intra muscular **fat** (IMF) content of double-muscled meat (Oliván *et al.*, 2004). Aldai *et al.* (2006) found that, when IMF content was high, there was a concomitant lower result for juice loss from raw meat, measured as the expressible juice under pressure. A rapid **pH** fall or a lower pH would tend to cause protein denaturation and greater drip loss (Offer & Knight, 1988).

Meat tenderness

Meat tenderness can be attributed to a person's perception, such as softness to tongue, resistance to tooth pressure and adhesion, or to the force required to shear the meat through the test that is known as Warner–Bratzler Shear Force test (**WBSF**; Bratzler, 1932).

Sources of tenderness variation in meat for instance may be ascribed to animal's **age**, **sex**, **liveweight**, **breed** and *ante-mortem* **stress**. Tenderness varies, mainly due to changes to the myofibrillar protein structure of muscle, in the period between animal slaughter and meat

consumption (Muir *et al.*, 2000). For example, if the carcass is refrigerated too hastily immediately after slaughter, muscle fibres contract severely, and the result is "cold-shortening" which will require a high force to shear the fibres after cooking (Razminowicz *et al.*, 2006).

Muir *et al.* (2000) and Monson *et al.* (2005) argued that meat tenderness is globally a function of the **collagen** content, heat stability and the **myofibrillar** structure of muscle. These factors appear to be affected mainly by the rate of growth of the animal rather than breed *per se*. Similarly, Wheeler and Koohmaraie (1991) and Koohmaraie *et al.*, 2002 suggested that sarcomere length, connective tissue and proteolysis of myofibrillar proteins could explain most of the variation observed in aged meat, with proteolysis being the main biochemical factor contributing to the variation in tenderness. In fact, meat tenderness improves with **ageing** of the muscle. Ageing can be used to decrease shear force values during *post-mortem* storage, as a result of the proteolysis of myofibrillar proteins, which is mediated by calpains (Koohmaraie, 1996) and other protein systems present in the muscular cell, such as proteasome, cathepsins, caspases and heat shock proteins. This tenderization through ageing involves several aspects that affect myofibrillar fragmentation, including animal characteristics, pH and *pre-rigor* conditioning (Sañudo *et al.*, 2004).

Different **breeds** of cattle have a wide spectrum of fibre types in muscles (Campo *et al.*, 2000; Gil *et al.*, 2001), but these are not always reflected by differences in instrumental analyses using Warner Bratzler or sensory panels (Sañudo *et al.*, 2004). Indeed, several authors have reported no differences in WBSF values due to breed when animals are slaughtered at the same age (Muchenje *et al.*, 2008; Muir *et al.*, 2000; Revilla & Vivar-Quintana, 2006). On the contrary, Stolowski *et al.* (2006) reported significant breed- and breed-by-ageing interaction effects on meat tenderness, with the animals with higher levels of Angus blood being tenderer than those with lower levels of Angus blood. Sañudo *et al.*, 2004 found that differences between breed types for most WBSF values were more pronounced at the lower carcass weight than at higher carcass weights. These last authors also reported significant differences in WBSF values among breeds at short ageing times, but the differences disappeared at 21 days, implying that longer ageing times tend to homogenize the product, especially in the heavier animals. The authors concluded that a higher slaughter weight and longer ageing time could make the product more homogeneous, independently of the breed type. Seideman *et al.* (1986) reported significant breed effects on total and insoluble collagen, which could be more important than weight or even production system in determining meat tenderness.

Pasture beef turned out to have a WBSF higher than conventional beef (Razminowicz *et al.*, 2006). However, French *et al.* (2000) found no difference in WBSF between beef samples produced on grass-based and concentrate-based diets.

Future investigations

Since the mitochondrion has a key role in the metabolism of the cell and in the regulation of the apoptotic process, future investigations in meat tenderness process could be focalize on **mitochondrion** functions and on the molecules/factors that act in this fundamental organelle. In fact, despite mitochondria have a pivotal role during apoptosis, studies regarding its *post-mortem* fate and its role in the tenderization process of beef are lacking (Ouali *et al*., 2013).

The *post-mortem* period is characterized by a structural and functional alteration of mitochondrion. In this situation, there is an increase of the mitochondrial outer membrane permeabilization (MOMP) with a dissipation of transmembrane potential and the release of **apoptotic factors** that are normally present in the intermembrane space. The irreversible MOMP is considered a point of no return of apoptosis, that can start at the outer mitochondrial membrane, owing to the pore-forming activity of pro-apoptotic **Bak** and **Bax** proteins (member of Bcl-2-family) or can result from the calcium-dependent permeability transition (PT) of the inner membrane, leading to mitochondria swelling (Galluzzi *et al*., 2012). Another member of Bcl-2-family, **Bik,** can influence the levels of Ca^{2+} in the endoplasmic reticulum, and thus determine whether the released Ca^{2+} is sufficient to overload mitochondria and induce cell death.

In addition to Bcl-2-family factors, other mitochondrial proteins are involved in the apoptotic process. For example, the high-temperature-requirement protein (**HTRA2**) and the second mitochondria-derived activator of caspase (**SMAC**), that mediate the caspase cascade activation, as well as of the apoptosis-inducing factor (**AIF**) and the endonuclease G (**ENDOG**) that mediate DNA fragmentation, about which contradictory results have been reported (Becila *et al*., 2010; Cao *et al*., 2014).

Moreover, the occurrence of mitochondria **fission**, which is associated with MOMP, could be assessed by the analysis of dynamin-related protein 1 (**Drp1**) and of mitochondrial fission protein 1 (**Fis1**). In addition, levels of cyclophilin D (**CyPD**), which is a mitochondrial matrix protein favoring PT (Giorgio *et al*., 2013) could be determined.

It becomes likely that other processes such as mitochondrial **autophagy** should be activated the dying muscle cells, as recently suggested by the finding autophagy markers in *post-mortem* bovine muscle (Sierra & Oliván, 2013). Therefore, the level of **Bnip3** (Bcl-2 nineteen kilodalton interacting protein 3), which modulates mitochondrial autophagy (Webster *et al.*, 2013) and its translocation to mitochondria, could be also evaluated.

Because of their potential capacity to predict the beef tenderness, the above-mentioned proteins could be studied, at the mRNA expression level and at the protein content level, to a better understanding of the role of mitochondria in meat quality.

Moreover, other specific proteins will be taken into consideration in next years in relation to meat tenderness development: annexins and serpines.

Annexins are members of large structurally related and calcium sensitive protein family. Expressed in all eukaryotic cells, they participate in a variety of cellular processes including apoptosis and intracellular signalling. Changes in the concentration of two annexin isoforms, annexin A1 and annexin A6, have been reported in *post-mortem* muscle, a change probably related to apoptosis development and meat tenderization (Bjarnadottir *et al*., 2012; Zhao *et al*., 2010).

In particular, **annexin A1** is rapidly translocated to the cell surface as an "eat me" message to promote the removal of cells that have undergone apoptosis, but its exportation from the cytoplasm to the cell surface is dependent on caspase activation. At the outer plasma membrane leaflet, it co-localizes with phosphatidylserine and where it is also required for efficient clearance of apoptotic cells (Arur *et al.*, 2003).

As reviewed by Cornely *et al*., (2011), **annexin A6** is involved in a large set of biological processes and promotes apoptosis. Mitochondrial fission is an early event during apoptosis, occurring before caspase activation. This process is mediated by binding of Drp1 to the outer mitochondrial membrane, leading to a preliminary release of small amounts of cytochrome *c* (Chlystun *et al*., 2013; Suen *et al*., 2008). In *post-mortem* muscle, annexin A6 may act as a brake to apoptosis through inhibition of Drp1. Because of this function of annexin A6 in apoptosis, a lower abundance of this protein in tender meat would be expected as compared to tough meat, a proposal in good agreement with the conclusions of Bjarnadottir *et al*. (2012), who observed lower annexin A6 levels in tender meat.

Serpins, an acronym of SERine Protease Inhibitors, were discovered in the beginning of the 80's; this superfamily comprises the largest family of protease inhibitors identified to date. The two first serpins (70 and 75 kDa), that are able to inhibit strongly initiator (caspase 8) and effector (caspase 3) caspases (Herrera-Mendez *et al.*, 2009), were purified from bovine

skeletal muscle (Herrera-Mendez *et al.*, 2006; Tassy *et al.*, 2005) and characterized. They were designed bovSERPIN A3-1 and bovSERPIN A3-3 (Pelissier *et al.*, 2008). Serpins have been suggested to be a good marker of meat tenderness (Zamora *et al*., 1996; 2005). The Serpin A3-like group were assumed to be associated not only with meat tenderness (Gagaoua *et al.*, 2012; Zamora *et al*., 1996, 2005), but also to other quality attributes including drip loss and extent of pH drop *post-mortem* (te Pas *et al*., 2013).

References of the introduction

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Aim of the thesis

The connective tissue of a muscle, together with its water and lipids content are important factors in the determination of tenderness of meat derived from it. However, especially in cooked meat, where the collagen contribution is reduced, the myofibrillar structure of the muscle and its degradation during maturation are fundamental to the ultimate tenderness of the meat.

In the recent past years, some proteins and proteases systems have been the focus of research interest for their involvement in meat tenderization process, both for their role in myofibrillar degradation and in their functions in the process of apoptosis that occurs during *post-mortem* in slaughtered animals. Among these proteins, calpain/calpastatin system, caspases and heat shock proteins (Hsp) are amongst the most important factors involved. Calpain/calpastatin system is an enzymatic system that acts in physiological turnover and then in the degradation of myofibrillar proteins. Caspases are pro-apoptotic factors and are involved in degradation of myofibrillar proteins; on the contrary, Hsp are anti-apoptotic factors and have functions in the protection of myofibrillar proteins. Contrary to calpains, the role of caspases and Hsp is less known and attracts research attention.

It was shown by researchers that these proteins/proteases systems act differently in different types of muscles and in different type of fibres that constitute the muscles itself, leading to a different tendency to maturation of the muscles and then to a different degree of tenderness of the meat. Moreover, it has been shown that different species and breeds of animals show a different presence/activity of these protein/enzymatic systems, leading to a different extent of tenderness of the meat. Finally, the different levels of tenderness observed at different stage of life of an animal could be linked to a different presence/activity of these protein systems in the muscle, aside from the structural modifications that physiologically occur during the ageing of the animal.

Based on these considerations, my thesis work was centered on the investigation of these important protein systems in three different experimental conditions usefull for studing the influence of some main factors affecting tenderness. Firstly, we focused on the involvement of the protein systems of interest in the maturation of two different types of bovine muscles, a fast-contracting, glycolytic muscle and a slow-contracting, oxidative muscle. Secondly, we considered the role of these protein systems in the same muscle (*Longissimus dorsi*, LD) of two different bovine species. Finally, choosing the caprine species as a relatively fast growing

ruminant species, we evaluate the evolution of these protein systems in LD muscle at different ages of the experimental animals.

In conclusion, we have tried to highlight the role of calpain/calpastatin system, some caspases and some Hsp in the determination of a different degree of tenderness in three representative conditions that are important for the animal breeding sector.

Experimental section

First study

Early *post-mortem* expression of genes related to tenderization in two Italian Simmental young bulls' skeletal muscles differing in contractile type

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Early *post-mortem* expression of genes related to tenderization in two Italian Simmental young bulls' skeletal muscles differing in contractile type

Abstract

The early *post-mortem* expression of eight genes potentially involved in meat ageing process and the tenderness of two Italian Simmental young bulls' (*Bos taurus*) skeletal muscles differing in their contractile type were evaluated. Samples of *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscles were collected from 17 bulls. The mRNA abundances of Calpain-1, Calpain-2, Calpastatin, Caspase 3, Caspase 9, Heat Shock Protein 27 (Hsp27), Hsp40 and Hsp70 were detected by quantitative PCR (qPCR). The Myosin Heavy Chain-slow and -fast isoform content, the pH_{48h}, and the lipid content of the muscles were in line with the contractile and metabolic type. In comparison with the fast LL, the slow IS showed a lower Calpain-1/Calpastatin mRNA content ratio after slaughtering and a higher Warner-Bratzler Initial Yield value after 7 days of ageing. Hsp27 and Hsp70 mRNA abundances were significantly lower in LL than IS, highlighting their potential role in the ageing process of bovine muscles.

Keywords: calpain, calpastatin, caspase, heat shock protein, warner bratzler share force.

Introduction

Tenderness is the most important factor influencing consumer satisfaction for beef palatability (Miller *et al*. 2001). This quality is the result of a complex process still not clearly understood (Ouali *et al.* 2013) and depends on many factors such as breed, age, amount and solubility of collagen, intramuscular fat content, pH *post-mortem* decline, fibre type characteristics and consequently type of muscle (Seideman & Koohmaraie 1987). Anyhow, ageing plays a key role in muscle tenderization, which involves the activation of several intracellular proteolytic systems; it is now believed that many of the factors responsible for differences in meat tenderness control the rate and extent of *post-mortem* proteolysis of myofibrillar proteins (Ouali *et al.* 2006).

Among the different proteolytic system that participate to the cell dismantling process, it is long ago recognized that the proteolytic system of Calpains 1 and 2 and their inhibitor Calpastatin directly contributes to beef tenderness (Koohmaraie *et al.* 1991; Geesink *et al*. 2000; Volpelli *et al.* 2005) and the at-death enzyme/inhibitor ratio is still considered one of the best predictors of meat tenderness (Ouali *et al.* 2013).

More recently, it was hypothesised that the hypoxic/ischemic conditions, which are induced through the process of slaughtering and exsanguination, could activate the apoptosis in the *post-mortem* skeletal muscle (Sentandreu *et al.* 2002; Herrera-Mendez *et al.* 2006). It was actually demonstrated, by histochemical analyses, that, at least in rat, muscle cells commit suicide soon after animal bleeding through apoptosis (Becila *et al.* 2010). Caspases are the major effectors of apoptosis and are able to degrade a large number of muscle proteins (Earnshaw *et al.* 1999; Fischer *et al*. 2003). Caspase 3 is an effector Caspase, while Caspase 9 is an initiator one, involved in the intrinsic pathway of apoptosis (Earnshaw *et al*. 1999). Herrera-Mendez *et al.* (2006) suggested that predominant apoptotic pathway active in *post-mortem* skeletal muscle is the intrinsic one. Consistently, it was later found (Pulford *et al*. 2009) that Caspase 9, during the first 3 hours *post-mortem*, had double the activity of Caspase 8, which is selectively involved in the extrinsic pathway of apoptosis. In spite of their central role in apoptosis, the involvement of Caspases in meat tenderization, by means of proteolytic degradation, is still debated (Underwood *et al*. 2008; Mohrhauser *et al*. 2011).

The anoxia state also activates cell survival processes, such as the increase in the concentration of several Heat Shock Proteins (Hsps). In normal cells, they act as molecular chaperones during protein assembly and transport, folding and unfolding, and in the refolding of damaged proteins (Ellis & van der Vies 1991). In particular, Hsp40 is a co-chaperon of Hsp70 and is supposed that would assist the latter in the folding and compartmentalization of nascent proteins (Vos *et al.* 2008). Hsp27 is a small Hsp that is involved in the regulation and stabilization of myofibrillar proteins and protection of actin filaments (Lomiwes *et al*. 2013). In addition, Hsps are effective at key regulatory points in the control of apoptosis (Arrigo *et al.* 2002; Beere 2004). Hsp27 and the complex Hsp70-40 were identified to inhibit directly the intrinsic and extrinsic apoptotic pathways (Gotoh *et al*. 2004; Ouali *et al.* 2006). Because their anti-apoptotic function and their protective actions on myofibrillar proteins, Hsps have been proposed to have a potential role in meat tenderization process (Herrera-Mendez *et al*. 2006; Lomiwes *et al*. 2014).

Among the factors influencing early *post-mortem* proteolytic degradation, muscle type plays a recognised role. Many authors reported that slow-twitch muscles are less prone to tenderization in comparison to fast-twitch ones, mainly ascribing this difference at the Calpain/Calpastatin system activity (Ouali & Talmant 1990; Monin & Ouali 1991; Muroya *et al*. 2012). In addition, the recent observation that Hsp27, Hsp40 and Hsp70 are more abundantly expressed in oxidative than glycolytic muscles (O'Neill *et al*. 2006; Cassar-Malek *et al.* 2011; Guillemin *et al.* 2011) prompted the authors to suggest that Hsps may have a role

in tenderness, although the muscle tenderness degree had not been experimentally evaluated in these studies (Cassar-Malek *et al*. 2011; Guillemin *et al*. 2011).

Italian Simmental (*Bos taurus*) breed is the most important dual purpose breed reared in Italy. Due to its high standards in milk production and its excellent reproductive parameters, associated with the high value of calves and beef, the breed is gaining an increasing economic importance for breeders. For this reason, it is important to identify the factors controlling the quality of its meat. Aim of this study is to examine the early *post-mortem* mRNA expression of Calpains 1 and 2, Calpastatin, Caspase 3 and 9, Hsp 27, 70 and 40, in relation with the ageing rate in Italian Simmental young bulls. According to our knowledge, the expression of these genes related to meat tenderness has not yet been studied in this breed. We chose two muscles having different contractile specificities, and then likely different tendencies to tenderization, to investigate, in our conditions, which of these genes were potentially involved in this complex process.

Materials and methods

Animal and sampling

All procedures meet the requirements of the European Community Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.

Seventeen Italian Simmental young bulls (18 months old) were randomly chosen from one farm, fed corn silage-based diets and slaughtered at 694±11.6kg (mean±se) of weight at slaughter. Samples of approximately 5 g of *Longissimus lumborum* muscle (LL, 3^{th} - 4^{th} lumbar vertebra) and *Infraspinatus* muscle (IS) were obtained within 20 minutes of exsanguination, frozen in liquid N_2 and stored at -80ºC until proteomic and transcriptomic analysis. Animal were slaughtered at an EU-licenced abattoir following standards procedures.

After carcass chilling at 4ºC for 48h (final temperature reached in 24 h) pH was measured by a pH-meter (HI 8424; Hanna Instruments, Padua, Italy) equipped with a glass electrode (5232; Crison, Barcelona, Spain) and samples of LL and IS were collected for fat and collagen analysis. Then the meat was aged at 4°C for 7 days. After this ageing period, meat samples were taken for shear force evaluation.

Myosin Heavy Chain-slow (MyHC-slow) and -fast (MyHC-fast) content determination

Proteins extraction and quantification

One hundred mg of muscle were minced at 4°C, suspended in 1 mL of the extraction buffer (60 mM Tris/HCl, pH 7.5, 0.1 M dithiothreitol [DTT], 2% w/v of Sodium-dodecyl-sulphate

[SDS]) and added with 2 µL of anti-proteases (Protease Inhibitor; Sigma-Aldrich, St. Louis, MO, USA). The samples were homogenized by Ultra-Turrax T25 Digital (IKA, Staufen, Germany) at 13000 rpm for 3x30s and centrifuged at 5600g for 4 min at room temperature. They were then heated at 98°C and shaken for 30 min at room temperature. Finally, they were centrifuged at 5600 g for 10 min at room temperature. The protein content was determined by absorption at 280 nm on a spectrophotometer (UV-250; Shimadzu, Kyoto, Japan). The samples were diluted 1:10 with water and their absorption was compared with a calibration curve obtained with solutions of bovine serum albumin (BSA) dissolved at known concentrations in 0.2% SDS.

SDS-PAGE

The stacking gel was prepared with 4% (v/v) acrylamide:N,N'-methylenebisacrylamide at 37.5:1 (w/w) ratio, solubilized in 0.125M Tris/HCl buffer, pH 6.8, with 0.1% (w/v) SDS. The running gel contained 13% of acrylamide:N,N'-methylenebisacrylamide at 37.5:1 (w/w) ratio, solubilized in 0.025M Tris/HCl, pH 8.8, with 0.1% (w/v) SDS and 0.25M glycine. 0.1% ammonium persulfate and 0.0015% (v/v) TEMED were added to start gel polymerization. Electrophoresis was performed at 250V and 15mA. Protein molecular weights were estimated by running standard proteins (Bio-Rad, Hercules, CA, USA) of known molecular weight in separated lanes.

Western-blot

Proteins separated via SDS-PAGE were transferred to a 0.2 µm pore size nitrocellulose membrane (Schleicher & Schuell Bio Science, Dassel, Germany) using a semi-dry blotting apparatus (TE 22; Amersham Biosciences, GE Healthcare Life Sciences, Uppsala, Sweden). The transfer was performed at 2.5 mA/cm^2 for 1 h in the transfer buffer. After staining with Ponceau S solution (ATX Poceau S red staining solution; Fluka, Sigma-Aldrich), to verify transfer efficiency, the nitrocellulose sheets were cut at the level of 50 kDa. The two parts were saturated with 3% (w/v) non-fat dry milk in PBS buffer plus 0.1% Tween 20 for 1 h at room temperature and then incubated overnight separately with the antibodies. The lower part with antibodies against Actin (43 kDa) (polyclonal anti-Actin, 1:5000; Sigma A2066; Sigma-Aldrich), while the upper part with antibodies against MyHC (223 kDa), namely against MyHC-slow (I) (monoclonal anti-MyHC skeletal slow, 1:4000; Sigma M8421; Sigma-Aldrich) or anti-MyHC-fast (II) (monoclonal anti-MyHC skeletal fast, 1:4000; Sigma M1570; Sigma-Aldrich). Blots were then rinsed three times with PBS buffer plus 0.1% Tween 20 and

incubated for 1.30 h with peroxidase coupled to anti-mouse-IgG (1:8000; Sigma-Aldrich) for both anti-MyHC antibodies, and to anti-rabbit-IgG (1:15000; Sigma-Aldrich) for the anti-Actin antibody, at room temperature. The membranes were then washed three times in PBS buffer plus Tween 20 and developed with SuperSignal West Dura (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Densitometric analysis

High-resolution images of the films and gels were acquired and processed using the program ImageQuant TL (GE Healthcare Life Sciences). The bands were hand-selected and their intensity was subtracted from that of the background. The level of MyHC-slow and MyHC-fast protein was quantified by measuring the intensity of the bands and normalising them to actin.

Fat analysis

Extraction of total lipids was performed according to the procedure of Folch *et al.* (1957): 2 g of minced meat were homogenized in 40 mL of chloroform-methanol mixture (2:1 v/v) using an Ultra-Turrax homogenizer (T 25 basic; IKA) and were subsequently filtered under vacuum through Whatman 1820-047 filter paper (GE Healthcare Life Sciences). The extract was washed with 10 mL of 0.88% (w/v) KCl, mixed vigorously for 1 min and then left overnight at room temperature. The organic phase was separated and the solvents were evaporated at 40°C with a Univapo Vacuum Concentrator 100EHC (Elettrofor Scientific Instruments, Rovigo, Italy). The fat amount was calculated by difference between the weight before and after evaporation and expressed as a percentage of the initial weight.

Collagen analysis

Total collagen content was determined by a method based on Bonnet and Kopp (1984) and Reddy and Enwemeka (1996). Two g of muscle was treated with 15 mL of perchloric acid, 70%, at 100°C for 4 h. Then the samples were diluted to 50 mL volume with distilled water and filtered (paper 008; Durieux, France). To create a standard curve, standard solutions of Hydroxyproline were made from a stock solution (1 mg/mL) and added with NaOH. Samples and standard solutions were added with an oxidant solution (chloramine-T) and kept 25 min at room temperature. Finally solutions were added with Ehrlich reactive (4-dimethylaminobenzaldehyde) and kept 20 min at 65°C. Absorbance of standard solution and samples was read at 550 nm on a spectrophotometer (Uvikon XS; Secomam, Alès Cedex,

France). Hydroxyproline concentration of samples was deduced on the base of the standard curve originated by standard solutions. Collagen content was determined as Hydroxyproline content (Hydroxyproline is estimated as 12.5% of collagen).

Rheological analysis

Ten cylindrical cross-section sample replicates of 15 mm in diameter along the fibre axis were cut from the raw LL and IS muscles of each animal. The samples were shared at right angles to the direction of muscle fibres using a Warner-Bratzler (WB) device, with a triangular hole (60°) in the shear blade (1 mm thickness), mounted on a Lloyd TA Plus texture analyser (Lloyd, Borgnor Regis, UK) operating at a crosshead speed of 100 mm/min. From the WB deformation curves, two parameters were recorded: the initial yield (YD, in N) taken as a measurement of the myofibrillar component of tenderness (Møller 1981; Purchas & Aungsupakorn 1993) and the peak yield force (PY, in N), the maximum peak yield of the force-deformation curve. PY-IY values was considered as an index of the connective tissue contribution to meat tenderness (Purchas & Aungsupakorn 1993).

RNA extraction, retro-transcription and quantitative-PCR (qPCR)

RNA extraction

Total RNA was extracted from 40 mg muscle (powdered with a mortar and a pestle in liquid N) using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, DNase treatment included. Concentration and purity of RNA extracted were assessed using spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific). Integrity of RNA (presence of intact 18S and 28S rRNA bands) and absence of genomic DNA was assessed by agarose gel (1% in TBE buffer) electrophoresis, comparing with MW standards.

Retro-transcription

To obtain cDNA, the iScript cDNA Synthesis kit (BioRad) was used. Each 20 µL of reaction contained 4 µL of reverse-transcription reaction mix, 1 µL of iScript reverse transcriptase (MMLV-derived RNase H+), a volume of RNA solution to have 50 ng/µL RNA final concentration, nuclease-free water to final volume. The mixture was held 5 min at 25°C, 1 h at 42°C, and 5 min at 85°C before being cooled on ice. The cDNA concentration and purity was verify using NanoDrop 2000c.

Primers validation

A qualitative PCR was carried out on 6 randomly chosen samples to validate all primers pair specificity, for both genes in exam (Calpain-1, Calpain-2, Calpastatin, Caspase 3, Caspase 9, Hsp27, Hsp40, Hsp70 and reference genes [Table 1]). The PCR was performed using the Bio-Rad CFX96 system (BioRad), on a reaction volume of 20 μL, containing 0.3 μL of each forward and reverse primer (0.3 μM), 10 μL of iQ SYBR Green Supermix (BioRad), 8.4 μL of sterile water, 1 μL of cDNA.

Amplification conditions included 1 cycle of 3 min at 95°C, 40 PCR cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, then 1 min at 95°C, followed to a melt curve of 55-95°C with 0.5°C increments every 5 s. Aspecific amplifications and primers-dimers formation were excluded by checking melt curves. Amplicons length was verified by agarose gel (1.5% in TBE buffer) electrophoresis, comparing with MW standards (Table 1).

Table 1. Primer sequences (5' to 3'), amplification products of gene in exam and reference genes

Gene	Primers Forward-Reverse, Amplicon length (A; bp), Accession Number (AN), Efficiency (E), R^2
Capn1	F: AACCGGATCCGGAATTACCTGTCCATCTTC R: GTAACCACTTAAACAAGTCAAAGGTCACCA A: 282; AN: NM_174259; E: 0.98; R^2: 0.99
Capn2	F: CGAGGACATGCACACCATTGGCTTCG R: TCCTCGCTGATGTCAATCTCGTCAATGTTG A: 314; AN: NM_001103086.1; E: 1.00; R^2: 0.99
Cast	F: CACAGAAGCCAAGGCTATTCC R: TTTACAGCCTGTTTTTTGTGTCTTTTC A: 87; AN: NM_001030318; E: 0.97; R^2: 0.99
Casp3	F: AGAACTGGACTGTGGTATTGAGA R: CACAAAGAGCCTGGATGAAC A: 167; AN: NM_001077840.1; E: 1.00; R^2: 0.99
Casp9	F: CCTGTGGTGGAGAGCAGAAAG R: CATCTGGCTCGTCAATGGAA A: 134; AN: NM_001205504.1; E: 0.99; R^2: 0.99
Hsp27	F: CGTTGCTTCACTCGCAAATA R: TACTTGTTTCCGGCTGTTCG A: 210; AN: NM_001025569.1; E: 0.92; R^2: 0.99
Hsp40	F: GGACTGACCATTGCTGCTG R: CAAACCCACCTCTGTAATAGC A: 138; AN: NM_001034458.1; E: 1.00; R^2: 0.99
Hsp70	F: AACAAGATCACCATCACCAACG R: TCCTTCTCCGCCAAGGTGTTG A: 274; AN: NM_00174550; E: 0.98; R^2: 0.99
Cyclophilin	F: GGATTTATGTGCCAGGGTGGTGA R: CAAGATGCCAGGACCTGTATG A: 119; AN: NM _00178320; E: 1.00: R^2: 0.99
β-actin	F: CTCTTCCAGCCTTCCTTCCT R: GGGCAGTGATCTCTTTCTGC A: 177; AN: NM_00173979; E: 0.96; R^2: 0.99
GAPDH	F: TCATCCCTGCTTCTACTGGC R: CCTGCTTCACCACCTTCTTG A: 177; AN:NM_001034034; E: 0.93; R^2: 0.99
RPLP0	F: CAACCCTGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA A: 226; AN: NM_001012682; E: 0.93; R^2: 0.99

Abbreviations used: Capn1: Calpain-1; Capn2: Calpain-2; Cast: Calpastatin; Casp3: Caspase 3; Casp9: Caspase 9; Hsp27: Heat Shock Protein 27; Hsp40: Heat Shock Protein 40; Hsp70: Heat Shock Protein 70. Cyclophilin, β-actin, GAPDH, RPLP0: reference genes.

Quantitative PCR

The qPCR was performed using the Bio-Rad CFX96 system (BioRad), on a reaction volume of 20 µL, containing 0.3 µL of each forward and reverse primer (0.3 µM), 10 µL of iQ SYBR Green Supermix (BioRad), 8.4 µL of sterile water, 1 µL of cDNA. Amplification conditions included 1 cycle of 3 min at 95°C, 40 PCR cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, then 1 min at 95°C followed to a melt curve of 55-95°C with 0.5°C increments every 5 s. Aspecific amplifications and primers-dimers formation were excluded by checking melt curves.

Each sample was analysed in triplicate and relative gene expression was calculated according to the efficiency-corrected method (Pfafll 2001). β-actin, Cyclophilin, Glyceraldehyde-3-P dehydrogenase (GAPDH), and Ribosomial protein large P0 (RPLP0) were treated as reference genes and used for normalization of qPCR data (M<0.10). Data were presented in fold-change ratio having as reference IS. Primers efficiency was calculated and verified using the standard curve obtained by amplification of serial dilution of the pooled cDNA (Pfafll 2001) (Table 1).

Statistical analysis

The statistical analysis was performed using SPSS for Windows (v. 7.5.21; SPSS Inc., Chicago, IL, USA). Normality of data distribution was tested by the Kolmogorov-Smirnov test and, where appropriate, non-parametrically-distributed data were transformed for parametric testing. A paired samples t-test was used to evaluate the differences between muscles. The correlations between variables were determined by Rho Spearman's or Pearson's test when appropriate.

Results and discussion

Several studies reported that slow-twitch muscles are less prone to tenderization and show a slower *post mortem* proteolysis in comparison to fast-twitch ones (Ouali & Talmant 1990; Monin & Ouali 1991; Muroya *et al*. 2012). We therefore chose two muscles characterized by a different distribution of fiber types, i.e. *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscles, which were first analysed for their content of Myosin Heavy Chain (MyHC) isoforms. Indeed, it was explained that type I muscle fibres have exclusively the MyHC-slow isoform, while type IIa and IIb fibres have exclusively MyHC-fast (Billeter *et al.* 1980). The results of Western-blot analysis of MyHC isoforms are reported in Table 2 and Figure 1.

Table 2. Chemical and physical characteristics of *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscles

		IS	LL	SEM
Myosin Heavy Chain[1]:				
Fast		0.55^{B}	1.04^{A}	0.171
Slow		1.05^{a}	0.25^{b}	0.167
Fat	g/100g	4.22^{α}	3.18^{β}	0.308
pH_{48h}		5.73^{A}	5.52^{B}	0.036
Collagen	mg/g	3.56^{A}	1.56^{B}	0.057
Warner-Bratzler parameters:	N			
IY		17.4^{a}	13.9^{b}	0.639
PY		30.3^{A}	20.2^{B}	0.941
PY-IY		12.9^{A}	6.3^{B}	0.655

Abbreviations used: IY: initial yield; PY: peak yield.
[1]Expressed relative to actin. $^{A, B:}$ $P \leq 0.01$; $^{a,b:}$ $P \leq 0.05$; $^{\alpha,\beta}$: $P = 0.08$.

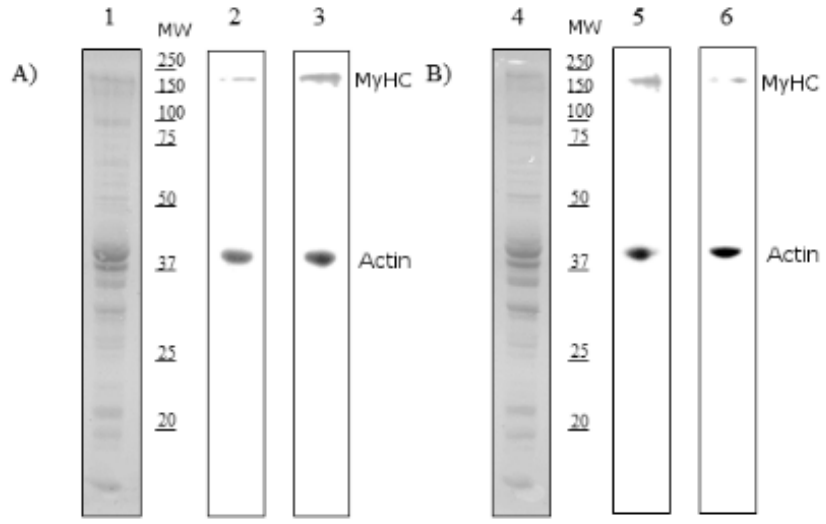

Figure 1. Representative Ponceau S-stained membranes and Western blot membranes relative to one animal. Protein extracts (10 μg) of LL (A) and IS (B) muscles were separated via SDS-PAGE, transferred to nitrocellulose membrane and stained with Ponceau S solution (lanes 1 and 4). After de-staining each lane was cut at the level of 50 kDa and the lower part was incubated with antibodies against Actin, while the upper part with antibodies against MyHC-slow (lanes 2 and 5) or anti-MyHC-fast (lanes 3 and 6); MW: molecular weight; MyHC: Myosin Heavy Chain.

As expected, IS muscle had a significantly lesser MyHC-fast content ($P \leq 0.01$), and a significantly greater MyHC-slow content ($P \leq 0.05$) than LL muscle. Such difference is consistent with the fibre pattern defined by Totland and Kryvi (1991) in bull, according to which LL is a muscle rich in type II fibres, whereas IS is rich in type I fibres.

Based on what reviewed by Lefaucheur (2010) low-contracting muscles with a predominance of type I fibres have an oxidative metabolism and a low glycogen content; instead, fast-contracting muscle with high abundance of type II fibres have a high glycogen content and a glycolytic metabolism, apart from the IIa type that shows an intermediate metabolism (oxido-glycolytic). Most abundant glycogen may ensure a lower ultimate pH, due its conversion in lactic acid (Young *et al*. 2004). Furthermore, it is often stated that lipid content is greater in slow fibres than in fast ones (Howald *et al*. 1985), because of their adaptation to prolonged efforts. The chemical and physical parameters determined in LL and IS (Table 2) were in line with their contractile and metabolic type. Indeed IS was numerically richer (4.22 *vs.* 3.18 mg/100g fresh tissue; $P=0.08$) in intramuscular lipids and showed a greater collagen content (3.56 *vs.* 1.56 mg/g fresh tissue; $P \leq 0.01$) than LL, which in turn attained a lower ultimate pH (5.52 vs. 5.73; $P \leq 0.01$), indicative of a faster ageing process (Young *et al*. 2004).

In our trial, IS showed a higher Warner-Bratzler Peak Yield share force, the maximum peak yield of the force-deformation curve (PY; 30.3 *vs.* 20.2 N; $P<0.01$) than LL, consistently with Torrescano *et al.* (2003). Moreover, the highest mechanical hardness of IS was not only a consequence of the background toughness due to connective tissue of muscle. Indeed, both the Initial Yield (WB-IY), which is a measurement of the myofibrillar component of tenderness (Møller 1981; Purchas & Aungsupakorn 1993), and the PY-IY value, that is considered an index of the connective tissue contribution to meat tenderness (Purchas & Aungsupakorn 1993), were both significantly higher for IS than LL (IY: 17.4 *vs.* 13.9 N, $P \leq 0.05$; PY-IY: 12.9 *vs.* 6.3 N, $P \leq 0.01$, respectively; Table 2). Thus, in addition to the structural effect of collagen, also the myofibrillar component played a key role in determining the differences in tenderness between the two muscles, likely because of a lesser pronounced *post-mortem* proteolysis in IS than LL, as already observed in slow muscles with respect to the fast ones by several authors (Ouali & Talmant 1990; Monin & Ouali 1991; Muroya *et al*. 2012).

Table 3. Fold change ratio in relative RNA expression of genes of *Infraspinatus* muscle (IS) and *Longissimus lumborum* (LL)

Gene	IS	LL	SEM
Capn1	1.00^{α}	1.33^{β}	0.190
Capn2	1.00	1.05	0.138
Cast	1.00^{α}	0.87^{β}	0.093
Capn1/Cast	1.00^{a}	1.53^{b}	0.094
Casp3	1.00	1.21	0.161
Casp9	1.00	0.94	0.086
Hsp27	1.00^{A}	0.53^{B}	0.074
Hsp40	1.00	1.02	0.097
Hsp70	1.00^{a}	0.68^{b}	0.129

Abbreviations used: Capn1: Calpain-1; Capn2: Calpain-2; Cast: Calpastatin; Casp3: Caspase 3; Casp9: Caspase 9; Hsp27: Heat Shock Protein 27; Hsp40: Heat Shock Protein 40; Hsp70: Heat Shock Protein 70.

$^{A, B}$: P<0.01; ^{a,b}: P<0.05; $^{\alpha, \beta:}$ P<0.10.

Calpain-1 and Calpain-2 are calcium-dependent proteases that are considered responsible for *post-mortem* muscle proteolysis (Geesink *et al*. 2000; Volpelli *et al*., 2005), although Calpain-1 is believed to have the most important role in the meat tenderization process (Koohmaraie *et al*. 1987; Geesink *et al*. 2006). Moreover, a positive correlation between mRNA expression and activity of Calpain-1 and its endogenous reversible and competitive inhibitor Calpastatin has been reported (Li *et al*. 2009). In our trial Calpain-1 mRNA abundance was slightly higher in LL than IS (P=0.08), while Calpain-2 transcript content was similar between muscles (P>0.05; Table 3). We also found a general moderate negative correlation between Calpain-1 mRNA content and WB-IY (r=0.287; P=0.09). Moreover, when the Calpain-1/Calpastatin mRNA ratio was analyzed, a higher value was found in LL than IS (P<0.05; Table 3), while the abundance of Calpastatin transcript was higher in IS than LL (P<0.05). Other authors reported a relation between Calpain/Calastatin system activity and contractile type in cattle, pig and sheep (Ouali & Talmant 1990; Muroya *et al.* 2012), founding a higher Calpain/Calpastatin ratio in fast-twitch glycolytic than slow-twitch oxidative muscles. Furthermore, Calpastatin activity has been shown to be less in fast glycolytic muscles than oxidative slow muscles (Whipple & Koohmaraie 1992; Sazili *et al*. 2005).

Overall, in our study, the tendency of higher Calpain-1 mRNA abundance, higher Calpain-

1/Calpastatin transcript ratio and lower Calpastatin mRNA content recorded at slaughter in LL compared to IS, could contribute to explain the different WB-IY observed, in accordance with previous reports (Ouali & Talmant 1990; Monin & Ouali 1991; Muroya *et al*. 2012).

After the process of slaughter and exsanguination, all cells undergo anoxia and receive no more nutrients. In such conditions, muscle cells can decide to die by initiating the apoptotic process (Herrera-Mendez *et al*. 2006; Pulford *et al.* 2009; Becila *et al*. 2010). Caspases are peptidases playing a key role in this process (Earnshaw *et al.* 1999; Ouali *et al.* 2006). In our trial differences between muscles in Caspase 3 and Caspase 9 mRNA content were not found ($P>0.05$). Instead positive correlations were found between Calpain-1 and Caspase 9 transcripts amount both in IS (r=0.680; $P=0.03$) and LL (r=0.507; $P=0.04$; data not reported). In accordance with our findings, no difference in Casp3 mRNA expression was found in pigs (Kemp *et al.* 2006) and cattle (Yang *et al.* 2012). Although a different post-translational regulation of Caspases in the two muscle cannot be excluded, our data seem to rule out that modulation of the expression levels of this class of proteases plays a key role in meat tenderization also in the Italian Simmental breed. Interestingly, it was already pointed out the lacking of association between Warner-Bratzler shear force at slaughter and Caspase 3 activity (Underwood *et al.* 2008), as well as that Calpain-1, not Caspase 3, was responsible for the degradation of key myofibrillar proteins during beef ageing (Mohrhauser *et al.* 2011).

The stress condition during slaughter might induce the increase of synthesis of Hsps that have specific anti-apoptotic roles in the cell (Arrigo *et al.* 2002; Beere 2004). In particular, Hsp70, its co-chaperon Hsp40, and the small Hsp27 are involved in the intrinsic pathway, as demonstrated in different mammalian cell cultures (Li *et al.* 2000; Gotoh *et al*. 2004; Voss *et al*. 2007). In our trial LL showed lower Hsp27 ($P<0.01$), Hsp70 ($P<0.05$), and similar Hsp40 ($P>0.05$; Table 3) mRNA levels than IS. Moreover, a general slight/moderate positive correlation between Hsp27 and WB-IY was found (r=0.307; P=0.07). These results concerning Hsp27 and 70 are consistent to data recently obtained in other bovine breeds. Indeed in two studies on the transcriptomic analysis of *Longissimus thoracis* of Charolais young bulls was found a negative correlation between Hsp27 and Hsp70 expression and tenderness sensory scores (Bernard *et al.* 2007; Hocquette *et al.* 2012). These results fit well also with the positive correlation of Hsp27 protein levels and shear force values found in *Longissimus dorsi* muscle of Korean cattle (Kim *et al*. 2008), and with results of a study who reported lower levels of Hsp27 in a tenderer group of Charolais and Limousin young bulls' *Semitendinosus* muscle (Bouley *et al*. 2004). Moreover, in electrically stimulated *Longissimus dorsi* of Norwegian Red young bulls a lower abundance of Hsp70 in comparison to non-

stimulated muscles was determined, indicating that electrical stimulation, a known tenderization technique, could indirectly accelerate apoptosis and *post-mortem* tenderization via its actions on Hsp70 (Bjarnadóttir *et al*. 2011).

Interestingly, Pulford *et al.* (2008) demonstrated the precipitation of small Hsps at pH values 6.2 and lower, due to their isoelectric points. Further studies demonstrated that Hsp precipitation leads to the lack of myofibrillar protection against muscle proteases (Pulford *et al.* 2009; Lomiwes *et al.* 2013). In particular, the role of small Hsps, such as Hsp27, in protection of myofibrillar proteins was recently confirmed (Lomiwes *et al.* 2014). In our experiments, due to the lower pH of LL and to its lower expression of Hsp27, it is reasonable to suggest that both factors favor a greater degradation of myofibrillar proteins leading to a more tender meat.

The lack of effect of muscle type on Hsp40 transcript amount in our experiment was unexpected, because this gene was proposed as a negative marker for beef tenderness (Bernard *et al.* 2007; Hocquette *et al.* 2012). However, Hocquette *et al*. (2012) underlined the difficulty to extend the results out of their experimental conditions (breed, gender, management, environmental conditions, etc.). Moreover, Cassar-Malek *et al.* (2011) found higher expression of Hsp40 in the higher oxidative muscles at protein level, but not always at transcript one, probably because translational mechanisms and/or mRNA stability.

Conclusions

Tenderization process of beef is complex and depends on many factors, related to the animal (i.e. breed, age, management) and to the muscle characteristics (fat and collagen content, fibres type and proteolytic systems activity). Our study showed as LL and IS of Italian Simmental young bulls have different ageing rate, confirming the tendency of fast-fibres muscles to have a greater tenderization level. The differences between muscles appear to be linked to Calpain-1/Calpastatin mRNA ratio at slaughter, confirming the role of Calpain/Calpastatin system in meat tenderization. Moreover, the difference in tenderness between these two muscles appears to be also due to the difference in Hsp27 and Hsp70 mRNA abundance, highlighting the potential role of these proteins in the ageing process of bovine muscles.

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Annex 1

Animal Science Journal

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ORIGINAL ARTICLE

Early *post mortem* expression of genes related to tenderization in two Italian Simmental young bulls' skeletal muscles differing in contractile type

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ABSTRACT

The early *post mortem* expression of eight genes potentially involved in meat ageing process and the tenderness of two Italian Simmental young bulls' (*Bos taurus*) skeletal muscles differing in their contractile type were evaluated. Samples of *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscles were collected from 17 bulls. The messenger RNA (mRNA) abundances of calpain-1, calpain-2, calpastatin, caspase 3, caspase 9, heat shock protein 27 (Hsp27), Hsp40 and Hsp70 were detected by quantitative PCR. The myosin heavy chain-slow and -fast isoform content, the pH_{48h} and the lipid content of the muscles were in line with the contractile and metabolic type. In comparison with the fast LL, the slow IS showed a lower calpain-1/calpastatin mRNA content ratio after slaughtering and a higher Warner-Bratzler Initial Yield value after 7 days of ageing. Hsp27 and Hsp70 mRNA abundances were significantly lower in LL than IS, highlighting their potential role in the ageing process of bovine muscles.

Key words: *calpain, calpastatin, caspase, heat shock protein, Warner Bratzler shear force.*

Second study

Assessment of calpain and caspase systems activities during ageing of two bovine muscles by degradation patterns of αII spectrin and PARP-1

(Animal Science Journal, 2015, doi: 10.1111/asj.12473 - Annex 2)

Assessment of calpain and caspase systems activities during ageing of two bovine muscles by degradation patterns of αII spectrin and PARP-1

Abstract

The activities of calpain and caspase systems during ageing in *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscle of Italian Simmental young bulls (*Bos taurus*) were assessed. Samples from 10 animals were collected within 20 min of exsanguination (T0), after 48 h (T1) and 7 days (T2) *post-mortem*. Calpain and caspase activity was evaluated based on the formation of αII spectrin cleavage products of 145 kDa (SBDP145) and 120 kDa (SBDP120), respectively. Caspase activity was also assessed by the presence of poly (ADP-ribose) polymerase-1 (PARP-1) cleavage product. At T0, LL showed higher level of SBDP145 than IS ($P < 0.01$), while SBDP120 and PARP-1 degradation products were similar between muscles. At T1, no difference was found in the level of SBDP145 between muscles, while SBDP120 and PARP-1 cleavage products were not detected. At T2 neither αII spectrin nor PARP-1 cleavage products were found. LL and IS showed different proteolysis after slaughter that was greater influenced by calpain than caspase activity, which was detectable only in the early *post-mortem*.

Keywords: bovine muscle, calpain, caspase, ageing.

Introduction

Tenderness is one of most appreciated properties of beef by consumers, which are willing to pay more for more tender beef (Riley *et al*. 2009). Despite tenderness is affected by many factors, the degradation of cytoskeletal and myofibrillar proteins seems to be the most important one during ageing (Ouali *et al*. 2013). However, proteolysis mechanisms during ageing are still debated (Mohrhauser *et al*. 2014). It is widely accepted that the calpain system, a family of intracellular cysteine proteases, does contribute to meat tenderization, specifically calpain-1 and its endogenous reversible and competitive inhibitor calpastatin (Koohmaraie *et al*. 1987; Geesink *et al*. 2006).

Several authors (Ouali *et al*. 2006; Kemp *et al*. 2010; Ouali *et al*. 2013; Kemp & Parr 2012) more recently suggested as calpain could not be the only proteolytic enzyme active in the *post-mortem* affecting meat quality, and, at this regards, highlighted a cross-talk between calpain and caspase systems.

Caspases are a family of cysteine proteases involved in skeletal muscle metabolism; caspase-3

and 7 are effectors caspases acting in both extrinsic and intrinsic pathways of apoptosis (Earnshaw *et al.* 1999), being caspase-3 the most active one (Elmore 2007). Caspases are activated early in hypoxia, when mitochondria are mainly implicated in the development of ischaemic damage (Solaini & Harris 2005), which may be similar to that developed by the hypoxic conditions of *post-mortem* (Kemp *et al.* 2006). However, the role of caspase-3 during *post-mortem* has been questioned (Underwood *et al.* 2008; Cao *et al.* 2010).

Aim of this study was to assess the activities of calpain and caspase during ageing in the *Longissimus lumborum* muscle (LL) and the *Infraspinatus* muscle (IS) of Italian Simmental young bulls, which are a fast- and a slow-twitch muscle, respectively (Totland & Kryvi 1991; Lefaucheur 2010). We recently found that LL has a greater tenderization level than IS in Italian Simmental young bull and that such a difference is linked to calpain-1/calpastatin mRNA ratio, but not to caspase-3 mRNA level at slaughter (Saccà *et al.* 2015). This prompted us to assess, in these two muscle types, the calpain and caspase enzymatic activity over the *post-mortem* period, to actually evaluating their contribution to meat ageing.

Materials and methods

Experimental design and sample collection

All procedures meet the requirements of the European Community Directive, 86–609-EC for Scientific Procedure Establishments.

Ten Italian Simmental young bulls, of about 18 months of age, were randomly chosen from one farm, fed with corn silage-based diets and slaughtered at an EU-licensed abattoir, following standard procedures (average weight, 700 ± 10.2 kg, mean ± SE). After cutting, the carcasses were chilled and kept at 4°C until the end of ageing. At slaughter, within 20 min of exsanguinations, and after 48 h and 7 days, approximately 10 g of samples of *Longissimus lumborum* muscle (LL; $3^{th}/4^{th}$ lumbar vertebra) and *Infraspinatus* muscle (IS) were collected, frozen in N_2, and stored at -80 ºC until analyses.

SDS-PAGE and Western Blotting analysis

The calpain and caspase activity was measured by following the changes in the levels of their specific substrates αII spectrin and poly (ADP-ribose) polymerase-1 (PARP-1), and the related degradation products, as in Kemp *et al.* (2006). Total proteins were extracted from 100 mg of each muscle. Samples were homogenized 3 times for 30 s with Ultra-Turrax T25 Digital (IKA, Staufen, Germany) in 0.5 mL extraction buffer, that contain 0.05 M DTT, 2 % w/v SDS, 60 mM Tris-HCl pH 7.5 and antiproteases (Protease Inhibitor Cocktail, Cat. N.

P2714; Sigma-Aldrich, St. Louis, MO, USA): 2 mM AEBSF, 0.3 µM Aprotinin, 116 µM Bestatin, 14 µM E-64, 1 µM Leupeptin, 1 mM EDTA. Samples were than boiled 2 min at 98 °C, mixed 30 min at room temperature and centrifuged 10 min at 5600 *g*. Protein content of the final supernatant was determined by the near UV absorbance (280nm) method (Walker 2002). Equal protein quantity (30 µg) of each sample was subjected to 8 % SDS-PAGE (Laemmli 1970). Samples of both muscles from each animal taken at specific sampling time were analysed on the same gel, in order to assess the differences between muscles within sampling time. Proteins separated by SDS-PAGE were stained with NOVEX® Colloidal Blue Staining Kit (Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) or transferred to nitrocellulose membranes using a semi-dry blotting apparatus (TE 22; Amersham Biosciences, GE Healthcare Life Sciences, Uppsala, Sweden). The nitrocellulose sheets were saturated with 3 % (w/v) non-fat dry milk in PBS buffer and incubated overnight with αII spectrin antibody diluted 1:250 (Spectrin αII (C-3): sc-48382; Cruz Biotechnology Inc., Santa Cruz, CA, USA) or PARP-1 antibody diluted 1:500 (PARP-1 (H-250):sc-7150; Santa Cruz Biotechnology Inc.). Immunoreactive bands were detected by enhanced chemiluminescence assay (Pierce Protein Biology Products, Thermo Scientific, Rockford, IL, USA) using horseradish peroxidise-conjugated anti-mouse-IgG and analysed by densitometry using ImageQuant software (GE Healthcare Life Sciences). The apparent masses of the protein bands were estimated from a calibration curve obtained by plotting the migration distances of high range standard proteins (Precision Plus Protein Standards; Biorad, Hercules, CA, USA) versus their known molecular masses. Linearity and specificity of the two antibodies against the full-length antigens and their respective degradation products were achieved.

Statistical analysis

The statistical analysis was performed using SPSS for Windows, version 7.5.21 (SPSS Inc., Chicago, IL, USA). Normality of data distribution was tested by the Shapiro-Wilk test. Differences between muscles within time of sampling were carried out using paired samples t-test procedure.

Results and discussion

It is well known that the cytoskeletal protein αII spectrin and the nuclear PARP-1 are substrates of calpain and caspase enzymes, which produce specific cleavage products. In particular, specific cleavage of αII spectrin by caspase-3 generates 150 and 120 kDa

degradation products (SBDP150 and SBDP120, respectively; Warren et al. 2005), of which SBDP120 is widely used as a marker for caspase-3-mediated proteolysis (Nath et al. 2000). AlphaII spectrin can also be cleaved by calpains, producing another 150 kDa peptide and a specific 145 kDa peptide (SBDP145), whose level increases upon proteases activation (Warren et al. 2005). Moreover, among the numerous proteins degraded during apoptosis, PARP-1 was identified as the first protein to be proteolyzed, and the appearance of the 89 kDa PARP-1 peptide fragment is generally considered as a good marker for the caspase-3 and 7 activity (Soldani & Scovassi 2002; Kemp et al. 2006). Indeed, a positive correlation was found between caspase activity and protein levels of PARP-1, αII spectrin and their degradation products during post-mortem in porcine muscle (Kemp et al. 2006). In the present experiment, the above mentioned degradation products of calpain and caspase activity were detected by immunoblots of LL and IS muscle samples taken over the post-mortem period, as shown in Figure 1. The statistical analysis of the immunoblot data from 10 Italian Simmental young bulls obtained at slaughter and at 48 h are reported in Table 1 and 2, respectively.

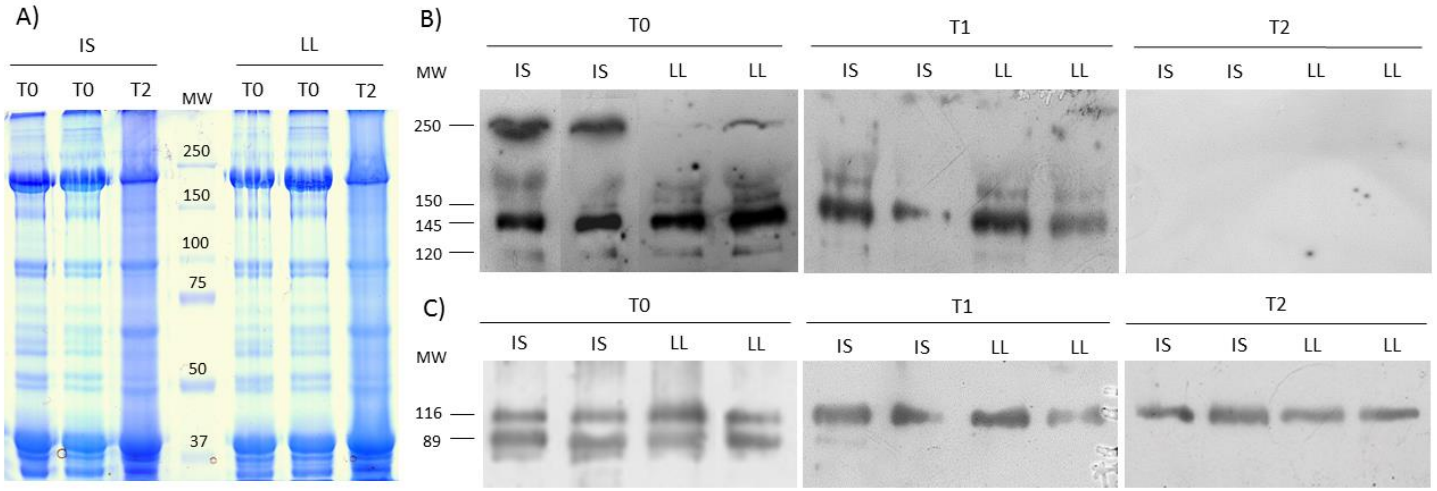

Figure 1. Representative electrophoretic pattern of muscle protein extracts and Western blotting against αII spectrin and poly (ADP-ribose) polymerase-1 (PARP-1). Protein extracts (30 µg) of *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscles at 20 min (T0) and 7 days (T2) from slaughter were separated on 8% SDS-PAGE and stained by Novex staining (panel A). The same protein extracts at T0, as well as those taken at 48h (T1) and 7 days (T2), after separation by 8% SDS-PAGE, were transferred to nitrocellulose membrane and separately incubated with antibodies against αII spectrin (panel B) or with antibodies against PARP-1 (panel C). MW: molecular weight markers (kDa).

Table 1. Cleavage products of αII spectrin and poly (ADP-ribose) polymerase-1 (PARP-1) in *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscle at slaughter. Data were calculated as percentage on the total area of the immunoreactive bands revealed in each sample.

	LL	IS	SEM
αII spectrin cleavage products (%)			
250kDa full-length	11.4^{A}	70.1^{B}	1.96
150kDa length fragment	5.4	4.5	2.35
145kDa length fragment	78.8^{B}	21.0^{A}	1.22
120kDa length fragment	4.4	4.4	1.60
PARP-1 cleavage products (%)			
116 kDa full-length	52.0	52.3	3.65
89 kDa length fragment	48.0	47.7	3.65

^{A,B} Within the same row, means with unlike letters differ significantly at $P < 0.01$.

Table 2. Cleavage products of αII spectrin and poly (ADP-ribose) polymerase-1 (PARP-1) in *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscle after 48 h from slaughter. Data were calculated as percentage on the total area of the immunoreactive bands revealed in each sample.

	LL	IS	SEM
αII spectrin cleavage products (%)			
250kDa full-length	nd	nd	-
150kDa length fragment	34.1	20.3	14.91
145kDa length fragment	65.9	79.7	14.91
120kDa length fragment	nd	nd	
PARP-1 cleavage products (%)			
116 kDa full-length	100	100	-
89 kDa length fragment	nd	nd	-

nd: not detected.

At slaughter the full-length αII spectrin level (250 kDa) was higher in IS than LL ($P < 0.01$; Table 1). This result is in agreement with previous studies (Whipple & Koohmaraie 1992; Joo *et al*. 2013) who found, in the early *post-mortem* period, a lower proteolysis in slow twitch with respect to fast twitch fibres. Moreover, SBDP145 level was significantly higher in LL than in IS ($P < 0.01$), indicating that calpains, and most probably calpain-1, was more active in LL than in IS. This result is consistent to the higher calpain-1/calpastatin mRNA ratio found in LL than IS at slaughter (Saccà *et al.* 2015). Conversely, SBDP120 level, and consequently the caspase-3 activity, was comparable in the two muscles ($P > 0.05$), in

accordance with the similar caspase-3 mRNA levels found in LL and IS (Saccà *et al.* 2015). The PARP-1 cleavage product analysis confirmed that the activity of caspase-3 (and 7) was similar. Indeed, as showed in Table 1, the 89 kDa PARP-1 fragment was detected and it was almost identical between muscles ($P > 0.05$). These results confirm that apoptosis process takes place in LL and IS in the early *post-mortem*, and that caspase-3 may contribute to the degradation of cytoskeletal proteins. Our results are in agreement with the findings of Cao *et al*. (2010) that, measuring the caspase-3 activity by a fluorometric assay, have not revealed differences between *Longissimus*, *Semitendinosus*, and *Psoas minor* muscles of bulls collected at 30 minutes from slaughter. SBDP145 level was higher than SBDP120 both in LL ($P < 0.01$) and IS ($P < 0.01$) suggesting that the activity of calpains was higher than that of caspase-3 in both muscles at slaughter (Table 1). However, a different reactivity of the antibodies used for Western Blotting against SBDP120 and SBDP145 cannot be excluded. Table 1 shows that the level of SBDP150 is very low and similar in LL and IS. This result is rather surprising and possibly suggests that this fragment is quickly degraded to SBDP145 and SBDP120 by calpains and caspase-3, respectively.

After 48 h from slaughter, the presence of the full-length αII spectrin has not been detected in both muscles (Table 2; Fig. 1). The αII spectrin cleavage product SBDP145 was still present, and its level was similar between muscles ($P > 0.05$). Taking into account these results and those obtained at slaughter, the calpain activity seems delayed in IS with respect to LL. The underlying mechanism is not clear, but it is consistent with the lower calpain-1/calpastatin mRNA ratio found in IS than LL at slaughter (Saccà *et al*. 2015) and the lower proteolysis observed in slow twitch fibres (Joo *et al.* 2013). In this regards, it is worth mentioning that also Camou *et al*. (2007) observed a quick reduction of calpain-1 during *post-mortem* that was faster in LL than *Triceps brachii*, which is a slightly slower muscle (Totland & Kryvi 1991; Lefaucheur 2010). At this time of ageing, SBDP120 was not detected in both muscles. Consistently, only the full-length PARP-1 (116 kDa) was found, indicating that caspase-3/7 was not active at 48 h from slaughter. Conversely, the 89 kDa degradation product was no longer detectable, much probably due to the activity of other proteases still to be identify. In accordance with our results, Kemp *et al*. (2006) observed that formation of this fragment was quickly followed by its disappearance during *post-mortem* in porcine muscle, indicating that the caspase system activity declines early after the initial phase of ageing.

Detection of caspase-3 activity in ageing is still controversial and factors affecting its modulation/inactivation remain to be elucidated. In fact, Huang *et al*. (2014) found that in *Longissimus thoracis* muscle of Luxi × Simmental crossbred bulls, the activity of caspase-3

after three days *post-mortem* was less than 70% than that recorded at slaughter. Conversely, Cao *et al*. (2013) found that caspase-3 was active also at 7 days *post-mortem* in *Longissimus* muscle of crossbred bulls. The detection of the αII spectrin cleavage product SBDP150, whose level was similar between muscles ($P > 0.05$), suggests that it could derive from calpains, which were probably still active. Also Kemp *et al*. (2006) highlighted the presence of SBDP150 during ageing in pig. In this study, no correlation between SBDP150 level and caspase activity was found, suggesting that the increasing of SBDP150 during ageing could be mainly due to calpain rather than caspase activity. Also Delgado *et al*. (2001) detected calpain-1 activity till 72h *post-mortem* in skeletal muscles of lamb.

As expected, based on the finding that full-length αII spectrin was not already found after 48 h, after 7 days of ageing neither SBDP150, nor SBDP145 were detected (Fig. 1). Considering these results, the activity of calpains cannot be evaluated at 7 days of ageing through the αII spectrin cleavage products detection. At this sampling time only the full-length PARP-1 was found, indicating that caspase-3/7 was not active (Fig. 1).

In summary, the development of a tender meat in LL than IS is related to a different *post-mortem* proteolysis which is greater influenced by calpain than caspase activity. Moreover, caspase seems active only in the early *post-mortem*, having not detected its specific degradation product at 48 h and 7 days from slaughter.

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Annex 2

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RAPID COMMUNICATION

Assessment of calpain and caspase systems activities during ageing of two bovine muscles by degradation patterns of αII spectrin and PARP-1

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ABSTRACT

The activities of calpain and caspase systems during ageing in *Longissimus lumborum* (LL) and *Infraspinatus* (IS) muscles of Italian Simmental young bulls (*Bos taurus*) were assessed. Samples from 10 animals were collected within 20 min of exsanguination (T0), after 48 h (T1) and 7 days (T2) *post mortem*. Calpain and caspase activity were evaluated based on the formation of αII spectrin cleavage products of 145 kDa (SBDP145) and 120 kDa (SBDP120), respectively. Caspase activity was also assessed by the presence of poly (adenosine diphosphate-ribose) polymerase-1 (PARP-1) cleavage product. At T0, LL showed higher levels of SBDP145 than IS ($P < 0.01$), while SBDP120 and PARP-1 degradation products were similar between muscles. At T1, no difference was found in the level of SBDP145 between muscles, while SBDP120 and PARP-1 cleavage products were not detected. At T2 neither αII spectrin nor PARP-1 cleavage products were found. LL and IS showed different proteolysis after slaughter that was influenced more by calpain than caspase activity, which was detectable only in the early *post mortem* period.

Key words: *ageing, bovine muscle, calpain, caspase.*

Third study

Comparison of meat physical quality traits and the expression of tenderness-related genes between Goudali zebu breed and Italian Simmental x Goudali crossbreed

(Awaiting submission to a journal)

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Abstract

The aim of this trial was to compare some meat physical quality traits and the expression of some tenderness-related genes between Goudali (G, *Bos indicus*) breed and Italian Simmental x Goudali (SimGoud, SG, *Bos taurus* x *Bos indicus*) crossbreed. Twenty-five G and 25 SG bulls bred in the Western Highland Plateau Savannah of Cameroon were considered. Physical quality traits of *Longissimus thoracis* (LT) such as drip loss, color parameters, cooking loss and rheological properties were determined together with pHu (ultimate pH). The mRNA early *post-mortem* abundance of calpain-1 (Capn1), calpain-2 (Capn2), calpastatin (Cast), caspase 3 (Casp3), caspase 9 (Casp9), αB-crystallin (Cryab), heat shock protein 27 (Hsp27), Hsp40 and Hsp70 was detected by quantitative PCR (qPCR). The Capn-1, Cast, Hsp27 and Hsp70, early *post-mortem* protein content was determined by ELISA. The meat of G bulls had higher values of lightness and hue angle and lower values of redness. Moreover, the meat of G had more water losses. None of the other variables considered was significantly different between G and SG breeds, except for the expression of Hsp70, both at the mRNA and at protein level, which was higher in G, probably in relation to the adaptation to tropical climate of the breed.

While crossbreeding with Italian Simmental breed changed the color and ameliorated the water retention capacity of G meat, tenderness and the expression of tenderness-related genes, both at mRNA and at protein level, was not significantly different between the pure local breed and the crossbreed. Crossbreeding Goudali with Italian Simmental, to improve the meat productivity of Goudali genotype, in terms of growth rate and carcass quality, has limited impact on meat traits in tropical conditions.

Keywords: Bos indicus, *Bos taurus* x *Bos indicus*, meat quality, tenderness, gene expression.

Introduction

The Goudali (G) is a *Bos indicus* breed widespread in Cameroon, with good tolerance to heat and which shows resistance to local ecto- and endoparasites, high fertility, maternal performance and foraging ability (Nfor *et al*., 2014a; 2014b; Pamo, 2008). However, these animals present limitations in terms of growth performance, carcass and meat quality, and genetic improvement is therefore desirable. In the past, various experiences of crossbreeding

between zebu and European or American cattle breeds were conducted (Highfill et al., 2012; Koger, 1980; Strydom, 2008) to ameliorate productive performances of *Bos indicus* breeds or to increase disease resistance and climatic tolerance of *Bos taurus* in tropical and sub-tropical regions. Consequently, many studies have focused on the differences in meat traits between zebu breeds, *Bos taurus* breeds and their crosses. The pH of meat seems not to be conditioned by the genotype when *Bos indicus*, *Bos taurus* and their crossbreeds are considered (Gama *et al*., 2013; Bressan *et al.*, 2011) or when also local unimproved breeds (*Bos taurus africanus*) are included in the comparison (Muchenje *et al*., 2008; Muchenje *et al*., 2009; Strydom, 2008). Generally, the meat color is darker in local African breeds with respect to *Bos indicus* x *Bos taurus* crossbreeds or *Bos taurus* breeds, probably because of relatively higher stress level at the moment of slaughter in local breeds, especially when the animals are raised on natural pasture (Muchenje *et al*., 2009b; O'Neill *et al*., 2006). Bressan *et al*. (2011) found no differences in cooking loss of meat between *Bos indicus* and *Bos taurus* breeds. Similarly, Muchenje *et al*. (2008) found no differences in drip loss between *Bos Taurus*, *Bos indicus* x *Bos Taurus* and *Bos taurus africanus*, or in cooking loss in the same genotypes (Muchenje *et al*., 2009). Meat tenderness difference between European cattle breeds and zebu breeds has been largely examined (O'Connor *et al*., 1997; Shackelford *et al*., 1991; Whipple *et al*., 1990; Crouse *et al*., 1989; Wheeler *et al*., 1990). In general, as the percentage of *Bos indicus* increases in crossbreeds, the variability of tenderness tends to increase and tenderness tends to decrease, although there are some contrary findings (Du Plessis and Hoffman, 2007; Frylinck, 2009; Hadlich, 2007; Muchenje *et al*., 2008; Strydom & Frylinck, 2005).

Many enzymatic systems are known to control the quality traits of meat and its tenderness in particular (Ouali *et al*., 2006), among which the calpain system has been considered the most important (Koohmaraie, 1992, 1996; Wheeler, Cundiff, Koch, & Crouse, 1996). This system consists of two calcium dependent proteases, calpain-1 and calpain-2 and their competitive inhibitor calpastatin. Shackelford *et al*. (1991) and Whipple *et al*. (1990) indicated that a higher level of calpastatin was responsible for lower meat tenderness in zebu cattle. Wheeler *et al*. (1990) also found a lower calpain-1 protein expression in Brahman breed (*Bos indicus*) when compared to Hereford breed (*Bos taurus*), which resulted in lower meat tenderness in *Bos indicus* animals.

Calpain/calpastatin system is not the sole determinant in the tenderization process. More recently, Cao *et al*. (2010) and Becila *et al*. (2010) provided evidences that cell death in *post-mortem* muscle occurs via an apoptotic process. Caspases are the major effectors of apoptosis and are able to degrade a large number of muscle proteins (Earnshaw *et al*., 1999; Fischer *et*

al., 2003). Caspase 3 and caspase 9 are involved in the intrinsic pathway of apoptosis (Earnshaw *et al*., 1999) that is the predominant apoptotic pathway active in *post-mortem* skeletal muscle (Herrera-Mendez *et al*., 2006). In spite of their central role in apoptosis, the involvement of caspases in meat tenderization, by means of proteolytic degradation, is still debated (Mohrhauser *et al*., 2011; Saccà *et al*., 2015a, 2015b; Underwood *et al*., 2008).

The anoxic state that characterizes *post-mortem* condition also activates cell survival processes, such as the increase in the concentration of several heat shock proteins (Hsp). Because of their anti-apoptotic function and their protective actions on myofibrillar proteins, Hsp have been proposed to have a potential role in meat tenderization process (Herrera-Mendez *et al*., 2006; Lomiwes *et al*., 2014). From a meat quality perspective, important Hsp are known. AlphaB-cristallyn is known to protect myofibrillar proteins from endopeptidases degradation by binding to myofibrils and stabilizing denaturing proteins in beef cattle (Pulford *et al*., 2009). Hsp27 is involved in the regulation and stabilization of myofibrillar proteins and protection of actin filaments (Lomiwes *et al*., 2013) and that was related to tenderness by many authors (Bernard *et al*., 2007; Hocquette *et al*., 2007; Saccà *et al*., 2015a). Hsp40 is known to be a co-chaperon of Hsp70. Hsp27 and the complex Hsp70-40 have been identified to directly inhibit the intrinsic and extrinsic apoptotic pathways (Gotoh *et al*., 2004; Beere, 2004, 2005; Li et al., 2000).

The Hsp are activated also in response to other stress factors such as heat, physical strain and oxidative stress (De Maio, 1999; Iwaki *et al*., 1993; Kregel, 2002). Owing to their long time adaptation to tropical climates, zebu breeds of cattle are able to regulate their body temperature in response to heat stress better than European breeds (Beatty *et al*., 2006; Gaughan *et al*., 1999). Differential expression of heat shock proteins under thermal stress may partially explain the relative stress tolerance of tropical breeds of cattle compared to European breeds. Among the Hsp, Hsp70 has a significant role in cell thermotolerance (Beckham *et al*., 2004) and animal survival (King *et al*., 2002).

Similarly to previous experiences, a crossbreeding program between the local Goudali and the Italian Simmental (*Bos taurus*) breeds was initiated in the year 2008 (Bessong *et al*., 2011; 2015), thanks to the collaboration between the Cameroon Livestock Development and Husbandry Corporation, known by its French acronym SODEPA (Societé de Développement et d'Exploitation des Productions Animales) and the Italian Simmental Breeders' Association (ANAPRI). The Italian Simmental x Goudali (SimGoud, SG, *Bos Taurus* x *Bos indicus*) crossbreed animals are expected to match good adaptation to the tropical environment with improved growth performances and meat quality traits.

The aim of this trial was to compare the physical meat quality traits and the expression, at mRNA and at protein level, of some tenderness-related genes between G breed and SG crossbreed, to evaluate the effect of the crossbreeding in tropical pastures breeding conditions on some important aspects of meat quality.

Material and methods

Animals and treatments

Fifty male bulls of two genotypes, 25 zebu Goudali (G, *Bos indicus*) and 25 SimGoud (SG, *Bos taurus* x *Bos indicus*) crossbreed, from 20 to 41 months, were selected from the Dumbo Ranch (Latitude 060 42' N, Longitude 0100 25' E) in Cameroon. The 25 SG bulls originated from five artificial insemination breeding herds, where G cows were fertilized with semen from 13 different Italian Simmental (IS) bulls. The experimental SG animals were selected from the progeny of five IS bulls. The 25 G bulls were derived from pure breed calves born by Goudali cows on natural mount with pure G bulls in five commercial herds of Dumbo ranch, at about the same week with those produced by crossbreeding, and introduced immediately after calving into the artificial insemination breeding herds with their dams. After calving, each bull was identified by use of a plastic ear-tag and a corresponding rumen transponder. The experimental herds were submitted to the standard zoo-veterinary care of the ranch and were fed by grazing natural herbage of the Western Highland Plateau Savannah plus NaCl supplementation. At the end of the trial, the animals were weighed and moved initially on-foot for 8 days over 208 km to Bamenda, and then loaded in unspecialized animal transport trucks, as is commonly practiced by cattle traders, to Douala, in an 8 hours drive, over 306 km. To alley stress, the animals were rested for five weeks at the Douala Cattle market lairage, during which they were grazed intermittently on native pastures on the outskirts of the town, close to the market. After this, the animals were slaughtered in the SODEPA industrial slaughterhouse at Douala, following standard procedures. Growth performances and production traits of the experimental animals were discussed by Bessong *et al*. (2015).

Sample collection and first physical measurements

Immediately after slaughter, samples (5 g) of *Longissimus thoracis* (LT) muscle were obtained within 30 minutes of exsanguination, after the separation of the two half carcass and incision in the left side, frozen in liquid N_2 and stored at -80ºC until proteomic and transcriptomic analysis.

After chilling for 24 h, the LT of the 7^{th} - 8^{th} ribs section (LT7) and of the 8^{th} - 9^{th} ribs section (LT8) were extracted from the left side half carcass and the ultimate pH (pHu) was measured, in 3 different point per muscle, by a pH-meter (HI 8424; Hanna Instruments, Padua, Italy) equipped with a glass electrode (5232; Crison, Barcelona, Spain). Approximately 100 g of LT7 were cut and weighed; after that, the cuts were suspended for 24 h at 4°C and then weighed again to determine the drip loss.

After 48 h of ageing at 4°C, color of the LT8 was measured, on 5-10 points/loin, after a blooming time of 45 min, using a portable spectrophotometer Minolta CM 2600d (Konica Minolta, Tokyo, Japan) with an 8 mm aperture, Standard Illuminant D65 light source and 10 viewing angle geometry. The values recorded, according to the standard conditions of the Commission International d'Eclairage (CIE; 1976) included L* (Lightness), a* (Redness), b* (Yellowness), Chroma* (color saturation) and Hue* (angle) scores. Samples of LT8 were aged at 4°C for 5 days until cooking loss determination.

Cooking loss and Warner Bratzler Shear Force determinations (WBSF)

Slices of LT8 muscle of 2 cm thickness were cooked in plastic bags, until reaching 75°C at the core, using a water bath, according to the procedure described by Honikel (1998). The internal temperature was monitored by a thermocouple (Thermocouple Thermometer HD 9016, Delta Ohm, PD, Italy). Each slice of meat was weighed before and after cooking (after quick drying with a paper towel). The total loss of fluid was calculated by difference between the weight before and after cooking and expressed as a percentage of the initial weight.

After two days at 4°C, the Warner-Bratzler Shear Force test was performed on cooked meat. Ten cross-section cylinders of 15 mm in diameter were cut along the fibers axis for each LT sample. The cylinders were sheared perpendicularly of muscle fibers using a Warner-Bratzler (WB) device, with a triangular hole (60°) in the shear blade (1 mm thickness), mounted on a Lloyd TA Plus texture analyser (Lloyd, Borgnor Regis, UK), operating at a crosshead speed of 100 mm/min. From the WB deformation curves, two parameters were recorded: the peak yield force (PY, in N), that is the maximum peak yield of the force-deformation curve and the final yield (FY, in N), that is the second peak yield of the force-deformation curve. The first peak is taken as a measurement of the myofibrillar component of tenderness and the second is taken as a measurement of the connective tissue component of tenderness (Møller, 1981).

RNA content analysis

RNA extraction

Total RNA was extracted from 40 mg muscle, powdered with a mortar and a pestle in liquid N, using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, DNase treatment included. Concentration and purity of RNA extracted were assessed using spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA). Integrity of RNA (presence of intact 18S and 28S rRNA bands) and absence of genomic DNA were assessed by agarose gel (1% in TBE buffer) electrophoresis, comparing with MW standards.

Retro-transcription

To obtain cDNA, the iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA) was used. Each 20 µL of reaction contained 4 µL of reverse-transcription reaction mix, 1 µL of iScript reverse transcriptase (MMLV-derived RNase H+), a volume of RNA solution to have 50 ng/µL RNA final concentration, nuclease-free water to final volume. The mixture was held 5 min at 25°C, 1 h at 42°C, and 5 min at 85°C before being cooled on ice. The cDNA concentration and purity was verified using NanoDrop 2000c.

Primers validation

A qualitative PCR was carried out on 6 randomly chosen samples to validate all primers pair specificity, for all the genes in exam (Calpain-1 (Capn1), Calpain-2 (Capn2), Calpastatin (Cast), Caspase 3 (Casp3), Caspase 9 (Casp9), αB-crystallin (Cryab), Heat shock protein (Hsp27), Hsp40, Hsp70 and reference genes (Table 1)). The PCR was performed using the Bio-Rad CFX96 system (BioRad), on a reaction volume of 20 µL, containing 0.3 µL of each forward and reverse primer (0.3 µM), 10 µL of iQ SYBR Green Supermix (BioRad), 8.4 µL of sterile water, 1 µL of cDNA.

Amplification conditions included 1 cycle of 3 min at 95°C, 40 PCR cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, then 1 min at 95°C, followed to a melt curve of 55-95°C with 0.5°C increments every 5 s. Aspecific amplifications and primers-dimers formation were excluded by checking melt curves. Amplicons length (Table 1) was verified by agarose gel (1.5% in TBE buffer) electrophoresis, comparing with MW standards.

Quantitative-PCR (qPCR)

The qPCR was performed using the Bio-Rad CFX96 system (BioRad), on a reaction volume

of 20 μL, containing 0.3 μL of each forward and reverse primer (0.3 μM), 10 μL of iQ SYBR Green Supermix (BioRad), 8.4 μL of sterile water, 1 μL of cDNA. Amplification conditions included 1 cycle of 3 min at 95°C, 40 PCR cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, then 1 min at 95°C followed to a melt curve of 55-95°C with 0.5°C increments every 5 s. Aspecific amplifications and primers-dimers formation were excluded by checking melt curves.

Each sample was analyzed in triplicate and relative gene expression was calculated according to the efficiency-corrected method (Pfafll, 2001). Cyclophilin, β-actin, Glyceraldehyde-3-P dehydrogenase (GAPDH), and Ribosomial Protein Large P0 (RPLP0) were treated as reference genes and used for normalization of qPCR data ($M<0.10$). Data were presented in fold-change ratio having G breed as reference. Primers efficiencies (Table 1) were calculated and verified using the standard curve obtained by amplification of serial dilutions of the pooled cDNA (Pfafll, 2001).

Table 1. Primer sequences (5' to 3'), amplification products of gene in exam and reference genes.

Gene	Primers Forward-Reverse, Amplicon length (A; bp), Accession Number (AN), Efficiency (E), R^2
Capn1	F: AACCGGATCCGGAATTACCTGTCCATCTTC R: GTAACCACTTAAACAAGTCAAAGGTCACCA A: 282; AN: NM_174259; E: 0.979; R^2: 0.986
Capn2	F: CGAGGACATGCACACCATTGGCTTCG R: TCCTCGCTGATGTCAATCTCGTCAATGTTG A: 314; AN: NM_001103086.1; E: 0.968; R^2: 0.997
Cast	F: CACAGAAGCCAAGGCTATTCC R: TTTACAGCCTGTTTTTTGTGTCTTTTC A: 87; AN: NM_001030318; E: 0.978; R^2: 0.997
Casp3	F: AGAACTGGACTGTGGTATTGAGA R: CACAAAGAGCCTGGATGAAC A: 167; AN: NM_001077840.1; E: 0.957; R^2: 0.996
Casp9	F: CCTGTGGTGGAGAGCAGAAAG R: CATCTGGCTCGTCAATGGAA A: 134; AN: NM_001205504.1; E: 0.945; R^2: 0.980
Cryab	F: CCGCCTCTTTGACCAGTT R: AGAGGCCAGTGTCAATCC A: 134; AN: NM_174290; E: 0.991; R^2: 0.997
Hsp27	F: CGTTGCTTCACTCGCAAATA R: TACTTGTTTCCGGCTGTTCG A: 210; AN: NM_001025569.1; E: 0.936; R^2: 1.000
Hsp40	F: GGACTGACCATTGCTGCTG R: CAAACCCACCTCTGTAATAGC A: 138; AN: NM_001034458.1; E: 0.984; R^2: 0.998
Hsp70	F: AACAAGATCACCATCACCAACG R: TCCTTCTCCGCCAAGGTGTTG A: 274; AN: NM_00174550; E: 1.015; R^2: 0.996
Cyclophilin	F: GGATTTATGTGCCAGGGTGGTGA R: CAAGATGCCAGGACCTGTATG A: 119; AN: NM _00178320; E: 0.992: R^2: 0.998
β-actin	F: CTCTTCCAGCCTTCCTTCCT R: GGGCAGTGATCTCTTTCTGC A: 177; AN: NM_00173979; E: 0.961; R^2: 0.997
GAPDH	F: TCATCCCTGCTTCTACTGGC R: CCTGCTTCACCACCTTCTTG A: 177; AN: NM_001034034; E: 0.974; R^2: 0.999
RPLP0	F: CAACCCTGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA A: 226; AN: NM_001012682; E: 0.987; R^2: 0.999

Abbreviations used: Capn1: Calpain-1; Capn2: Calpain-2; Cast: Calpastatin; Casp3: Caspase 3; Casp9: Caspase 9; Cryab: alpha B-crystallin; Hsp27: Heat Shock Protein 27; Hsp40: Heat Shock Protein 40; Hsp70: Heat Shock Protein 70. Cyclophilin, β-actin, GAPDH, RPLP0: reference genes.

ELISA analysis of Hsp27 and Hsp70

Proteins extraction and quantification

Protein extraction was made according to Pulford *et al*. (2008), with modifications. Two hundred milligrams of frozen meat sample were pulverized under liquid nitrogen in a mortar using a pestle. The powder was homogenized, using a vortex, in 1 ml of ice-cold extraction buffer (25 mM HEPES pH 7.5, 0.1% Triton X-100, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol) and 10 ul of Protease Inhibitor Cocktail (P8340, Sigma-Aldrich, Saint Louis, MO, USA), to produce a whole muscle homogenate. The homogenate was centrifuged at 20000 g for 10 min at 4°C and the supernatant collected (sarcoplasmic fraction).

Protein concentration of muscle extracts were determined by the method of Bradford (1976) using Bradford reagent (Sigma-Aldrich).

Quantification of Hsp27 and Hsp70

Quantification of Hsp27 and Hsp70 was made according to Lomiwes *et al*. (2014), with some modifications, by indirect enzyme-linked immunosorbent assay (ELISA). Sarcoplasmic fraction was adjusted to concentrations of 4 μg mL^{-1} protein with coating buffer (10 mM Na_2HPO_4, 15 mM NaCl; pH 7.4). One hundred microliters of samples was dispensed into 96 well Costar® high binding polystyrene plates (Corning Inc., 3590) in duplicate. Plates were placed on an oscillating shaker overnight at 4 °C. Following coating, the contents of wells were discarded, then wells were blocked with 200 μL/well of 1% BSA wash buffer (10 mM Na_2HPO_4, 15 mM NaCl, 0.1% Tween 20; pH 7.4) for 1 h at room temperature, then washed three times with 200 ul/well wash buffer. Following washing, 100 μL/well of mouse monoclonal antibody (anti-Hsp27, Hytest 4HSP27 and anti-Hsp70, Hytest 4HSP70; Hytest, Turku, Finland), each diluted to 1:10000 with 1% BSA in assay buffer (0.01 M PBS, 0.01% Tween20), were added and left to incubate for 2 h at room temperature, then washed as previously described. Goat anti-mouse IgG HRP conjugate (A4416, Sigma-Aldrich) was diluted to 1:5000 in assay buffer (1% BSA) and 100 μL/well aliquots were dispensed and left to incubate for 1 h at room temperature. Following washing, 100 μL/well 3,3',5,5'-tetramethybenzidine (TMB) substrate (T0440, Sigma-Aldrich) was dispensed and left to incubate for 30 min. The reaction was stopped by dispensing 100 μL/well of 2 N H_2SO_4. The absorbance was measured at 450 nm using a Tecan Sunrise plate reader (Tecan Group Ltd., Männedorf, Switzerland). Relative quantity of Hsp27 and 70 was measured by optical density. Data were presented as fold-change ratio having G breed as reference.

ELISA analysis of Calpain-1 and Calpastatin

Proteins extraction and quantification

Protein extraction was made according to Geesink and Koohmaraie (1999), with modifications. Two hundred milligrams of frozen meat sample were pulverized under liquid nitrogen in a mortar using a pestle. The powder was homogenized, using a vortex, in 1 ml of ice-cold extraction buffer (50 mM Tris/HCl, pH 8.3, 10mM EDTA, 0.05% v/v 2-mercaptoethanol) and 10 ul of Protease Inhibitor Cocktail (P8340, Sigma-Aldrich). The homogenate was centrifuged at 30000 g for 1 h at 4°C and the supernatant collected.

Protein concentration of muscle extracts were determined by the method of Bradford (1976) using Bradford reagent (Sigma-Aldrich).

Quantification of Calpain-1 and Calpastatin

Quantification of Calpain-1 and Calpastatin was made according to Doumit *et al*. (1996), with modifications, by indirect enzyme-linked immunosorbent assay (ELISA). Muscle extracts were adjusted to concentrations of 4 µg/mL protein with Tris-buffered saline (TBS). One hundred microliters of samples was dispensed into 96 well Costar® high binding polystyrene plates (Corning Inc., 3590) in duplicate and incubated for 2 h at 37°C. Wells were emptied and washed five times with TBS containing 0.01% Tween-20 (TTBS), then incubated for 1 h at 37°C with 100 mL/well of mouse monoclonal anti-calpain antibody (C0355, Sigma-Aldrich) diluited 1 µg/mL in 1% BSA-TTBS, or with 100 mL/well of mouse monoclonal anti-calpastatin antibody (C270, Sigma-Aldrich) diluted to 1:5000 in 1% BSA-TTBS. Wells were then washed five times with TTBS and goat anti-mouse IgG HRP conjugate (A4416, Sigma-Aldrich), diluited 1:1000 in 1% BSA-TTBS, was applied for 1 h at 37°C. Following five washes with TTBS, 100 mL/well 3,3',5,5'-tetramethybenzidine (TMB) substrate (T0440, Sigma-Aldrich) was applied. After approximately 10 min at room temperature, the reaction was stopped by adding 100 mL/well of 2 N H_2SO_4. The absorbance was measured at 450 nm using a Tecan Sunrise plate reader (Tecan Group Ltd., Männedorf, Switzerland). Relative quantity of Capn1 and Cast was measured by optical density. Data were presented as fold-change ratio having G breed as reference.

Statistical analysis

The statistical analysis was performed using SPSS for Windows (v. 7.5.21; SPSS Inc., Chicago, IL, USA). The normality of the data distribution was tested using the Shapiro-Wilk test. Where appropriate, non-parametrically-distributed data were log-transformed for

parametric testing. The correlations between variables were determined by Rho Spearman's or Pearson's test when appropriate. The effect of genotype was evaluated by the analysis of covariance using 'genotype' (G *vs*. SG) as a fixed factor and 'age' as a covariate, an intra-class covariate when the intra-genotype coefficients were significantly different. The intraclass covariate was considered suitable for the age factor correction, because of the equal distribution of ages within genotypes. In tables, the genotype means were adjusted to a covariate mean age of 31 months.

Results and discussion

Table 2 presents color parameters of LT muscle of G breed and SG crossbreed. G breed showed a higher lightness (L*) value with respect to SG. The L* value of SG crossbreed (32.5) is not so far from L* value declared by Corazzin *et al*. (2012; 33.6) for IS bulls, but below the lowest value reported by Muchenje *et al*. (2009b; 33.2) for L* parameter. The lightness of G breed (36.7) appeared high for a *Bos indicus* breed, as showed by other authors (Bressan *et al*., 2011; 32.3, for *Bos indicus* at pasture; Nfor *et al*., 2014; 29.5 on average), even if Strydom *et al*. (2008) reported higher values of L* (39.0) for a *Bos indicus* crosses (Brahman) with respect to Simmental crosses (37.8). Moreover, Gama *et al*. (2013) found a reduction of lightness as effect of crossbreeding between *Bos indicus* and *Bos taurus* bred at pasture.

Table 2. Color parameters of *Longissimus thoracis* muscle belonging to *Goudali* (G) and *Simmental* × *Goudali* (SG) crossbreed bulls.

				Age coefficient	
	G	SG	MSE	G	SG
L*	36.7[a]	32.5[b]	0.67	-0.35**	-0.42**
a*	11.4[b]	14.2[a]	0.55	0.14	0.36**
b*	14.8	15.4	0.50		
Chroma	18.8	21.2	0.71	0.24*	
Hue	52.7[a]	47.7[b]	1.61	-0.21	-0.40***

[a,b]: P<0.05; *: P<0.10; **: P<0.05; ***: P<0.01.

In comparison with SG crosses, the redness was lower and the hue angle was higher for G breed, showing its higher tendency to yellow color. The redness in G breed (11.4) was higher than the values reported by Nfor *et al*. (2014) for zebu breeds (7.2 on average), but lower than redness recorded by Bressan *et al*. (2011) for *Bos indicus* breeds (19.8). In any case, our

values of redness, both for G and for SG (14.2), were within the limits showed by Muchenje *et al*. (2009b; 11.1 – 23.6) that considered both European and African breeds in their review.

Lightness and hue diminished and redness increased with the age of bulls, according to different rates in the two genotypes. Chroma values showed a tendency to augment with the age, showing the same trend in G and SG. These evolutions agree with the well-known change in meat color during animal growth, because of the age-dependent changes in muscle myoglobin content (Lawrie & Ledward, 2006). The low color change rates of the local genotype may indicate its late maturing condition in comparison with the exotic crossbreed. A delay in sexual maturity in *Bos indicus* in comparison to *Bos taurus* has been already highlighted (Brito *et al*., 2004).

Table 3 shows pHu data, rheological parameters and water losses values of G and SG meat. The pHu was not different between genotypes (5.58 on average). The pHu (<5.6) and L* values (>32) were substantially within the expected ranges (Lawrie & Ledward, 2006; Diaz *et al*., 2006) that would not result in dark firm dry (DFD) meat. The five weeks of rest before slaughter seems to be effective in reducing the stress of the travel from the farm to the slaughterhouse and to reconstitute the glycogen stores. Similarly to our trial, other authors did not find pH differences between *Bos taurus* and *Bos indicus* (Bressani *et al*., 2011; 5.86 on average) or between these genotypes and their crosses (Gama *et al*., 2013). Also Muchenje *et al*. (2008; 2009) did not find pHu differences between *Bos taurus* and *Bos taurus* x *Bos indicus* breeds (5.7 on average). The pHu diminished with bull maturity, at a higher rate in G than SG, likely because of a lesser susceptibility to pre-slaughter stress of older cattle.

Table 3. Ultimate pH (pHu), Warner Bratzler Shear Force (WBSF) and water losses of *Longissimus thoracis* muscle belonging to *Goudali* (G) and *Simmental* × *Goudali* (SG) crossbreed cattle.

				Age coefficient	
	G	SG	MSE	G	SG
pHu	5.57	5.59	0.014	-0.010**	-0.004**
WBSF (N)					
PY	51.3	55.9	1.63		
FY	45.9	49.7	1.42		
Water losses (%)					
Drip loss	3.70^{a}	2.86^{b}	0.177		
Cooking loss	36.69^{A}	32.57^{B}	0.394	-0.137*	

PY: peak yield force, FY: final yield force. ^{a,b}: P<0.05; $^{A, B:}$ P<0.01; *: P<0.10; **: P<0.05.

Concerning the rheological parameters, differences between G and SG were not found (P>0.05), neither for the miofibrillar nor for the connective component of the shear force (Table 3). Although it is commonly accepted that *Bos taurus* gives more tender meat compared to *Bos indicus* (Crouse *et al*., 1989; Wheeler *et al*., 1990; Shackelford *et al*., 1991), there are some evidence in the opposite direction. Indeed, Strydom (2008) and Frylink *et al*. (2009) reported higher WBSF for Simmental crosses in comparison with Brahman crosses (*Bos indicus*) and Nguni crosses (*Bos taurus africanus*). Du Plessis and Hoffman (2007) also reported no differences in shear force resistance of three days aged loin muscle from Nguni, Brahman and Simmental crosses aged between 18 and 30 months, showing that breed effect on meat shear force was not significant in natural pasture of South African arid subtropics. Also in our condition (tropical climate, natural pasture feeding, high physical activity) differences between genotypes have not been highlighted.

The values of WBSF for G breed (51.3 N, PY, maximum peak yield) are much lower than the values reported for G breed by Nfor *et al*. (2014; 112 N), probably because of the lower average age and better-controlled management conditions of animals in our study. Corazzin *et al*. (2012) reported values of WBSF of 47.9 N for Italian Simmental breed reared in stable. Our values of WBSF were 53.6 N on average between G and SG. The slightly higher shear force values in loin of bulls finished on pasture may have been due to increased collagen cross-linking, associated with increased exercise (Purslow, 2005). In any case, our values were fully included between limits reported by Muchenje *et al*. (2009b), both at 2 and at 21 days of ageing.

In a number of studies in South Africa (De Bruyn, 1991; Frylinck & Heinze, 2003; Marais, 2007), the poor tenderness quality of Simmental meat was questioned. In particular, De Bruyn (1991) found no significant differences between shear force resistance of Afrikaner, Bonsmara, Charolais and Hereford meat, while the toughness values were significantly higher for Brahman and Simmental meat. Moreover, Frylinck *et al*. (2009) hypothesized that, under specific environmental challenges, both Simmental and *Bos indicus* contributed to the poor tenderness of the crossbreed meat.

Water losses (Table 3) are different between our two genotypes, with SG crossbreed having lower values of both drip loss and cooking loss and thus lower losses of water before being eaten, i.e. a positive commercial characteristic. Even if the pH was comparable between the two genotypes, the differences in meat color and water losses could be related to a different

muscle fibers organization (Offer & Knight, 1988). Indeed, Gagaoua et al. (2015) reminded that more tightly packed fibers led to an increase of water-holding capacity and darkness of meat, because of a less scatter light.

The values of cooking loss for G breed (36.7%) were slightly lower than the values reported by Nfor *et al*. (2014) for the Cameroonian *Bos indicus* breeds (38.4% on average). Conversely, the values of cooking loss, which showed a tendency to decrease with bull age, were higher for both genotypes (G: 36.69%; SG: 32.57%) than those showed by Corazzin *et al*. (2012) in young bulls of Simmental breed (30.35%). Muchenje *et al*. (2009b) reported a maximum value of cooking loss of 34.54%, with respect to which only our SG crossbreed complies.

The values of RNA expression are shown in Table 4. No significant differences were found between genotypes, except for the value of Hsp70 that is more expressed in G breed. Even the age effect reached the threshold of significance only for CRYAB (0.587 for G, $P < 0.05$) the expression of which increased with the age of G (data not tabulated).

Table 4. Fold change ratio in relative RNA expression of genes in *Longissimus thoracis* muscle belonging to *Goudali* (G) and *Simmental* × *Goudali* (SG) crossbreed cattle.

Gene	G	SG	MSE
Capn1	1	1.26	0.411
Capn2	1	1.29	0.518
Cast	1	1.62	0.409
Capn1/Cast	1	0.77	0.154
Casp3	1	0.99	0.391
Casp9	1	0.98	0.205
Cryab	1	1.23	0.406
Hsp27	1	1.13	0.413
Hsp40	1	1.25	0.189
Hsp70	1^{a}	0.46^{b}	0.148

Abbreviations used: Capn1: Calpain-1; Capn2: Calpain-2; Cast: Calpastatin; Casp3: Caspase 3; Casp9: Caspase 9; Cryab: alpha B-crystallin; Hsp27: Heat Shock Protein 27; Hsp40: Heat Shock Protein 40; Hsp70: Heat Shock Protein 70. ^{a,b}: $P<0.05$.

The Hsp27 is a good promising protein for its relation with meat tenderness (Bernard *et al*., 2007; Hocquette *et al*., 2007; Saccà *et al*., 2015) so we have analyzed this protein also by ELISA, to verify the result obtained by mRNA analysis. The ELISA analysis (Table 5) confirmed the lack of difference in the expression of Hsp27 between the two breeds

highlighted by mRNA analysis, and also confirmed the different content of Hsp70, that is more present in G breed. Because of the environmental adaptation, zebu breeds of cattle are better able to regulate body temperature in response to heat stress than European breeds (Beatty *et al*., 2006; Gaughan *et al*., 1999). Among the Hsp, Hsp70 has a significant role in cell thermotolerance (Beckham *et al*., 2004), indeed Gaughan *et al*. (2013) found a strong relationship between Hsp70 concentration and ambient temperature and photoperiod. Our results on Hsp70 can therefore be explained by a better adaptation of G to tropical climate. Recently, Gagaoua et al. (2015) underlined the correlation between Hsp70 and the color of young bulls meat. Even in the present study, the genotypic difference in Hsp70 expression is associated with differences in meat color, but their relationship did not follow the framework suggested by these authors. Probably, differences in animals considered (Blond d'Aquitaine young bulls) and breeding conditions (intensive rearing in European climate) of this trial with respect to our experiment led to a different relation between Hsp70 expression and color of meat.

Table 5. Fold change ratio in relative protein quantity of *Longissimus thoracis* muscle belonging to *Goudali* (G) and *Simmental* × *Goudali* (SG) crossbreed cattle.

Protein	G	SG	MSE
Capn1	1	0.97	0.071
Cast	1	0.99	0.037
Capn1/Cast	1	0.98	0.034
Hsp27	1	1.45	0.235
Hsp70	1^{a}	0.81^{b}	0.053

Abbreviations used: Capn1: Calpain-1; Capn2: Calpain-2; Cast: Calpastatin; Hsp27: Heat Shock Protein 27; Hsp70: Heat Shock Protein 70. ^{a,b}: $P<0.05$.

Concerning the mRNA expression of calpains and calpastatin, the major effectors of tenderness process in meat, the results are in accordance with the rheological measurements. In fact, no significant differences occurred between the two genotypes for both shear force values and the expression levels of genes related with tenderness. The ELISA analysis (Table 5) confirmed the lack of difference in the expression of both Capn1 and Cast between the two breeds that was highlighted by mRNA analysis. Many authors reported a higher calpastatin activity and/or a lower calpain-1 expression in *Bos indicus* than *Bos taurus*, resulting in lower rates and amount of *post-mortem* tenderization (Shackelford *et al*., 1991; Whipple *et al*.,

1990, Wheeler *et al*., 1990; Ibrahim *et al*., 2008; Pringle *et al*., 1997). The lack of difference between SG and G in the expression of calpain and calpastatin genes, sustained by the lack of difference in share force values, may be related to the extreme environmental factors (climate, feeding regime and management stresses) that characterized our experimental conditions. Similarly, Marais (2007) considering Brahman, Nguni and Simmental crosses highlighted a lack of correspondence between the genotypic makeup and the phenotypic expression of tenderness genes caused by environmental factors.

Conclusions

As it was expected in the crossing program, the crossbreeding with Italian Simmental breed improved the meat productivity of Cameroonian Goudali breed in terms of growth rate and carcass quality (Bessong *et al*., 2015). Unfortunately, the crossing seems to have limited impact on meat traits, at least in tropical environment and in the working pre-slaughter conditions that may have differently influenced the two genotypes, smoothing the outcomes of crossing. Indeed, although it caused a reduction of water losses of meat, it had no effects on meat tenderness and on the expression of the related genes. Based on its higher Hsp70 expression, the local Goudali breed should respond better to the tropical climate, where it has evolved over the years, with respect to the SimGoud crossbreed.

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Fourth study

Meat quality traits and expression of tenderness-related genes in *Longissimus lumborum* of Alpine young goats at different age

(Awaiting submission to a journal)

Meat quality traits and expression of tenderness-related genes in *Longissimus lumborum* of Alpine young goats at different age

Abstract

The aim of this trial was to evaluate some meat quality parameters and the early *post-mortem* expression of genes involved in tenderization process in *Longissimus lumborum* (LL) of Alpine young goats (*Capra hircus*) at different age. Samples of LL were collected at slaughter of 32 Alpine goats, divided in 9 suckling kid (Sk) of 1.3 months of age, 16 chevon of 4.6 months of age (Ch) and 7 post-puberal goat (PPu) of 8 months of age. Animal and carcass parameters (live weight gain [LWG], live weight, carcass weight, fat deposits) and some quality traits of LL meat (lipid content, ultimate pH [pHu], color parameters, cooking loss and shear force) were determined. The mRNA early *post-mortem* abundances of calpain-1 (Capn1), calpain-2 (Capn2), calpastatin (Cast), caspase 3 (Casp3), caspase 9 (Casp9), αB-crystallin (Cryab), heat shock protein 27 (Hsp27), Hsp40 and Hsp70 were detected by quantitative PCR. Sk type had the higher LWG and the slimmer carcasses. Meat of PPu group was the darker and the tougher. The redness of meat increased with age of goats. The same was for the expression of Capn1, Capn2, Cryab, Hsp27 and Hsp70. Sk group had also the lower expression of Casp3. Calpains expression is limited in younger classes probably because their great muscular growth. The same could be for Casp3 in Sk group. The higher expression of Hsp in PPu group, probably also related with hormonal condition, could overhang the action of calpains, leading to a tougher meat.

Keywords: Meat quality, calpains, calpastatin, caspases, heat shock proteins, gene expression.

Introduction

Goat meat can be considered as a lean meat with favourable nutritional quality, sensory particularities and a consumption linked to cultural and geographical factors (Webb *et al.*, 2005). For marketing purposes, goat meat could be divided into two classes, i.e. capretto, which derives from suckling kids, producing a carcass of 6–12 kg, with pink flesh, and chevon, which derives from older goats to produce a carcass of 16–22 kg (Dhanda *et al.*, 2003). Adult goats for meat consumption are preferred in the Indian subcontinent as well as in Africa and South America (Naudé & Hofmeyr, 1981). In Italy, the "capretto" type, i.e. four to seven-week old kid fed on milk (Piasentier *et al.*, 2000; 2005), is the traditional and, still, the main meat product (Boyazoglu & Morand-Fehr, 2001) from goat kids. Less frequently,

chevon is considered for consumption. Capretto meat is appreciated for the milk aroma and delicate taste; instead, chevon meat acquired an increasing intensity of goat flavour and livery notes, partially related to feeding regime and fatty acid profile (Borgogno *et al*., 2015). There were indications that puberty and/or increasing age caused a raise in the characteristic "goaty" meat aroma, (Young *et al.*, 1997). Even tenderness decrease during young goat growth (Schönfeldt *et al*., 1993). However, a consumers' niche preferred chevon over capretto (Borgogno *et al*., 2015), in relation to food habits and cultural factors.

Flavour, tenderness, odour and juiciness affect goat meat acceptability (Naudé & Hofmeyr, 1981; Borgogno *et al*., 2015), while color is important especially in capretto carcasses, where flesh should be pale or pink. However, in general, tenderness is the most important factor influencing consumer satisfaction for meat palatability, at least for beef (Miller *et al*., 1995). Tenderness is affected by many factors such as species, breed and age of animal, and, at muscle level, by the amount of collagen, intramuscular fat content, pH *post-mortem* decline, size and type of muscle fibers (Seideman & Koohmaraie, 1987; Maltin *et al*., 2003). Moreover, enzymatic systems involved in protein degradation are important in meat *post-mortem* tenderization process (Ouali *et al*., 2006; Kemp *et al*., 2010). Calpain proteolytic system hydrolyze myofibrillar proteins and cause the main structural changes during meat ageing (Veiseth *et al*., 2001; Koohmaraie & Geesink, 2006). This system consists of two calcium-dependent proteases, calpain-1 and calpain-2 and their competitive inhibitor calpastatin that play an important role even in goat muscles tenderization (Nagaraj & Santhanam, 2006).

Becila *et al*. (2010) provided evidences that the hypoxic/ischemic conditions that occur during slaughter of animals could activate cell death in *post-mortem* muscle via an apoptotic process. Caspases are the major effectors of apoptosis and are able to degrade a large number of muscle proteins (Earnshaw *et al*., 1999; Fischer *et al*., 2003). The intrinsic pathway of apoptosis is the predominant in *post-mortem* skeletal muscle (Herrera-Mendez *et al*., 2006) and it takes place for the action of caspase 9 and 3 (Earnshaw *et al*., 1999), an activator and an effector caspase, respectively. Despite their central role in apoptosis, the involvement of caspases in proteolytic degradation that causes meat tenderization is still debated (Mohrhauser *et al*., 2011; Saccà *et al*., 2015a, 2015b). The increased expression of caspase 3 in apoptosis of various goat tissues was highlighted (Tao *et al*., 2014; Gu *et al*., 2015a, 2015b; Zhuang *et al*., 2015), but, to our knowledge, the relation between caspases and meat goat ageing has not yet been considered.

The state of stress caused by ischemia that characterizes *post-mortem* condition could activates cell survival processes that involve the increase in the concentration of several heat shock proteins (Hsp). In relation to their anti-apoptotic function and their protective actions on myofibrillar proteins, Hsp have been proposed to have a potential role in meat tenderization process (Herrera-Mendez *et al*., 2006; Ouali *et al*., 2006). AlphaB-cristallyn can bind and protect myofibrillar proteins from endopeptidases degradation (Pulford *et al*., 2009) and can regulate intermediate filaments structure (Lomiwes *et al*., 2014). Hsp27 is involved in the stabilization of myofibrillar proteins and protection of actin filaments (Lomiwes *et al*., 2013) and was related to tenderness by various authors (Hocquette *et al*., 2007; Saccà *et al*., 2015a). Moreover, Hsp27 and the complex Hsp70-40 have been identified as regulators and suppressors of the apoptotic process by several authors (Li *et al*., 2000; Beere, 2005). About goats, Hsp27 and Hsp70 was reported to be involved in thermal stress resistance (Dangi *et al*., 2012; Mohanarao *et al*., 2014), but, to our knowledge, the relation between Hsp and meat quality in goat has not yet been examined.

Despite numerous reports on beef, little information is available on the expression of tenderization genes in goat, especially for caspases and heat shock proteins. Moreover, although the age dependent change in meat tenderness is due to the modification that occurs in muscle fibers and collagen structure, little is known about the variation in the expression of proteins involved in tenderization process during animal growth. Goat is a ruminant relatively fast growing species that allows following the ageing of animals from birth to maturity and studying the evolution of tenderness in parallel with the expression of genes of interest involved in tenderization process. The aim of the trial was to compare meat quality traits and the mRNA expression of tenderness-related genes among young goats of different age.

Material and methods

Animals and treatments

Thirty-two young goats of Alpine breed, born in six farms of the Friuli Venezia Giulia region (North-East Italy) were considered. Animals were randomly allotted into three groups, which included 9 suckling kid (Sk), 16 chevon (Ch) and 7 post-puberal goat (PPu). After the birth, the kids were suckled by their dams in the farm of origin up to weaning at an average age of 1.3 months, when the Sk group was slaughtered. After weaning, the remaining kids were brought into the experimental farm of the University of Udine and kept in multiple boxes, on

straw, until slaughter. Animals were fed on a mixed diet comprising 400 g/d meadow hay and 800 g/d commercial concentrate (18% crude protein), weighed bi-weekly to determine, by linear regression, the live weight gain (LWG) and finally weighed the day before slaughtering. The slaughter occurred at an average age of 4.6 months for the Ch group and at an average age of 8 months for the PPu group (Rodrigues *et al*., 2012).

Sample collection and measurements

Animal were slaughtered and dressed using standard commercial techniques in an EU-licensed abattoir; all procedures met the requirements of the European Commission Directive, 86-609-EC for Scientific Procedure Establishments. For all the analysis, the right *Longissimus lumborum* (LL) was considered. After slaughter, samples (5 g) of LL muscle were obtained within 20 minutes of exsanguination, frozen in liquid nitrogen and stored at -80ºC until transcriptomic analysis.

Twenty-four hours after slaughter, the carcasses were weighed to determine the cold carcass weight (CCW) and visually evaluated for subcutaneous and kidney fat deposition (Colomer Rocher et al., 1987). Ultimate pH (pHu) was measured in five points/loin, using a pH-meter (Hanna, HI 8424, Padova, IT) equipped with a glass electrode (Crison, 5232, Barcelona, E). The samples for fat content analysis were taken from the LL and immediately stored at −20°C until analysis. After 48 h of ageing at 4°C, color of the LL was measured, on five points/loin, after a blooming time of 45 min, using a portable spectrophotometer Minolta CM 2600d (Konica Minolta, Tokyo, J) with an 8 mm aperture, Standard Illuminant D65 light source and 10 viewing angle geometry. The values recorded, according to the standard conditions of the Commission International d'Eclairage (CIE; 1976) included Lightness (L*), Redness (a*) and Yellowness (b*). Samples of LL were aged at 4°C for 7 days until cooking loss and Warner-Bratzler Shear Force (WBSF) determination.

Cooking loss and WBSF determinations

Slices of LL muscle of 2 cm thickness were cooked in plastic bags, until reaching 75°C at the core, using a water bath, according to the procedure described by Honikel (1998). The internal temperature was monitored by a thermocouple (Thermocouple Thermometer HD 9016, Delta Ohm, Padova, IT). Each slice of meat was weighed before and after cooking (dried with paper). The cooking loss (CL) was calculated as difference between the weight before and after cooking, and expressed as a percentage of the initial weight.

The Warner-Bratzler Shear Force test was performed on cooked meat after CL determination.

Seven cross-section cylinders of 15 mm in diameter were cut along the fibers axis for each LL sample. The cylinders were sheared perpendicularly of muscle fibers using a Warner-Bratzler (WB) device, with a triangular hole (60°) in the shear blade (1 mm thickness), mounted on a Lloyd TA Plus texture analyser (Lloyd, Borgnor Regis, UK), operating at a crosshead speed of 100 mm/min. From the WB deformation curves, two parameters were recorded: the peak yield force (PY, in N), that is the maximum peak yield of the force-deformation curve and the final yield (FY, in N), that is the second peak yield of the force-deformation curve. The first peak is taken as a measurement of the myofibrillar component of tenderness and the second is taken as a measurement of the connective tissue component of tenderness (Møller, 1981).

Fat analysis

Extraction of total lipids was performed according to the procedure of Folch *et al.* (1957): 2 g of minced meat were homogenized in 40 mL of chloroform-methanol mixture (2:1 v/v) using an Ultra-Turrax homogenizer (T 25 basic; IKA, Staufen, D) and were subsequently filtered under vacuum through Whatman filter paper (1820-047, GE Healthcare Life Sciences, Little Chalfont, UK). The extract was washed with 10 mL of 0.88% (w/v) KCl, mixed vigorously for 1 min and then left overnight at room temperature. The organic phase was separated and the solvents were evaporated at 40°C with a Univapo Vacuum Concentrator (100EHC, Elettrofor Scientific Instruments, Rovigo, IT). The fat amount was calculated by difference between the weight before and after evaporation and expressed as grams of lipids per 100 g of muscle tissue.

RNA content analysis

RNA extraction

Total RNA was extracted from 40 mg muscle (powdered with a mortar and a pestle in liquid nitrogen) using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, D) according to the manufacturer's protocol, DNase treatment included. Concentration and purity of RNA extracted were assessed using spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA). Integrity of RNA (presence of intact 18S and 28S rRNA bands) and absence of genomic DNA were assessed by agarose gel (1% in TBE buffer) electrophoresis, comparing with molecular weights (MW) standards.

Retro-transcription

To obtain cDNA, the iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA) was used. Each 20 μL of reaction contained 4 μL of reverse-transcription reaction mix, 1 μL of iScript reverse transcriptase (MMLV-derived RNase H+), a volume of RNA solution to have 50 ng/μL RNA final concentration, nuclease-free water to final volume. The mixture was held 5 min at 25°C, 1 h at 42°C, and 5 min at 85°C before being cooled on ice. The cDNA concentration and purity was verified using NanoDrop 2000c.

Primers validation

A qualitative polymerase chain reaction (PCR) was carried out on 6 randomly chosen samples to validate all primers pair (Table 1) specificity, for all the genes in exam: calpain1 (Capn1), calpain2 (Capn2), calpastatin (Cast), caspase 3 (Casp3), caspase 9 (Casp9), αB-crystalline (Cryab), heat shock protein 27 (Hsp27), Hsp40, Hsp70 and reference genes (Cyclophilin, β-actin, Glyceraldehyde-3-P dehydrogenase [GAPDH] and Ribosomial Protein Large P0 [RPLP0]). The PCR was performed using the Bio-Rad CFX96 system (BioRad), on a reaction volume of 20 μL, containing 0.3 μL of each forward and reverse primer (0.3 μM), 10 μL of iQ SYBR Green Supermix (BioRad), 8.4 μL of sterile water, 1 μL of cDNA. Amplification conditions included 1 cycle of 3 min at 95°C, 40 PCR cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, then 1 min at 95°C, followed to a melt curve of 55-95°C with 0.5°C increments every 5 s. Aspecific amplifications and primers-dimers formation were excluded by checking melt curves. Amplicons length (Table 1) was verified by agarose gel (1.5% in TBE buffer) electrophoresis, comparing with MW standards.

Quantitative PCR (qPCR)

The qPCR was performed using the same instrument, the same reaction reagents and volumes, and the same amplification conditions of the primers validation phase, on the samples for every gene considered (in exam and reference). Each sample was analyzed in triplicate and relative gene expression was calculated according to the efficiency-corrected method (Pfaffl, 2001). Cyclophilin, β-actin, GAPDH and RPLP0 were treated as reference genes and used for normalization of qPCR data (gene stability measure < 0.10). Data were presented in fold-change ratio having as reference the Sk group. Primers efficiencies (Table 1) were calculated and verified using the standard curve obtained by amplification of serial

dilutions of the pooled cDNA (Pfaffl, 2001).

Table 1. Primer sequences, amplification products and amplification efficiency of gene in exam and reference genes.

Gene	Primers sequence, 5' to 3'; Amplicon length (A; bp); Accession Number (AN); Efficiency (E; %); R^2
Capn1	F: AACCGGATCCGGAATTACCTGTCCATCTTC R: GTAACCACTTAAACAAGTCAAAGGTCACCA; A: 278; AN: NM_001285663.1; E: 95.0; R^2: 0.993
Capn2	F: CAGAGCTTCCAGGAGAACTATGC R: TGCCGAGTGCACGAAGAG; A: 128; AN: XM_005690587.1; E: 100.1; R^2: 0.997
Cast	F: CACAGAAGCCAAGGCTATTCC R: TTTACAGCCTGTTTTTTGTGTCTTTTC; A: 87; AN: NM_001009788; E: 97.6; R^2: 0.995
Casp3	F: AGAACTGGACTGTGGTATTGAGA R: CACAAAGAGCCTGGATGAAC; A: 159; AN: NM_001286089.1; E: 94.9; R^2: 0.990
Casp9	F: CCTGTGGTGGAGAGCAGAAA R: CATCTGGCTCGTCAGTGGAA; A: 134; AN: XM_005690814.1; E: 98.5; R^2: 0.985
Cryab	F: CCGCCTCTTTGACCAGTT R: AGAGGCCAGTGTCAATCC; A: 132; AN: XM_005689436.1; E: 97.6; R^2: 1.000
Hsp27	F: CCTGGACGTCAACCACTTC R: GCTTGCCAGTGATCTCCAC; A: 76; AN: JQ957566.1; E: 97.5; R^2: 0.998
Hsp40	F: GTGGTAGCAGTTGGACTGACCAT R: CTGAAGGCAGATTTTGGTAGACTTT; A: 124; AN: XM_005675220.1; E: 96.8; R^2: 0.997
Hsp70	F: GACGACGGCATCTTCAAG R: GTTCTGGCTGATGTCCTTC; A: 131; AN: JN604433.1; E: 99.6; R^2: 0.991
Cyclophilin	F: GGATTTATGTGCCAGGGTGGTGA R: CAAGATGCCAGGACCTGTATG; A: 120; AN: XM_005679322.1; E: 95.0; R^2: 0.994
β-actin	F: CTCTTCCAGCCTTCCTTCCT R: GGGCAGTGATCTCTTTCTGC; A: 177; AN: JX046106.1; E: 96.8; R^2: 0.999
GAPDH	F: TCATCCCTGCTTCTACTGGC R: CCTGCTTCACCACCTTCTTG; A: 176; AN: XM_005680968.1; E: 96.9; R^2: 0.997
RPLP0	F: CAACCCCGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA; A: 224; AN: XM_005709526.1; E: 93.2; R^2: 0.997

Capn1 = calpain-1; Capn2 = calpain-2; Cast = calpastatin; Casp3 = caspase 3; Casp9 = caspase 9; Cryab = αB-crystallin; Hsp27 = heat shock protein 27; Hsp40 = heat shock protein 40; Hsp70 = heat shock protein 70. Cyclophilin, β-actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ribosomal Protein Large P0 (RPLP0) = reference genes.
F = Forward; R = Reverse.

Statistical analysis

Univariate analysis was performed using SPSS for Windows (7.5.21 version; SPSS Inc., Chicago, IL, USA), while multivariate analysis was performed using The Unscramble X, 10.3 package (CAMO Software AS., Oslo, N). The normality of the data distribution was tested using the Shapiro-Wilk test. Where appropriate, non-parametrically-distributed data were transformed for parametric testing. Discriminant PLS (PLS-DA; Chevallier *et al*., 2006) was applied for checking the efficacy of RNA expression data (X matrix) to discriminate the goat types, the matrix (Y matrix) of which was made by defining a dummy variable for each of them. Live weight, carcass weight, meat characteristics and individual gene expression were subjected to one-way analysis of variance with 'goat meat type' as a fixed effect, and to Tukey-Kramer test for unequal sample size as post-hoc test. The effect of goat meat type on fatness scores was studied using the Kruskal-Wallis test and the non-parametric Mann–Whitney U-test with Bonferroni adjustment as the post-hoc test.

Results and discussion

Animal and carcass characteristics, together with the lipid content of LL, are summarized in Table 2. LW and CCW of the three classes are in line with the literature data regarding Apine breed (Piasentier *et al*., 2000; Mahgoub *et al.*, 2012; Borgogno *et al.*, 2015). The Sk group showed a higher LWG compared to the other age classes with a significant growth rate decrease between the Sk and the Ch group. Mahgoub *et al.* (2012) reported that the weaning is the phase during which goats tend to gain most weight relative to time, compared with any other phase in the growth process. Sk was the leaner group, indeed fat deposits were statistically or numerically (kidney fat) lower in the youngest goats. The other two groups showed a comparable level of fatness, confirming the leaner nature of goat carcasses (Webb *et al.*, 2005).

Table 2. Animal and carcass characteristics and lipid content of *Longissimus lumborum* of young goats.

	Sk	Ch	PPu	SEM
Animal, No.	9	16	7	-
Average age, months	1.3	4.6	8.0	-
LWG, g/day	196[a]	134[b]	124[b]	5.28
LW, kg	11.4[c]	22.7[b]	32.4[a]	0.485
CCW, kg	5.4[c]	10.0[b]	14.8[a]	0.277
Subcutaneous score[1], points	1.6[b]	2.2[a]	2.0[a]	0.060
Kidney score[2], points	1.8	2.2	2.1	0.084
Intramuscular fat, %	1.4[b]	2.8[a]	2.2[ab]	0.160

Sk = suckling kid; Ch = chevon; PPu = post-puberal goat.
LWG = live weight gain; LW = live weight; CCW = cold carcass weight.
[1] From 1 (low fat cover) to 5 (very high fat cover) (Colomer Rocher *et al*., 1987).
[2] From 1 (little) to 3 (excessive) (Colomer Rocher *et al*., 1987).
[a,b] $P \leq 0.05$.

Ultimate pH and physical parameters of LL meat are shown in Table 3. The pHu values were in line with those reported in previous experiments for goat meat (Piasentier *et al*., 2005; Webb *et al*., 2005). Meat color is an important trait in goat carcasses evaluation, with pink or pale red expected for kids (Dhanda et al., 1999). Lightness and yellowness were higher in Sk and Ch groups compared to PPu one. Moreover, Sk showed the lowest a* value probably because these animals were fed exclusively on natural milk. Redness reflects the presence of myoglobin and the availability of iron (Sañudo *et al*., 2012) in meat. An increase in redness was then observed with goat age, in accordance with the parallel rise of the muscle myoglobin content (Berge *et al*., 2003; Lawrie & Ledward, 2006). A decrease in L* and an increase in a* values during the growth of goats were also reported by Dhanda *et al*. (1999) and Kannan *et al*. (2003).

Table 3. Ultimate pH, color characteristics, cooking loss (CL) and Warner-Bratzler Shear Force (WBSF) parameters of *Longissimus lumborum* of young goats.

	Sk	Ch	PPu	SEM
pHu	5.87	5.86	5.79	0.034
L*	40.7[a]	39.4[a]	30.5[b]	0.477
a*	4.2[c]	6.1[b]	6.9[a]	0.165
b*	11.5[a]	11.3[a]	9.1[b]	0.160
CL, %	18.1	16.6	15.2	0.902
WBSF parameters, N				
PY	28.0[b]	30.9[b]	38.2[a]	1.444
FY	25.2	24.9	30.8	1.327

Sk = suckling kid; Ch = chevon; PPu = post-puberal goat.
PY: peak yield force; FY: final yield force.
[a,b] $P \leq 0.05$.

The cooking loss percentage (Table 3) was not different among age classes, but a numerically decreasing tendency was observed, similarly that it was found in other studies (Arain, 2010), probably because of the higher content of water in meat of younger animals. Meat shear force was significantly different for the PPu group compared with the younger classes, but only for the myofibrillar component (PY), showing solely a numerical tendency for the connective component (FY). Other authors (Dhanda *et al*. 1999, 2003; Kannan *et al*., 2003) have already reported the decrease in muscle tenderness with age/body weight in goats. Unexpectedly, PY values of Sk meat were relatively high, being not statistically different from the Ch group. This relatively lower tenderness could be explained by different reasons, as highlighted by Berge *et al*. (2003) in lamb meat. Indeed, the suckling kids could have suffered a greater cold shortening due to their lighter and leaner carcasses. Moreover, Berge *et al*. (2003) hypothesized that young animals could also have a lower calpain and/or a higher calpastatin activity compared to older ones that can lead to a lower meat tenderness.

A multivariate analysis was performed in order to verify the presence of gene expression profiles distinguishing the experimental groups of animals. A PLS-DA was thus carried out and the correlation loadings of X (standardized values of RNA amount) and Y (goat types) variables along factor 1 and factor 2 were plotted in Figure 1. All the genes apart from two (Cast and Hsp40) were located within or very close the outer ring of the plot, indicating that the first two components of the model explained at least 50% of the original variance of their RNA expression. The same happened for the goat-type's correlation loadings that fell within the outer ring of the plot. The two dimensional plot may thus be considered a viable visualisation of the significant correlations within the most part of genes considered and

between them and the goat types. Genes close to each other have a high positive correlation while those in opposed quadrants have a tendency to be negatively correlated.

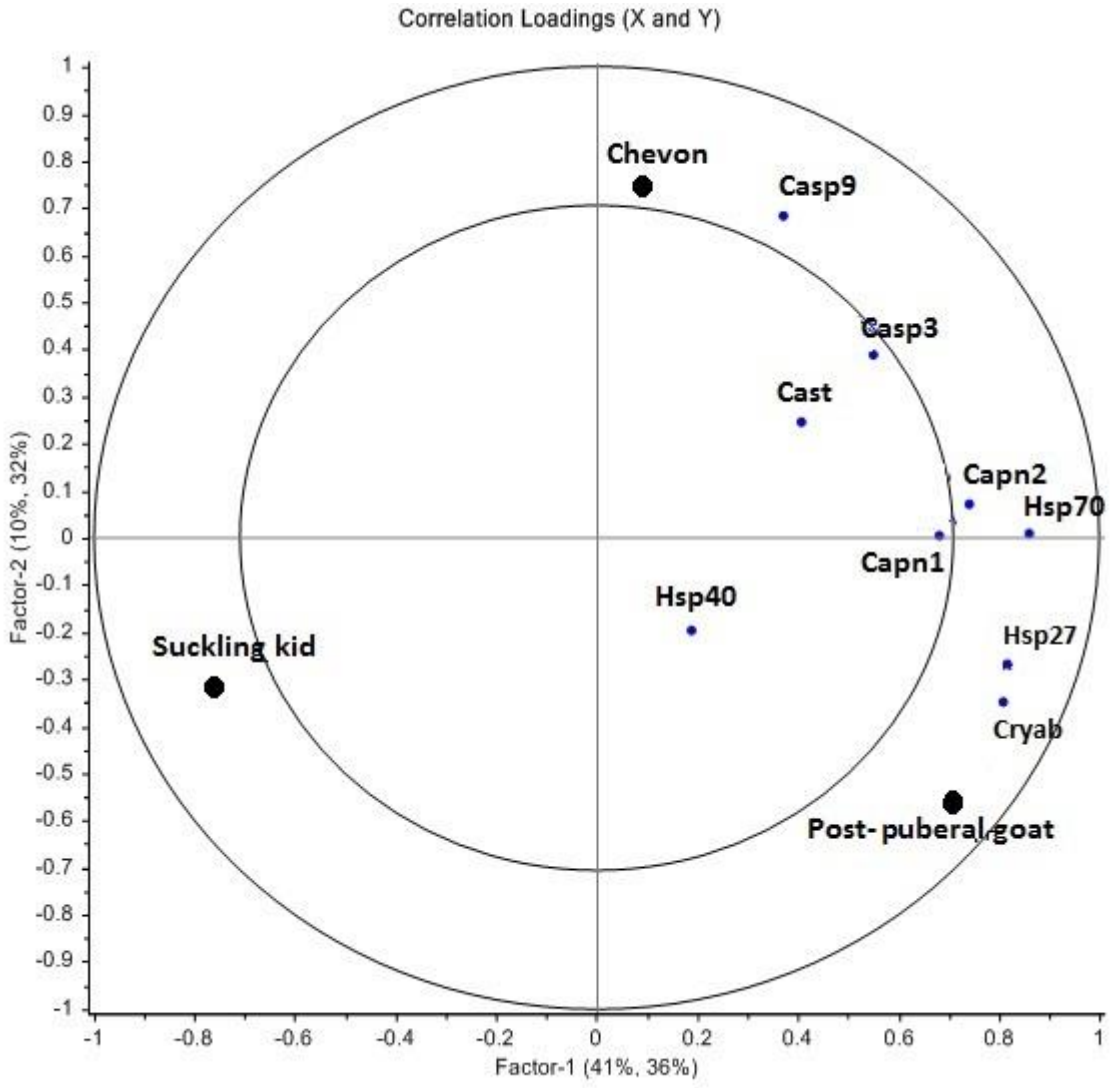

Figure 1. Correlation loadings on the two first factors of a PLS-DA model for the classification of goat types according to age (suckling kid, chevon and post-puberal goat; Y-matrix) based on their gene's expression profile (X-matrix).

It can be observed that Cryab and Hsp27 genes were high positively correlated, as expected in relation to their similar function in the cell (Lomiwes *et al.*, 2013). The same condition can be observed for Capn1 and Capn2 genes, basing on their cooperative role (Koohmaraie & Geesink, 2006). The Hsp70 was close to both the previous pairs. All these five genes were located near factor 1. Van Ba and Hwang (2013) examining bovine skeletal muscle satellite

cells, found a crosstalk between Capn1 and Hsp systems (Hsp27, Hsp70 and Hsp90). These protein systems are probably closely related in the physiological cell survival contrasting apoptosis.

The goat classes were clearly discriminated by their genes expression profiles because they loaded far from each other in three different quadrants of the plot. The PPu group position was close and thus positively associated with high levels of Cryab and Hsp27. Its projection along factor 1 fall very near Capn1, Capn2 and Hsp70. Along the same factor1, Sk group was in opposite position respect to the same genes (Cryab, Hsp27, Hsp70, Capn1 and 2). The Ch type compared to Sk and PPu goats had an intermediate position along factor 1 that can be thus regarded as the age factor. By contrast, Ch type compared to Sk and PPu goats had an opposite location along factor 2 and loaded very close to its positive half. This factor was highly correlated with Casp9 (0.73). Moreover, it was also slightly correlated with Casp3 (0.42) and negatively with Cryab (-0.37) and Hsp27 (-0.30) and thus may be regarded in same way associated to apoptosis.

The scores of the individual animals are plotted in Figure 2 that confirms the good separation of goats of different age due to the expression of the examined genes, the individual average values of which are reported in Table 4. Capn1 and Capn2 showed an increasing trend with the age of animals, whereas the Capn1/Cast ratio was higher in PPu group compared to younger animals. Capn1 and Capn2 are calcium-dependent proteases greatly responsible for *post-mortem* tenderization, being involved in muscle proteolysis (Veiseth *et al*., 2001; Koohmaraie & Geesink, 2006), while Cast is their competitive inhibitor. However, calpains play also an important physiological role in intracellular protein turnover. The rate of skeletal muscle growth is determined, to a large extent, by the balance between level of protein synthesis and level of protein degradation (Goll *et al*., 1998) that involves the calpain system (Goll *et al*., 2008). In animals with fast growing muscles, as they were the experimental kids in comparison with the older goats, muscle proteins synthesis largely overcome degradation. The associate attenuation of calpains action in the cell can result from a lower transcription of the relative RNA messenger.

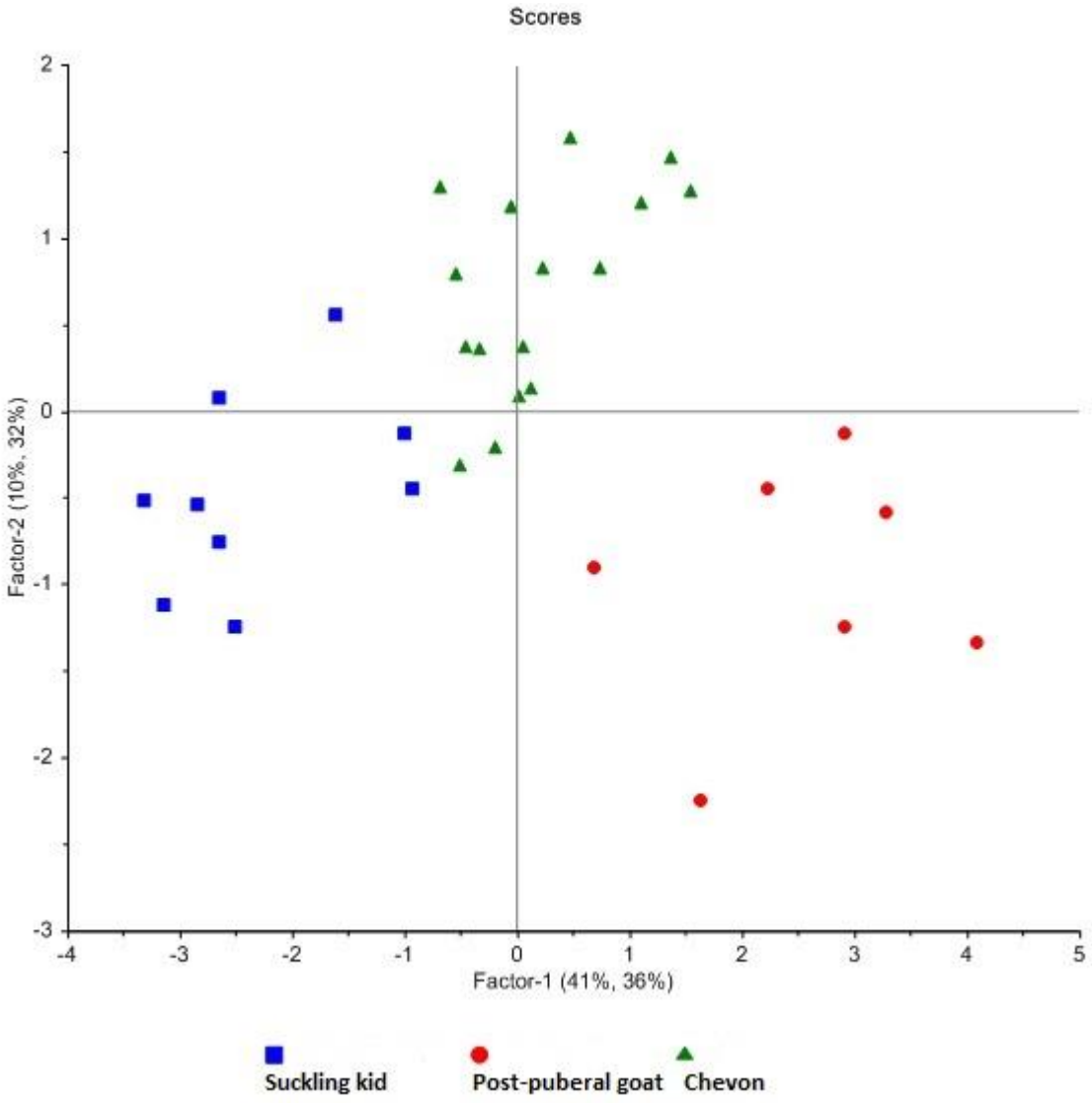

Figure 2. PLS-DA regression scores, showing the individual goats distribution in the bi-factorial space of their gene's expression profile. Individual goats are denoted by different symbols according to their age (suckling kid, chevon and post-puberal goat).

Table 4. Fold change ratio in relative RNA expression of tenderness-related genes in *Longissimus lumborum* of young goats.

	Sk	Ch	PPu	SEM
Capn1	1.00^{c}	1.34^{b}	1.78^{a}	0.001
Capn2	1.00^{c}	1.45^{b}	1.91^{a}	0.001
Cast	1.00	1.30	1.29	0.001
Capn1/Cast	1.00^{b}	1.13^{b}	1.45^{a}	0.054
Casp3	1.00^{b}	1.84^{a}	1.80^{a}	0.000
Casp9	1.00^{b}	1.36^{a}	1.19^{ab}	0.000
Cryab	1.00^{c}	2.21^{b}	5.61^{a}	0.093
Hsp27	1.00^{c}	1.81^{b}	3.10^{a}	0.039
Hsp40	1.00	1.11	1.34	0.009
Hsp70	1.00^{c}	1.72^{b}	2.28^{a}	0.003

Sk = suckling kid; Ch = chevon; PPu = post-puberal goat.
Capn1 = calpain-1; Capn2 = calpain-2; Cast = calpastatin; Casp3 = caspase 3; Casp9 = caspase 9; Cryab = αB-crystallin; Hsp27 = heat shock protein 27; Hsp40 = heat shock protein 40; Hsp70 = heat shock protein 70.
^{a,b} $P \leq 0.05$.

In pigs, Li *et al*. (2009) verified that activities of total protein synthesis increase over neonatal development before weaning, while protein degradation decrease in association with a reduced expressions of calpains. Sk group showed also a low level of caspases than older goats. Muscle cells die by apoptotic process after slaughtering of animals (Ouali et al., 2006; Becila et al., 2010) and the intrinsic pathway of this process, which involves Casp9 (initiator) and Casp3 (effector), is the major in the muscle cell (Herrera-Mendez et al., 2006). Similarly to calpains, caspases could be less expressed in younger animals because of the necessity of lower protein degradation in early postnatal stages of life. Other works have highlighted a crosstalk between calpains and caspases systems (Neumar et al., 2003; Kemp et al., 2013; Van Ba & Hwang, 2013; Van Ba et al., 2015), reporting a cooperation/compensation of the two enzyme systems. Powers (2009) highlighted that calpains and caspase 3 operate in concert by degrading both cytoskeletal proteins and actomyosin complexes in muscle tissue.

Interesting results were found for Hsp mRNA expression. For all the Hsp except Hsp40 a significant increasing trend can be observed with animal age. It was speculated that Hsp expression could be stimulated after slaughter in response to the muscle cell stress (Herrera-Mendez *et al*., 2006; Ouali *et al*., 2006). It is known that various Hsp, i.e. Hsp27 and 70, have

an anti-apoptotic function (Creagh *et al.*, 2000; Li *et al*., 2000; Beere, 2005). Hsp27 and Cryab may also interact with various intermediate filaments of the myofibrillar organization, protecting them to degradation (Lomiwes *et al*., 2014). Due to their anti-apoptotic action and to their protective role on myofibrillar structures, Hsp can contribute to produce a tougher meat. Indeed, in our study, the maximum expression of Hsp can be found in PPu class, which also had the higher values of shear force. The tenderization is a complex process not only on the exclusive control of calpain system. Thus, considering the expression of calpains, caspases and Hsp together with the WBSF values, it seems that the overexpression of small Hsp in older goats can overcome the actions of calpains and caspases, leading to a tougher meat. This effect can be obtained both protecting the myofibril against the calpain and/or caspases action, than inhibiting the apoptosis promoted by caspases. Furthermore, we can suppose that the higher values registered for Hsp in PPu class can be also due to the hormonal changes that occurred during puberty and sexual maturation. Moreover, the increase of Hsp with age could represent the physiological evolution of the resistance of animal (cells) to environmental stresses. The Ch group, in which the Hsp are not so expressed like in the post-puberal goats, the apoptotic process was more noticeable.

The role of calpains, caspases and Hsp systems in the muscle cell homeostasis is complex and not yet fully understood. Such as calpains are involved in myoblasts differentiation and growth (Goll *et al*., 2003; Theil *et al*., 2006), also caspases and Hsp are referred as involved in these processes (Fernando *et al*., 2002; Lanneau *et al*., 2007), being moreover in relation with calpains expression (Van Ba & Hwang, 2013, 2014). Then, all these proteins can be involved in muscle development and myofibrillar turnover, and consequently in meat ageing process, in ways that have to be yet deepened and clarified.

Conclusions

Suckling kids showed lighter and leaner carcasses, with paler and tenderer meat than older goats. Although tenderer, the meat of younger goats showed the lower expression of calpains, probably because a lower degradation level of myofibrillar proteins that characterised their rapid growth phase. Similar considerations can be do for caspases, Casp 3 in particular. Conversely, the tougher meat of the older animals may be due to the higher expression of Hsp that can overhang the action of proteases systems acting in the muscle cell. Physiological conditions of pre- and post-puberal goats could be the basis for the diverse expression of the genes considered and probably involved not only in meat tenderization process.

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General conclusions

Meat tenderness is the most important eating factor that influences consumer preference. Tenderization process is complex and depends on many factors, related to the animal's (i.e. breed, age, management) and to the muscle characteristics (fat and collagen content, fibres type and proteolytic systems activity). For these reasons, meat tenderness is a very variable trait, but, for example, simple parameters such as the pH or the shear force of a muscle can help in the prediction of meat texture at the point of consumption. Currently, there is a growing need to identify *ante-mortem* markers that can predict the ultimate tenderness of an animal's meat and to use these markers for the genetic selection of animals during husbandry. In this sense, modern technologies of bio-molecular analysis can be useful, both at genomic and at proteomic level, providing the possibility to identify genes and proteins that are in relation with the desired trait. The research can be conducted at a broad level, considering the total transcriptome or proteome of the muscle cell, otherwise, it can be more targeted to specific cellular mechanisms and factors.

At the beginning, the studies were focused on proteins that are more closely related to the meat tenderization process, such as the enzymatic systems responsible for physiological protein degradation in muscle, i.e. calpains, cathepsins and proteasome. Afterwards, the studies have considered the programmed cell death process called apoptosis, which starts just after slaughter taking into account the factors related to this process, such as caspases and heat shock proteins (Hsp). These latter proteins are also directly involved in the protection of proteins in the physiological conditions of muscle cell.

Our research wanted to investigated the role of a set of protein factors involved in meat tenderization process that are the calpain/calpastatin system, some important caspases members (3 and 9) and some Hsp (27, 40, 70 and αB-crystallin) in different experimental conditions that involve different muscle types, different species of animals and different animal ages in the same species.

Our work confirm the primary role of the calpain/calpastatin system in the maturation process of meat, and in particular the importance of calpain-1, which is positively related to tenderness of meat. However, during the higher muscle growth phase, in younger animals, this system can be inhibited to reduce the myofibrillar degradation. Moreover, it was highlighted a less important role of caspases enzymes in meat maturation, even if confirming their activity in early *post-mortem* moment, as characteristic of the apoptotic process.

Anyway, similarly to calpains, the caspases expression could be limited in the phase of maximum growth of muscles.

Finally, even more interesting results were found relating to the involvement of Hsp in the tenderization of muscle. Indeed, in our studies, we have highlighted a constant negative relation between Hsp expression and tenderness of meat. In particular Hsp27 and Hsp70, but also αB-crystallin, were more expressed in more tougher meat, and their expression showed a tendency to increase with the age of the animal. Hsp70 also confirmed their role in thermotolerance of animals, instead Hsp40 did not show the importance highlighted in other works. We can conclude that these proteins could be considered for further studies to find molecular markers of meat tenderness.

New scenarios are opening for the comprehension of the muscle tenderization mechanisms. Specifically, more in-depth studies on the apoptotic system in the muscle cell in general, and the role of the mitochondrion in this process in particular are needed. In addition to a more complete knowledge of the physiology of the muscle cell, more indications on the factors involved in meat maturation can derive from these studies. The highlighted factors may be taken into account in future as genetic selection markers for animals with suitable meat tenderness levels.

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Main activities

Coursis:

- Joint PhD Program in Molecular Biology. University of Udine, University of Trieste, SISSA and ICGEB. Lessons of December 9-11, 2014, University of Udine, Udine, Italy.

Congress partecipations:

- PRION 2014 - Animal TSE Workshop. SISSA and University of Udine. May 27, 2014, University of Udine, Udine, Italy.

- Annual Meeting of the Italian Group of Biomembranes and Bioenergetics (GIBB), June 18-20, 2015, University of Udine, Udine, Italy.

- "Sostenibilità ambientale ed economica dell'allevamento confinato in Italia e del pascolo consociato con coltivazione arborea in Brasile: due sistemi a confronto". October 9, 2015. Department of Agricultural and Environmental Sciences, University of Udine, Udine, Italy.

Congress presentations:

- Contribution to the oral presentation: N. Pizzutti, G. Lippe, A. Fabbro, E. Saccà, M. Corazzin, E. Piasentier, "The caspase and calpain proteolytic systems in *Longissimus dorsi* and *Infraspinatus* muscles of Italian Simmental young bulls". Proceedings of the Animal Science and Production Association (ASPA) 20th Congress, June 11-13, 2013, Bologna, Italy. [Italian Journal of Animal Science, PAGE Press Publications, Pavia, Italy, Vol. 12 (Suppl. 1), p. 15, Abstr. C-037].

- Contribution to the oral presentation: E. Piasentier, E. Saccà, M. Corazzin, N. Pizzutti, S. Bovolenta, "Comparison of the expression of some genes involved in beef tenderization in two bovine skeletal muscles". Proceedings of the 59th International Congress of Meat Science and Technology (ICOMST), August 18-23, 2013, Izmir, Turkey.

- Poster presentation: M. Borgogno, M. Corazzin, H. Mousavikhorshidi, S. Favotto, E. Saccà, O. Bearzi, D. Furlan, E. Piasentier, "Sensory properties and consumer acceptability of traditional capretto and chevon", 60th International Congress of Meat Science and Technology (ICOMST), August 17-22, 2014, Punta del Este, Uruguay.

- Contribution to the oral presentation: Corazzin M., Piasentier E., Saccà E., Bazzoli I., Bovolenta S., "Produzione del vitellone di razza Rendena con metodo biologico: primi risultati di una sperimentazione in Val Rendena". 20th Congress of SoZooAlp - Società italiana per lo studio e la valorizzazione dei sistemi zootecnici alpini - "I formaggi protagonisti della zootecnia alpina", November 7-8, 2014, Gemona del Friuli, Udine, Italy.

- Poster presentation: M. Borgogno, S. Favotto, E. Saccà, M. Martinez-Alvaro, E. Piasentier, "Indice di qualità per la valorizzazione della carne pezzata rossa italiana", 5th National Congress of SISS – Società Italiana di Scienze Sensoriali - November 26-28, 2014, San Michele all'Adige, Trento, Italy.

- Poster presentation: M. Borgogno, M. Corazzin, E. Saccà, S. Favotto, S. Salvador, S. Bovolenta, E. Piasentier, "Prediction of eating quality of Italian Simmental beef form butcher's steak appearance judgment", 21st Congress of the Animal Science and Production Association (ASPA), June 9-12, 2015, Milano, Italy.

- Poster presentation: W.O. Bessong, M. Corazzin, E. Saccà, C. Biondokin, G. Menta, E. Piasentier, "Carcass and meat characteristics of young bulls from local Goudali (Zebu) cattle of Cameroon and its crosses with Italian Simmental". 61st International Congress of Meat Science and Technology (ICOMST), August 23-28, 2015, Clermont-Ferrand, France.

- Contribution to the oral presentation: E. Saccà, M. Corazzin, N. Pizzutti, G. Lippe, E. Piasentier, "Calpains, caspases, heat shock proteins and tenderness of beef". Annual Meeting of the Italian Group of Biomembranes and Bioenergetics (GIBB), June 18-20, 2015, Udine, Italy.

- Poster presentation: Borgogno M., Corazzin M., Saccà E., Favotto S., Salvador S., Bovolenta S., Piasentier E., "Prediction of eating quality of Italian Simmental beef from butcher's steak appearance judgment". Proceedings of the Animal Science and Production Association (ASPA) 21st Congress, Milano, Italy, June 9-12, 2015. [Italian Journal of Animal Science, Page Press, Pavia, Italy, Vol. 14 (Suppl. 1), P-003, Abstr. 88].

- Poster presentation: M. Borgogno, E. Saccà, "La qualità della carne: dai geni alla tavola", PhD EXPO 2015, June 18, 2015, University of Udine, Udine, Italy.

Teaching:

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Publications:

- E. Saccà, M. Corazzin, N. Pizzutti, G. Lippe, E. Piasentier, 2015. "Early *post-mortem* expression of genes related to tenderization in two Italian Simmental young bulls' skeletal muscles differing in contractile type". Animal Science Journal, 86, 992-999.

- E. Saccà, N. Pizzutti, M. Corazzin, G. Lippe, E. Piasentier, 2015. "Assessment of calpain and caspase systems activities during ageing of two bovine muscles by degradation patterns of αII spectrin and PARP-1". Animal Science Journal, doi: 10.1111/asj.12473

- M. Borgogno, M. Corazzin, E. Saccà, S. Bovolenta, E. Piasentier, 2015. "Influence of familiarity with goat meat on liking and preference for capretto and chevon". Meat Science, 106, 69-77.