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Early degradation of myosin light chains beneficially impacts human health of sea bream (*Sparus aurata*) consumers

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“C’è solo un bene il sapere,

Solo un male, l’ignoranza”

(Socrate)

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ACRONYMS AND ABBREVIATION

ACE: angiotensin-converting enzyme

ACE-I: angiotensin-converting enzyme- inhibitory

Ab: antibody

FAO: Food and Agricultural Organization

GI: gastrointestinal

GERD: gastroesophageal reflux disease

MLC: myosin light chain

MLC3: myosin light chain 3

SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

WB: Western Blot

ABSTRACT

This work investigated on the pre-slaughter changes in sea bream muscle proteins as affected by fish pre-slaughter fasting in comparison to full feeding and how these changes could affect the presence of active peptides and the gastric digestibility of sea bream fillet. In the early post-mortem, actin fragments were detected in both the samples regardless of the feeding regime, while only in the fasted fish samples myosin light chains were found markedly degraded in the early *post-mortem*. On the other hand, degradation was also evident in the full feeding samples, where myosin light chains were no longer visible during *post mortem* storage at 4 °C, consistent with the natural instability of these fish proteins in comparison to the mammal ones. ACE-inhibitor gastric-resistant bioactive peptides (VF, MF, VAF), which derived from myosin light chains, were found in both the samples, regardless of the feeding regime, with one of these peptides (VF) seeming in larger amount in the fasted samples.

By using static standardized in-vitro digestion methods, digestibility of myosin was found to increase to a greater extent in the fasted samples at pH 4.0, when individual test enzymes were applied under conditions of drug-induced high pH occurring in GERD patients. Differences in myosin gastric digestibility were also apparent at pH 3.0, while at this pH actin was found susceptible to full proteolysis, irrespective of the feeding regime. Therefore, it appeared that under conditions which caused an early degradation of the myosin light chain and possibly an impaired myosin integrity, gastric digestibility of the main myofibrillar proteins was much higher in the altered gastric milieu. In conclusion, sea bream feed restriction, other than decreases the environmental impact of fish aquaculture, seems to act on fish proteins, resulting in nutritional benefits for hypertensive and GERD patients.

1 Introduction: General characteristics of sea bream (Sparus aurata)

Biology

Sea bream (Figure 1.1) belongs to the class *Actinopterygii*, order *Perciformes*, family *Sparidae*, genus *Sparus*, species *S. aurata*. It is a sea-bone fish that tolerates considerable changes in salinity, but it is extremely sensitive to low temperatures (temperatures below 5°C can be fatal) and to oxygen deficiencies (Giordani & Melotti, 1984). Sea bream is present in the western and northern Mediterranean Sea and in the eastern Atlantic. It is a strictly coastal fish, it lives on hard and sandy bottoms and is particularly widespread on the border between the two substrates. Sea bream is a very important and valuable species from the commercial and organoleptic point of view, along with the red snapper (*Dentex dentex*) and pagro (*Pagrus pagrus*). The body is oval, the head is robust and the front profile of the muzzle is very pronounced (Tortonese, 1975). The mouth, with full lips, is small and the upper part (jaw) is slightly prominent compared to the lower one (mandible). It is equipped with molariform teeth, which, together with the jaws, make this fish capable of crushing the shell of the crustaceans and the shells of molluscs. The fish scales, present all over the body with the exception of the snout, the pre-orbital and the orbital areas, are well evident, as well as the lateral line (Giordani & Melotti, 1984; Fischer et al., 1987). The coloration is typical of fish that lives on seabed, with silvery-gray sides (often even thin black lines are present). The back is light gray with blue-golden reflections, while the dorsal fin has gray-blue reflexes and is furrowed in the middle by a black line. The characteristic golden band present between the eyes, interposed between two thin black bands, which is much more evident in adult specimens, gives the name to the species (Giordani & Melotti, 1984).

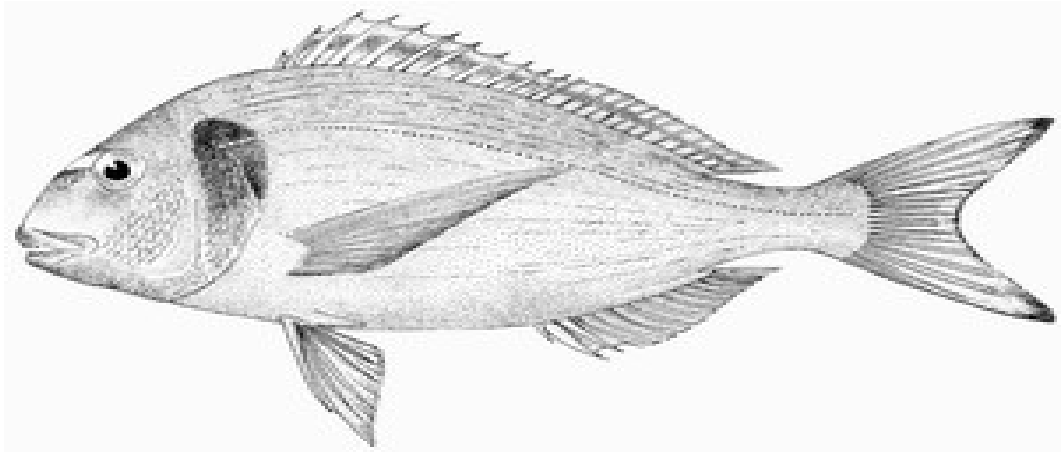

Fig. 1. 1 - Sea bream (*Sparus aurata* L.) (FAO, 2018)

Sea bream is a fairly long-lived species that can reach 20 years of age, a size of 70 cm (generally it is common between 20-50 cm) and the weight of 5-6 kg (Fischer et al., 1987). Sea breams are proterandic hermaphrodites: during the first phase of life, up to about 2 years of age most of the individuals undergo the sexual inversion which gives the female characteristics. In males, sexual maturity is around 20-30 cm, while in females is around 35-40 cm. Reproduction (with several cycles of egg laying) occurs between October and December, at temperatures ranging between 14° and 16 °C (Bini, 1970). The fertilized eggs have a diameter of 1 mm, are pelagic and equipped with an oily drop to promote buoyancy (Alessio & Gandolfi, 1975). After hatching, the young have a transitory yellowish color, up to the length of 45-50 mm and migrate in lagoon waters or in estuaries where they find the trophic conditions to grow quickly (Figure 1.2).

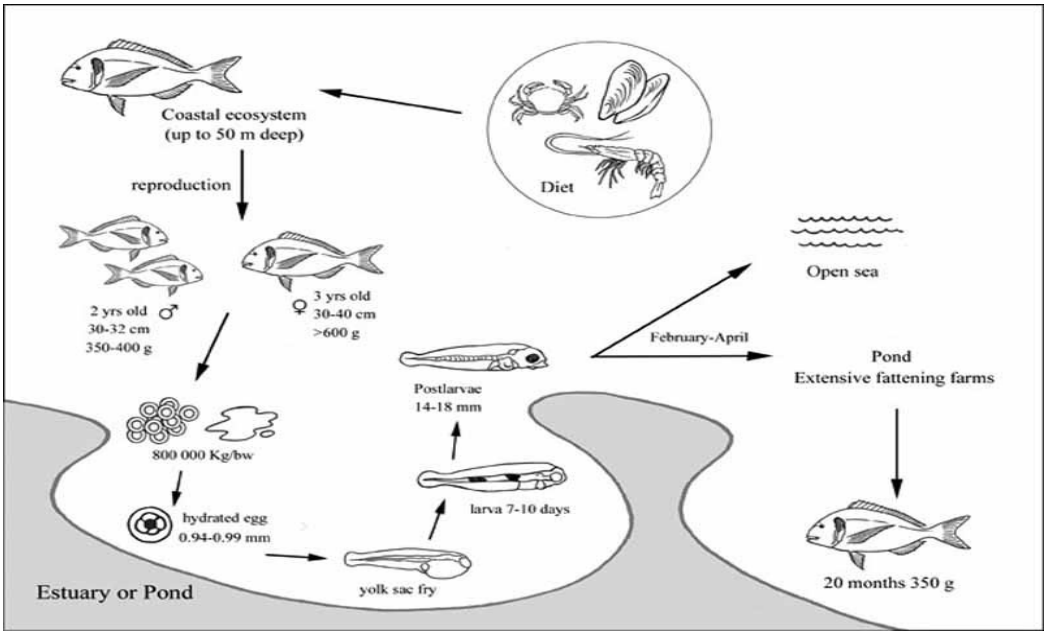

Fig. 1. 2 - *Sparus* aurata reproduction cycle - extended system (FAO, 2018)

1.1 Fishing, breeding and diet

In Italy, most of caught fish comes from Tyrrhenian Sea, Sicilian Sea and Sardinian Sea. According to the EC 1967/06 regulation, the minimum size of the caught fish is 20 cm, even if in some periods of the year it is allowed to catch juveniles for breeding. However, catches at sea represent a smaller portion of the total marketed. Most of the sea bream found on the markets comes from farms built in the lagoon valleys of the upper Adriatic (lagoon of Grado and Marano in Friuli-Venezia Giulia) and in the coastal ponds of Sardinia (15%) and Tuscany (25%), or from offshore cages in Sicily (20%) (Table 1.1) (Figure 1.3).

Region	Production (tons)
Friuli-Venezia Giulia	170
Veneto	23
Emilia-Romagna	16
Liguria	410
Toscana	1.692
Lazio	1.805
Puglia	390
Sardegna	979
Sicilia	1.345
TOTAL	6.830

Table 1. 1 – Production of sea bream in Italy (Source: Unimar / MiPAAF, 2015)

Fig. 1. 3 - Distribution of the facilities raising sea bream and sea bass (Unimar census, 2009)

There are three basic types of farming: extensive farming, semi-intensive farming, and intensive farming. These methods are very different, especially with regard to the density of fish farming and the supply of food (Olivieri, 1995; FAO, 2018).

- *Extensive breeding* (fish farming, coastal lagoons, and salted ponds) is based on the migratory behavior of juveniles entering the lagoons for growing. They are captured by means of fixed tools placed on the border between the lagoon and the sea. This practice provides a very limited and unpredictable supply; many modern production units rely on both young subjects caught in the wild and young subjects raised in nurseries. In this type of breeding, man do not interfere with important energy contributions, as the trophic contribution is totally dependent on the environment. Sea bream reaches the commercial size of 350 g in 20 months and is bred together with mullet, eels and sea bass. The total production of this type of breeding is between 30-150 kg / ha / year, depending on the area's production: in the lagoons of north-eastern Italy the production of sea bream is quantified between 15-30 kg / ha / year; in extensive fish farms the density of fish does not exceed 0.0025 kg / mc.
- *Semi-intensive breeding* represents a development of the extensive one. The management of the environment is piloted by the man who intervenes with energy supplies external to the systems, in the form of nourishment, or by introducing pre-fattened young subjects, to minimize mortality and reduce breeding times. This type of farming can include the use of fertilizers to increase the availability of food in the natural environment or the introduction of artificial feed and supplemental oxygen. This farming system is usually carried out through enclosures in limited areas in the lagoons. Final production can vary widely, depending on the size of the young subjects and the amount of feed. The density in semi-intensive systems normally does not exceed 1 kg / mc and the production varies between 500-2400 kg / ha / year.
- *Intensive breeding* includes large quantities in high density tanks (over 15-45 kg/mc). It can be realized either in open sea (floating or submerged cages), or in tanks on the ground. Although the densities of the cages (10-15 kg/mc) are lower than those of the tanks, there are great advantages that make these types of breeding more profitable: there are no energy costs for pumping, aeration or post-breeding water treatment. It is still not possible to control the temperature if it needs a longer breeding period or the need for stocks of older juveniles. Compared to the previous farm systems, in tanks the environment is totally controlled by man and requires considerable water supplies, oxygenation and aeration systems to keep the level of dissolved oxygen high. Intensive growth involves breeding, larval and pre-fattening breeding and needs the presence of different structures such as hatchers (for laying eggs), fattening farms and ground tanks or cages in the open sea, where the specimens are fed up to reach the commercial size of 300-400 g in optimal conditions (18-26 °C). Food is distributed by automatic feeders for small fish (1-3 g) or by hand for

larger fish at two-hour intervals, increasing the percentage of artificial feed (150-300 μm of particles) (FAO, 2018). To avoid the differentiation of fish development, the calibration of the feed is required at least two or three times per cycle.
In the early 1980s, knowledge of the nutritional needs of this species was virtually non-existent. Over the years, technologies have evolved and, in the mid-1990s, extruded feeds began to be produced extensively. With the extrusion technique the flexibility on the number, type and quantity of formulations has increased and the physical characteristics of the feeds and the digestibility of the ingredients have been improved. With the new feed formulations, the sea bream's diet is more flexible and no longer fixed. Until 2013, the ban on the feeding of animal meal caused a series of changes in the formulation of feed, which resulted in proteins and vegetable fat sources implementation. Vegetables are more tolerated by sea bream and sea bass than cold water species, such as trout (Robaina et al., 1995; Pereira et al., 2003; De Francesco et al., 2007).
Since the 1980s, there has been a progressive increase in production from breeding facilities, thanks to the widespread use of artificial reproduction plants. Italian production has remained relatively stable between the 9,000 and the 10.00 tons between 2003 and 2011. Subsequently it decreased constantly due to the strong competition from Greece and Turkey, reaching 7,360 tons in 2015, the lowest level since 2000 (Table 1.2), or 7% of the EU production (down from 10% in 2011 and 13% in 2002) (Olivieri, 1995; AAVV, 2017).

Country	2002	2003	2005	2007	2009	2011	2012	2013	2014	2015
Cyprus	1.266	1.181	1.465	1.404	2.572	3.065	3.121	4.444	2.919	3.656
France	1.361	1.100	1.900	1.392	1.648	1.500	1.300	1.477	1.105	1.502
Greece	37.944	55.000	50.000.	79.000	90.000	63.000	75.000	72.000	71.000	65.000
Italy	8.000	9.000	9.500	9.800	9.600	9.700	8.700	8.400	8.200	7.360
Portugal	1.855	1.449	1.519	1.930	1.383	1.200	1.000	1.500	1.500	1.400
Spain	11.335	12.442	15.577	22.320	23.690	16.930	19.430	16.800	16.230	16.231
Croatia	700	1.000	1.200	1.500	2.000	1.793	2.105	2.466	3.640	4.500
UE-28	62.461	81.172	81.161	117.346	130.893	97.188	107.656	110.087	104.594	99.649
Turkey	11.681	16.735	27.634	33.500	28.362	32.187	30.743	35.701	41.873	48.000
Total	74.142	97.907	108.795	150.846	159.255	129.375	138.399	145.788	146.467	147.649

Table 1. 2 – Evolution of the sea bream aquaculture production (ton) (FEAP, 2016)

1.2 Marketing and nutritional characteristics

Sea bream is marketed both fresh and frozen. It is attractive from a price point of view and maintains a positive image in the minds of consumers. According to ISMEA (Institute of Services for the Agricultural Food Market) data relating to the total quantities consumed fresh, sea bream is the main species consumed in 2015 (9%) and has significantly increased its production between 2005 and 2010, with a market share coming from 7.7 % in 2005 at 8.9% in 2010. About half of Italian families (51%) consume fish at least once a week: 55% prefer fresh and thawed fish; 23% frozen fish, 18% preserved fish and 4% the fish gastronomy department (salted and smoked) (Olivieri, 1995). In the choice of products, initially the consumer was influenced exclusively by the price; nowadays there are other parameters that take on an increasingly significant weight: quality, freshness, nutritional value, novelty and convenience in the preparation. In general, Italian consumers prefer references with a high content of services, already clean and easy to be prepared.
According to the National Research Institute for Food and Nutrition (INRAN) database, the meat of the farmed bream is rich in proteins (19.7 g / 100 g of edible portion) and low in fat (8.4 g / 100 g). These characteristics make the gilthead fish easily digestible. Among the mineral salts, phosphorus (1050 mg / 100 g) and calcium (30 mg / 100 g) are noteworthy. As reported in Table 1.3, the wild sea bream has a higher protein content and lower fat content than the farmed sea bream fillet.

Chemical composition	Fresh sea bream fillet (*Sparus aurata*)	Farmed sea bream fillet (*Sparus aurata*)
Energy (Kcal/KJ)	121/505	159/665
Edible portion (%)	100	100
Water (g)	73.2	69.1
Proteins (g)	20.7	19.7
Lipids (g)	3.8	8.4
Cholesterol (mg)	64	68
Total lipids (%)	3.8	8.4
Total saturated (%)	0.88	1.94
Total monounsaturated (%)	0.93	2.78
Total polyunsaturated (%)	1.21	2.21
Polyunsaturated/saturated ratio	1.4	1.1
Avaiable carbohydrates (g)	1	1.2
Solubre sugar (g)	1	1.2

Table 1. 3 – Nutritional value of 100 g of fillets of fresh sea bream and fillets of fresh farmed sea bream (INRAN, 2009)

1.3 Fish quality

Quality is one of the most abused words in food science, especially in aquaculture. The concept of quality applies to a wide range of fish traits, including meat, skin, up to the impact of the animal on the environment. The fish quality that refers to the attributes of meat (muscle) is evaluated through the consumer attractiveness (Robb, 2002).

The quality of the raw material is very important. Fish is a highly perishable food product due to its biological composition and for this reason it must be managed with care in order to extend its shelf-life. In recent years, retailers and producers have had to cope with an increase in demand, caused both by the change in the lifestyle of consumers seeking healthy products, and by the globalization that has increased the supply of fish species (Alasalvar et al., 2002). Studies on fish meat by Johansson et al. (2000) showed that consumers choose fish products based on visual impression (40%), flavor (40%) and texture (20%). A fundamental aspect of the fish quality is

freshness that cannot be assessed by a single method. Subjective (sensory) and objective (non-sensory) methodologies can be used to evaluate freshness (Figure 1.4). Sensory methods are rapid and non-destructive systems (except for cooked products), applicable to any species. They are divided into: discriminating, descriptive and affective methods. The choice of the method depends on the purpose of the application of the sensory analysis and at what level of the supply chain quality must be evaluated. The Tasmanian Food Research Unit (TFRU, in the book Seafoods: Quality, Technology and Nutraceutical Applications, 2013) and the EU tables (Report from the commission to the european parliament and The council, Brussels, 2018) on the possibility of introducing certain requirements regarding the protection of fish at the time of killing are the most used sensory evaluation methods for analyzing the quality of raw fish in inspections and industry, while for cooked fish the Torry table is used. These methods allow us to have a valid idea of what consumers want from the fish product, because they provide immediate information on quality. Sensory evaluations have the disadvantage that they cannot be objective and documented; for this reason, they are used in association with objective methods. The non-sensory methods used to determine the freshness of the fish are divided into:

- chemical/biochemical methods: evaluation of ATP decomposition compounds (K value), trimethylamine (TMA), total nitrogen-based compounds (TVB-N), evaluation of biogenic amines, measurement of volatile compounds and lipid oxidation;
- physical methods: texture analysis, water content and color;
- microbiological methods: total bacterial load (TVC) and counts of specific deterioration organisms (SSO).

These analytical techniques do not depend on the senses of man as they are the result of instrumental or laboratory methods and have the advantage of not being influenced by the variability of the operator and are easily reproducible and reliable. However, the disadvantage of these systems is that they are not attributable to all quality attributes (Alasalvar et al., 2002; Abbas et al., 2008).

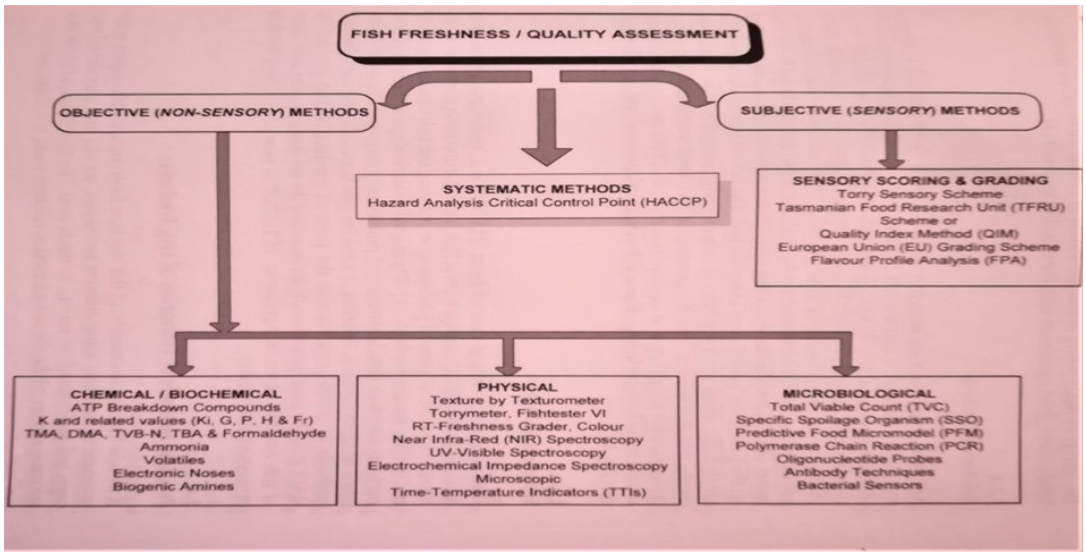

Fig. 1.4 - Methods for evaluating freshness and quality of fish (modified from Alasalvar et al. 2002)

To evaluate the freshness of the sea bream stored in ice (Alasalvar et al., 2001) and of the rainbow trout (*Oncorhynchus mykiss*) (Alasalvar et al., 2002) subjective methods were used in association to non-sensory methods. In the specific case of sea bream, the degree of freshness evaluated by the TFRU table (Table 1.4) showed a good time-dependent correlation with K values and hardness.

Parameters being assessed		**Demerit points**			
		0	**1**	**2**	**3**
Appearance		Very bright	Bright	Slightly bright	Dull
Skin		Firm or elastic	Soft		
Slime		Absent	Slightly slimy	Slimy	Very slimy
Stiffness		Pre-rigor	Rigor	Post-rigor	
Eyes	Clarity	Clea	Slightly cloudy	Cloudy	
	Shape[a]	Normal	Flat sunken	Sunken or swollen	
	Iris	Visible	Slightly visible	Not visible	
	Blood	No blood	Slightly bloody	Bloody	Very bloody
Gills	Colour[a]	Dark red	Red	Brown	Dark brown or grey
	Mucus	Absent	Slight	Moderate	Excessive or sticky
	Smell	Neutral	Fishy	Stale	Spoilt
Belly	Discoloration	White iridescent	Some yellowish	Yellow	Excessive yellow
	Firmness	Firm	Soft	Sunken	Burst
Vent	Condition	Normal	Slight break and darkening	Excesive	
	Smell	Fresh	Neutral	Fishy	Spoilt

Table 1. 4 – Sensory evaluation for cultured sea bream (modified by Alasalvar et al., 2001). The shape of the eyes and the color of the gills are not good indicators for sea bream stored on ice.

The pH, which is another important parameter for the fish quality, along with texture, has been found significantly correlated with fish freshness. According to Abbas et al. (2008), low pH values in the first ice- storage period reflected good nutritional status of the fish; at the end of this storage period, the pH increased (7 and 12 days of storage for sea bream and sea bass) along with muscle deterioration, associated to an increase in the production of metabolites from alkaline bacteria, free fatty acids, thiobarbituric acid (TBA) and nitrogen-based compounds (TVB-N).

Water Holding Capacity (WHC) represents a physical method for assessing fish freshness and it is a parameter that influences the cooking process and organoleptic sensations during chewing (Tejada et al., 2002). According to Flos et al. (2002), WHC, which is evaluated on the amount of water maintained in the muscle after being subjected to a pressure, expresses the content of muscular water that can be influenced by any loss of fluids and dehydration suffered by the fish in the post-harvest management. *Rigor mortis* causes fluid leakage that determines the decrease of sea bream WHC (Flos et al., 2002). Significant differences were observed between farmed and wild fish WHC (Sañudo et. al., 1993). Through nuclear magnetic resonance (NMR) it was possible to localize most of the water within myofibrils (Damez et al., 2008; Erikson et al., 2012).

1.4 Fish metabolism

Metabolism represents the set of energy transformation processes associated with the transformation reactions of matter (chemical, mechanical, electrical and thermal energy). Animals get energy for the cellular functions from both the aerobic and anaerobic metabolisms. Aerobic metabolism requires oxygen for the oxidation of carbohydrates, lipids and proteins. The anaerobic metabolism, on the other hand, generates pyruvic acid, which may be than transformed in lactic acid. This mechanism is used when high amounts of energy are needed quickly, for example during swimming (Robb, 2002). The environmental conditions, the size of the animal, the growth and reproduction phases affect the metabolism of the fish, so that small and young fish require more energy than adult and larger ones; furthermore, oxygen levels and water temperature determine an increase in fish metabolism. For example, according to Requena et al. (1997), the increase in temperature from 20 to 28 °C causes an increase in energy demand in sea-bream, associated to an increase in the rate of oxygen consumption, compared to levels measured at 20 °C.
In response to the reduction or absence of food, metabolism adapts allowing fish to survive even for long periods. Numerous species can remain without food for many months and then they can recover their metabolic reserves with re-feeding; these species can modify their energy reserves (and their body's constituents) to survive periods of food absence. It is however important to distinguish between natural fasting and experimental fasting. Natural fasting is a phenomenon that can occur naturally in the environment due to low food supply, during fish migration, overwintering or during reproduction, throughout which fish demonstrate the ability to survive without food for lengthy periods (Navarro et al., 1995). In the specific case of sea bream, there have been numerous studies related to the effect of fasting. From these studies it was observed that sea bream is more resistant to fasting (20 days at 14 °C) than to the decrease in temperatures (8-12 °C for 15 days) (Sala-Rabanal et al., 2004). Food deprivation can negatively affect energy metabolism, and consequently the response or adaptation to stress, which requires a lot of energy. Food restriction causes significant weight loss and rapid depletion of energy deposits in the liver, with significant loss of lipids, glycogen and proteins and with a significant increase in water content in the liver. These changes are transposed in the nuclear dimension of the hepatocytes, but re-feeding rapidly compensates for the hepatic energy reserve that is rapidly reconstructed, while some plasma proteins, for example transtiretin (TTR), are not completely restored (Power et al., 2000). According to Company et al. (1999), fasting (23-27 °C for 18 days) after a hypocaloric diet (46:17 of proteins: lipids) causes hypoglycemia in gilthead bream due to the decrease in the part of fat with the progressive decrease of glucose levels in the plasma. Some studies

have shown that fasting (24-28 ° C for 3 weeks) can affect the quality of the gilthead fish without body loss (Grigorakis et al., 2005). In other studies, the absence of food (18-23 °C for 1-8 days) was found to produce significant differences in texture: the compression strength increased as the fasting period progressed. Other results indicate that the starvation period before slaughter improves the shelf life and quality of the product on the market (Ginés et al., 2002). However, although the physical quality of the fish improved with fasting, microbiological quality and shelf life decreased with a consequent increase in microbial deterioration of the sea bream (Álvarez et al., 2008). Color was found negatively affected by fasting. When sea bream was stored in ice, discoloration of sea bream (mainly on the dorsal parts of the fish) was observed (Grigorakis et al., 2005; Álvarez et al., 2008).

1.5 Structure of the fish muscle and its conversion to meat

The musculature in fish is used to swim, as well as to promote digestion, nutrition, circulation and secretion. To accomplish all these tasks, it occupies 30 to 50% of the body mass. In fish, as in all other vertebrates, there are three types of muscle tissue: skeletal muscle tissue, heart muscle tissue and smooth muscle tissue. The first two types of muscle tissue (skeletal and cardiac) are striated and regulated by the central nervous system. They present the characteristic light and dark bands on the optical microscope consisting of red aerobic and white anaerobic fibers of elongated shape, which serve for contraction (Swartz et al, 2009). Striated muscle tissue is distinguished on the basis of its functions in skeletal striated muscles and striated cardiac muscles: the first allows locomotion and protects the skeletal structure; it is also present in some parts of the digestive tract and near the gills, where short and powerful contractions are necessary. The striated cardiac tissue, on the other hand, forms the tunic of the heart constituting the contractile component (Johnson, 1999). Smooth (involuntary) muscle tissue is regulated by the autonomic nervous system and constitutes the tissues of the digestive system, the ducts of the glands connected to the throat and to the swim bladder (blood vessels, genital organs). It contracts slowly, but resists fatigue and remains contracted longer. This type of muscle has no striations, although the cells that compose it also contain actin and myosin (Padoa, 1991). The skeletal muscle of the fish is highly developed especially at the level of the trunk and tail and represents the part of the edible fraction, which varies according to the species, the size, the state of maturation and the physiological conditions of the animal. Most of the muscles are located in the axial part, which develops from the head to the tail, along the sides of the fish, and consists of myomers, which are muscle segments with contractile myofibrils. In bony fish, the number of myomers is almost always equal to the number of vertebrae. A connective septum between the vertebrate bodies and the skin (vertical medial myoseptum) separates the myomers from the left side from the right side, while the horizontal myoseptum separates the axial musculature in dorsal epiaxial musculature and ventral hypoaxial musculature (Bremner & Hallet, 1985; Robb, 2002).

The axial musculature generates an undulatory movement thanks to an alternate contraction of the muscles of each side of the body, which, with the help of the tail, pushes the fish forward. The axial musculature has both a white and a red color, due to the structure of the fibers that take the name of fast fibers and slow fibers (Bassani & Della Libera, 1991). The fundamental difference between white and red fibers is the different amount of mitochondria, myoglobin and vessels. The slow (red) fibers contain fats (energy reserve to draw on) and myoglobin (responsible for the dark color). They have an aerobic mechanism, are much vascularized, and guarantee a

prolonged and intense swimming. The red fibers develop on the surface and along the sides; the fast (white) fibers are free of myoglobin and have a low amount of fat. They are suitable for rapid and powerful contraction, but having a predominantly anaerobic metabolism, they undergo oxygen debt and do not allow prolonged swimming. To return to being reused, the white muscles need a resting and oxygenation phase to eliminate the accumulated metabolites. White fibers are particularly abundant and developed both on the surface and in depth

1.5.1 Myofibrillar proteins

Striated muscle tissue consists of myocytes or muscle fibers, which are wrapped in a membrane (sarcolemma) surrounded by a sheath of connective tissue. Each fiber is made up of elongated polynuclear cells with myofibril-rich cytoplasm. The sarcomere (contractile unit of the muscle) is repeated several times inside the fiber, and is formed by different proteins with a contractile function.
The proteins of the myofibrils include (Nelson & Cox, 2010):

- Proteins with a contractile function (actin and myosin, which make up 80% of the total proteins);
- Proteins with regulatory function (tropomyosin and troponin);
- Structural proteins (actinin, desmin, nebulin etc.);
- Enzymatic proteins that support muscle metabolism (catalase, cytochromes, peroxidases, glycolytic enzymes).

Myofibrillar protein content in fish is similar to that of mammals (Delbarre-Ladrat et al., 2006). These proteins, which are responsible for muscle contraction, make up 55-60% of the fish muscle, to which they give consistency (Goll et al., 2008).
Myosin is the main sarcomere protein (65% of total myofibrillar proteins) and interacts reversibly with actin. Myosin has a molecular weight of 470 kDa and contains six polypeptide chains: two heavy chains (220 kDa) and four light chains (14 -22 kDa, two chains for each lobe) (Venugopal & Shahidi, 1996; Lanier et al., 2013).
The heavy chains are composed of three parts: a globular head where the ATPase site and the actin binding site are located, a flexible neck region where myosin light chains are bound and a long filamentous or tail, which contributes to the formation of the thick filament (Craig & Woodhead, 2006), as shown in Figure 1.5.
In fish, myosin light chains (MLC) exists in two forms: the alkaline chains (MLC1/3, non-phosphorylable) and the regulatory chains (MLC2, phosphorylable), both

include different isoforms that are differently expressed in different cells or in the same cells at different stages of development. For example, MLC2 presents three isoforms expressed in fast skeletal muscle (MLC2f), in slow skeletal muscle (MLC2s), in smooth muscle and in non-muscle cells (Xu et al., 1999). LC1F (27 kDa), LC2F (18.5 kDa) and LC3F (16.9 -19.5 kDa) are present in the white muscles (fast) of sea bream, while LC1S (26.5 kDa) and LC2S (20.5 kDa) were found in red muscles (slow) (Carpene et al., 1998). Myosin light chains are chosen as an identification parameter of animal species due to its specific composition and due to its molecular weight variability (Focant et al., 1976; Seki et al., 1980; Rowleson et al., 1985).

The myosin head contains the binding site for ATP, whose hydrolysis releases the energy for contraction (movement and force development): the occupation of the nucleotide site causes the opening and closure of the slit that binds actin and generates rotation of the head around the neck of the protein.

The tail region has predominantly binding functions with shuttle molecules for intracellular transport, or with other myosin molecules. In muscle fibers, the myosin tail region is involved in binding to other myosins, to form the thick filament backbone.

Myosin has several isoforms, the most important being myosin II. Myosin II, present in the sarcomere of striated muscles, has the primary function in muscle movement. Myosin in fish is very sensitive to temperature, which affects the expression levels of various isoforms (Iwami et al., 2002).

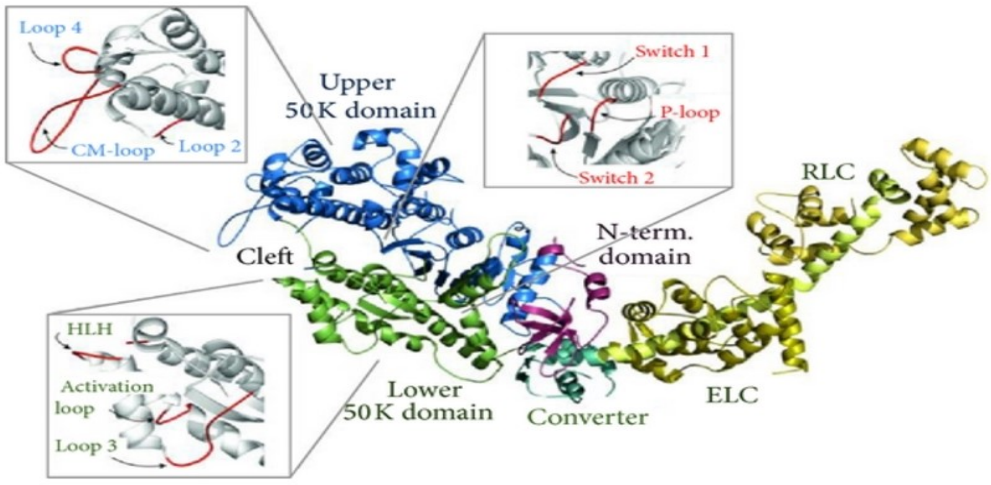

Fig. 1.5 - Structure of the myosin molecule (ResearchGate ©, 2008-2018)

The major component of the thin filament is actin (Figure 1.6), a filamentous protein (F-actin) with a diameter of about 8 nm, which represents 15-30% of the total myofibrillar proteins. The globular monomeric form (G-actin), which contains an ATP molecule and a bivalent ion, has a molecular weight of 42 kDa. In the muscle, the G-actin monomers are assembled by polymerization, stimulated by ATPase activity to form a fibrous actin chain. In this way the filaments maintain a head-to-tail orientation, in which the ATP-binding G-actin identifies the positive end, while the ADP-binding G-actin the negative one (Barni et al., 2008). The F-actin consists of two chains that wrap around to form a helical structure. In the helicoidal groove of the F-actin there is tropomyosin that, together with troponin, performs regulatory functions in the contraction. Actin interacts with myosin heads and stimulates ATPase activity of myosin for muscle contraction (Figure 1.7).
In vertebrates, three different actin isoforms have been identified: alpha-actin, beta-actin and gamma-actin. Alpha-actin is typical of skeletal muscle, cardiac muscle and smooth muscle. Beta-actin is localized in the cytoplasm of all cell types and gamma-actin is present in both smooth muscle and cytoplasm (Bergeron et al., 2010).

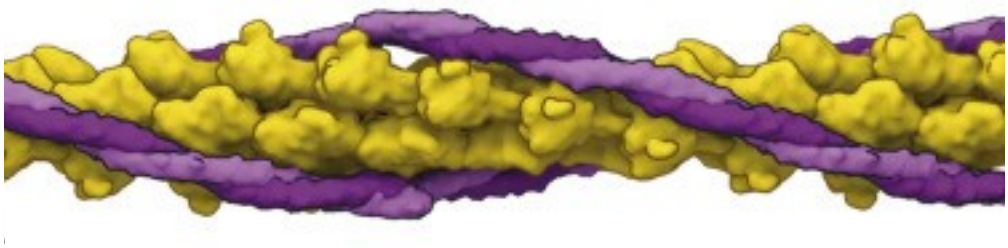

Fig. 1.6 - Thin filament (Based on Protein Data Bank [www.rcsb.org] file 1AX2, created by Roberto Dominguez, University of Pennsylvania)

1.5.2 Muscle contraction

Muscle contraction is the result of a series of coordinated intracellular changes leading to the movement of the muscle fiber and, consequently, of the muscle itself. Contraction occurs in all types of muscle; it is best represented in skeletal muscle, where there is a metameric structure, the sarcomere. The sarcomere is the morpho-functional and contractile unit of the skeletal and cardiac striated muscle. The main peptide components are actin and other structural proteins that allow and promote the maintenance of the structure (troponin and tropomyosin).
Some microstructures can be recognized under the microscope (Venugopal & Shahidi, 1996):
- Band I (region in which there are only thin filaments);
- Band A (region where there are both filaments);
- Zone H (region where there are only thick filaments);
- Line M (dark line that runs in the center of the sarcomere, on which the filaments of the myosin are inserted);
- Line Z (beginning and end of the sarcomere, line on which the filaments of the actin are inserted).
The mechanisms of muscular contraction are shown in Figures 1.7 and 1.8.

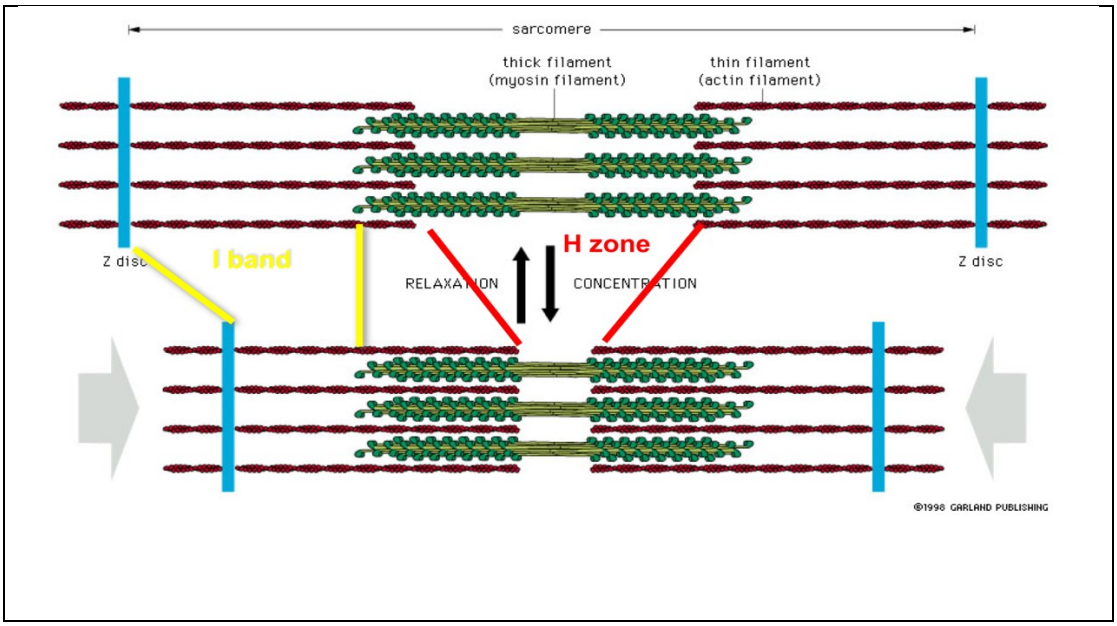

Fig. 1.7 - Muscle contraction

During contraction, the thin filament slides over the thick ones and this event occurs after a series of well-coordinated cellular processes. The nerve impulse reaches the

neuromuscolar plate; it releases acetylcholine that reaches the sarcolemma and excites it, modifying its permeability to sodium ions (depolarization). Through the T system, excitation reaches all myofibrils and the sarcoplasmic reticulum surrounding them; calcium ions are released from the from the terminal cisternae of the sarcoplasmic reticulum. These calcium ions reach the thin filaments of actin and block the inhibitory proteins, troponin and tropomyosin. The actin can thus slide on the filaments of myosin, binding itself to its transversal bridges at the level of which the ATPase enzyme, splitting the ATP into ADP^{+} and phosphate, delivers the energy necessary for the sliding. The sarcomeres are shortened, so the myofibrils are shortened and, consequently, the muscle fibers, generating the contraction. Once the excitation, with the repolarization of the sarcolemma, is over, the calcium returns into the sarcoplasmic reticulum. Without calcium ions troponin and tropomyosin prevent the establishment of actin-myosin bonds, leading to the relaxation of the muscle (Barbone, 2010; Nelson & Cox, 2010).

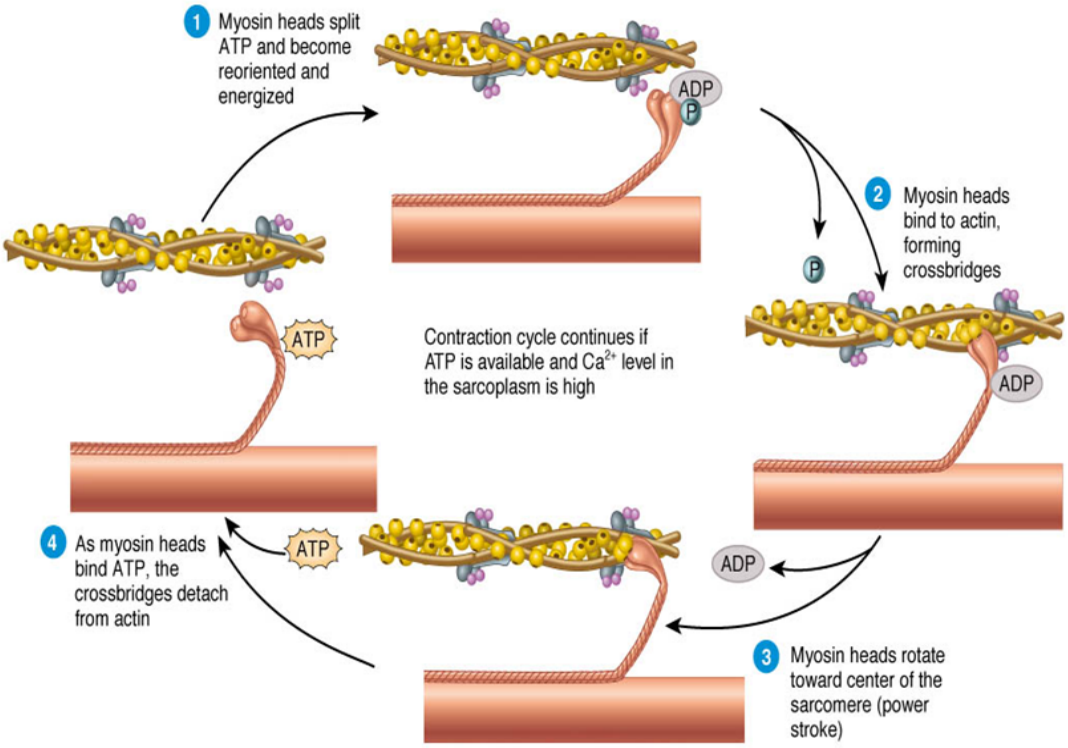

Fig. 1.8 - Molecular mechanism of muscle contraction (Nelson & Cox, 2010)

1.5.3 Conversion of muscle into meat

After the death of the fish, respiration ceases. When oxygen is no longer present, the body may continue to produce ATP via anaerobic glycolysis, generatic lactic acid. This final product of glycolysis accumulates in the muscle and leads to a decrease in pH. However, pH decrease is less important in fish than in mammals, and its final level is rarely less than 6.0 (Delbarre-Ladrat et al., 2006).
ATP is required to cause separation of the actin-myosin cross-bridges during relaxation of muscle. When the body's glycogen is depleted, the ATP concentration diminishes, and the body enters *rigor mortis* because it is unable to break those bridges.
In *rigor mortis* myosin heads continue binding with the active sites of actin proteins via adenosine diphosphate (ADP), and the muscle is unable to relax until further enzyme activity degrades the complex. Normal relaxation would occur by replacing ADP with ATP, which would destabilize the myosin-actin bond and break the cross-bridge.
All these events occur during the post-mortem period and the maximum rigidity tension in non-stressed fish is observed after 1-3 days. In fish, the *rigor* phase is influenced by species, diet, physical activity and animal stress in the *ante mortem*. For example, the increased muscle activity of sea bream and sea bass caused by crowding in the nets not only causes unnecessary suffering to the fish, but also causes a rapid decrease in ATP reserves, an increase in the level of lactic acid and a decrease in muscle pH during post mortem (Smart, 2000; Bagni et al., 2007). Furthermore, pre-slaughter stress exposes fish meat to the oxidation of polyunsaturated acids (PUFAs) and can lead to the formation of reactive oxygen metabolites (ROM), causing alterations to nucleic acids, proteins and lipids, especially in the case of sea bream. According to Bagni et al. (2007), the killing method strongly influences the development of rigor mortis: asphyxia in both chilled and subaerial water are highly stressful methods with respect to stunning and killing. For example, the maximum rigor of tension develops in gilthead sea bass and in the sea bass slaughtered by asphyxia already after 6 hours post mortem, while in salmon suppressed through stunning only after 18-24 hours.

1.5.4 Proteases: calpains and cathepsins

The fish muscle undergoes minor structural changes in *post mortem* compared to the myofibrils of other mammals: beef and sheep myofibrils breaks between bands I and costamers after 7 days of storage, compared to fish in which these interruptions do not occur. Fish cytoskeletal proteins, despite being degraded *post mortem*, are structurally more stable (Delbarre-Ladrat et al., 2006).
The degradation process is mainly based on the action of different proteases ((acid (cathepsins), neutral (calpain - Ca^{2+} - dependent - and Ca^{2+} -independent proteases) and alkaline)), which are a wide range of enzymes belonging to the hydrolases that split the peptide bond between the amino group and the carboxyl group of two adjacent amino acids (Fauconncau et al., 1995; Cleveland et al., 2009). Generally, proteases are present in all tissues, but the major activities are observed in the intestine and liver. However, these enzymes are present in muscle tissues, mostly localized in intracellular fluids, in sarcoplasm or into intracellular organelles. When fish are alive these proteases have a role in protein turnover, but during *post mortem* there is the loss of regulatory mechanisms (Foegeding et al., 1996).
Calpains, which belong to the family of intracellular neutral cysteine proteases (Figure 1.9) are activated by a series of processes, including the increase in the intracellular concentration of Ca^{2+}, phosphorylations and proteolytic events. There are two forms of calpains distributed in the cytoplasm of all the cells of the entire animal kingdom and they are the micro (μ) -calpains and the milli (m) -calpains. This distinction is linked to the concentration of calcium ions necessary to activate the calpins themselves: the micro needs micromolar concentrations of calcium, the milli needs millimolar concentrations (Ladrat et al., 2000). Calpains are heterodimers, consisting of two subunits: one greater than 80 kDa and one smaller than 28 kDa. The smaller subunit has a regulatory role as a chaperone (Suzuki et al., 1986). In the major subunit there is the active site that needs the minor subunit to have maximum activity (Tsuji & Imahori, 1981). Calpain have an optimal pH of 6.9-7.5 and an optimal temperature of 30 °C (Kolodziejska & Sirorski, 1996). Calpastatines are their main endocellular inhibitors. During *post mortem* calpains mediate the detachment of intact actin and myosin filaments, which cause meat tenderization.

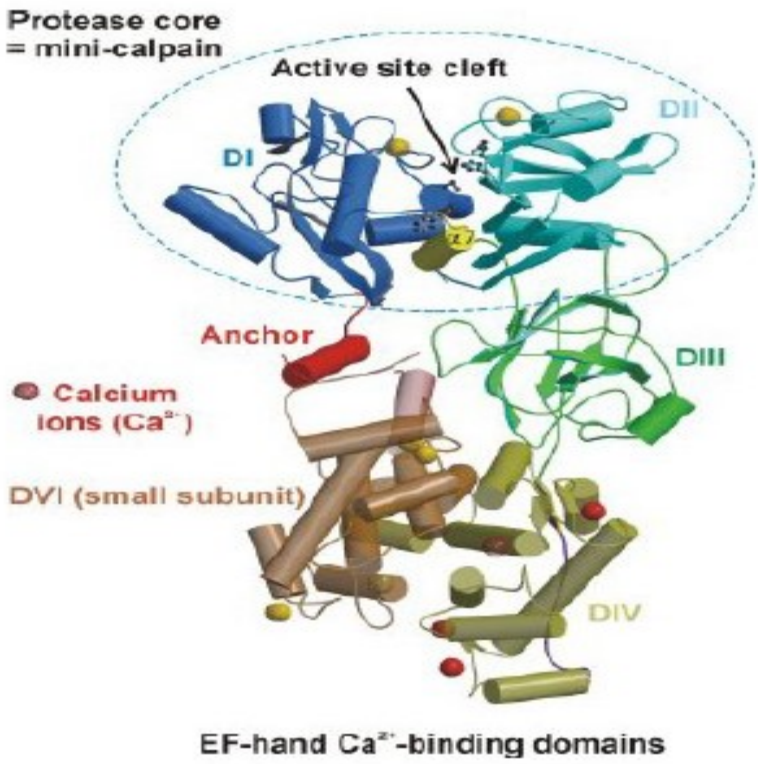

Fig. 1.9 - Structure of calpains (ResearchGate ©, 2008-2018)

Cathepsins are acid proteases localized in lysosomes. The *post mortem* proteolysis by cathepsins occurs after the calpain action. The calpains cause cell lysis and release of cathepsins both in the cytoplasm and in the intracellular spaces. Lysosomes contain 13 types of cathepsins (Goll et al., 1983), which are distinguished by their active site (aspartic, cysteine, serine and metalloproteases). Those that play a fundamental role in fish *post mortem* are the cathepsins B (EC 3.4.22.1), L (3.4.22.15), H (3.4.22.16), D (3.4.22.5). Cathepsins B, H and L are regulated by cystatins, prosthetic inhibitors (Turk & Bode, 1991).

The most interesting studies on the mechanisms involving calpains and cathepsins in *post mortem* processes are listed in chronological order in Table 1.5.

Authors	Fish/Animal	Enzymes	Study
Ladrat *et al.* (2000)	Sea bass	Calpain	Evaluation of polymorphism and biochemical aspects; evaluation of *post mortem* degradation of meat.
Ladrat *et al.* (2003)	Sea bass	Cathepsins B, D, L	In vitro proteolysis of myofibrillar and sarcoplasmic proteins of white muscles
Delbarre-Ladrat *et al.* (2004)	Sea bass	Calpain	Study of the proteolytic potential in white muscles during the *post mortem* phase through freezing: time-dependent changes of the components of the calpain system.
Delbarre-Ladrat *et al.* (2004)	Sea bass	Calpain and cathepsin	Contribution of calpins and cathepsins in the degradation of muscle proteins.
Chéret *et al.* (2005)	Sea bass	Calpain and cathepsin B, D, H, L	Effects of high pressure on the main proteolytic enzymes involved in the degradation of muscle tissue during storage.
Chéret *et al.* (2007)	Beef e Sea bass	Calpain and cathepsin B, D, H, L	Calpain and cathepsin activity in *post mortem* in fish and meat.
Caballero *et al.* (2009)	Sea bream	Calpain	*Post mortem* changes in the sea bream muscle during ice storage.
Ayala *et al.* (2010)	Sea bream	Calpain and cathepsin	Changes in muscle structure and texture of the sea bream Sparus aurata L. during *post mortem* storage.
Salmerón *et al.* (2013)	Sea bream	Calpain	Characterization and expression of members of the calpain family in relation to nutritional status, diet composition and texture of sea bream meat.

Table 1. 5 – Main contributors to the study of proteases in sea bass and sea bream

1.6 Bioactive peptides from animal products

With the generic term of bioactive peptides (BAPs) are named short sequences of amino acids, which derive from the hydrolysis of proteins present in beef, pork, chicken, and in marine organisms. Drying, maturing, refining, maturation and fermentation are special procedures useful to give flavor to meat products but are also important for the release of bioactive peptides from the starting proteins. Once released, during gastrointestinal digestion, peptides would play a bioactive role beyond their nutritional value. A number of functions have been described, including antioxidant, antihypertensive, antibacterial, opioid-like, and antithrombotic effect (Zhang et al., 2010; Hu et al., 2016). The regulation of the immune, gastrointestinal and neurological responses made by the bioactive peptides is essential for their activities in terms of prevention of hypertension, obesity, diabetes and other metabolic disorders.

Following the theory of structural functionality, the different activities of peptides are predictable from the amino acid composition, the type of terminal amino acid, the length of the chain, the total molecular weight, the hydrophobic properties and the spatial structure (Li & Yu, 2015). In general, anti-hypertensive peptides would mostly consist of hydrophobic amino acids and at the N-terminal level they would have more aromatic or alkaline amino acids (Escudero et al., 2013a). Commonly, a low isoelectric point (PI, 3-6) of bioactive peptides is associated with better antioxidant and anti-hypertensive activity (Park et al., 2008; Lafarga et al., 2014). At the same time, the presence of tyrosine (Tyr), glutamate (Glu) and aspartic acid (Asp), for their properties of attracting electrons, would increase the antioxidant activity of the peptide sequence. LDQW, LPHSGY, LLGPGLTNHA and LPHSGY are antioxidant peptides with leucine (Leu) in N-terminal position that would improve the interaction between the peptide and fatty acid (Je et al., 2005; Byun et al., 2009; Sadat et al., 2011; Li & Yu, 2015). Furthermore, based on their structural properties, many bioactive peptides possess numerous different properties that would allow us to guarantee multiple applications in the production of functional foods (Korhonen & Pihlanto, 2006; Dziuba & Dziuba, 2010).

Being sensitive to changes in temperature and pH, protein structures can be easily destroyed during meat processing, and even during seasoning, drying, salting, fermentation, storage, freezing and cooking. Therefore, bioactive peptides would result by changes in temperature and pH that could damage the protein spatial structure or break the amino acid chains (Lafarga et al., 2014). Furthermore, even the enzymes present inside the meat would favor the breaking of the protein chain and the generation of small peptides during the maturation process of the carcass (Huff-

Lonergan et al., 2010). It was seen that the amounts of bioactive peptides and free amino acids increased during a 14-day maturation process of fresh beef (Bauchart et al., 2006). In the meat processing industry, cold storage is common and aims to keep the freshness of meat products intact. However, freezing the meat, as well as temperature changes during the freezing process, could lead to the formation of ice crystals that can destroy supramolecular structures. Therefore, depending on the temperature and the storage time, some peptides could be generated during the freezing of meat products (Leygonie et al., 2012). To favor the degradation of the protein structure and the production of bioactive peptides, the most commonly process is represented by enzymatic hydrolysis. Since different enzymes have specific target sites on the sequence of a particular protein, the type of enzyme used is quite crucial for peptide release (Mora et al., 2014). A number of enzymes, such as pepsin, trypsin are used in research to simulate gastrointestinal digestion and hydrolysis of muscles of fish, chicken, duck, pig, deer and of some meat-based products such as cured ham (Saiga et al., 2003; Escudero et al., 2012; Lafarga & Hayes, 2014). In the food industry, Alcalase, Flavourzyme and Protamex are the most widely used to obtain peptides from duck, fish, chicken, pork, goat and "pancetta" proteins (Yanfeng & Li, 2006; Lee et al., 2010; Wang et al., 2015; Mirdhayati et al., 2016). A protease complex was also used for the hydrolysis of gelatin proteins from duck and bovine skin (Kim et al., 2001; Lee et al., 2012). Thanks to the activity of exogenous enzymes, peptides would also be released from seasoned meat products. Peptide production is influenced by the type of muscle, maturing methods and processing conditions including time, temperature, humidity and salt content (Escudero et al., 2013a). Recently, some antioxidant and anti-hypertensive peptides have been purified from hams (Spain, Parma, Jinhua and Xuanwei hams), and representative sequences have been identifyed in each of them (Escudero et al., 2013b, Xing et al., 2016; Zhu et al., 2017; Zuo et al., 2017). During the maturing process, cathepsins B, L and D play a major role in degrading myofibrillar proteins, aided by peptidyl-peptidase, aminopeptidase and carboxypeptidase in the further rupture of peptide chains with the generation of oligopeptides and free amino acids (Mora et al., 2011; Xing et al., 2018a). A long period of maturation would therefore induce an increase in the yield of peptides and amino acids, while the production of shorter chain peptides would be concentrated during the final stages of the ham curing (Zhang et al., 2009; Mora et al., 2011).

1.6.1 Identification of bioactive peptides in meat proteins

Thanks to the increased knowledge in bioinformatics (Figure 1.10), the "in silico predictive analysis" has become a new method to predict and identify bioactive peptides in various proteins. Unlike the previous processes that included hydrolysis and purification in the laboratory, the "in silico predictive analysis" is mainly based on the use of bioinformatics tools, such as the BIOPEP, ProtParam, UniProt, Blast and PeptideCutter databases. This method helps to trace the release of peptides from parental proteins and also to predict their potential bioactivity according to their amino acid sequences (Dziuba and Dziuba, 2010; Lafarga & Hayes, 2014). Recently, predictive analysis has been used to trace the release of bioactive peptides from porcine and bovine-derived proteins and is considered a theoretical basis for further verification of their bioactivity in empirical experiments (Lafarga et al., 2014; Sayd et al., 2016). During the bioinformatic analyzes conducted on beef, 1743 peptides were identified, along with 71 proteins (Sayd et al., 2016).

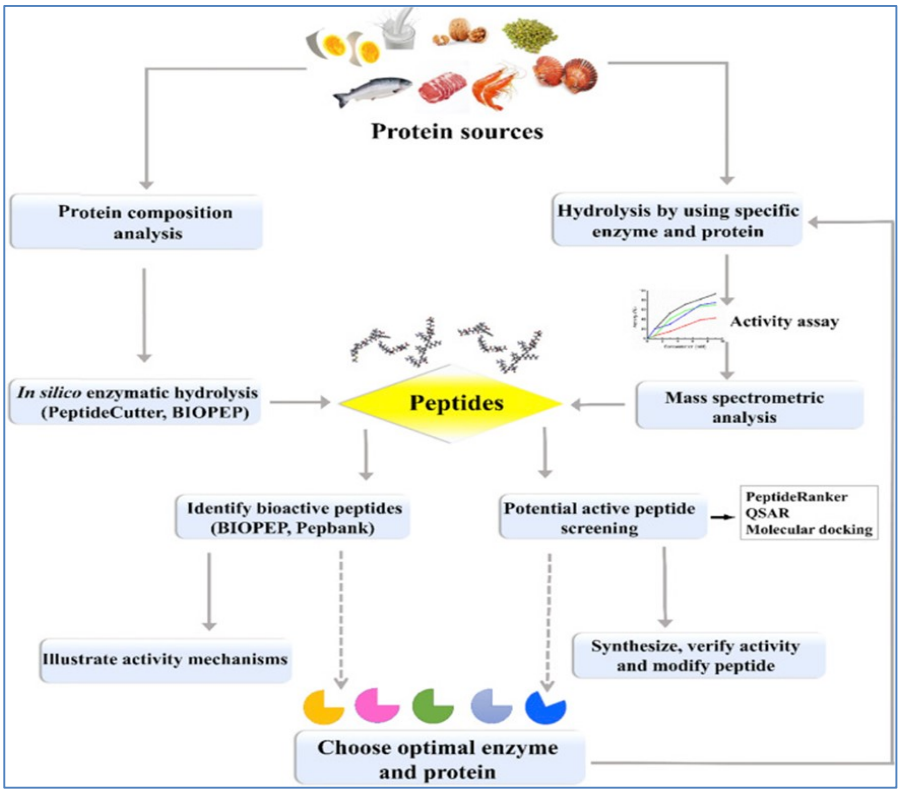

Fig. 1.10 Novel bioinformatics approaches for identifying and validating food protein-derived bioactive peptides (BAPs) (Tu et al. 2018).

1.6.2 The functions of peptides: ACE inhibitory peptides

The angiotensin-converting enzyme (ACE), a trans-membrane peptidase, performs multiple functions in organisms, including the degradation of bradykinin, the cleavage of a powerful vasodilator and the participation in the renin-angiotensin system (Ahhmed and Muguruma, 2010). Starting from the inactive form angiotensin I, ACE converts it into a powerful vasoconstrictor, angiotensin II (Ang II), which represents a risk factor for the increase in blood pressure. Therefore, inhibition of ACE activity would be an effective therapy for the treatment of clinical hypertension and cardiovascular diseases (Iwaniak et al., 2014). In recent years, the study of anti-hypertensive peptides has become a key topic in the field of studies conducted on the animal, marine and plant kingdom (Lee & Hur, 2017). In most studies, the inhibitory potency of anti-hypertensive peptides is generally assessed by the IC_{50} value and the animal model for blood pressure control. ACE inhibitory peptides are extensively studied and some of their structural features are shown in Table 1.6.
After hydrolysis by thermolysin (EC 3.4.24.27), Arihara et al. (2001) identified two ACE inhibitory peptides with MNPPK and ITTNP sequence in pig muscle, and this was the first report conducted on antihypertensive peptides derived from pork meat. Oral administration of pig hydrolysates and synthetic MNPPK and ITTNP sequences was then verified in spontaneously hypertensive rats (SHR) in which, 210 and 2100 mg (hydrolyzed kg^{-1} body weight) significantly decreased systolic blood pressure (SBP). In the group undergoing treatment with MNPPK and ITTNP, there were hydrolyzed sequences identified as MNP, NPP, PPK, ITT, TTN and TNP. All peptides had ACE inhibitory activity, and PPK was shown to have the greatest antihypertensive effect on SBP tests (Nakashima et al., 2002). Katayama et al. (2007) isolated EKERERQ and KRQKYDI sequences in hydrolysed (via pepsin) of porcine skeletal troponin with IC_{50} values of, respectively, 552.5 and 26.2 μM. After 3 and 6 hours of administration in SHR rats, KRQKYDI's temporarily antihypertensive activity was shown to reduce diastolic blood pressure with a dose of 10 mg kg^{-1}. Similarly, Muguruma et al. (2009) purified the KRVITY sequence (IC50 value, 6.1 μM) in pepsin-based porcine skeletal myosin and noted significant antihypertensive activity after oral administration to SHR (10 mg kg^{-1}). In addition to degradation with a single enzyme, some ACE inhibitory peptides were generated by artificial intestinal digestion set up with a mix of enzymes. Escudero et al. (2010) identified a hydrolyzate derived from pork after digestion with pepsin and pancreatin. Among all the sequences, those KAPVA and PTPVP showed remarkable inhibiting effects on ACE, with IC_{50} values respectively of 46.56 and 256.41 μM. Escudero et al. (2012) chose three RPR, KAPVA and PTPVP peptides to test the antihypertensive activity on SHR rats.

The ACE inhibitory properties could be maintained at 80% even after in vitro digestion by pepsin, trypsin and chymotrypsin, this implies that a longer chain would not hinder antihypertensive bioactivity. In porcine liver hydrolysates ACE inhibitor peptides were identified and their oral administration in SHR, at a dose of 1 g per rat $^{-1}$, had a significant role in controlling blood pressure (Inoue et al., 2013). Recently, the ACE inhibitory peptides have been fractionated and separated also by dry-cured hams (Escudero et al., 2010; Mora et al., 2016; Zuo et al., 2017); in Spanish hams the AAATP sequence was the one with the greatest bioactivity, with an IC50 value of 100 μM (Escudero et al., 2012). After oral administration of AAATP to SHR rats, SBP was reduced by 25.62 mmHg within 8 hours (Escudero et al., 2013a). Furthermore, ACE inhibitory peptides in Spanish ham could be resistant to heat and gastrointestinal digestion (Escudero et al., 2014). During the evaluation of the transport on Caco-2 cell monolayer, an increase in the activity of AAPLAP and KPVAAP was found on the basal side, which reached 70% after 60 minutes from the beginning of the cellular transport (Gallego et al., 2016). Based on the activity of the membrane peptidase, most of the peptides were degraded into oligopeptides except for the KPVAAP which was absorbed with the intact peptide chain. In addition to pork, chicken and beef were also a good source of anti-hypertensive peptides (Table 1.7).

N-Terminus----------		----------C-Terminus
Hydrophobic residues	2-12 amino acids in length Peptide conformation important for longer peptides	C-terminal tripeptide Bulky hydrophobic residues Aromatic or branched side chains Proline at one or more positions Positively charged residues in position two, Arg, Lys Tyr, Phe, Trp, Leu L-configured residue in position three

Table 1. 6 – Some structural features of potent angiotensin converting enzyme (ACE) inhibitory peptides (Norris and Fitzgerald, 2013).

Fonte	Sequenze	IC50 (Mm)	Riferimenti
Muscolo di suino	MNPPK	945.5	Arihara et al. (2001)
	ITTNP	549.0	Arihara et al. (2001)
	EKERERQ	552.5	Katayama et al. (2007)
	KRQKYDI	26.2	Katayama et al. (2007)
	VKKVLGNP	28.5	Katayama et al. (2007)
	KRVITY	6.1	Muguruma et al. (2009)
	KAPVA	46.56	Escudero et al. (2010)
	PTPVP	256.41	Escudero et al. (2010)
	ER	667	Escudero et al. (2010)
	KLP	500	Escudero et al. (2010)
	RPR	382	Escudero et al. (2010)
	AAATP	100	Escudero et al. (2012)
	RPR	382	Escudero et al. (2012)
Muscolo di bovino	VLAQYK	32.06	Jang et al. (2004)
	GFHI	117	Jang et al. (2008)
	DFHING	64.3	Jang et al. (2008)
	FHG	52.9	Jang et al. (2008)
	GLSDGEWQ	50.5	Jang et al. (2008)
	AKGANGAPGIAGAPGFPGARGPSGPQ-GPSGPP	51.10	Banerjee and Shanthi (2012)
	PAGNPGADGQPGAKGANGAP	79.85	Banerjee and Shanthi (2012)
Pollo	LKA	8.5	Iroyukifujita et al. (2000)
	LKP	0.32	Iroyukifujita et al. (2000)
	LAP	3.5	Iroyukifujita et al. (2000)
	FQKPKR	14	Iroyukifujita et al. (2000)
	FKGRYYP	0.55	Iroyukifujita et al. (2000)

Table 1. 7 – Sources of anti-hypertensive peptides (https://www.ruminantia.it)

1.7 Protein digestion

Digestion means the splitting of nutrients into compounds that can be absorbed and then used for cellular metabolism. The digestive process includes the demolition of foods in simpler substances (amino acids, fatty acids, oligosaccharides, vitamins, nucleic acids, etc.), their absorption through the epithelium of the digestive tract and the expulsion of the waste substances in the form of feces. This task is carried out by the digestive system which consists of a series of hollow organs that form the alimentary canal, which crosses the whole body from the head to the perineum. Glands are attached to the digestive tract (eg salivary glands attached to the mouth; liver and pancreas attached to the duodenum). In order to assimilate proteins, the human body must break them down into peptides and free amino acids. In the absorption phase, the digestive products are transported from the small intestine to the liver by blood circulation (Figure 1. 11).

According to the 2007 FAO / WHO / UNU (United Nations University) report, a healthy diet includes the consumption of 0.83 g of high-quality protein per kg of body weight. The body digests 50 to 100 g of endogenous proteins a day, which are secreted into the lumen of the gastrointestinal tract. Most of the mix of exogenous and endogenous proteins (115-200 g / day) are effectively digested and absorbed by enterocytes in the form of free amino acids, di- and tripeptides. About 85% of the total proteins are absorbed up to the ileum and 10 to 20 g / day of proteins enter the colon. In the large intestine most of the undigested nitrogen is used for microflora growth.

Protein digestion begins at the stomach level, where gastric hydrochloric acid and pepsin denature and hydrolyse proteins. The stomach plays a minor but essential role in the digestion of proteins and has the task of preparing the polypeptides for the digestion and absorption processes that affect the small intestine, where the hormone cholecystokinin is produced for regulating the exocrine secretion of the pancreas and the sense of satiety (Figure 1.11). When food is present in the stomach the main gastric cells secrete inactivated pepsinogens. Pepsin activation occurs spontaneously at pH less than 5 following an intramolecular process involving proteolytic cleavage of an N-terminal segment of pepsinogen. Pepsin is classified as an endopeptidase because it attacks the peptide bonds of the polypeptide chain. The catalytic mechanism involves two carboxyl groups at the active site of the enzyme, placing pepsin in the carboxyl protease group. Pepsin prefers to break down peptide bonds involving the carboxyl groups of tyrosine, phenylalanine and tryptophan (Stipanuk & Caudill, 2013).

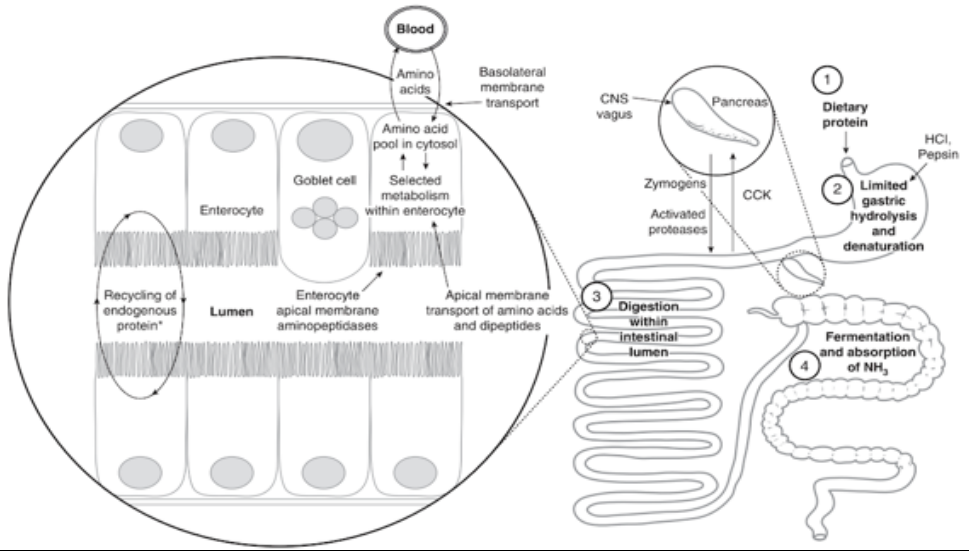

Fig. 1.11 - Digestion and absorption of proteins. CKK: cholecystokinin, CNS: central nervous system, HCl: hydrochloric acid (modified from Stipanuk & Caudill, 2013)

1.7.1 Bioactive peptide absorption

Many bioactive peptide preparations are produced by mimicking the digestive environment in the gastrointestinal (GI) tract, with protease treatment yielding the "active" peptides out of the native protein structure (Kitts et al, 2003; Rutherfurd-Markwick et al 2012; Hartmann et al 2007). Being generated through simulated digestion, some bioactive peptides, such as the egg protein derived tripeptide IRW, are naturally resistant to digestive enzymes (Hartmann et al 2007). This is a huge advantage in delivering bioactives through the oral route, as a lack of digestion in the GI tract ensures increased bioavailability and a better chance of exerting a significant effect on the body's physiology. On the other hand, some peptides such as LKPNM, derived from enzymatic digestion of bonito fish protein, are further metabolized into their active components in the GI tract (LKP, an anti-hypertensive tripeptide is released from LKPNM), which then exert the intended biological action upon absorption into the systemic circulation. This could be considered analogous to a pro-drug, which undergoes metabolism to yield the active ingredient (Fujita et al 1999).
Absorption from the GI tract is essential for a bioactive peptide to exert any systemic biological actions downstream. Traditionally, it was believed that all peptides and proteins were digested down to their constituent amino acids, and only these amino acids were capable of absorption across the intestinal epithelial barrier. Indeed, the absorption of larger entities such as peptides and proteins were only considered as pathological phenomena, and a key culprit in food allergies. However, it is apparent now that many peptides do cross the intestinal epithelium under normal conditions, enter into the circulation, and exert systemic effects (Muheem et al, 2016). Several mechanisms have been postulated to explain the intestinal uptake of peptides from the GI lumen, as detailed in the review by Lundquist et al. (2016). Briefly, the key mechanisms are as follows: paracellular transport through intercellular tight junctions; direct penetration of the epithelial cell membranes; endocytosis/phagocytosis by cells; and last, but not least, active transport by specific carrier proteins. Each of these mechanisms may occur alone or in association with others, while the same peptide may utilize one or more different approaches. In summary, the absorption of intact peptides, either alone or as part of a protein hydrolysate, is an exciting area of research that is critical for the successful oral use of these compounds.

2 Aim of the research

The present study was designed to investigate the impact of sea bream starvation before slaughtering (in comparison to a full ration control) on:

A) the quality characteristics of fish muscle, comprising processes such as proteolysis of the main myofibrillar proteins;

B) human gastric digestibility of fillets in relation to gastric pH, including conditions relevant to the treatment of the gastroesophageal reflux disease (GERD);

C) the release in the gastric digests of bioactive peptides exhibiting ACE inhibitory activity.

3 Materials and methods

3.1 Animal feeding and sampling

Sea bream (*Sparus aurata*) was farmed in the aquaculture facility of the Department of Agri-Food, Environmental and Animal Sciences of the University of Udine, located in Pagnacco (UD). Fish with an average weight of 338g were kept in the tanks for a 5 weeks adaptation period, after which they were subjected to two different feeding regimes for 21 days:
- the Control group was given a quantity of feed considered adequate for optimal growth.
- the group of fish indicated as Fasting was fasted.
Two tanks were set up for each diet, with 21 individuals each. A single type of extruded fish feed was used for feeding the sea bream, the *Prestige6G*, produced by *AIA Italian Agriculture Food Sp.* (Veronesi group).
At the end of the breeding period, the fish were slaughtered, eviscerated and filleted. For each group of fish, portions of about 1g of the upper dorsal musculature have been taken. The sampling took place at different times from slaughter (t0) and the samples were immediately frozen in liquid nitrogen, to allow storage at -80 ° C until the analysis was started.
The trial complied with the EU directive 2010/63, regarding the protection of animals used for scientific purposes.

3.2 pH measurement

The pH values were measured using a pH meter 213 model (Hanna Instruments, Rhode Island, USA), with the electrode inserted into the epaxial muscle after carrying out a lateral incision. Measures were taken in the neck, dorsal and caudal area on three fish for each feeding procedure.

3.3 Protein extraction and protein content determination

For protein elettrophoretic analysis the muscle tissue of the sea bream was treated to extract the protein fraction, as described by Piñeiro et al. (1999); to this method small modifications have been made in order to maximize the extraction of myofibrillar proteins.

From each sample, which was kept at 4 °C to prevent the activation of proteolytic processes, about 100 mg of muscle tissue were taken and added with 1 mL of extraction buffer containing 60 mM Tris / HCl, 2% (w/v) of sodium dodecyl sulphate (SDS), 100 mM 1,4-dithiothreitol (DTE) (Sigma-Aldrich, Saint Louis, DTE, Missouri, USA) pH 7.5 and 2 µl of anti-protease (Protease Inhibitor, Sigma-Aldrich, Saint Louis, Missouri, USA). The samples were homogenized using Ultra-turrax® T25 Digital (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 13,000 rpm for two repetitions of 30 seconds each, interspersed with a 30-second pause. The homogenated samples were centrifuged at 5600g for 4 minutes at room temperature and the supernatants were heated to 98 °C, shaken for 30 min and centrifuged as before. Finally, the supernatant was frozen and stored at -80 °C.
The quantitative determination of the protein concentration of the extracts was carried out using the UV-VIS spectrophotometer, evaluating the absorption at a wavelength of 280 nm. This value was chosen for the spectroscopic properties of proteins: at the level of the molecular structures of the aromatic amino acids (tryptophan, tyrosine and phenylalanine) the aromatic ring shows an absorption peak at 280 nm (Aitkein & Learmonth, 2002). In this assay quartz cuvettes (Sigma-Aldrich, Saint Louis, Missouri, USA) were used, as they are invisible to UV. The light absorption of the sea bream samples (Sparus aurata) was compared with that of a calibration line built with a solution of bovine albumin (BSA) at a known concentration, dissolved in aqueous solutions of 2% SDS.

3.4 Polyacrylamide gel electrophoresis (SDS-PAGE)

The muscle protein extracts, as well as the *in vitro* digested muscle samples, were separated by the use of polyacrylamide gel electrophoresis (Figure 3.12) in denaturing conditions (SDS-PAGE) according to the method described by Piñeiro et al. (1999).
The SDS-PAGE technique is based on the handling of charges in an electric field, where the SDS anionic detergent eliminates the effect of the native protein charge and binds to the proteins, changing their structure and giving a negative charge proportional to their mass. This mechanism allows the separation of proteins only based on their mass, regardless of their isoelectric point and amino acid composition. The polyacrylamide gel behaves like a sieve and determines a greater speed of migration of the smaller proteins compared to the larger ones according to Stokes law.
The protein extracts were added with an equal volume of the Laemmli Sample Buffer 2X (125 mM Tris / HCl pH 6.8, 4% w/v SDS, 20% v/v Glycerol), containing 10% (w/v) β-mercaptoethanol and 0.004% (w/v) bromophenol blue (Sigma-Aldrich, Saint

Louis, Missouri, USA), which has the function of making the protein extracts visible during the electrophoretic run. The extracts were treated for 5 minutes at 98 ° C with the Thermoblock (PBI International, TD 150 P2, Milan, Italy), to facilitate protein denaturation and color fixing.

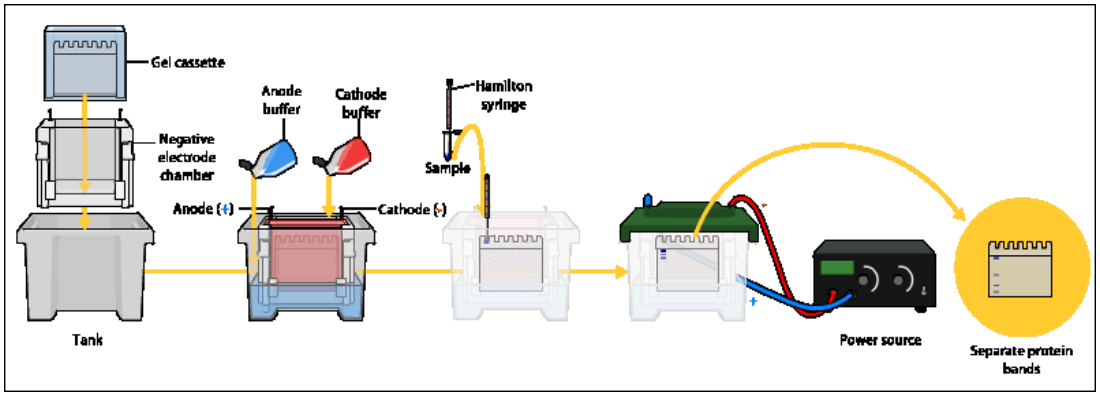

Fig. 3.12 - SDS-PAGE Electrophoresis (Protocols® Online, 2016).

The gel (10x10 cm) used was prepared using a 40% acrylamide solution (Bio-Rad Laboratories, Hercules, California, U.S.A.) and 2% bis-acrylamide (Bio-Rad Laboratories, Hercules, California, U.S.A.). The composition and quantities of the solutions used for the preparation of the running gel and stacking gel are specified in (Table 3.8).

	***Running gel* 13% (mL)**	***Stacking gel* (mL)**
Acrylamide solution	2	0.2
Bis-acrylamide solution	1.1	0.125
1.5 M Tris / HCl solution, pH 8.8	1.55	/
0.5 M Tris / HCl solution, pH 6.8	/	0.50
Milli-Q water	1.6	1.25
Aps 10%	20 µl	10 µl
SDS 10%	60 µl	20 µl
Temed	9 µl	5 µl

Table 3. 8 – Composition of running gel and stacking gel.

the sea bream samples and molecular weight standards (Bio-Rad, Precision Plus Protein Standard, Hercules, California, USA) were loaded in the wells. The run was carried out using a buffer, called running buffer (Tris / HCl 0.15 g / L, pH 6.8, glycine 0.72 g / L, SDS 0.05 g / L) and setting to a current of 15 mA and a potential difference of 150 V for about one hour and a half. A recirculation of running water was used in order to avoid overheating of the instrument and possible damage to the migration.
After the electrophoretic run, to visualize the bands corresponding to the proteins that completed their run, the gel was first immersed in a Fixer solution (consisting of 8% v/v acetic acid, 46% v/v methanol and 46% of milli-Q water) for an hour in agitation and then in a blue solution of Coomassie (consisting of 0.025% w/v of Coomassie Blu G250, 10% v/v acetic acid, 90% v/v of milli-Q water) in agitation over-night.
After destaining with a solution of 10% v/v of acetic acid for 1-2 hours, the gel was analyzed using a high-resolution imaging instrument (Syngene, G: BOX, Cambridge, United Kingdom), associated to a software for rapid densitometric analysis.
When specified, the apparent masses of the protein bands were estimated from a calibration curve obtained by plotting the migration distances of standard proteins (Precision Plus ProteinTM Dual Color Standards – Biorad, Hercules, California, USA) versus their known molecular masses.
When gel bands of interest had to be identified by mass spectrometry, the NOVEX Colloidal Blue Staining Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was

used for gel staining at the end of the eletctrophoretic run. After destaning, the gel bands (0.5 cm width and band size-dependent thickness) were manually excised and sent by mail to Dr. Barbara Prandi, University of Parma. The gel bands were then cut into small pieces; after destaining, reduction and alkylation of the free thiols with iodoacetamide, the gel pieces were subjected to standard in-gel digestion (25 ng mL^{-1} trypsin in 25 mM NH_4HCO_3 and 2.5 mM in $CaCl_2$ overnight at 37 °C). The solutions obtained from the digestion were dried under N_2 flux (Prandi et al., 2013). The dried samples obtained from in-gel digestion were dissolved in 0.1% (v/v) formic acid and analysed by a Dionex Ultimate 3000 micro HPLC coupled with an LTQOrbitrap (Thermo Fisher Scientific, San José, CA, USA) mass spectrometry equipped with a conventional ESI source.

3.5 Western Blotting e Immunostaining

When specified, the Western Blotting was used after SDS-PAGE to recognize by immunodetection actin or myosin in the the electrophoretically separated proteins. After the electrophoretic run, the gel was immersed in the transfer buffer, called trans blot buffer (25 mM Tris / HCl, 192 mM glycine, 20% v/v methanol pH 8.1-8.3) together with two spongy supports. The preparation of the gel transfer cassette consists of the assembly and overlapping of various components: at the base a spongy support followed by a rectangle of absorbent paper, the gel, the nitrocellulose membrane, an additional rectangle of absorbent paper and another spongy support. The cassette was placed inside the XCell II ™ Blot Module (Life Technologies Corporation, Mexico) taking care to put the nitrocellulose membrane well adherent to the gel (Figure 3.13).
The parameters used for the transfer were 150 mA with a potential difference of 30 V for a time of 45-60 minutes. To avoid overheating of the instrument, the outside of the transfer chamber was filled with milli-Q water.
At the end of the electroblotting, the protein transfer efficiency was evaluated by incubating the membrane for about two minutes with an ATX Ponceau S Red staining solution (Sigma-Aldrich, Saint Louis, Missouri, USA) which has a high sensitivity and solubility in water.
The membrane was washed with milli-Q water to remove traces of the dye and subsequently was immersed in a 3% powdered milk-based blocking solution (PBS solution, milli-Q water, Tween 0.1%) and left under stirring at room temperature for about an hour (The blockage serves to saturate the membrane sites where no proteins have been transferred, in order to avoid the formation of false positives).

Immunodetection consists in incubating with a primary antibody able to recognize the proteins of interest, followed by the incubation with a secondary antibody conjugated to horseradish peroxidase, which specifically binds the primary antibody When the degradation of actin was followed, before proceeding with the immunostaining, the membrane was cut at 37 kDa and the two parts were incubated separately with different dilutions of the primary antibodies, so as to increase the sensitivity against the actin fragments. For myosin this step has not been applied.
Two primary monoclonal antibodies against the actin carboxy terminal (Abcam, Anti-Alpha Skeletal Muscle Actin antibody ab97378, San Francisco USA and Sigma-Aldrich, Monoclonal Anti-Actin antibody produced in mice, Saint Louis, Missouri , USA), a monoclonal antibody against the actin amino terminal (Abcam, Anti-Alpha Skeletal Muscle Actin antibody ab97373, San Francisco, USA) and a polyclonal antibody against heavy and light chains of myosin (Sigma-Aldrich, Anti-Myosin Skeletal antibody produced in rabbit M 7523, Saint Louis, Missouri, USA) were used. These antibodies were diluted in water milli-Q, respectively:
- 1:2000 when proteins >37 kDa were incubated with the anti-actin C-terminal antibody;
- 1:3000 when proteins >37 kDa were incubated with the anti-actin N-terminal antibody;
- 1:1000 when proteins<37 kDa were incubated with the anti-actin C-terminal or N-terminal antibody;
- 1:1000 when the anti-myosin antibody was used.
When the gastric-chyme was analysed (see paragraph 4.5), these antibodies were diluted in water milli-Q:
- 1: 2000 for the actin C-terminal antibody;
- 1: 1000 for the anti-myosin antibody.

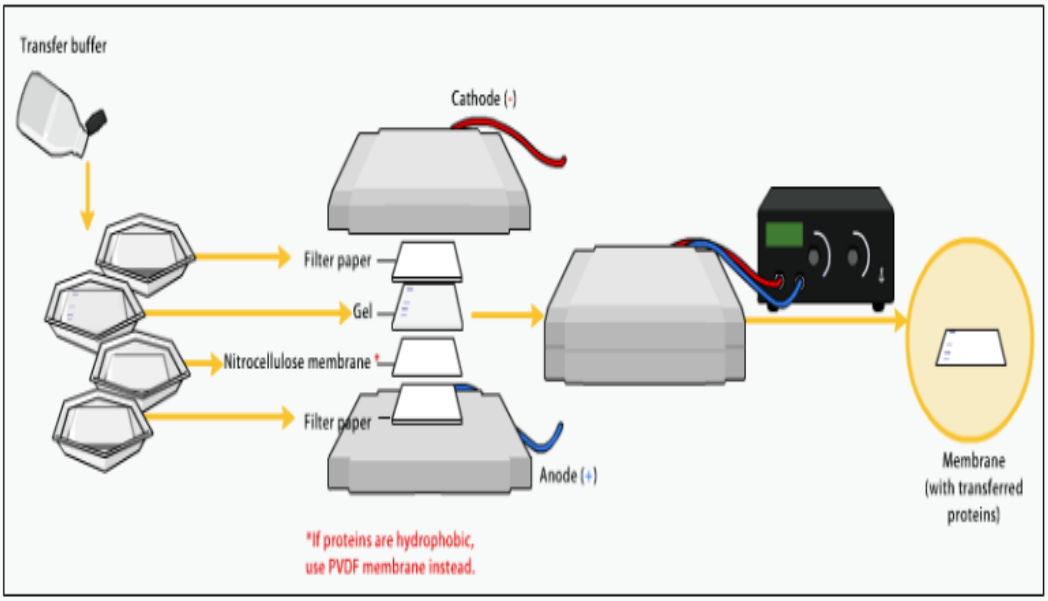

Fig. 3.13 - Protein transfer for immunostaining (Protocols® Online, 2016)

After incubation at 4°C over-night and after the three washes of five minutes each with PBS-Tween-20 at 0.1%, the membrane was incubated for an hour and a half by shaking with the secondary antibody Goat Anti-Rabbit IgG or Goat Anti-Mouse IgG (HRP conjugated) (Abcam). These secondary antibodies were diluted 1: 10,000 in milli-Q water. After further 10 minutes washings each with 0.1% PBS-Tween-20, the detection was performed using the chemiluminescence method (Figure 3.14) with the high-resolution imaging instrument for fluorescence and chemiluminescence (Syngene, G: BOX, Cambridge, United Kingdom), associated with a rapid setting software (Syngene, GeneSys, Cambridge, United Kingdom).
Chemiluminescence is a non-radioactive chemical reaction that produces a white light, which in Western Blotting occurs when the chemiluminescent substrate bound to a specific antibody of the target protein undergoes an enzymatic reaction, due to the horseradish peroxidase HRP (Syngene © Online, 2009 -2018). The membranes were incubated in agitation for about 1 minute with a 1:1 solution of luminol and hydrogen peroxide (Thermo Scientific, Chemiluminescent Western Blotting, Waltham, Massachusetts, USA) and then inserted for two minutes in the darkroom, in contact with a transilluminator UV (Syngene, Transilluminator, Cambridge, United Kingdom), in order to detect the bands recognized by the secondary antibody. The secondary antibody conjugated to the horseradish peroxidase allows the conversion of a soluble substrate (luminol) into an insoluble one (aminophthalate), which is deposited on the protein identified by the primary antibody and emits light, according to the following chemiluminescence reaction:

$$luminol + 2H_2O_2 + OH^- \longrightarrow aminophtalate + N_2 + 3H_2O + light$$

The signal acquired inside the transilluminator has been converted into a digital image using the GeneSys program, which allows you to view the bands recognized by the secondary antibody.

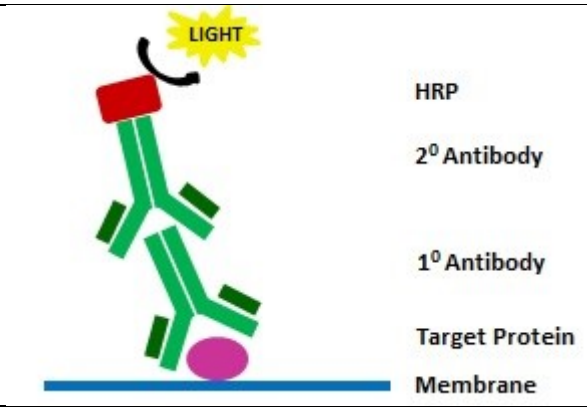

Fig. 3.14 - Western Blot Chemiluminescence (Syngene © Online, 2009-2018)

3.6 In vitro simulation of gastric and intestinal digestion

In vitro digestion is an experimental approach used to study the gastro-intestinal behavior of food and drugs. It has the advantage of being fast, inexpensive, little laborious and has no ethical restrictions. The in vitro gastric digestion of proteins was carried out following two different methods: the method by Wen (Wen et al, 2015) and the method by Minekus (Minekus et al., 2014). In performing both methods, digestion conditions at the level of the oral cavity have not been simulated, as protein degradation begins only in the stomach.
When the method of Wen (Wen et al., 2015) was used, fish fillet samples were treated with pepsin alone in 10 mM HCl. Instead, following the method of Minekus et al. (2014) the samples were subjected to the action of pepsin in a buffer that simulated the composition of the gastric fluid, defined Simulated Gastric Fluid (SGF), while the gastric samples-chyme was then mixed with the Simulated Fluid Intestinal (SIF) added with bile and pancreatin enzymes to simulate the intestinal conditions.

3.6.1 In vitro simulation of gastric digestion according to the method of Wen et al. (2015)

Samples of 0.5g from dorsal musculature were taken, added with 1 mL of milli-Q water and homogenized using Ultra-turrax® T25 Digital (IKA®-Werke GmbH & Co. KG, Staufen, Germany) operating at 13,000 rpm for three repetitions of 30 seconds each interspersed with a 30-second pause. The homogenated samples were added with:
- pepsin in a 1:32 ratio to the weight of the fillet. A 4% (w/v) pepsin solution (Sigma Aldrich, Saint Louis, Missouri, USA) in 10 mM HCl was used. The pH was then adjusted to 2 with a 1M HCl solution to simulate the gastric digestion of a healthy person;
- pepsin as before, but adjusting the pH to 4 with 1 M HCl to simulate the gastric digestion under conditions of drug-induced high pH occurring in patients suffering from gastroesophageal reflux (GERD);
- or with 10 mM HCl solution alone.
Samples were incubated at 37 °C for one or two hours under continuous agitation. At the end of the incubation, pepsin was inactivated by adjusting the pH to 7.5 with a 1M NaOH solution. After adding three volumes of ethanol, the samples were stored for 12 hours at 4°C. Subsequently the samples were centrifuged (Beckam Coulter Life Science, Avanti ™ J-25 Centrifuge, USA) at 10,000 rpm for 20 minutes at a

temperature of 4°C. The precipitates were separated and stored at -30°C for subsequent analyzes.

The bicinconinic acid method (BCA method) was applied to determine the protein content of the precipitates. In this type of assay bicinconinic acid (Sigma-Aldrich, Saint Louis, Missouri, USA) and pentahydrate cupric sulfate (Sigma-Aldrich, Saint Louis, Missouri, USA) are used to obtain a BCA Working Reagent solution. The calibration line is set by preparing a solution of bovine albumin (BSA) of known concentration. Readings are taken at a wavelength of 562 nm using the UV-VIS spectrophotometer (Shimadzu, UV-VIS Spectro Photometer UV-2501 PC, Kyoto, Japan), and the UVProbe software (Shimadzu, UVProbe, Photometric, Druisburg, Germany).

For electrophoretic and immunochemical analyzes the precipitates were suspended in 0.5 ml of extraction buffer (Tris / HCl 60 mM pH 6.8, SDS 2%, DTE 100 mM) (Figure 3.15).

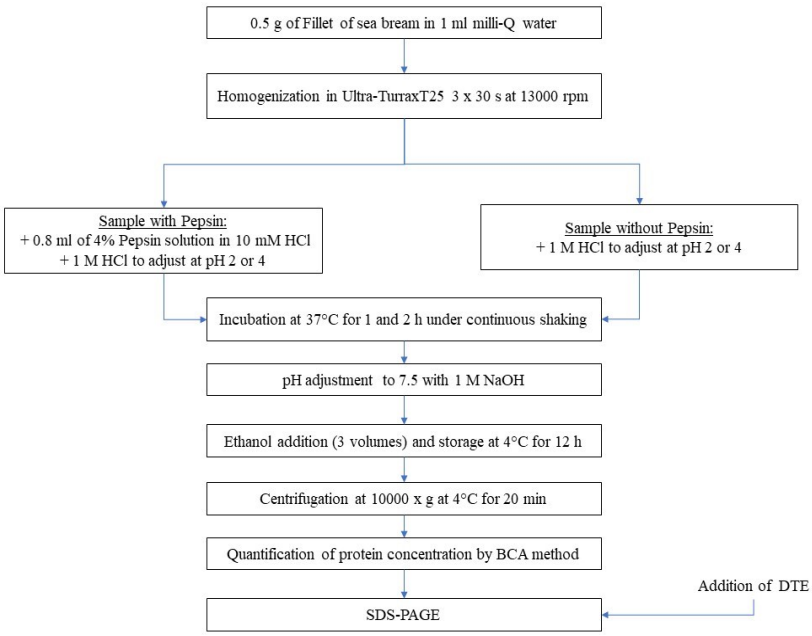

Fig. 3.15- In vitro simulation of gastric digestion of sea bream fillet (Wen et al., 2015)

3.6.2 In vitro simulation of gastric and intestinal digestion according to the method of Minekus et al. (2014)

The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) according to the method of Minekus et al. (2014) consist of a solution of electrolytes, enzymes, $CaCl_2$ and water. The concentrations and volumes of the stock solutions and the final concentration of each constituent of SGF and SIF are shown in (Table 3.9).
Simulated digestion was conducted in parallel at pH 3, to mimic a healthy condition, or 4, to mimic the GERD condition.
The samples, consisting of 1g of sea bream muscle, were added with 0.8 ml of SGF (pH 3 or pH 4) and 0.4 ml of H_2O and homogenized by Ultra-turrax® T25 Digital (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 13000 rpm for for three repetitions of 30 seconds each interspersed with a 30-second pause. After adding 1.8 ml di SGF containing 1.46 mg/ml of pepsin (2500 U/mg) (Sigma Aldrich, Saint Louis, Missouri, USA) (prepared in SGF at pH 3 or 4) to the homogenized samples, these were incubated at 37 °C for 2 h in continuous agitation. At the end of incubation with pepsin, an aliquot of 1.5 ml was taken from each sample, to which 1.5 ml of SIF were added. Subsequently the samples were incubated at 37 ° C for another two hours to simulate intestinal digestion, together with pancreatin (Sigma Aldrich, Saint Louis, Missouri, USA), which was added based on the trypsin activity (100U/ml in the final mixture) and 10 mM bile salts (Sigma Aldrich, Saint Louis, Missouri, USA). After checking the pH and eventually adjusted to 7 with NaOH, the samples were placed at 95 °C for 5 minutes. Finally, 3 volumes of ethanol were added and stored over-night at 4 °C.
The following day, the gastric- and intestinal-chyme samples were centrifuged (Beckam Coulter Life Science, Avanti ™ J-25 Centrifuge, USA) at 10,000 rpm for 20 minutes at 4°C. The precipitates were suspended in 1 ml of extraction buffer (Tris / HCl 60 mM pH 6.8, SDS 2%, DTE 100 mM) and used for the analysis by SDS-PAGE and Western blotting.
When the gastric-chyme was analysed for the presence of bioactive peptides by mass spectrometry, the 3 ml samples were heated at 95 °C for 5 minutes and added with 6 ml of 0.15 M HCl and 0.25 ml of a solution of 1 mM Phe-Phe, used as internal standard. After centrifugation for 30 minutes at 8000 g at 4°C, the supernatants were filtered through 0.45 µm membranes (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) and sent by mail to Dr. Barbara Prandi, University of Parma.

			SGF		SIF	
			pH 3		pH 7	
Constituent	Stock conc.		Vol. of stock	Conc. in SGF	Vol. of stock	Conc. in SIF
	$g\ L^{-1}$	$mol\ L^{-1}$	mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$
KCl	37.3	0.5	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	12.5	25	42.5	85
NaCl	117	2	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.5	0.5	—	—
For pH adjustment						
	$mol\ L^{-1}$		mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$
NaOH	1		—	—	—	—
HCl	6		1.3	15.6	0.7	8.4

$CaCl_2(H_2O)_2$ is not added to the simulated digestion fluids, see details in legend

	$g\ L^{-1}$	$mol\ L^{-1}$	$mmol\ L^{-1}$	$mmol\ L^{-1}$
$CaCl_2(H_2O)_2$	44.1	0.3	0.15 (0.075*)	0.6 (0.3*)

[a] * in brackets is the corresponding Ca^{2+} concentration in the final digestion mixture.

Table 3. 9 – Composition of the SGF and SIF buffers according to Minekus et al. (2014).

4 Results and discussion

4.1 Quality characteristics

To assess the effect of different feeding regimes on the quality of fish fillets, sea bream (*Sparus aurata*) were divided into four tanks containing 21 individuals each and were subjected to full regime diet (C) for 21 days or to fasting (F), the latter consisting of 100% reduction of the feed regime. The trial was conducted at the aquaculture facilities of the Department of Agri-Food, Environmental and Animal Sciences of the University of Udine. At the end of the breeding, sea bream was sedated, slaughtered and stored at 4 °C. (Figure 4.16) shows the muscle pH valus in the first hours of storage during post-mortem.

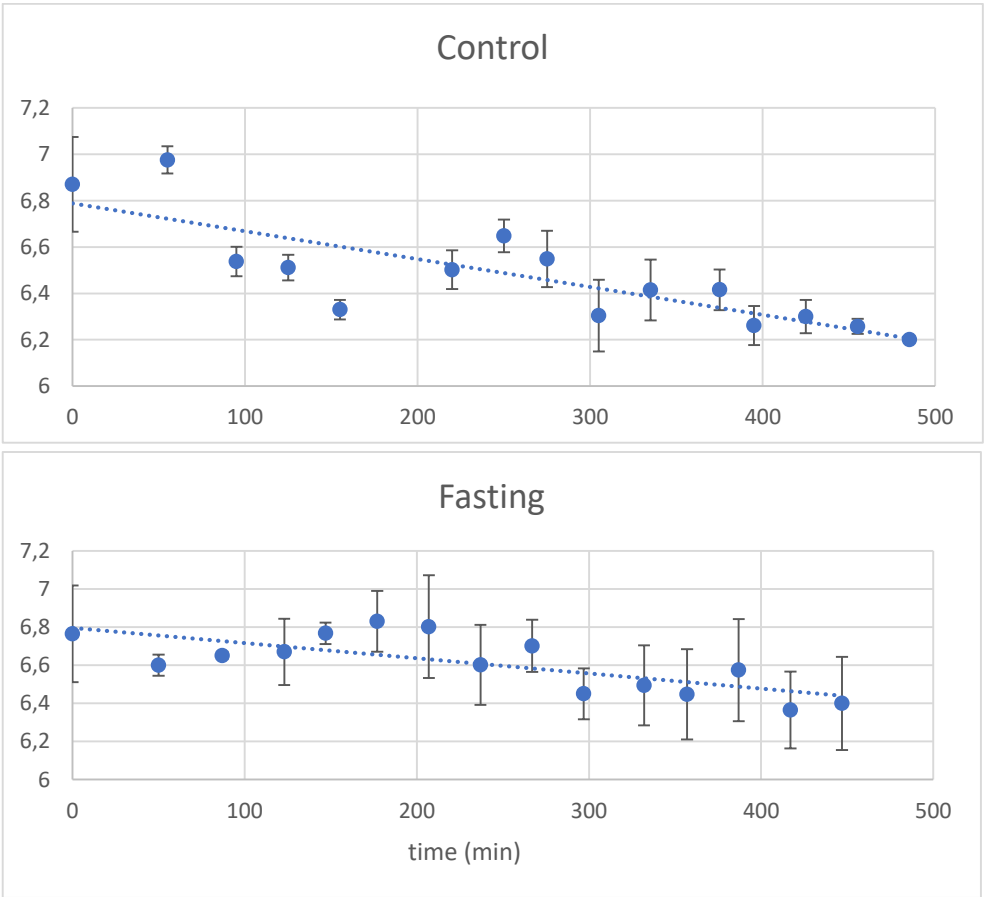

Fig. 4 .16 - Effect of different dietary regimes on muscle pH in fillets of sea bream (Sparus aurata). Control and Fasted samples were stored on ice and pH analyzed at different times after slaughter. Measures were taken in the neck, dorsal and caudal area on three fish for each feeding procedure. The bars show the standard deviations.

Both the two different dietary regimes are characterized by pH variability, which reflected in high standard deviations. However, variability was more evident in the fasted samples. The behavior of the fasted group is probably due to an altered glucose metabolism, would therefore seem to represent the heterogeneity of the response of individual subjects to fasting. Muscle pH decreased markedly throughout storage time and then stabilized at pH 6.2-6.4.

In breeding snappers (*Dentex dentex*) both lactate levels and muscle pH were weakly affected by fasting (5 weeks at 25 ± 2 ° C) (Suárez et al., 2010). In addition, fasting snapper fillets showed a high firmness of the muscle and no difference in the WHC (Water Holding Capacity) compared to controls. The length of the fasting period was found critical for its effect on pH. According to Álvarez et al. (2008), sea bream subjected to short periods of fasting (24 and 48 hours) showed higher values of muscle pH at the time of slaughter compared to fasted bream for longer periods (72 hours). In particular, the highest pH values were found after 14 and 21 days of storage on ice at 4 °C. Stress conditions also seem to strongly influence muscle pH. According to Mørkøre et al. (2008), in Atlantic salmon (*Salmon Salar*) the decrease in muscle pH (6.2) during storage at 5°C was slower (48 h) in fed or fasting groups in the absence of stress. In fact, fasting or fed fish subjected to stress reached the final pH after 12-24 hours.

In fasted fish, the altered metabolic activity could result in increasing susceptibility of the muscular proteins to the action of the different endogenous proteases, which are known to be activated by fasting conditions, resulting in greater proteolysis, which, in turn, could affect the digestibility of sea bream fillet. To test whether sea bream fasting influenced the muscle protein degradation at slaughter and during post-mortem storage, the protein components of the muscle were analyzed as described in the following paragraphs.

Profile of proteins extracts from sea bream fillet were obtained by using two techniques:

- separation by denaturing electrophoresis on polyacrylamide gel (SDS-PAGE).
- protein identification by Western Blotting, using specific antibodies.

The combination of these approaches offers the possibility of combining the resolution of the separations obtained with SDS-PAGE with the sensitivity of specific antibodies. With this combination it was possible to detect protein degradation in muscular post mortem and in vitro digestion of sea bream fillets subjected to different feeding regimes.

4.2 Profile of protein extracts of sea bream fillets

4.2.1 SDS-PAGE analyses

The samples from sea bream, which have been subjected to different feeding regimes, were quickly frozen immediately after slaughtering. The protein fraction was extracted by a method that requires the use of SDS in order to maximize protein recovery (Piñeiro et al., 1999). Following SDS-PAGE denaturing electrophoresis at 13%, the protein patterns were detected by Coomassie's Blue coloring. Protein profiles of the samples taken at slaughter (t0) were similar in control and fasted fillet except for a band at about 16 kDa (Figure 4.17). This 16 kDa band appears clearly visible in the three samples of Control sea bream, while it appears visibly reduced in the three samples of fasted sea bream. Based on the electrophoretic behavior, it was hypothesized that the 16 kDa band could correspond to the light chain 3 of the myosin (MLC3).

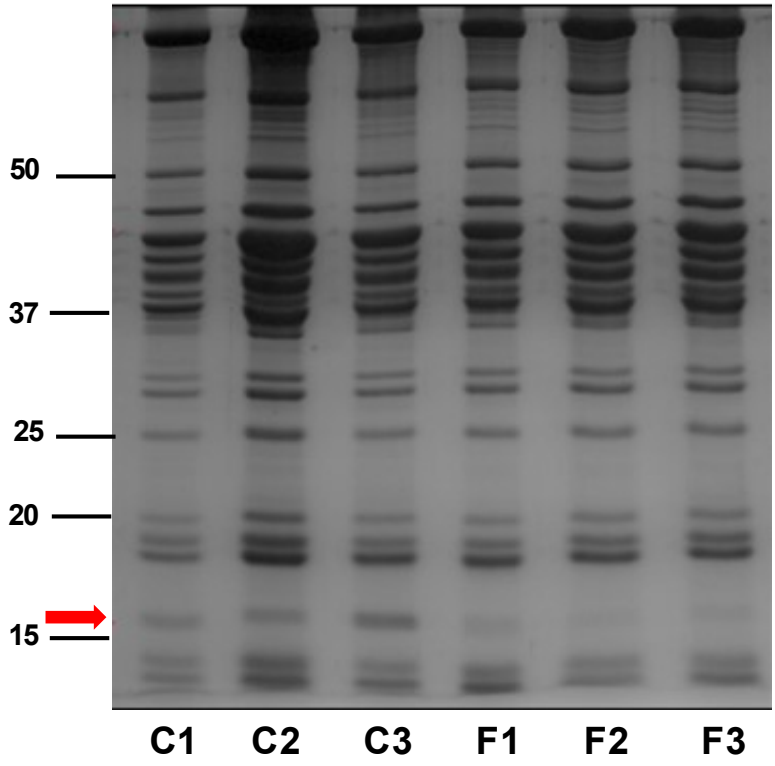

Fig. 4 .17 – Representative SDS-PAGE protein profiles at slaughter of muscle extracts from three control (C1, C2, C3) and three fasted (F1, F2, F3) sea bream samples. The protein extracts were subjected to 13% SDS-PAGE. Arrow indicates the 16 kDa band.

When an electrophoretic run was performed on samples stored at 4 °C for different times after slaughtering (3, 7 and 24h post-mortem), it was confirmed, for the fasted samples, the early loss of myosin light chain (Figure 4.18). The disappearance of the 16 kDa band may be due to adaptations to fasting, in terms of reduced synthesis or increased proteolysis of this protein. Since it is known that fasting can activate endogenous proteolysis also in sea bream (Sala-Rabanal et al., 2004), *ante mortem* degradation appears a more plausible explanation. Degradation was also evident in the Control samples, where myosin light chain was no longer visible in all samples during post-mortem storage at 4 °C.

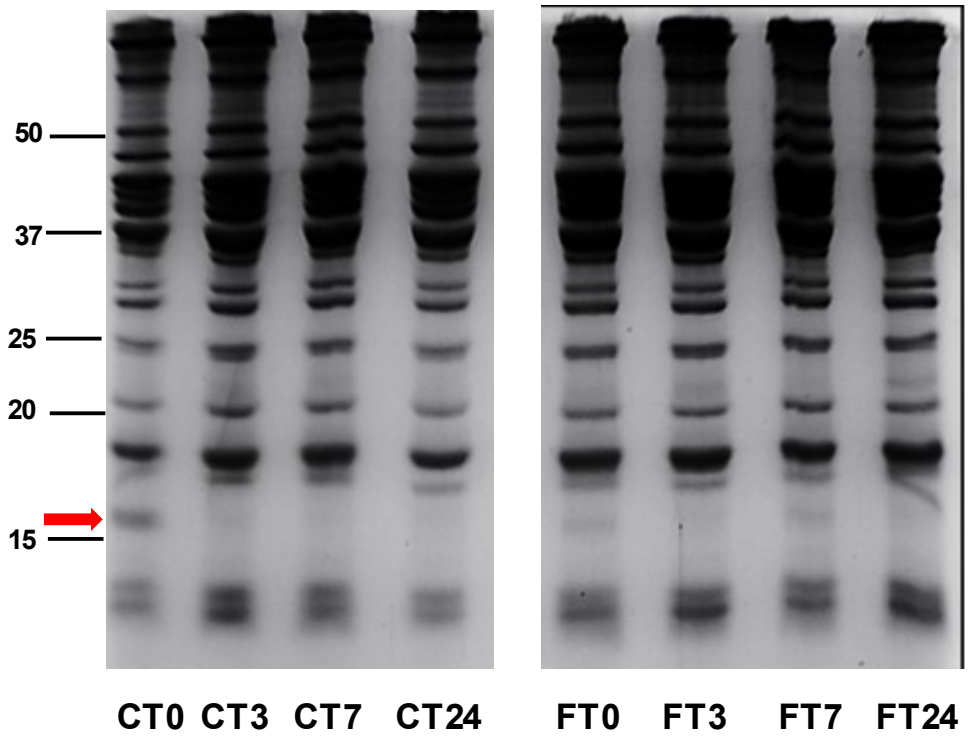

Fig. 4 .18 – Representative SDS-PAGE protein profiles of muscle extracts from control (C) and fasted (F) sea bream obtained at different times from slaughter (time 0: T0; time 3h: T3; time 7h: T7; time 24h: T24). The protein extracts from fillets stored on ice were subjected to 13% SDS-PAGE in three independent experiments. Arrow indicates the 16 kDa band.

4.2.2 Western Blotting analysis

Since no specific antibodies for fish exist on the market, analyses by Western blotting were carried out using antibodies against human actin and myosin. It was therefore compared the amino acid sequences of the human MLC3 isoform and of the human α-actin with respect to the fish Danio Rerio, the most widely used fish model for genetic studies (Figures 4.19 and Figure 4.20). The alignment concerning actin showed how much the primary structure of this protein is conserved, with only two mutated amino acids between *Homo Sapiens* and *Danio Rerio*.
For myosin light chain 3 a 61% identity was obtained, making plausible its immunodetection in fish by an antibody targeted agains human protein.

Score	Expect	Method	Identities	Positives	Gaps
787 bits(2032)	0.0	Compositional matrix adjust.	375/377(99%)	377/377(100%)	0/377(0%)

```
Query  1    MCDEDETTALVCDNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEA  60
            MCD+DETTALVCDNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEA
Sbjct  1    MCDDDETTALVCDNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEA  60

Query  61   QSKRGILTLKYPIEHGIITNWDDMEKIWHHTFYNELRVAPEEHPTLLTEAPLNPKANREK  120
            QSKRGILTLKYPIEHGIITNWDDMEKIWHHTFYNELRVAPEEHPTLLTEAPLNPKANREK
Sbjct  61   QSKRGILTLKYPIEHGIITNWDDMEKIWHHTFYNELRVAPEEHPTLLTEAPLNPKANREK  120

Query  121  MTQIMFETFNVPAMYVAIQAVLSLYASGRTTGIVLDSGDGVTHNVPIYEGYALPHAIMRL  180
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Sbjct  121  MTQIMFETFNVPAMYVAIQAVLSLYASGRTTGIVLDSGDGVTHNVPIYEGYALPHAIMRL  180

Query  181  DLAGRDLTDYLMKILTERGYSFVTTAEREIVRDIKEKLCYVALDFENEMATAASSSSLEK  240
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Sbjct  181  DLAGRDLTDYLMKILTERGYSFVTTAEREIVRDIKEKLCYVALDFENEMATAASSSSLEK  240

Query  241  SYELPDGQVITIGNERFRCPETLFQPSFIGMESAGIHETTYNSIMKCDIDIRKDLYANNV  300
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Sbjct  241  SYELPDGQVITIGNERFRCPETLFQPSFIGMESAGIHETTYNSIMKCDIDIRKDLYANNV  300

Query  301  MSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWIT  360
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Sbjct  301  LSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWIT  360

Query  361  KQEYDEAGPSIVHRKCF  377
            KQEYDEAGPSIVHRKCF
Sbjct  361  KQEYDEAGPSIVHRKCF  377
```
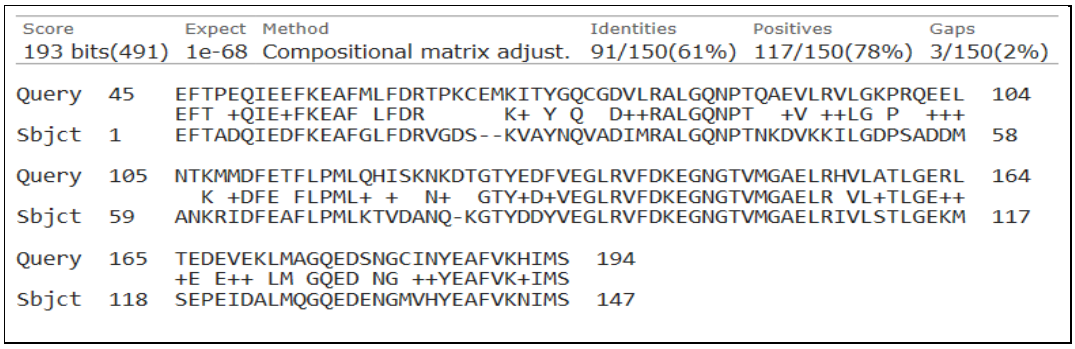

Fig. 4.19- Alignment of the skeletal muscle α-actin of zebra fish (Danio Rerio) (query)with α-actin of human skeletal muscle (Homo Sapiens) (UniProt ©, 2002-2018).



Score	Expect	Method	Identities	Positives	Gaps
193 bits(491)	1e-68	Compositional matrix adjust.	91/150(61%)	117/150(78%)	3/150(2%)

```
Query  45   EFTPEQIEEFKEAFMLFDRTPKCEMKITYGQCGDVLRALGQNPTQAEVLRVLGKPRQEEL  104
            EFT +QIE+FKEAF LFDR       K+ Y Q  D++RALGQNPT  +V ++LG P  +++
Sbjct  1    EFTADQIEDFKEAFGLFDRVGDS--KVAYNQVADIMRALGQNPTNKDVKKILGDPSADDM  58

Query  105  NTKMMDFETFLPMLQHISKNKDTGTYEDFVEGLRVFDKEGNGTVMGAELRHVLATLGERL  164
              K +DFE FLPML+ +  N+  GTY+D+VEGLRVFDKEGNGTVMGAELR VL+TLGE++
Sbjct  59   ANKRIDFEAFLPMLKTVDANQ-KGTYDDYVEGLRVFDKEGNGTVMGAELRIVLSTLGEKM  117

Query  165  TEDEVEKLMAGQEDSNGCINYEAFVKHIMS  194
            +E E++ LM GQED NG ++YEAFVK+IMS
Sbjct  118  SEPEIDALMQGQEDENGMVHYEAFVKNIMS  147
```

Fig. 4.20- Alignment of the skeletal muscle myosin light chain 3 (MLC3) of zebra fish (Danio Rerio) (query) with myosin light chain 3 of human skeletal muscle (Homo Sapiens) (UniProt ©, 2002-2018).

Sea bream samples were then analyzed by Western Blotting, using monoclonal antibodies against both actin (both C-terminal and N-terminal) and myosin.
(Figure 4.21) shows the immune detection of total myosin in fillets of Control and Fasting sea bream immediately after slaughter. The presence of the 16 kDa immunostained band was confirmed in the control samples, whereas the antibody did not detect any band at that molecular weight in the fasted samples.
MLC3 was identified, although in lower quantities, in both wild and farmed bream (Carpene et al. 1998). The molecular weight showed in the study by Carpene et al. (1998) was higher (19.5 kDa) than that reported by the UniProt © database for sea bream MLC3 (16.9 kDa). However, there still are discrepancies about the molecular weight of MLC3 in different fish species (Ochiai et al., 1990; Rowlerson et al., 1985).

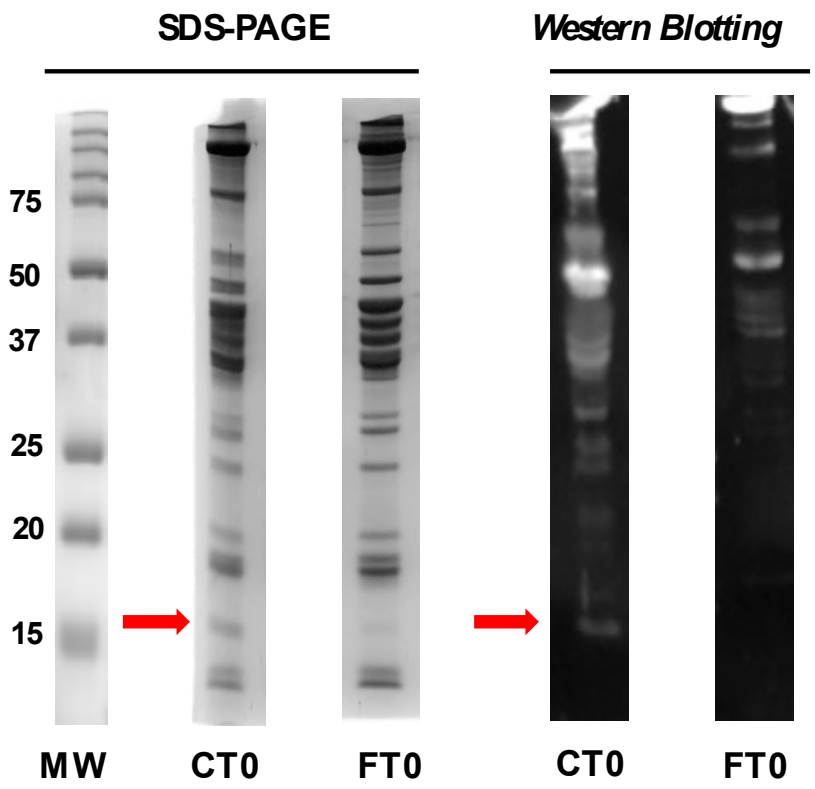


Fig. 4.21- SDS-PAGE and Western Blotting against total myosin of control and fasted sea bream muscle samples at time of slaughter. The protein extracts were subjected to SDS-PAGE at 13% and then to Western Blotting in three independent experiments. Arrow indicates the 16 kDa band.

It is known that the proteolytic action of calpains and cathepsins leads to softening of the meat and changes in texture, which can lead to a decrease in product quality, but also to the formation of peptides, some of which may exhibit potential bioactive properties. In rainbow trout (*Oncorhynchus mykiss*) cathepsins B and D were found to degrade light-chain myosins MLC1 and MLC2 (Godiksen et al., 2009).
In addition to myosin, actin degradation during post mortem was evaluated by Western Blotting using both an antibody against the N-terminal and the C-terminal portion of actin. As shown in (Figure 4.22), there were no differences between the two dietary regimens with respect to the intact actin content, detected at 42 kDa (intact actin molecule weight) with both antibodies, and to actin fragments recognized by the antibody against C-terminal portion. In particular, the three main C-terminal fragments, which were identified at 35, 28 and 20 kDa, did not show any changes at different post-mortem times.

On the contrary, the N-terminal monoclonal antibody (panel B) showed actin fragments at different molecular weights: 33 kDa for the control samples and 26 kDa for the fasted samples.

Actin, like myosin, is degraded by the synergistic action of proteases. However, several studies have reported a reduced susceptibility of actin than myosin light-chains to the action of proteases. In the study by Caballero et al. (2009), carried out in gilthead fish, the actin was detected even more than 10 days after slaughter during storage at 4°C, demonstrating how actin persists despite muscle deterioration. Similar results have been also found in carp, where, at different temperatures and times of muscle storage, actin, tropomyosin and heavy chain myosin were stable, unlike light-chain myosin (Jasra et al., 2001).

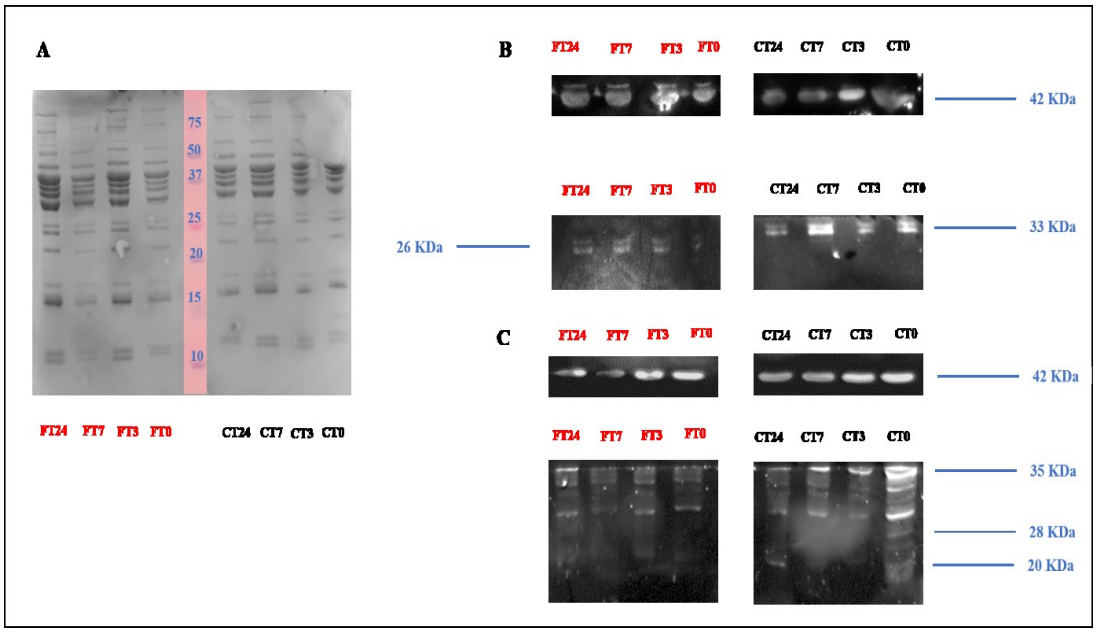


Fig. 4.22- SDS-PAGE and Western Blotting against actin of control and fasted sea bream muscle samples at different times from slaughter. The protein extracts were subjected to SDS-PAGE at 13% (A) and then to Western Blotting using antibodies against actin N-term (B) or against actin C-term (C) in three independent experiments.

4.2.3 Mass spectrometry identification of proteins

To confirm that identity of 16 kDa proteins, the bands were isolated and submitted to identification by mass spectrometry at the University of Parma with the collaboration of Prof. Stefano Sforza and Dr.ssa Barbara Prandi. Whith this aim, the samples were subjected to SDS-PAGE, and gels were stained by using the Colloidal Blue Staining kit method. Figure 4.23 shows an example of the Colloidal Blue stained gel, which was cut by means of sterile scalpel to sample the 16 kDa portions of gel. These gel samples were stored in pure ethanol and sent to Parma, where they were analyzed by FT-ICR / Orbitrap.

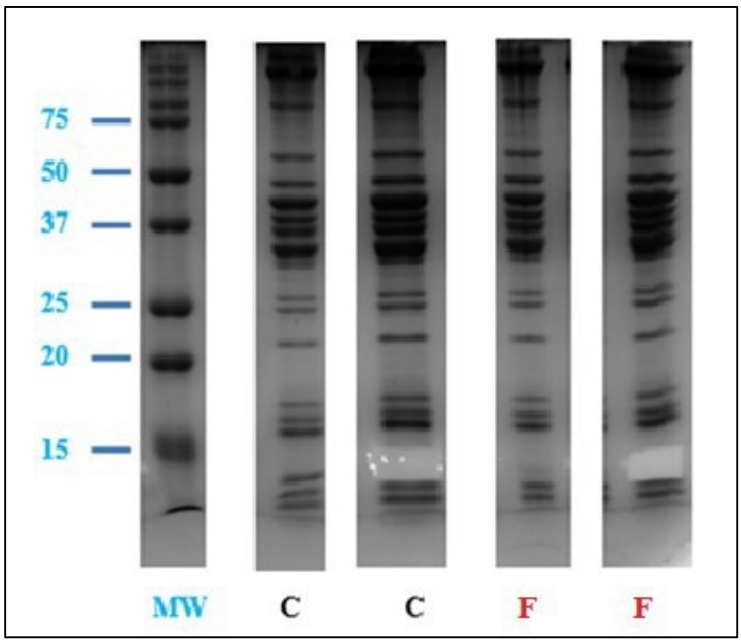
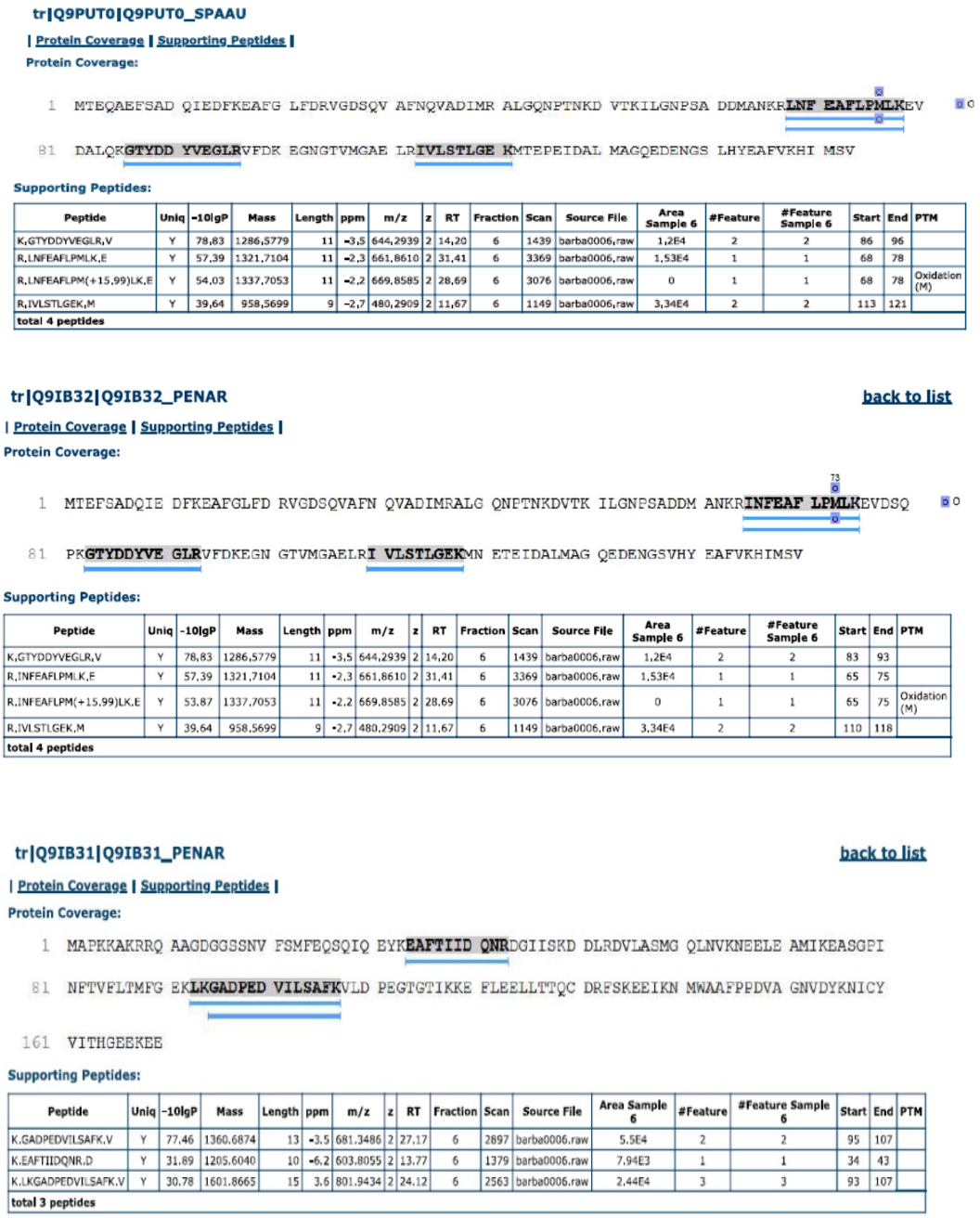


Fig. 4.23– Representative SDS-PAGE of protein extracts from control and fasted muscle samples at slaughter. The protein extracts were subjected to 13% SDS-PAGE, followed by staining using Colloidal Blue Staining kit. The bands at 16 kDa were manually excised from three control and fasted muscle samples for mass spectrometry analyses.

In the 16 kDa bands of the Control samples, the presence of the fast skeletal myosin light chain 3 from *Sparus aurata* (Accession number Q9PUTO - coverage 20%) was confirmed (Figure 4.24). Myosin light chain 3 (Accession number Q9IB32 - coverage 21%) and myosin light chain 2 (Accession number Q9IB32 - coverage 15%) were also identified. As expected, in the bands obtained from two fasted samples myosin light chain proteins were not identified, thus supporting their absence in the electrophoretic patterns.



tr|Q9PUT0|Q9PUT0_SPAAU

| Protein Coverage | Supporting Peptides |

Protein Coverage:

1 MTEQAEFSAD QIEDFKEAFG LFDRVGDSQV AFNQVADIMR ALGQNPTNKD VTKILGNPSA DDMANKRLNF EAFLPMLKEV

81 DALQKGTYDD YVEGLRVFDK EGNGTVMGAE LRIVLSTLGE KMTEPEIDAL MAGQEDENGS LHYEAFVKHI MSV

Supporting Peptides:

Peptide	Uniq	-10lgP	Mass	Length	ppm	m/z	z	RT	Fraction	Scan	Source File	Area Sample 6	#Feature	#Feature Sample 6	Start	End	PTM
K,GTYDDYVEGLR,V	Y	78,83	1286,5779	11	-3,5	644,2939	2	14,20	6	1439	barba0006.raw	1,2E4	2	2	86	96	
R,LNFEAFLPMLK,E	Y	57,39	1321,7104	11	-2,3	661,8610	2	31,41	6	3369	barba0006.raw	1,53E4	1	1	68	78	
R,LNFEAFLPM(+15,99)LK,E	Y	54,03	1337,7053	11	-2,2	669,8585	2	28,69	6	3076	barba0006.raw	0	1	1	68	78	Oxidation (M)
R,IVLSTLGEK,M	Y	39,64	958,5699	9	-2,7	480,2909	2	11,67	6	1149	barba0006.raw	3,34E4	2	2	113	121	
total 4 peptides																	

tr|Q9IB32|Q9IB32_PENAR back to list

| Protein Coverage | Supporting Peptides |

Protein Coverage:

1 MTEFSADQIE DFKEAFGLFD RVGDSQVAFN QVADIMRALG QNPTNKDVTK ILGNPSADDM ANKRINFEAF LPMLKEVDSQ

81 PKGTYDDYVE GLRVFDKEGN GTVMGAELRI VLSTLGEKMN ETEIDALMAG QEDENGSVHY EAFVKHIMSV

Supporting Peptides:

Peptide	Uniq	-10lgP	Mass	Length	ppm	m/z	z	RT	Fraction	Scan	Source File	Area Sample 6	#Feature	#Feature Sample 6	Start	End	PTM
K,GTYDDYVEGLR,V	Y	78,83	1286,5779	11	-3,5	644,2939	2	14,20	6	1439	barba0006.raw	1,2E4	2	2	83	93	
R,INFEAFLPMLK,E	Y	57,39	1321,7104	11	-2,3	661,8610	2	31,41	6	3369	barba0006.raw	1,53E4	1	1	65	75	
R,INFEAFLPM(+15,99)LK,E	Y	53,87	1337,7053	11	-2,2	669,8585	2	28,69	6	3076	barba0006.raw	0	1	1	65	75	Oxidation (M)
R,IVLSTLGEK,M	Y	39,64	958,5699	9	-2,7	480,2909	2	11,67	6	1149	barba0006.raw	3,34E4	2	2	110	118	
total 4 peptides																	

tr|Q9IB31|Q9IB31_PENAR back to list

| Protein Coverage | Supporting Peptides |

Protein Coverage:

1 MAPKKAKRRQ AAGDGGSSNV FSMFEQSQIQ EYKEAFTIID QNRDGIISKD DLRDVLASMG QLNVKNEELE AMIKEASGPI

81 NFTVFLTMFG EKLKGADPED VILSAFKVLD PEGTGTIKKE FLEELLTTQC DRFSKEEIKN MWAAFPPDVA GNVDYKNICY

161 VITHGEEKEE

Supporting Peptides:

Peptide	Uniq	-10lgP	Mass	Length	ppm	m/z	z	RT	Fraction	Scan	Source File	Area Sample 6	#Feature	#Feature Sample 6	Start	End	PTM
K.GADPEDVILSAFK.V	Y	77.46	1360.6874	13	-3.5	681.3486	2	27.17	6	2897	barba0006.raw	5.5E4	2	2	95	107	
K.EAFTIIDQNR.D	Y	31.89	1205.6040	10	-6.2	603.8055	2	13.77	6	1379	barba0006.raw	7.94E3	1	1	34	43	
K.LKGADPEDVILSAFK.V	Y	30.78	1601.8665	15	3.6	801.9434	2	24.12	6	2563	barba0006.raw	2.44E4	3	3	93	107	
total 3 peptides																	

Fig. 4.24- Sequence coverage of myosin light chains identified in the 16 kDa bands excised from SDS-PAGE of protein extracts from sea bream muscle samples. The excised bands were analyzed by FT-ICR / Orbitrap.

Based on MS/MS data, we can speculate that myosin light chains were early degraded in Fasted fish, potentially giving rise to the formation of bioactive peptides.

4.2.4 HPLC-ESI-MS/MS identification of peptides

Bioinformatic analysis performed with the use of the BIOPEP search engine (Minkiewicz et al., 2008) showed that myosin light chains was a good source of di - and tripeptides with ACE inhibitory activity (Table 4.10).

Tripeptides and dipeptides	Q9PUTO	Q9IB31	Q9IB32	References	IC_{50} (uM) Biopep
MAP		X		biopep	0.80
GTG		X		biopep	5.54
VAF	x		x	Yu et al. (2018), biopep	35.80
AEL	x		x	biopep	57.10
AFL	x		x	biopep	63.10
SGP		X		biopep	77.72
LEE		X		biopep	100.00
VF	x	X	x	biopep	9.20
VK	x	X	x	biopep	13.00
HY	x		x	biopep	26.10
MF		X		biopep	45.00
RF		X		biopep	93.00

Table 4.10 - Bioinformatic analyses by BIOPEP search engine of ACE-inhibitory bioactive peptides from myosin light chains

In order to identify the ACE-inhibitory peptides, the fillet samples, after the in vitro simulated gastric digestion, were treated as described in M&M (3.6.2) and then separated by a reverse phase column in a HPLC (High Performance Liquid Chromatography) system coupled with electrospray ionization source (ESI) and mass spectrometry (MS) tandem detector. Analyses were performed at the University of Parma in collaboration with Prof. Stefano Sforza and Dr.ssa Barbara Prandi. Three peptides (Figure 4.25) were identified out of the 12 peptides observed by the bioinformatic analyses. These peptides (VF, MF, VAF) were resistant to gastric digestion, while the others were no longer detectable after digestion. It seems possible that the resistant peptides could be available *in vivo* where they could promote

potential health effects. Similar amounts of MF and VAF occurred in both the control and fasting digests, while the VF seemed in major amount in the fasted samples.

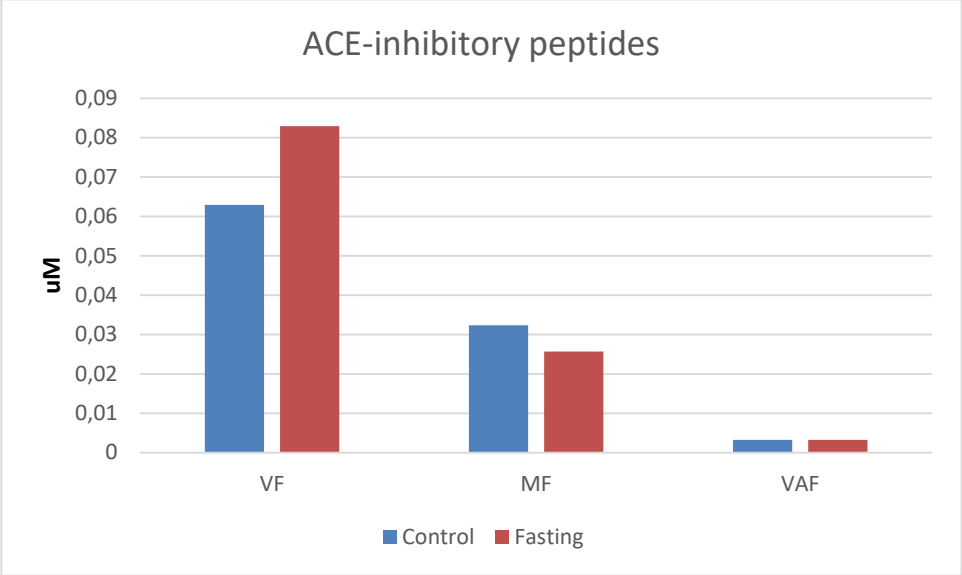


Fig. 4.25- Quantification of ACE-inhibitory peptides identified in two control and two fasted samples after gastric digestion.

4.3 Digestibility of raw sea bream fillet

4.3.1 In vitro simulation of gastric digestion according to the method of Wen et al. (2015)

To assess the impact on gastric digestibility of the increased degradation of myosin light chains observed in the fasted samples compared to controls, samples were subjected to simulated gastric digestion in vitro by incubating them with pepsin at pH 2 or pH 4, with the latter pH simulating a gastroesophageal reflux (GERD) drug treatment. The digested samples were treated with ethanol to extract the remaining protein fragments, which were analyzed by SDS-PAGE. (Figure 4.26) shows the results obtained in sea bream samples treated with pepsin at different incubation times (1-2 hours) and at different pH (2-4) (Figure 4.27 - 4.28). Pepsin at pH 2 degraded proteins in a comparable way in the fasted and control samples, generating fragments with molecular weights lower than 30 kDa. Furthermore, expecially after 1h digestion, the bands at molecular weights lower than 20 kDa appeared not properly distinguished, possible as a consequence of a multitude of peptide fragments generated by pepsin. A similar pattern was observed by Wen et al. (2015) analysing Zebrafish (Danio rerio).
Incubation with pepsin at pH 4 resulted in a more limited degradation, but much more marked in the fasted sea bream samples than that in control samples.

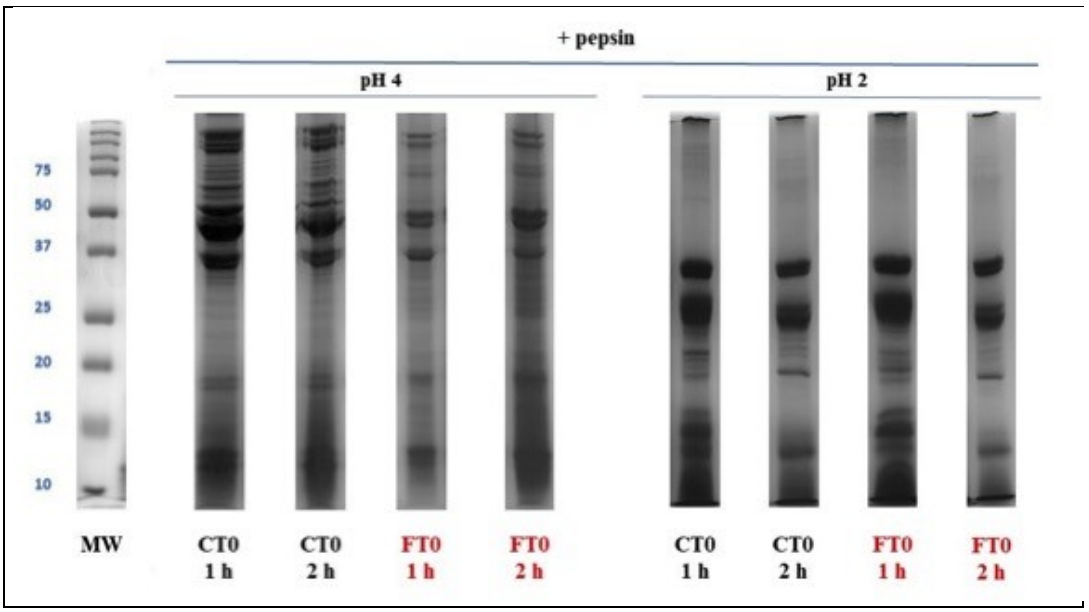


Fig. 4.26 – Representative SDS-PAGE protein profiles of raw fillets from three control and three fasted of sea bream samples at slaughter after (1 or 2 hours) in vitro gastric digestion (Wen et al., 2015) at pH 2 or at pH 4.

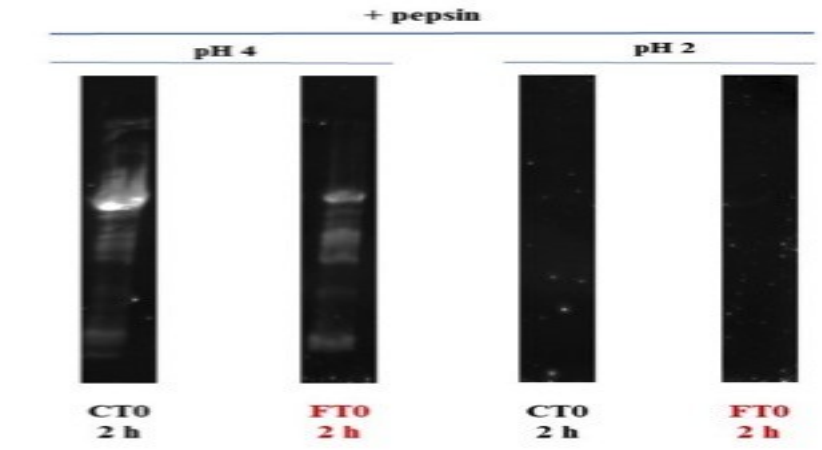


Fig. 4.27- Immuno-detection of α-actin in sea bream control and fasted raw fillet samples after (2h) in vitro gastric digestion at pH 2 or at pH 4, according to Wen et al. (2015).

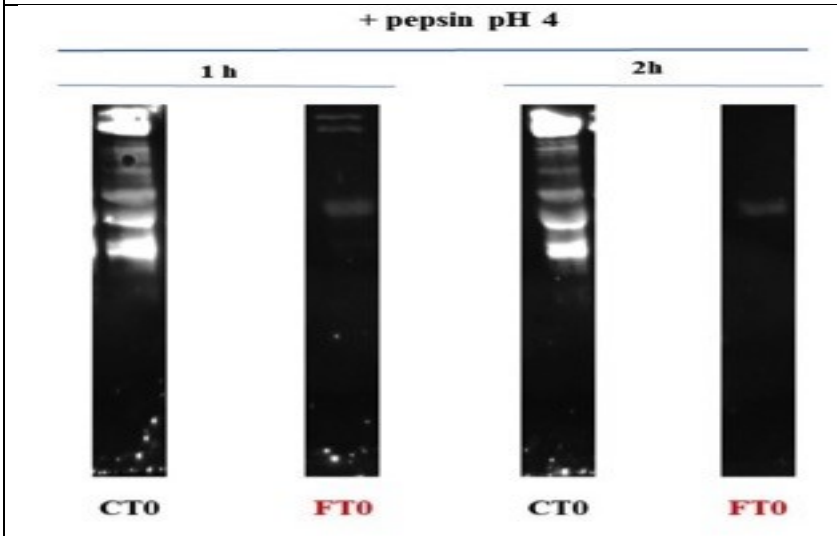


Fig. 4.28- Immuno-detection of total myosin in sea bream control and fasted raw fillet samples after (2h) in vitro gastric digestion at pH 4, according to Wen et al. (2015).

4.3.2 In vitro simulation of gastric digestion according to the method of Minekus et al. (2014)

In the gastric digestion by the method of Minekus (Minekus et al., 2014), simulated gastric fluid (SGF) was used in addition to pepsin. Simulated digestion was conducted in parallel at pH 3 and pH 4 and the residual protein fraction, separated by precipitation with ethanol, was analyzed by SDS-PAGE, as shown in Figure 4.29. Control and fasted samples digested at pH 3 did not show significant differences. In both type of samples, digestion generated fragments with molecular weights equal to or less than 30 kD. A marked band was observed at 30 kDa, while less properly distinguished bands were seen below 20 kDa. Also, the digestion at pH 4 generated similar bands in both the control and fasted samples, although in a wider range of molecular weights compared to pH 3. In particular, only the band around 30 kDa was present in both fractions obtained at pH 3 or 4.

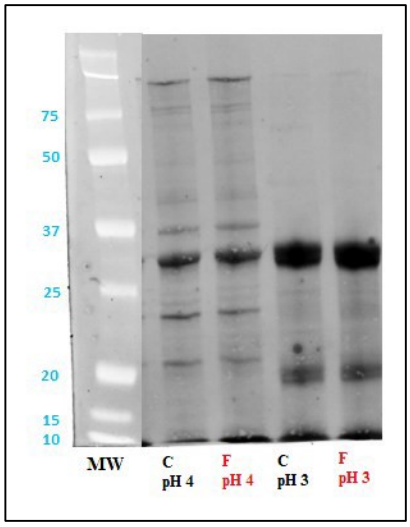


Fig. 4.29- Representative SDS-PAGE protein profiles of raw fillets from three control and three fasted of sea bream samples at slaughter after (2 hours) in vitro gastric digestion (Minekus et al., 2014) at pH 3 or at pH 4.

The samples separated by SDS-PAGE were subjected to Western blotting using a primary antibody against α-actin (Figure 4.30) and a primary antibody against total myosin (Figure 4.31). From Figure 4.30 it can be observed that at pH 3 the α-actin was completely degraded both in the control and the fasted samples, while at pH 4 the degradation of α-actin was limited and similar in both samples. On the other hand (Figure 4.31) degradation of myosin at both pH 3 and 4 was greater in the fasted samples than in the control ones. These results are consistent with those obtained by applying the simulated gastric digestion method according to Wen (Wen, et al., 2015). Both procedures confirmed that myosin was more sensitive to gastric degradation that was more marked in the fasted sea bream samples compared to the controls. This effect could be, at least in part, mediated by the degradation of myosin light chains by endogenous proteases.

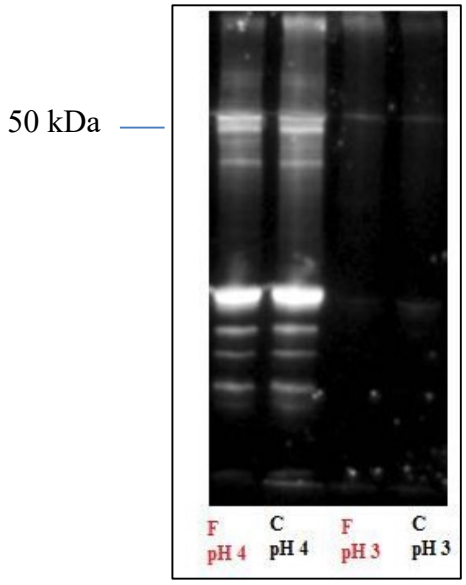


Fig. 4.30- Immuno-detection of α-actin in sea bream control and fasted raw fillet samples after (2h) in vitro gastric digestion at pH 3 or at pH 4 according to Minekus et al. (2014).

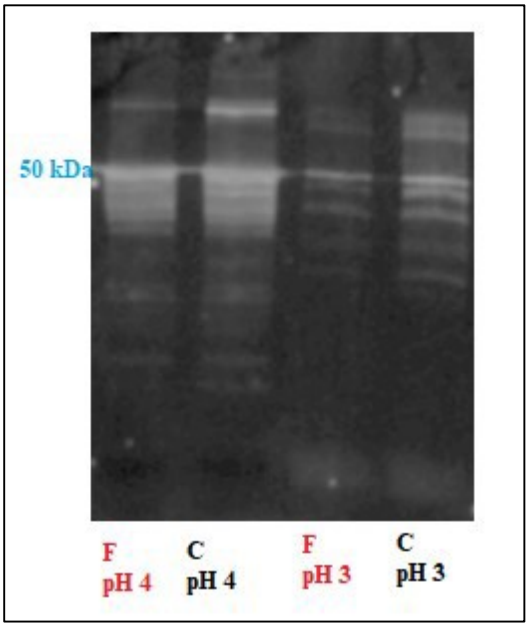


Fig. 4.31 - Immuno-detection of total myosin in sea bream control and fasted raw fillet samples after (2h) in vitro gastric digestion at pH 3 or at pH 4 according to Minekus et al. (2014).

Conclusions

Pre-slaughter sea bream fasting could be a very promising breeding practice as it would reduce management costs, without compromising the welfare of the raised subjects (Ashley, 2007) and the quality of fillets. In fact, pH, that is one of the main flesh quality attributes and it is known to have a great effect on conformation, thermal denaturation, and rheological properties of fish muscle proteins, particularly myosin (Tadpitchayangkoon et al., 2010) fell, even in the fasted samples, in the normal pH range of 6.2-6.8. On the other hand, the pre-slaughter fasting regime resulted in the early degradation of myosin light chains, which could be potential sources of bioactive peptides. Bioinformatic analysis with the use of the BIOPEP search engine, followed by mass spectrometry confirmed the presence of gastric-resistant bioactive peptides with ACE-inhibitory potential (VF, MF, VAF), derived from myosin light chains. These peptides were found in both the samples, regardless of the feeding regime, with one of these peptide (VF) seeming in larger amount in the fasted samples. These peptides, which are encrypted in the native protein sequences, remain inactive in the precursor proteins and only when they are released by the action of proteolytic enzymes, they may interact with selected receptors and regulate the body's physiological functions. Their potential activation mediated by the fish rearing conditions could therefore represent a strategy for the controlled hydrolysis of those susceptible proteins, such as myosin light chains, which can release bioactive peptides during gastrointestinal digestion. Once these bioactive peptides are liberated, they may play an important physiological role on regulation of the cardiovascular function, which includes a basic role in regulation of peripheral blood pressure via the renin-angiotensin system.

An important and neglected dimension of protein quality is its digestibility and absorption in humans. Applying static standardized in-vitro digestion methods, myosin digestibility was found to increase to a greater extent in the fasted samples at pH 4.0, when individual test enzymes were applied under conditions of drug-induced high pH occurring in GERD patients. Differences in myosin gastric digestibility were also apparent at pH 3.0, while at this pH actin was found susceptible to full proteolysis, irrespective of the feeding regime. Therefore, it appeared that under conditions which caused an early degradation of myosin light chains and possibly an impaired myosin integrity, gastric digestibility of the main myofibrillar proteins was much higher in the altered gastric milieu.

In conclusion, sea bream feed restriction, other than decreases the environmental impact of fish aquaculture, seems to act on fish proteins, resulting in nutritional benefits for hyperthensive patients, with regard to the regulatory effects of bioactive

peptides on the renin-angiotensin system components, and for the increasing number of people under acid- suppressant therapy for GERD, in relation to the enhanced gastric digestibility of the main myofibrillar proteins.

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