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Improvement of nutritional and technological characteristics of pork by supplementing the diet with extruded linseed and synthetic or natural antioxidants

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"You will come to know that what appears today to be a sacrifice will prove instead to be the greatest investment that you will ever make."

Gordon B. Hinckley

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ABSTRACT

Meat and meat products are important sources of protein, providing essential amino acids, fat, minerals, vitamins and other nutrients. However, in recent years, several epidemiological studies have associated red meat and processed meat consumption with an increased risk of cardiovascular disease and colorectal cancer. This awareness has moved consumers to become more health-conscious, and to prefer foods with a higher nutritional value, which can increase their health benefits. In this regard various studies proposed several strategies for improving meat quality in order to develop healthier meat and meat products. A good strategy for improving pork quality could be animal nutrition. In the swine production industry, fats and antioxidants in the diet are two key nutrients that impact the pork quality.

The aim of this research was to produce "healthier" pork by feeding pigs with ingredients rich in n-3 fatty acids as extruded linseed and addition of synthetic or natural antioxidants for improving meat quality. Linseeds are an important source of fat, especially unsaturated, with high percentages of essential fatty acids rich in n-3 and n-6. As for natural antioxidants, oregano possess effective antibacterial properties, potent antioxidant activity and also is a nutrient-dense spice. Grape skin is a wine-making by-product rich in polyphenolic compounds, which are characterized by a high antioxidant activity and the reuse of it can also reduce the environmental impact of the wine production chain.

Two experiments were performed to investigate the potential application of extruded linseed and synthetic or natural antioxidants on the growth performance and pork quality. The first experiment was made as a preliminary test, for the evaluation of the tested products.

The first experiment investigated the effect of inclusion of extruded linseed (5%) in pig diets supplemented with supra-nutritional levels of vitamin E (200 ppm) and Selenium (0.21 ppm) or natural antioxidants from grape skin extracts (3 g/kg of feed) on the growth performance, carcass characteristics, meat quality and shelf-life of *longissimus thoracis et lumborum* (LTL) muscle packaged in modified atmospheres (MAP) with oxygen (70% O_2/30% CO_2) or nitrogen (70% N_2/30% CO_2) in the inner gaseous atmosphere, during refrigerated storage. For the shelf-life study, colour parameters, pH, weight losses and oxidative stability (TBARS) were analysed. In addition, for the fresh muscle, drip loss, cooking loss, shear force, TBARS for raw and cooked muscle, crude protein, crude lipids, moisture and fatty acid composition were analysed. Moisture, crude fats and fatty acid profile were determined also for the backfat. No effect of dietary treatments was found on the growth performance and carcass characteristics. Further, drip and cooking losses, oxidative stability in raw and cooked muscle,

shear force and chemical composition (moisture, crude protein and lipid contents of LTL muscle) were affected neither by linseed nor by the antioxidants addition. Linseed inclusion in pig diets increased significantly the n-3 PUFA content and reduced the n-6/n-3 PUFA ratio in both backfat and LTL muscle. As expected, high concentration of oxygen in MAP brought an increase in oxidative products and yielded redder meat, irrespective of the dietary treatments, during refrigerated storage.

In the second experiment, in comparison with the first one, was included a diet with only extruded linseed (5%), and the diet with grape skin extract was supplemented with oregano extract (2 g/kg of feed). The effects of the diets on the same parameters of the previous experiment were evaluated, in addition to the microbial growth of LTL muscle packaged only in high oxygen MAP (35% CO_2, 65% O_2). The addition of extruded linseed and antioxidants supplementation in the pig diets did not affect the growth performance, carcass characteristics and qualitative parameters of pork. Shelf-life study showed that microbial growth tended to be higher in the control group respect to other groups. However, throughout the experiment all the microbiological counts, also in control group, were under the microbiological selling threshold (6 $\log_{10}$ CFU/g) of raw meat established by EFSA. Slightly differences were found for pH values during refrigerated storage, among dietary treatments, although statistically significant, are anyway negligible from biological point of view, as the recorded pH values are within a range of normality for pig meat. Lipid oxidation in LTL muscle increased with storage time for all dietary treatments, however no significant differences were found among the groups. Our results indicate that addition of vegetal extracts did not exert a relevant antioxidant effect on the LTL muscle respect to the control diet. However, it is interesting to note that vegetal extracts were as effective as vitamin E in preventing lipid oxidation. Linseed inclusion in pig diets increased significantly the n-3 PUFA amount in the backfat, perirenal fat and LTL muscle, and also reduced the n-6/n-3 ratio up to a level below 4, considered optimal for consumers' health.

Both experiments brought similar results, obtaining an increased n-3 PUFA content and a reduced n-6/n-3 PUFA ratio without impairing the pork quality.

Further experimental investigations are needed to explain the lack of antioxidant activity of vegetal extracts and to find the optimal supplementation dose.

RIASSUNTO

La carne è un'importante fonte di proteine, aminoacidi essenziali, grassi, minerali, vitamine ed altri nutrienti. Tuttavia, negli ultimi anni diversi studi epidemiologici hanno associato il consumo di carne rossa e carne rossa trasformata ad un aumentato rischio di malattie cardiovascolari e cancro del colon-retto. Perciò i consumatori sono diventati più consapevoli riguardo alle problematiche salutistiche ed interessati nel mantenere o migliorare la propria salute attraverso la loro dieta. A questo proposito vari studi hanno proposto diverse strategie per migliorare la qualità della carne al fine di sviluppare carne e prodotti a base di carne più sani. Il profilo nutrizionale e la qualità della carne suina possono essere migliorati attraverso la manipolazione della dieta degli animali. L'integrazione della dieta suina con acidi grassi n-3 ed antiossidanti, potrebbe essere una valida strategia per migliorare la qualità della carne suina.

Lo scopo di questa ricerca è stato quello di verificare l'effetto dell'impiego di semi di lino, ricchi di acidi grassi n-3, e l'aggiunta di antiossidanti sintetici o naturali nella dieta suina, al fine di migliorare la qualità della carne. I semi di lino sono un'importante fonte di grassi, soprattutto insaturi, con elevate percentuali di acidi grassi essenziali ricchi di n-3 e n-6. Per quanto riguarda gli antiossidanti naturali, l'origano possiede efficaci proprietà antibatteriche, antiossidanti ed è anche una spezia ricca di nutrienti. Le bucce d'uva sono un sottoprodotto enologico ricco di composti polifenolici, caratterizzati da un'elevata attività antiossidante ed il loro riutilizzo potrebbe ridurre l'impatto ambientale del settore vitivinicolo.

Sono stati condotti due esperimenti per studiare la potenziale applicazione dei semi di lino estrusi ed antiossidanti sintetici o naturali sulle caratteristiche della carcassa e sulla qualità della carne.

Il primo esperimento, preliminare al secondo, ha avuto come obbiettivo quello di studiare l'effetto dell'integrazione della dieta con semi di lino estrusi (5%) addizionati di livelli sopra-nutrizionali di vitamina E (200 ppm) e selenio (0.21 ppm) o estratto di bucce d'uva (3 g/kg mangime), valutando: le prestazioni di crescita, le caratteristiche della carcassa, la qualità del muscolo *longissimus thoracis et lumborum* (LTL), e la shelf-life di quest'ultimo confezionato in atmosfera modificata (MAP) con ossigeno (70% O_2/30% CO_2) o azoto (70% N_2/30% CO_2) a temperatura di refrigerazione, durante lo stoccaggio. Per lo studio della shelf-life, sono stati analizzati i seguenti parametri: pH, colore, perdita di peso e stabilità ossidativa (TBARS). Inoltre per il muscolo fresco, sono stati analizzati *drip* e *cooking loss,* tenerezza (WBSF), stabilità ossidativa per i muscoli crudi e cotti, le proteine ed i lipidi grezzi, umidità e la composizione acidica. L'umidità, i lipidi grezzi ed il profilo degli acidi grassi sono stati

determinati anche per il grasso sottocutaneo. Non è stato riscontrato alcun effetto dei trattamenti dietetici sulle prestazioni di crescita e le caratteristiche della carcassa. Inoltre *drip* e *cooking loss*, stabilità ossidativa nei muscoli crudi e cotti, tenerezza e composizione chimica (umidità, proteine e lipidi grezzi) non sono stati influenzati né dall'inclusione dei semi di lino né dall'aggiunta di antiossidanti. L'inclusione dei semi di lino nelle diete dei suini, ha aumentato in modo significativo il contenuto degli acidi grassi n-3 e ha ridotto il rapporto n-6/n-3 sia nel grasso sottocutaneo che nel muscolo LTL. Come previsto, il confezionamento in MAP ad alta concentrazione di ossigeno ha determinato un aumento dei prodotti ossidativi, mantenendo un colore più rosso della carne durante lo stoccaggio refrigerato, indipendentemente dal trattamento dietetico.

Nel secondo esperimento si è valutato, in aggiunta alle diete del primo, l'effetto di una dieta con l'inclusione di solo lino (5%), ed alla dieta che includeva l'estratto di bucce d'uva è stato aggiunto anche l'estratto di origano (2 g/kg mangime). Sono stati valutati, gli effetti delle diete sugli stessi parametri dell'esperimento precedente. A differenza del primo esperimento, è stata utilizzata un'unica formulazione di MAP ad alta concentrazione di ossigeno (35% CO_2, 65% O_2); ed inoltre è stata valutata l'evoluzione microbiologica durante il periodo di conservazione refrigerata del muscolo LTL. Le diete integrate con semi di lino estrusi ed antiossidanti non hanno influenzato l'accrescimento, le caratteristiche della carcassa e i parametri qualitativi della carne. Lo studio sulla *shelf-life* ha mostrato che la crescita microbica tendeva ad essere più elevata nel gruppo di controllo rispetto agli altri gruppi. Tuttavia, durante l'intero esperimento tutte le conte microbiche erano al di sotto del limite soglia per la vendita della carne cruda (6 $\log_{10}$ CFU / g), stabilito dall'EFSA. Durante lo stoccaggio refrigerato sono state riscontrate lievi differenze tra i trattamenti dietetici per i valori di pH, che tuttavia rientrano in un intervallo di normalità per la carne suina. L'ossidazione dei lipidi nel muscolo LTL è aumentata durante la conservazione per tutti i trattamenti dietetici, senza differenze significative tra i gruppi. I nostri risultati indicano che l'aggiunta di estratti vegetali non ha avuto un effetto antiossidante sul muscolo LTL rispetto alla dieta di controllo. Tuttavia, è interessante notare che gli estratti vegetali sono stati efficaci quanto la vitamina E nel prevenire l'ossidazione dei lipidi. L'inclusione dei semi di lino nelle diete dei suini ha aumentato significativamente la quantità di acidi grassi n-3 nel grasso sottocutaneo, grasso perirenale e nel muscolo LTL, ed ha anche ridotto il rapporto n-6/n-3 fino ad un valore inferiore a 4, considerato ottimale per la salute dei consumatori.

Entrambi gli esperimenti hanno condotto a risultati simili, ottenendo un maggiore contenuto di acidi grassi n-3 ed una riduzione del rapporto n-6/n-3, senza compromettere la qualità della carne.

L'assenza di effetti significativi sui parametri qualitativi può essere attribuito al basso livello del estratto vegetale nell'integrazione della dieta suina, per cui ulteriori ricerche sperimentali sono necessarie per trovare la dose di integrazione ottimale.

CHAPTER 1.

GENERAL INTRODUCTION

1.1. Evolution of meat consumption

In ancient times and during the Middle Age up to the Industrial Revolution, meat consumption has been present but insignificantly, more often reserved for the rich and the aristocrats. The fruits were much more consumed than nowadays, vegetables and, especially, cereals and derivatives were more consumed than animal products, including meat that, even in aristocratic environments, was present once or twice a day. In this context, the flesh began to become a symbol of preciousness, a sign of high social standing.

Meat consumption has been a very important evolutionary factor, this because, our ancestors had to learn to hunt. Hunting requires communication, planning, and use of tools, each of them requires greater mental abilities. At the same time, adding meat to the diet has therefore allowed to develop a series of morphological changes that helped to develop the language and Man's intellectual abilities. Also the controlled used of fire increased meat consumption opportunities as it increased food intakes. Meat was made more palatable through searing and roasting, also smoking preserved it for later consumption (Smil, 2002).

Another evolutionary factor was the domestication of many animal species that began about 11,000 years ago with sheep and goats and then progressed to cattle, pigs, horses and camels (Alvard & Kuznar, 2001).

The increase in meat consumption is a key indicator of food changes that has occurred as a result of economic development: today, animal products provide about one-third of the protein consumed by the world's population, and meat, in particular, is the main source of protein with high biological value.

The most consumed species are pork, chicken and cattle, though with a different annual trend: beef consumption is basically stable since over 20 years, while at the same time poultry consumption is almost twice as high (Fig. 1.1).

However, it is worthwhile to analyse either the individual regions of the world to see how and where people's eating habits have changed over time. To do this, in the Figure 1.2 we have the evolution of meat supply in European Union. Up to about fifty years ago, meat consumption was lower. Then, with the rapid economic development, the demand of meat has been gradually increasing, and in the '80s production has increased, particularly that of beef. From the '70s onwards the preference for pork has exceeded that for beef and veal, so that if in

1970 the average utilization of pork was 28.5 kg per capita and that of beef amounted to 21.4 kg per capita (FAO Data), in 2013 in EU was used 39 kg of pork, against the 14.9 kg of beef and 22.5 kg of poultry.

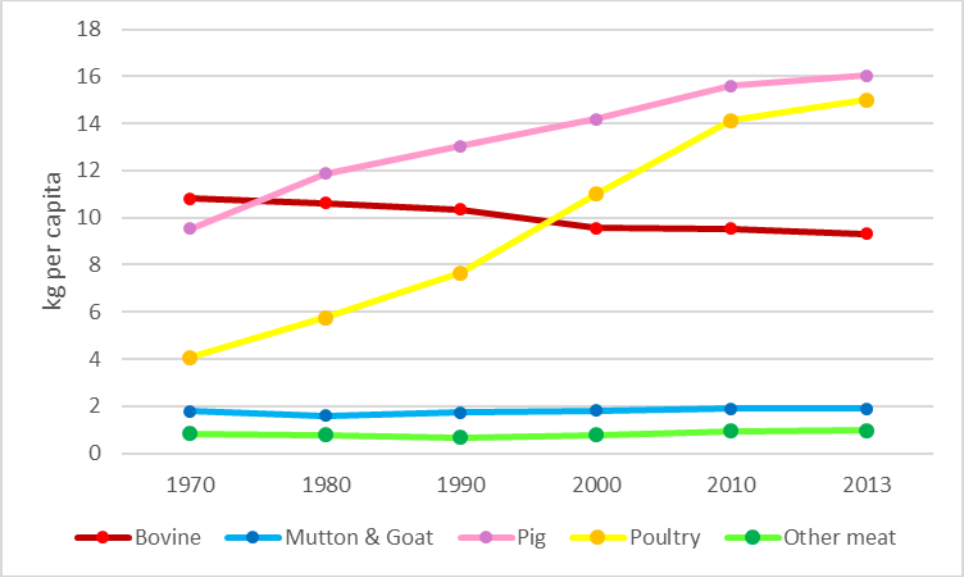

Figure 1.1. Evolution of meat supply (kg per capita) in the world (Source: FAO DATA)

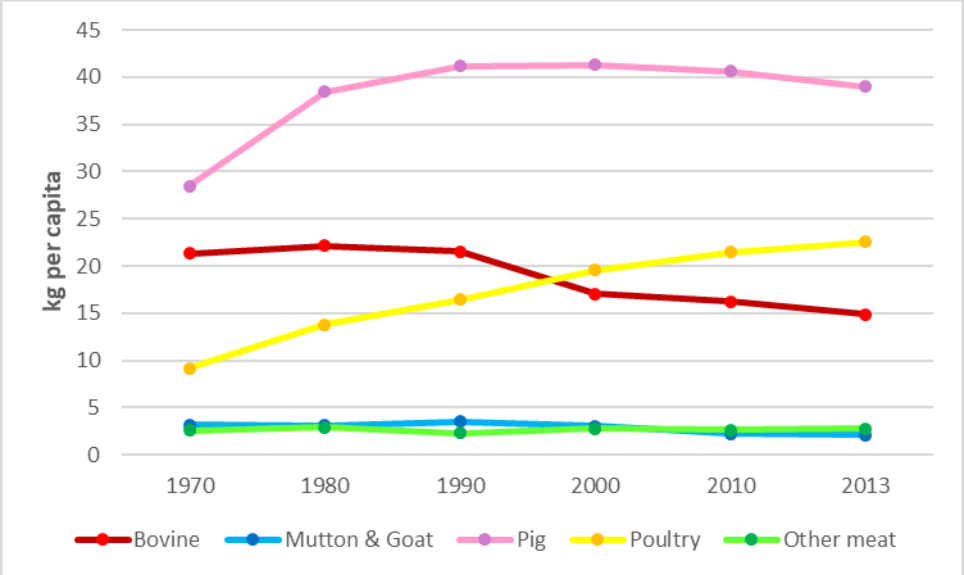

Figure 1.2. Evolution of meat supply (kg per capita) in EU (Source: FAO Data)

As the meat has a high cost, its consumption is not uniform all around the world. People from less developed countries are eating low quantities of any kind of meat. Also strong cultural or religious traditions impact the rate of meat consumption. For example, in India the major part of population is vegetarian. So, we can say that some countries are “big-meat eaters” (Fig. 1.3), who exceed the global average, while others consume less than a quarter of it.

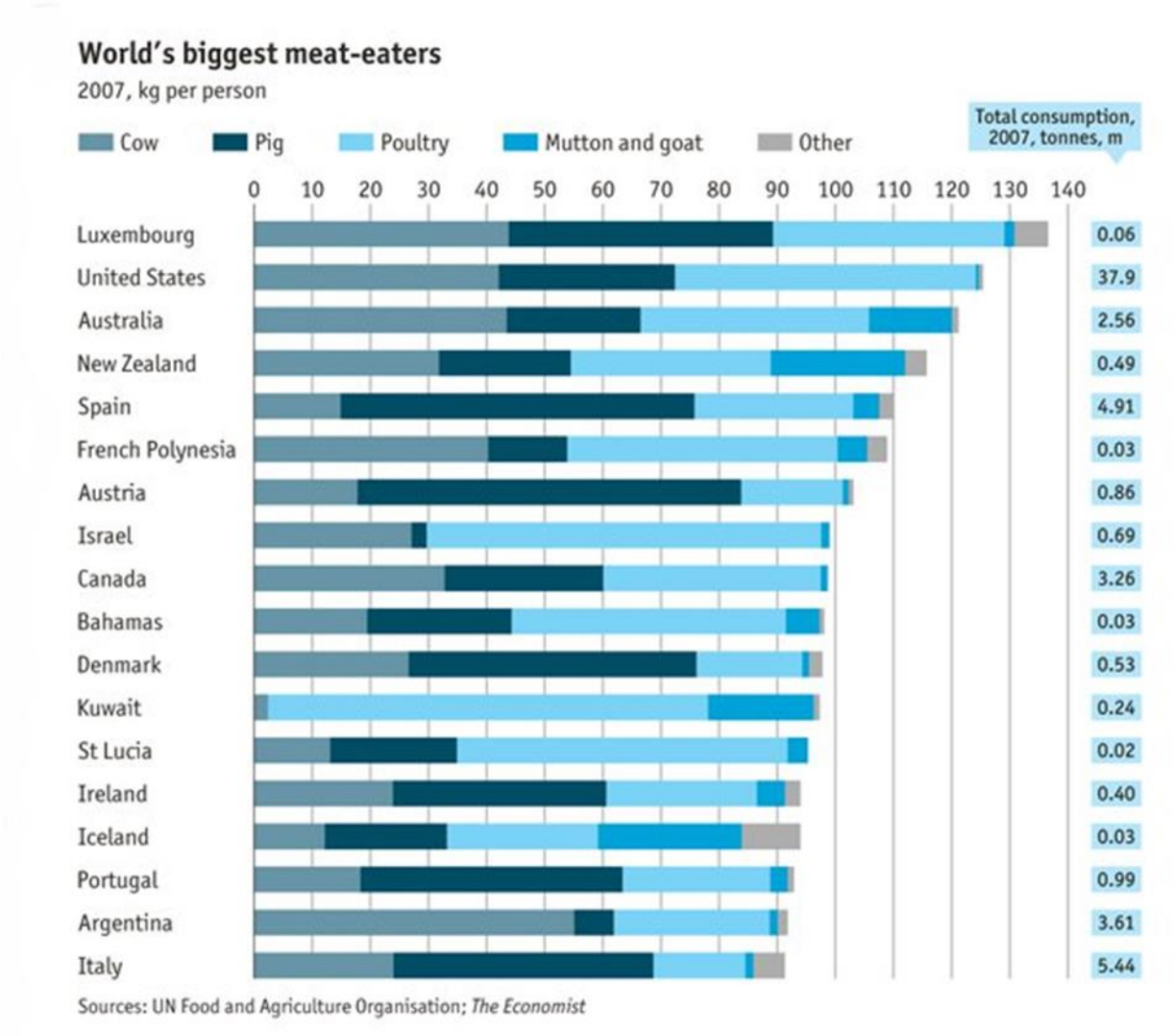

Figure 1.3. World's biggest meat-eaters. Source:US Department of Agriculture, 2007

1.2. Pig meat consumption

Pork is the most popular type of meat eaten in European Countries. During the years the pig consumption has continued to increase, but in the last 20 years it has stabilised. In Table 1.1 is represented the evolution of pork meat supply in different European Countries between 1970 and 2010.

In Italy, pigs are mainly raised in the Po valley. Lombardy is the leading region, with about 4 million heads reared, followed by Emilia Romagna, Piedmont and Veneto. Over 84% of the national pig heritage is concentrated in these 4 regions. The destination of meat produced by pig reared in Italy is for the production of fresh meat or derivatives products such as salami. Italy is one of the very few countries in the world to have a very marked salami culture. It is for this reason that pig farming is of primary importance in the Italian market, also as regards the export.

Table 1.1. Evolution of pork meat supply in different Europe countries between 1970 and 2010. (Data expressed as kg per capita by year). Source: FAO Data

Year	Pork meat					
	EU	Portugal	Spain	Italy	Germany	France
1970	28.5	10.9	13.8	12.9	44.9	30.5
1980	38.5	15.2	31.1	24.9	60.1	37.5
1990	41.1	28.7	47.9	31.9	59.5	33.8
2000	41.3	40.1	63.8	37.5	52.8	37.6
2010	40.6	42.8	49.4	41.9	54.2	34.5

In Italy, there is the production of two different types of pigs: the heavy one, with the highest lipid content used for processing, and the light one, leaner, used for fresh consumption. The so-called heavy pig weighs around 120-180 kg live weight, while the pigs slaughtered for fresh meat consumption are around 100-110 kg live weight (with local variations). Breeds and breeding systems are different for the two types and there are also differences in some sensory characteristics as well as some aspects of the composition. In the finished product intended for consumption, the quality is evaluated by examining the chemical composition (the content in nutrients), the sensory characteristics and the safety features of use. Safety depends on the presence of undesirable, harmful or toxic substances that may have different origins.

Recently, a chain of the so-called "intermediate pig" was organized which, slaughtered at the live weight of 125-135 kg, presents a shorter breeding cycle and leaner carcasses than the traditional "heavy" and therefore suitable for fresh consumption and cooked processing.

1.3. Muscle composition

Meat is generally defined as the part of the animal (cattle, pigs, sheep, goats etc.) that is consumed as food. It is composed of muscle mass, fat, connective tissue, and other tissues which are defined only after slaughter of the animals and the following chemical-physical modifications (Cobos & Diaz, 2014). According to European Union Legislation, meat may also refer to other edible tissues such as offal (liver, heart, kidney, brain etc.), including blood and viscera.

Meat consists of several tissues:

- Muscle tissue
- Connective tissue
- Adipose tissue
- Nervous tissue
- Blood vessels

- Epithelial tissue

All these tissues play a key role in the determination of meat quality.

Muscle is covered by a connective tissue sheath, named Epimysium (Fig. 1.4), which has the task to protect the muscle during the execution of the movement, which means to protect it from friction against other muscles and bones. Within this sheath originate perimysium and endomysium. Perimysium groups muscle fibres into bundles or fascicles and contains the larger blood vessels and nerves; endomysium covers each single fibre. Between the bone and the epimysium there is a unique structure named tendon, which binds muscles and bones (Lawrie & Ledward, 2006).

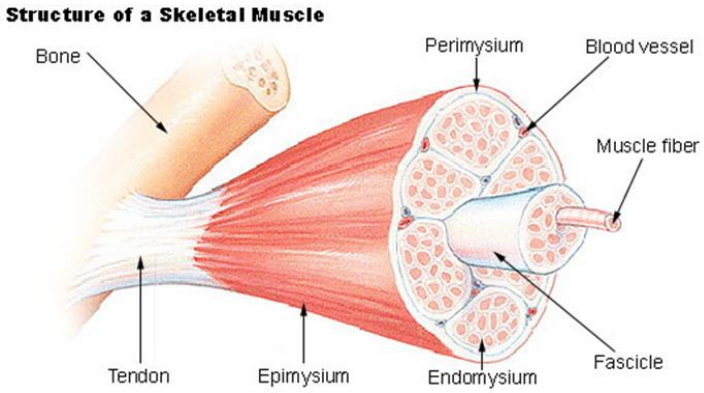

Figure 1.4. Structure of a skeletal muscle. Source: https://www.ck12.org/biology/skeletal-muscles/lesson/Skeletal-Muscles-BIO/

Muscle tissue is a conglomerate of muscle fibres. These muscle fibres are composed of elongated cells, with a diameter of 10-100 µm and a length up to 30 cm. Shorter are the fibres, more tender is the meat. Bigger is the amount of muscle tissue, more precious is the meat. These fibres are not isolated, but clustered in fascicles and wrapped in connective tissue. The cell membrane that surrounds the muscle fibre is called sarcolemma (Fig. 1.5). Inside the sarcolemma there are numerous nuclei surrounded by sarcoplasm, that contains the myofibrils, mitochondria, sarcoplasmic reticulum and other cellular elements. Each muscle fibre is composed by almost 1000 myofibrils wrapped by sarcoplasmic reticulum (which holds a reserve of calcium ions needed to cause a muscle contraction). Each myofibril consists of bundles of contractile filamentous proteins and is made up of short structural units known as sarcomeres. The sarcomeres represent the lowest contractile unit of the muscle. Coordinated contraction of millions of sarcomeres in a muscle is the basis of the muscle

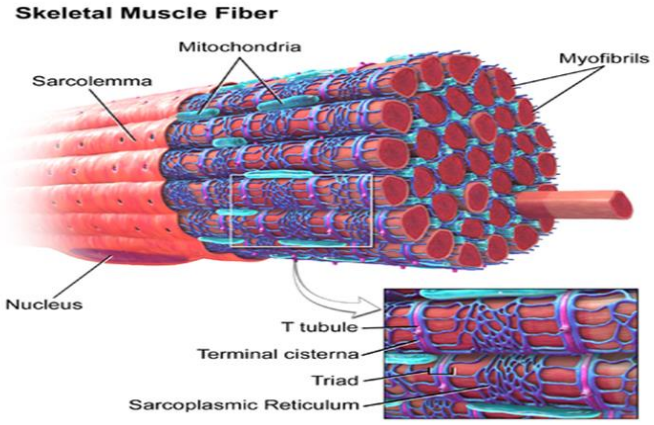

Figure 1.5. Skeletal muscle fibre. Source: https://biologydictionary.net/sarcomere/

mechanism activity. Sarcomeres are mainly composed of thin filaments of actin and thick filaments of myosin (Huff Lonergan *et al.*, 2010; Warner, 2016). These protein filaments, during muscle contraction, flow over each other and overlap, causing the shortening of myofibrils and, consequently, of muscle fibre (Figure 1.6). In this way, during the muscular contraction there is the flow of actin filaments to those of myosin. An important role for muscle contraction is also made by the sarcoplasmic reticulum, which wraps the myofibrils, penetrating inside them. In the Figure 1.6 are represented the events during a muscle contraction, where, the laterally projecting heads (cross bridges) of the thick myosin myofilaments come in contact with the thin actin myofilaments and rotate on them. This pulls the thin myofilaments towards the middle of the sarcomere beyond the thick myofilaments. The Z lines come closer together and the sarcomere becomes shorter. The length of the A band remains constant. Myofilaments stay at the same length. Free end of actin myofilaments moves closer to the centre of the sarcomere, bringing Z lines closer together. The I bands are shortening and H zone narrows. A similar action in all the sarcomeres results in shortening of the entire myofibril, and thereby of the whole fibre and the whole muscle. A contracted muscle becomes shorter and thicker and its volume remains the same (Cobos and Diaz 2014; Huff-Lonergan *et al.*, 2010; Lawrie and

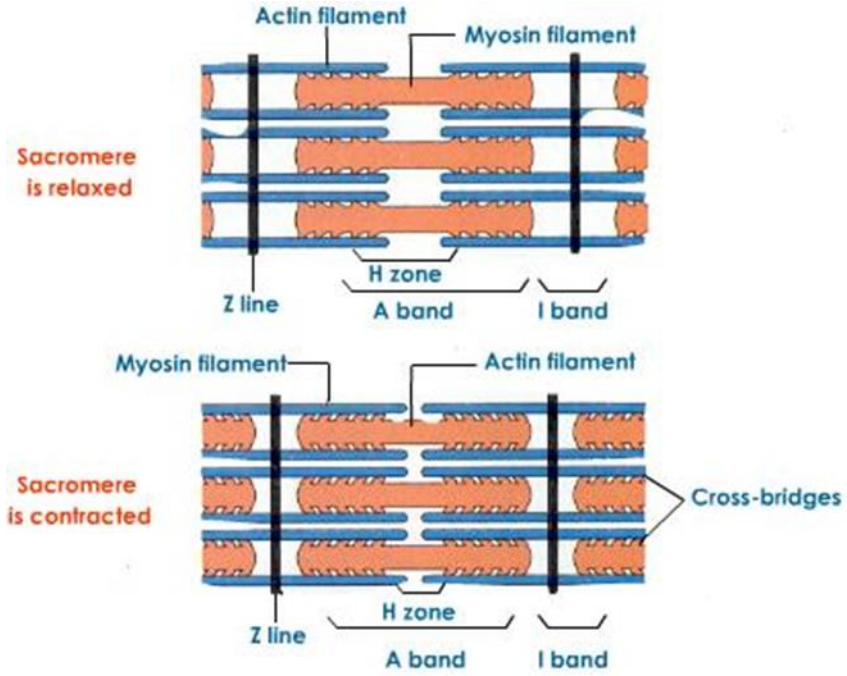

Figure 1.6. Events during Muscle Contraction. Source: https://medchrome.com/basic-science/physiology/mechanism-of-skeletal-muscle-contraction/

Ledward, 2006). For a contraction are necessary calcium, which is released from sarcoplasmic reticulum due to nerve impulse and biochemical reaction, and energy (ATP), that comes from diet and is stored in muscle and liver as glycogen.

1.4. Conversion of muscle into meat

After stunning, the brain is dead, but muscle is still alive, is still sending some nervous impulses. However, there are several changes in the biochemistry of the muscle, due to the lack of circulating oxygen, altered enzymatic reactions, cellular degradation and cessation of anabolic production of metabolites (Matarneh *et al.,* 2017). Hence, after the slaughter, the flesh of animals is not immediately edible, but it must pass a variable time during which the meat becomes suitable for consumption. Transformation of the muscle into meat occurs after the death of the animal. Muscle structure and composition undergo a sequence of physical and chemical modifications (Lawrie & Ledward, 2006; Warner, 2016).

A few hours after the slaughter, adenosine triphosphate (ATP) levels in skeletal muscle fibers have been completely exhausted, thus preventing myosin head detachment from actin. At the same time, as a consequence of ATP exhaustion in skeletal muscle fibers, the sarcoplasmic reticulum loses its ability to recall calcium ions from the sarcoplasm. As a result, the calcium ions already present in the sarcoplasm, as well as those that continue to leak out of the sarcoplasmic reticulum, trigger a sustained contraction in the fiber. Also, with less efficiency in energy generation, the lactic acid production is favored. Its accumulation results in a decrease of tissue pH from values around neutrality to 5.4-5.8; pH reduction is actually essential for good meat preservation, because the low pH reduces microbial proliferation (Matarneh *et al.,* 2017). Because no ATP is available, the cross-bridges between thick and thin filaments cannot detach. All skeletal muscles lock into a contracted position becoming rigid. This physiologic state, is termed *rigor mortis*, Latin for "stiffness of death", where muscle filaments can no longer slide over one another, and the muscle becomes inextensible. After a variable period of time, the resolution of rigor mortis happens with a progressive softening of the muscle tissues due to the action of proteolytic enzymes (Lawrie & Ledward, 2006; Matarneh *et al.,* 2017; Warner, 2016).

1.5. Pig carcass quality

Pigs are put through two distinct procedures when they are killed for meat consumption. First, they are stunned, and then they are slaughtered. Electricity and carbon dioxide are the principal stunning agents used today. The penetrating captive bolts is widely used as a back-up method when other methods fail (EFSA, 2004).

According to the EU rules, pig carcasses are classified taking into account the lean meat percentage. Six classes are used, identified with letters S E U R O P (EU Regulation 2013).

S (Superior) – more than 60% (and carcass weight less than 110 kg)

E (Excellent) – 55 to 60%

U (Very Good)– 50 to 55%

R (Good) – 45 to 50%

O (Fair)– 40 to 45%

P (Poor)– less than 40%.

In recent years, carcass composition changed, with increasing lean meat proportion and reducing fat content, improving the quality of pork and fat tissue. A good strategy for enhancing carcass quality could be animal feeding (Vehovský *et al.,* 2018).

1.6. Meat quality

Quality may be defined as a totality of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs (ISO 8402), or as a set of features of a product or service that satisfy customer needs (ISO 9000).

Meat quality is normally defined by the compositional quality (lean to fat ratio) and the sensory factors such as visual appearance, smell, firmness, juiciness, tenderness, and flavour. The nutritional quality of meat is objective, whereas sensory quality, as perceived by the consumer, is highly subjective (Heinz & Hautzinger, 2007).

Meat quality is influenced by several factors such as pre-slaughter effects, *post mortem (pm)* changes in muscle tissues, environment, processing and storage, etc. (Heinz and Hautzinger 2007; Joo *et al.,* 2013).

The quality of meat could be determined by:

- Technological quality traits;
- Sensory quality traits.

Technological meat quality includes the functional meat quality traits, such as pH, colour, water holding capacity (WHC), texture and amount of fat.

Sensory quality traits determine the meat quality at the point of consumption and include traits such as tenderness, juiciness and flavour.

1.6.1. Meat pH

The muscle of the live pig has a pH around 7.2. After death there is a gradual decrease in pH, the muscle becomes more acidic, because of the production of lactic acid by glycolysis. pH is generally measured within one hour of slaughter (initial pH or pH_{45min}) or within 24 hours

(ultimate pH or pH_{24h}). In normal meats the desired degree of acidity should be between 5.5 and 5.8. If it is lower we have PSE meat, if it is higher DFD (Fig. 1.7).

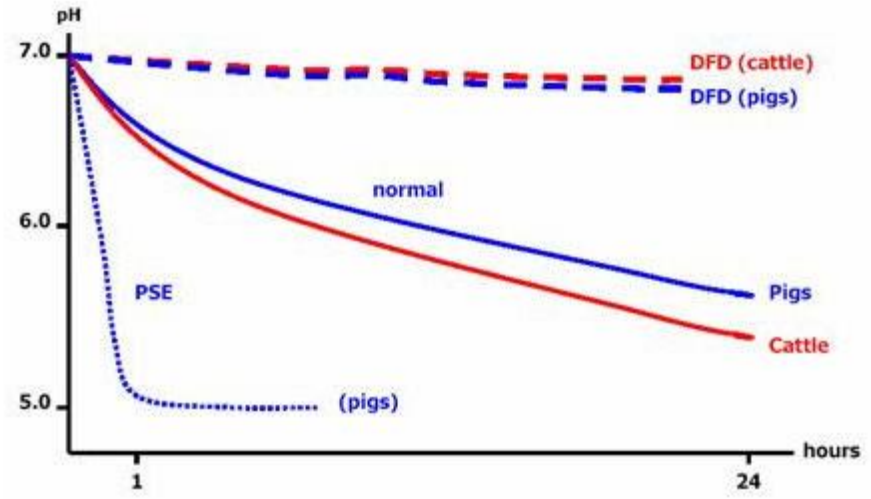

Figure 1.7. Changes in pH. Source: Heinz and Hautzinger (2007)

PSE stands for Pale Soft and Exudative. In PSE meat, glycolysis occurs much faster, and there is a rapid post mortem pH decline, while the muscle temperature is too high. The meat reaches a pH less than 5.8 in few seconds after death. *Post mortem* anaerobic glycolysis produces large amounts of lactic acid when the temperature is still high, with the denaturation of muscle proteins, reducing their ability to hold water (the meat drips and is soft and mushy). In the PSE meat there are soft and yielding muscles; aqueous and light surface. Compared to normal meat the PSE has increased losses of liquid, paler colour, increased risk of development of rancidity, reduced yield in product and soft texture (Fernandez *et al.*, 1994).

DFD stands for Dark Firm and Dry. In DFD meat, glycolysis does not occur in the normal measure, so the meat has a pH of more than 6.2 and sometimes up to 7. The DFD condition is associated with stress phenomena, but the mechanism is different from that of PSE meat. The cause lies in the prolonged stress before slaughter (24 hours or more) and the consequent reduction of a large part of glycogen stores before death, so that the extent of the *pm* anaerobic metabolism is reduced and the meat does not go to the normal acidification, lacking lactate. A pH between 6 and 6.2 is to be considered of attention, because it suggests a tendency to the DFD features (Adzitey & Nurul, 2011). The meat has a darker colour than normal, has a high myofibril volume and the light can penetrate deeply without being dispersed. The luminous waves will bring to the surface the colour of myoglobin, of the deep layers - purple red. Hence, the penetration of oxygen is limited while the enzymatic activity with oxygen consumption is high, so there is a faster formation of metmyoglobin. The cutting surface

appears dry as a result of a high WHC. DFD meats are suitable for the production of cooked or emulsified products, while they are unsuitable for raw or fermented products, and severely discounted at the marketplace (Guàrdia *et al.*, 2005; Holmer *et al.*, 2009).

1.6.2. Meat colour

The colour of the meat depends on many factors such as the animal species, the sex, the age and the type of feeding (Hunt *et al.*, 2012), but certainly the most important factors are two: the concentration of myoglobin in the muscle fibre and the structure of the fibres themselves. Myoglobin is a protein that transports oxygen to muscle tissues, and is therefore essential to ensure continuous energy reserves for muscle contraction. The molecule is very complex, it includes a non-protein group called the heme ring, which consists of a porphyrin molecule bound to an iron (Fe) atom (Fig. 1.8). The red colour of meat is given by the heme pigment, but the change in colour is linked to a single chemical element: iron (Mancini & Hunt, 2005).

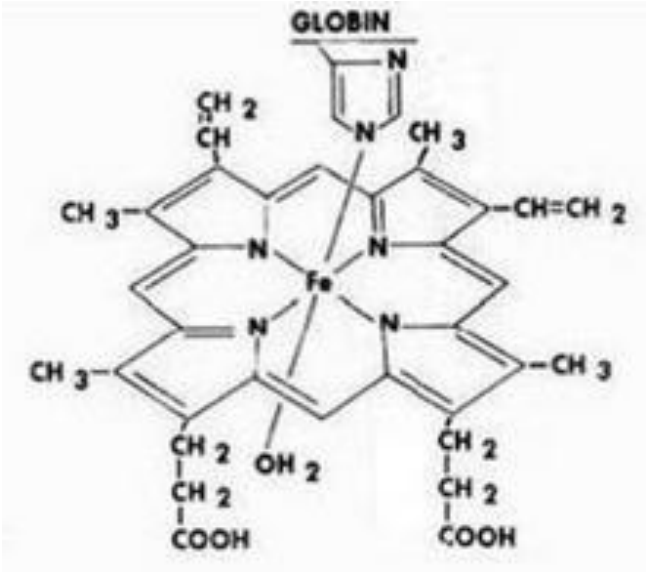

Figure 1.8. Structure of Myoglobin

Myoglobin's colour depends on oxygen levels. In fresh meat, myoglobin (Mb) has a purple colour and the iron atom is in the ferrous (Fe^{2+}) oxidation state. When it carries oxygen, it turns into oxymyoglobin (MbO_2) and becomes bright red, the iron is complexed and stabilized. But then it oxidizes and becomes Metmyoglobin (MMb) (Fig. 1.9). The iron atom is now in the ferric (Fe^{3+}) oxidation state, and the colour will be brown (Boles & Pegg, 1999; Richards, 2013).

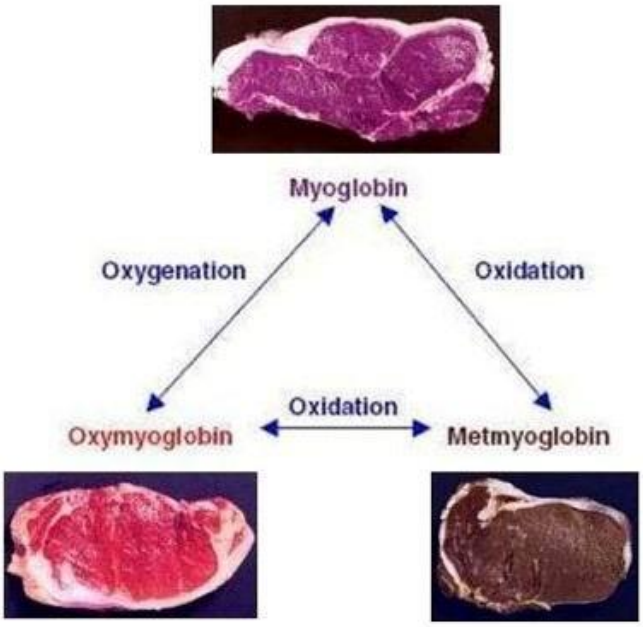

Figure 1.9. Interconversion of meat pigments. Source: Boles & Pegg, (1999)

The colour of the meat also depends on the structure of the muscle fibre. The meat with very tight muscle fibres, typical of muscles that work over time and continuously (slow-twitch), called "red fibres", will have a redder colour because richer in myoglobin, while muscle fibres that work in quick shots in a short time (fast-twitch), called "white fibres", will be poorer in myoglobin. Therefore, a high concentration of slow-twitch fibres in the animal muscle, make the meat to become darker (Listrat *et al.*, 2016).

Meat colour depends also on the animal species. Hence, beef has a higher concentration of myoglobin respect to pork, veal or lamb, resulting in a more intense colour. Also, the age of the animal influences the myoglobin concentration, older animals have darker pigmentation (Heinz & Hautzinger, 2007).

1.6.3. Water-holding capacity

Water holding capacity(WHC) of meat is defined as the ability of the *pm* muscle (meat) to hold all or part of its water, even though external pressures (e.g. gravity, heating, pressing) are applied to it (Huff-Lonergan & Lonergan, 2005; Watanabe *et al.*, 2018).

WHC is an attribute of the meat of great importance because it influences the appearance of the raw meat, its behaviour during cooking, the juiciness to mastication, and the response of meat to processing and seasoning. The WHC is in fact one of the qualitative criteria that allow distinguishing meat according to their technology attitude. Meat with low WHC, highlighted

by one considerable exudation of raw chilled meat, are considered undesirable, both for direct consumption and for processing, because tends to produce inferior processed products. Meat with high WHC such as DFD meat is unsuitable for most consumers, but it is suitable in many transformation processes (Warner, 2014).

In normal meat, WHC decreases with the decrease in pH, both due to the decreased availability of ionic bonds to bind water (lower ionization near the isoelectric point) and to the aggregation of myofibrils, which causes the expulsion of water. Therefore, WHC is influenced mainly by the pH and by others factors such as species, age and muscle function (Joo *et al.*, 2013; Listrat *et al.*, 2016).

1.6.4. Intramuscular Fat or Marbling

Marbling refers to visible fat found between muscle fibre bundles. It has a considerable effect on eating quality traits such as juiciness, tenderness, flavour and also on visual aspect of the meat. The quantity of intramuscular fat depends on many factors such as animal breed, slaughter weight, feeding strategy and growth rate (Joo *et al.*, 2013).

Intramuscular fat acts as a lubricant between muscle fibres, and decreases the density of proteins for a certain amount of meat: for these reasons a meat that contains a greater amount of marbling fat (fat infiltrated between the muscle fibres) will be more tender; meat which has little marbling may be dry and flavourless (Listrat *et al.*, 2016).

Meats with higher amount of intramuscular fat are in fact more sapid and tastier than those lean, especially because some of the compounds that influence the aroma and taste are fat-soluble. Also helps to keep the meat tender while cooking, keeping the right amount of water inside the meat. Hence, intramuscular fat plays an important role in the meat sensory characteristics, influencing juiciness, flavour and tenderness (Heinz & Hautzinger, 2007).

1.6.5. Tenderness

Tenderness is considered one of the most important parameters of meat quality. Tenderness, which gains a judgment only at the time of chewing, depends on both the composition of the meat itself and the technologies of slaughter and ageing (Joo *et al.*, 2013).

The tenderness of meat is the characteristic that most influences the level of consumer satisfaction. The perception of tenderness during consumption is defined by parameters such as softness, resistance to teeth pressure, the ease of fragmentation of the fibres, the adhesion between the fibres and finally the residue, that is the quantity of connective tissue that remains after mastication (Szczesniak & Torgeson, 1965).

Meat tenderness is mainly affected by the degree of proteolytic breakdown of myofibril proteins, fibre type composition of muscle, amount of connective tissue and the concentration of intramuscular lipid (Joo *et al.*, 2013; Maltin *et al.*, 2003).

In animals, different muscles perform different functions. Some are functional to locomotion and tend to be less tender, as the connective content and the cross-links of the collagen are higher. Other muscles, called attack muscles, do little work and, consequently, tend to be more tender. These muscles are the most valuable from a commercial point of view, as the muscles of the loins and of the vertebrae (for example *longissimus thoracis et lumborum*) (Joo *et al.*, 2013; Listrat *et al.*, 2016).

The connective tissue consists of fibrous proteins such as collagen and elastin, surrounded by a proteoglycan matrix. Collagen is the major component of the connective tissue, which has a tough, rigid structure. Its content is decisive, because it's the part that is less soluble and more resistant to cooking; its quantity and its resistance increase with the age of the animal and are certainly higher in male subjects, except for castrated animals. During the ageing and cooking process the collagen is broken down and denatured, forming a gelatine substance that makes the meat more tender (Maltin *et al.*, 2003; Nishimura 2010).

Meat tenderness is affected also by the structural modifications that occur during the transformation of muscle into meat. Proteolysis of muscle *pm* varies among different muscles. Koohmaraie (1995) suggested that proteolysis of myofibrillar proteins is the cause of meat tenderization. The function of these proteins is to maintain the structural integrity of myofibrils therefore, proteolytic degradation of these proteins is the cause of meat tenderization.

1.6.6. Juiciness

Juiciness, or the attitude of meat to produce liquid when it is chewed (quantity of water stored after cooking), is related to the degree of less or greater dryness of the meat.

Generally, juiciness is perceived in two ways: when it corresponds to the quantity of water that is felt in the mouth during the first chewing acts, or when is linked to saliva stimulation by fats.

The carcass undergoes a loss of fluids already in the slaughter phase. The meat is in fact made up of 75% of water, of which 65% in free form and 10% linked to proteins. Juiciness is positively related to WHC and the amount of intramuscular fat in the meat (Listrat *et al.*, 2016).

The human perception of juiciness is increased as the intramuscular fat content in meat increases. Therefore, juiciness depends mainly on WHC and intramuscular fat (Jeremiah *et al.*, 2003).

1.6.7. Flavour

Meat flavour is a function of the sensory sensations of odour and taste. Meat flavour can be influenced by species, sex, age, stress level, amount of fat, and diet of animal (Joo *et al.*, 2013).

Generally, most of meat flavour develops when it is cooked, since raw meat has little or no flavour. The flavour derives from the interaction of precursors from the fat and lean components of the meat. This interaction led to the formation of several volatile compounds. Several studies demonstrated that during thermal processes, meat could release more than 1000 volatile compounds. Each animal species have their own distinct flavour (Mottram, 1994, 1998). Flavour can also be added to meat through brining and marinating.

1.6.8. Safety

Meat is a very complex food system, with chemical and chemo-physical characteristics that allow the development of a great variety of microorganisms, above all bacteria, some of which are useful and indifferent, some altering and pathogens.

Naturally the meat has a microflora that is closely related to (Cerveny *et al.*, 2009; Dave & Ghaly, 2011):

- Chemical composition (water activity, content of nutrients, the structure of the foodstuff);
- Environment in which it is produced (conditions of rearing and slaughter);
- Conditions of transformation, distribution, conservation and consumption.

After slaughter, the carcasses are contaminated with microorganisms coming from the skin, faeces and intestine of the animal, as well as from the air, water, soil, operators and processing. Microbial populations that typically contaminate raw meat consist of a wide variety of species such as *Staphylococcus, Micrococcus, Pseudomonas, Moraxella, Psychrobacter, Corynebacterium, Flavobacterium, Chromobacterium, Enterobacteriaceae*, lactic bacteria, *Brochothrix thermosphacta*, *Bacillus*, yeasts and moulds (Omorodion & Odu, 2014).

Subsequently, the type of meat preservation determines a sort of selection of the microflora that initially contaminates the meat itself. In fact, only 10% of the microorganisms that are present on the meat is able to grow during the refrigerated storage phase, and of these only one part is able of causing alteration (Borch *et al.*, 1996).

From the beginning of the slaughter to the cutting of meat in small cuts, the number of microorganisms increases and the composition changes. At the end of the slaughter, before the refrigeration, on the surface of the carcasses the bacterial population ranges from 10^2 to 10^5 CFU/cm^2 depending on the hygiene of the slaughter. About 50% is given by Gram-negative bacteria, the other 50% is given by Gram-positive bacteria, moulds and yeasts (Cerveny *et al.*, 2009).

While the carcasses are hung in fridge the microbial charges increase slowly and progressively with few genera that take over from the others. The contamination occurs especially on the surface of the carcass with the development of Gram-positive microorganisms such as *Micrococcus spp., Lactobacillus spp., Bacillus spp.,* and enteric and environmental Gram-negative *Enterobacteria spp., Pseudomonas spp*. Part of these microorganisms comes from the intestine of the animals and is kept under control with correct slaughtering operations. Another part derives from the work environments (slaughtering, cutting) and is kept under control with adequate cycles of sanitizing the rooms (Omorodion & Odu, 2014).

It is well known that increasing the cutting degree of meat, increases the plasma liquids and the surfaces exposed to the contaminants. Bigger is the cutting degree, bigger is the microbial load, and this way decreases the shelf life of the product.

During the years, several methods of meat preservation were used including drying, smoking, brining, fermentation and refrigeration (Dave & Ghaly, 2011). However, today new preservation techniques are used in order to reduce the process of spoilage (addition of additives, packaging, dietary manipulation).

The most common methods for increasing shelf-life of meat during refrigerated storage are vacuum packaging and modified atmosphere packaging (MAP).

Vacuum packaging

In vacuum packaged meat, the air is removed before the pack sealing. However, 1% of oxygen remains in vacuum packages after prolonged storage (Cerveny *et al.,* 2009). The effectiveness of this technique is given by the utilization of proper packaging films of low gas permeability. Vacuum packaging retards microbial growth, prevent meat contamination and evaporative losses (Jeremiah, 2001).

Vacuum packaging is not widely used for retail packaging of red meat, since the oxygen elimination lead to the purplish-red colour of meat due to the deoxymyoglobin formation; but is used for poultry (Nassu *et al.,* 2010).

Modified atmosphere packaging

In MAP, meat products are packaged with an alternative atmosphere created by gas mixtures in different proportions, mainly oxygen (O_2) and carbon dioxide (CO_2), balanced with inert nitrogen. The purpose of this technique is to extend the preservation of meat quality, by blocking or slowing down the chemical (protein and lipid degradation) or biological (microbial growth) mechanisms that determine the product deterioration (Cerveny *et al.*, 2009; Jeremiah, 2001). Generally, CO_2 concentrations range from 10 to 40% to inhibit the microbial growth, and O_2 concentrations range from 60 to 80% in order to maintain the bright red colour of meat (Nassu *et al.*, 2010). It was shown by Jakobsen and Bertelsen (2000) that 20% of O_2 it is sufficient to maintain meat colour, and MAP with more than 55% of O_2 do not lead to a higher stability of colour, but, instead decreases the oxidative stability.

1.7. Meat nutritional value

Meat is the primary source of nutrients absent or scarcely found in vegetable products. It is therefore a food with high nutritional value and of fundamental importance in a proper nutrition, especially for the supply of certain macro-nutrients that differentiate it from other foods.

The chemical and nutritional composition of the meat is strongly influenced by many factors of productive order, including: animal species, genetic type, sex, slaughter age, nutrition techniques, breeding technology, and processing, preservation and distribution of the meat (Table 1.2). Also, the muscle type affects meat characteristics and its chemical composition (Cobos & Diaz, 2014).

Meat composition is represented by water (about 70-80%), proteins (about 20%, composed of essential amino acids), lipids (3-7%), mineral salts (2-4%, especially phosphates and chlorides, potassium, iron, magnesium, calcium and sodium) and sugars (0.5%, that after slaughter are fermented in lactic acid by bacteria) (Pereira & Vicente, 2013).

Water

In muscle tissue, water is present in and between myofibrils, sarcoplasm and muscle cell. Three types of water can be distinguished in meat: constitutional, entrapped and free. Constitutional water is linked to the proteins themselves, and very resistant to external stresses. Constitutional water is not affected by the action of aging and is not affected by freezing (it does not turn into ice) and cooking. The entrapped water is retained in the muscle by steric

effects or by attraction to constitutional water. It is susceptible to freezing and can be easily removed by drying. Also, is very sensitive to the muscle changes during ageing; a suddenly fall of pH could led to protein denaturation and loss of entrapped water.

Table 1.2. Meat composition of various species (value per 100 g). Source: Cobos and Diaz (2014)

	Unit	Veal	Lamb	Pork	Chicken	Turkey
Proximates						
Water	g	75.91	73.78	72.9	75.46	75.37
Protein	g	20.2	20.75	20.48	21.39	22.64
Fat	g	2.87	4.41	5.41	3.08	1.93
Ash	g	1.08	1.12	1.05	0.96	1.04
Vitamins						
C (ascorbic acid)	mg	0	0	0.9	2.3	0
Thiamin	mg	0.08	0.15	0.88	0.07	0.05
Riboflavin	mg	0.28	0.39	0.23	0.14	0.19
Niacin	mg	7.83	6.54	5.34	8.24	8.1
Pantothenic acid	mg	1.37	0.49	0.81	1.06	0.84
B6	mg	0.43	0.14	0.5	0.43	0.65
Folate	μg	13	0	9	7	7
B12	μg	1.4	2.71	0.71	0.37	1.24
E (α-tocopherol)	mg	0.26	0.21	0.17	0.21	0.09
Minerals						
Calcium	mg	15	8	6	12	11
Iron	mg	0.85	1.64	1.01	0.89	0.86
Magnesium	mg	25	19	25	25	27
Phosphorus	mg	211	202	229	173	190
Potassium	mg	328	171	369	229	235
Sodium	mg	86	46	55	77	118
Zinc	mg	3.23	3.01	2.27	1.54	1.84
Copper	mg	0.113	0.099	0.075	0.053	0.079
Manganese	mg	0.029	0.024	0.029	0.019	0.012
Selenium	μg	8.6	1.5	35.4	15.7	22.6
Cholesterol	mg	83	74	68	70	67

The free water is represented by the fraction that flows freely between the tissues and is maintained in the muscle by the weak capillary force. In the pre-rigor phase the free water is not easily visible, but can be released when the entrapped water moves from its position during rigor and post-rigor changes (Cobos & Diaz, 2014; Heinz & Hautzinger, 2007).

Proteins

Proteins are certainly the most important component of meat, and are of high biological value. Proteins are complex molecules made up of amino acids, which stand out in *essentials* (which the animal organism cannot synthesized and must therefore be introduced with the diet) and *non-essential* (that can be synthesized by the body). Both essential and non-essential are presented in Table 1.3. It is important to note that meat proteins, unlike most vegetable proteins, have very high digestibility and are therefore well absorbed by the body (Cobos & Diaz, 2014; Pereira & Vicente, 2013).

Table 1.3. Essential and non-essential amino acids (Pereira & Vicente, 2013)

Essential Amino Acids	Non Essential Amino Acids
Isoleucine	Alanine
Leucine	Asparagine
Lysine	Arginine
Methionine	Cysteine
Tryptophan	Aspartic Acid
Treonine	Glutamic Acid
Valine	Proline
Phenilalanine	Hystidine
	Tyrosin
	Serin
	Glycine

Meat protein can be divided in:

- *Myofibrillar*, which are salt-soluble proteins and represent more than half of total muscle protein (51.5%). Most abundant are myosin and actin, that determine the softness of the meat, the degree of water retention and therefore its tenderness. Also, myofibrillar proteins have the highest biological value (Heinz & Hautzinger, 2007).
- *Stromal*, connective tissue proteins (16%) including collagen and elastin. Collagen is the most abundant protein in mammals, while elastin can be found in lower amounts. These proteins have a low biological value. With the increase of animal age, collagen an elastin become more insoluble, increasing the hardness of meat (Heinz & Hautzinger, 2007).
- *Sarcoplasmic*, water soluble proteins (30%). Most representative are oxidative enzymes, heme pigments, mainly myoglobin, molecule responsible for the red

colouring of the meat. Sarcoplasmic proteins are good emulsifiers, but not as effective as myofibrillar proteins (Cobos & Diaz, 2014).

Beyond the proteins, in the meat are present also non-protein nitrogen compounds as free amino acids, dipeptides, oligopeptides, nucleotides, purinic and pyrimidine bases, creatine, creatinine, amines, urea, ammonia.

Vitamins and minerals

All vitamins are indispensable for the proper functioning of the body. Different categories of food contribute differently to vitamin supplements. Meat mainly contributes to the vitamins of Group B: thiamine (vitamin B1), riboflavin (vitamin B2), pantothenic acid (vitamin B5), folic acid (vitamin B9), niacin (vitamin B3), pyridoxine (vitamin B6) and cobalamin (vitamin B12). Red meats, in particular beef and lamb are good sources of vitamin B12, while pork is a good source of B1 (Cobos & Diaz, 2014). Group B vitamins are essential for a proper diet and their properties are very useful. They are important for the normal functioning of the nervous system, the transformation of carbohydrates into glucose, and for the metabolism of lipids and proteins. It is important to notice that vitamin B12 is found only in animal products and is involved in the regeneration of white blood cells. However, meat is not an important source of fat soluble vitamins A, D, E, K and vitamin C, these are present in small amounts (Cobos & Diaz, 2014; Heinz & Hautzinger, 2007; Pereira & Vicente, 2013).

Meat also contains an important variety of minerals. Each mineral participates in one or more vital functions of the organism and must be supplied in sufficient quantity through the diet. Minerals present in the meat include calcium, phosphorus, sodium, potassium, chlorine, magnesium with the level of each of these minerals above 0.1%, and trace elements such as iron, copper, zinc and others(Pereira & Vicente, 2013).

It is important to consider that vitamin amount could be influenced by the thermal process, as the meat most of the time is cooked. During cooking techniques vitamins are partially destroyed, which led to significant reduction of B vitamins.

Fats

Fats in meat animals are commonly classified as:

- Subcutaneous fat – beneath the skin;
- Intermuscular fat – between muscles;
- Intramuscular fat – between muscle bundles (marbling, which contributes to tenderness and flavour of meat).

The most important components of fat are fatty acids, which are subdivided into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. The main saturated fatty acids present in the meat are myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). Between PUFAs the most important are linoleic acid (C18:2n-6, LA) and α-linolenic acid (C18:3n-3, ALA), while between MUFAs the most important is oleic acid (C18:1n-9) (Webb & O'Neill, 2008).

Fats are indispensable for life, as are other nutritional principles. Their function is predominantly energetic, but they also perform other important functions as vehicle of essential micronutrients such as liposoluble vitamins and long chain PUFA, and as precursors of some hormones. Some PUFA are indispensable to the body. LA and ALA are termed *essential fatty acids,* although they cannot be synthesized by the body and must be introduced with the diet (Simopoulos, 2002).

Some animal species have higher quantities of fatty tissue, such as pigs, others lower quantities such as bovines. Fatty tissues from certain animal species are better suited for meat product manufacture, fats from other species less or not suited at all. Strong differences are also pronounced in older animals, with the well-known example of fat from old sheep, which most consumers refuse (Heinz & Hautzinger, 2007). Fat content can differ significantly within meat cuts and with feeding regime, also the species, genetic origin and age influence the final quality product (Webb & O'Neill, 2008).

In Table 1.4 are reported the fatty acid (FA) composition of adipose tissue and muscle of some animal species. FA profile of animal tissue depends on the FA present already in the tissue and FA content present in the feed. Dietary manipulation for improving FA profile depends on animal species. In monogastric, it is relatively easy to change the FA composition of the tissue, because dietary FA are absorbed unchanged from the intestine and incorporated into tissue lipids. In ruminants dietary FA are bio-hydrogenated by the rumen microorganisms, with the conversion of unsaturated fatty acids (UFA) to MUFA and SFA, and only a small quantity is absorbed into tissue lipids (Nieto & Ros, 2012a).

Recent studies reported that high values of n-6/n-3 PUFA ratio in human diets can affect human health. Therefore, there was a growing interest in enriching meat with n-3 PUFA, in order to reduce n-6/n-3 PUFA ratio and improve its nutritional value. Dietary guidelines recommend a n-6/n-3 ratio below 4 (Simopoulos, 2002). Hence, it is essential to increase the intake of n-3 PUFA and to decrease the n-6 PUFA in order to prevent several chronic diseases such as cardiovascular disease (CVD), hypertension, cancer, diabetes neurodegenerative disease, etc. (Simopoulos, 2010).

Table 1.4. Fatty acid composition (% of total fatty acids) of adipose and muscle tissue of pork, lamb and beef. Source: (Enser et al., 1996)

Fatty acid	**Adipose tissue**			**Muscle tissue**		
	Pork	***Lamb***	***Beef***	***Pork***	***Lamb***	***Beef***
C14:0	1.6	4.1	3.7	1.3	3.3	2.7
C16:0	23.9	21.9	26.1	23.2	22.2	25.0
C16:1	2.4	2.4	6.2	2.7	2.2	4.5
C18:0	12.8	22.6	12.2	12.2	18.1	13.4
C18:1n-9	35.8	28.7	35.3	32.8	32.5	36.1
C18:2n-6	14.3	1.3	1.1	14.2	2.7	2.4
C18:3n-3	1.4	1.0	0.5	0.95	1.37	0.70
C20:4n-6	0.2	ND	ND	2.21	0.64	0.63
C20:5n-3	ND	ND	ND	0.31	0.45	0.28
C22:4n-6	0.06	ND	ND	0.23	ND	0.04
C22:5n-3	0.22	ND	ND	0.62	0.52	0.45
C22:6n-3	0.16	ND	ND	0.39	0.15	0.05
n-6/n-3	7.6	1.4	2.3	7.2	1.3	2.1
P:S	0.61	0.09	0.05	0.58	0.15	0.11
Total	65.3	70.6	70.0	2.2	4.9	3.8

ND: not detected

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CHAPTER 2

MEAT AND HEALTH

Meat and meat products are recognized as highly nutritious foods in the diets. They supply a considerable amount of protein of high biological value, for their completeness in amino acids essential to human health, vitamins (mainly those of B complex), minerals of high bioavailability (especially iron and selenium), fatty acids rich in polyunsaturated n-3 and n-6, and bioactive nutrients such as creatine (energy source for muscles), taurine (beneficial for heart and muscles) and conjugated linoleic acid found in red meat, which provides health benefits in preventing several diseases (Jiménez-Colmenero *et al.*, 2012).

Meats may be classified according to the type of muscle they come from: red or white. The classification is based on the fat, cholesterol, and iron content. Red meat refers to fresh mammalian muscle meat (e.g. beef, veal, pork, lamb, mutton, horse, deer or goat meat), while white meat refers to poultry (chicken, turkey) (IARC, 2018). The epidemiological studies usually include in meat classification also processed meat, which refers to meat that has been transformed through salting, curing, fermentation, smoking or other processes that could enhance flavour or improve shelf-life (Boada *et al.*, 2016). Most processed meats contain pork or beef, but may also contain other red meats or poultry (WHO 2015).

However, despite its nutritional value, several epidemiologic studies have associated high consumption of meat, in particular red meat and processed meat, with an increased risk of CVD and several types of cancer (Egeberg *et al.*, 2013; Higgs, 2000; Klurfeld, 2015; Mccullough *et al.*, 2013).

CVD is the main cause of death in the Western world, and the second cause worldwide (Willerson & Ridker, 2004). Meat is often linked with CVD due to high fat amount, especially saturated fatty acids that increase low density lipoprotein cholesterol content. The mechanism responsible for health diseases generation have been proposed by Kennedy *et al.* (2009), who reported that high intake of SFA could increase white adipose tissue expansion and hypertrophy that leads to apoptosis. As a result of these phenomena, inflammatory proteins such as cytokines and chemokines are released, contributing to inflammation and insulin resistance in muscle, leading to an increased risk of CVD.

Micha *et al.* (2010) conducted a systematic review on the evidences that reported associations between red meat and processed meat with CVD. They analysed the effect of relative risk of CVD per 100g serving/day of red meat and 50g serving/day of processed meat. The authors found no association between red meat consumption and the risk of CVD, meanwhile processed meat consumption was linked with 42% higher risk of CVD. Micha *et al.* (2012) demonstrated that the risk of CVD depends on processing steps. They found higher risk of CVD for processed meat consumption and much lower or no risk for fresh meat consumption. This could be due to the addition of preservatives in processed meat such as salt and nitrates. Similar evidence were reported by recent studies (Bechthold *et al.*, 2019; Billingsley *et al.*, 2018; de Medeiros *et al.*, 2019; Kris-Etherton *et al.*, 2018), but most of them do not give an exact amount of meat intake associated with CVD. However, these studies lead to a reduction in meat consumption, being replaced by the consumption of vegetable fat rich in polyunsaturated fatty acids, discouraging in this way saturated fatty acids.

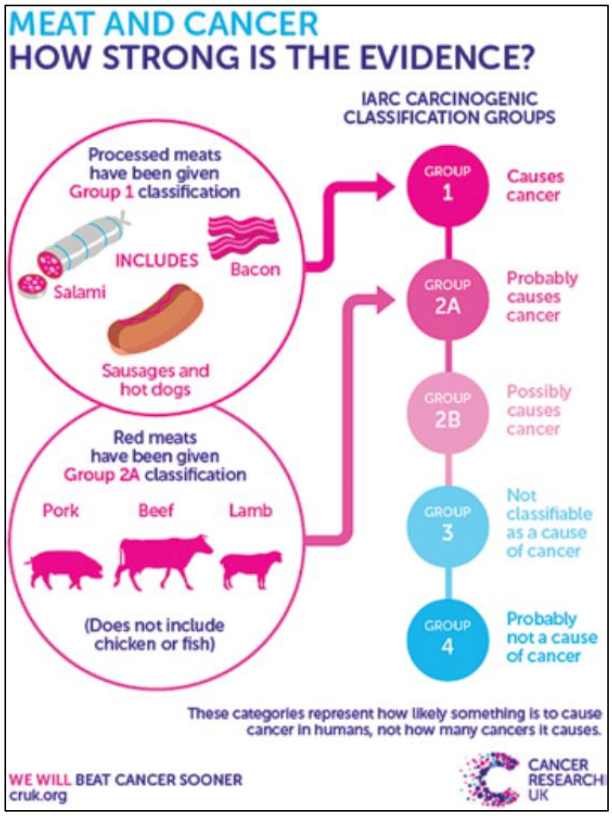

Figure 2.1. A diagram of classifying meat and processed meat as carcinogenic in accordance with IARC https://sites.tufts.edu/publichealth/files/2015/11/meat

Another strong evidence regarding red meat and processed meat consumption appear in 2015, when the International Agency for Research on Cancer (IARC) classified the consumption of red meat as probably carcinogenic to humans (Group 2A) and processed meat consumption as carcinogenic to humans (Group 1) (Fig. 2.1). "*The experts concluded that each 50 grams' portion of processed meat eaten daily increases the risk of colorectal cancer (CRC) by 18%.*" The risk of CRC in humans increases with the amount of meat consumed (IARC, 2018).

The classification was based on several types of epidemiological studies that investigated the association of consumption of red meat and processed meat with cancer.

One of the largest cohorts-study of humans that has been developed specifically to evaluate the association between diet and cancer is *The European Prospective Investigation into Cancer and Nutrition* (EPIC). The study included 366,521 women and 153,457 men from

10 European countries and took place between 1992 and 1998. They were subjected to a specific diet, regulated by the consumption of meat, and were monitored over the years for risk factors related to the development of CRC. The population was divided into two groups: one considered to have a high meat intake, or greater than 160 g/day, and one with a low meat intake, lower than 20 g/day. The authors showed that increasing red meat and processed meat consumption increased CRC. However, no relationship between poultry consumption and CRC risk was observed. Also, they reported that higher fish intake decreased significantly CRC risk (Norat *et al.,* 2005).

Results from other studies (Chen *et al.,* 2015; Cross *et al.,* 2010; English *et al.,* 2004) showed positive association between red meat and processed meat consumption and CRC. At the same time, they reported that elevated consumption of chicken and fish did not increase the risk of CRC.

In contrast to white meat, red meat and processed meat were found to be responsible for the increasing risk in CRC. Several studies reported that a possible cause could be the heme-iron content, however, the mechanism is still uncertain (Cross *et al.,* 2010; Egeberg *et al.,* 2013; Kabat *et al.,* 2007). Bastide *et al.* (2011) reported that the relationship between the risk of CRC and heme iron is based on the formation of N-nitroso compounds (NOC) and the formation of lipid oxidation end products (Fig. 2.2). NOC, such as nitrosamines and nitrosamides, are formed by N-nitrosation of amines and amides coming from the decarboxylation of amino acids by the gut. Heme iron from meat, when ingested, catalyses their formation starting from their intestinal precursors, yielding a higher amount of NOC in the gastrointestinal tract (Bastide *et al.,* 2015; Santarelli *et al.,* 2008).

NOC could also be developed during processing steps such as curing, drying, smoking, cooking and packaging. These treatments allow to improve quality characteristics, increase shelf-life and change flavour. However, NOC are known to be potentially carcinogenic and mutagenic, causing DNA damage.

The red meat is the major source of dietary iron, but not all red meats have the same amount of iron. The iron content is species dependent, the highest amount was reported for beef and lamb, and lower for pork and veal (Lombardi-Boccia *et al.,* 2002). Egeberg *et al.* (2013) suggested to consider the relationship between cancer and red meat subtype. They conducted a study on 53,988 participants, to evaluate associations between red meat and processed meat consumption for colon or rectal cancer in the Danish diet. They found no associations between red meat and processed meat consumption and risk for CRC. However, when considering red meat subtypes, they concluded that high lamb consumption increased colon cancer, while high

pork consumption increased rectal cancer. This study also suggested that replacing red meat with fish showed a significantly lower risk for colon cancer.

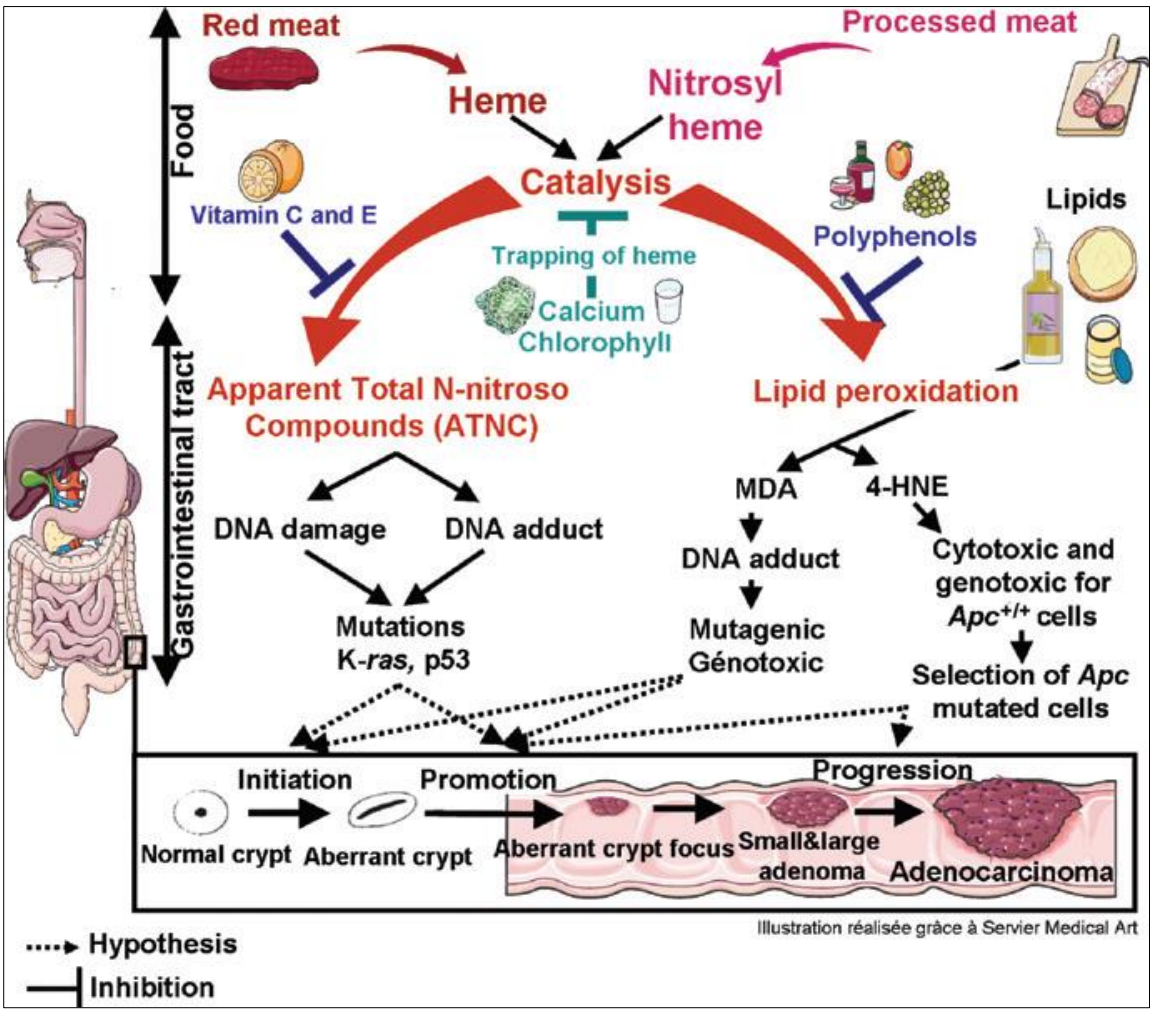

Figure 2.2. Catalytic effects of heme and its inhibition. Source: Bastide et al. (2011)

Heme iron catalyses also the formation of lipid oxidation end products, acting as a prooxidant. In particular, PUFA and cholesterol oxidation, with the production of numerous compounds such as hydroperoxides, ketones, cholesterol oxides and aldehydes. Some of these compounds are known to have mutagenic and carcinogenic effects and cytotoxic properties. The main products arising from lipid oxidation are malondialdehyde (MDA) and 4-hydroxynonenal (4–HNE). MDA is a toxic compound resulting from PUFA oxidation, which induce DNA damage forming mutagenic adducts (Niedernhofer *et al.*, 2003). 4-HNE is weakly mutagenic, even though is considered a toxic compound resulting from lipid oxidation. 4-HNE may induce DNA modifications, and cause deep changes in fundamental cellular processes, such as apoptosis and killing normal cells (Csala *et al.*, 2015).

Thermal processes that meats undergo, especially those at high temperature, generate toxic compounds such as heterocyclic amines (HAC) and the polycyclic aromatic hydrocarbons

(PAH). HAC are formed by pyrolysis of creatine with specific amino-acids. Their concentration is higher in overcooking meat, respect to the meat cooked at normal temperatures. PAH are produced by an incomplete combustion of organic compounds. This compound is characteristic for smoked or barbecued meat. PAH and HAC were recognised as potentially mutagens to human. They induce DNA damage, being cancer promoting (Cascella *et al.*, 2018; Santarelli *et al.*, 2008). However, the association between HAC, PAH and cancer risk is not completely clarified.

As reported above, the correlation between red meat and processed meat consumption and cancer involve different molecules: NOC, HAC, PAH and heme iron. The production of these compounds is determined by the methods of meat preparation, combination with other foods, and could also develop during processing steps.

Epidemiologic studies suggest that an excessive consumption of red meat and processed meat increases the risk of developing cancer, which is proportional to the amount and frequency of consumption. However, some studies reported non-significant association between fresh meat intake and CRC. Bernstein *et al.* (2015) found little evidence that high red meat consumption increases CRC, but observed a significant relationship between processed meat and CRC. World Cancer Research Fund (WCRF) recommended in 2007 a modest consumption of red meat, which contributes several nutrients to the diet, while processed meats should be consumed occasionally.

However, despite the correlation of red meat and processed meat consumption with major chronic diseases in our society, meat remain an important source of healthy components that provide a valuable amount of nutrients essential for optimal growth and development (Olmedilla-Alonso *et al.* 2013).

In conclusion, I think that consumption of meat of high quality and in right quantity (not excessive) does not increase the risk of chronic diseases. The development of these diseases depends also on several lifestyle risk factors such as physical activity, smoking, obesity, stress control and dietary habits, that when put together with high intake of red meat and processed meat could increase the risk of several pathologies.

In this regard numerous studies proposed several strategies for improving meat quality, in order to develop healthier meat and meat products. These strategies are based on animal production (genetic and nutrition), on technological strategies such as reduction of unhealthy components (sodium, nitrite, phosphate), and inclusion of bioactive compounds in meat and meat products. In this way, the improved meat and meat products could supply beneficial dietary compounds, improving their nutritive value, physio-chemical and sensorial properties.

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CHAPTER 3

THE OXIDATIVE PHENOMENA OF MEAT

3.1. Free radicals and oxidative stress

Biological systems are continuously subjected to stressing agents of both endogenous (metabolic pathway, enzymes) and exogenous (light, UV, heat) origin generating reactive species (Davies, 2012). The reactive species are free radical compounds which represent chemical species with an unpaired electron in their outer orbit, with high reactivity and chemical instability. They have the ability to react with various molecules from which they subtract or to which they give off an electron in an attempt to acquire stability (Halliwell & Chirico, 1993). Free radicals derive from three elements: oxygen, nitrogen and sulphur, with whom create reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS) (Carocho & Ferreira, 2013).

ROS are the most abundant class of radical species produced by organism and include a wide variety of chemical species (Fig. 3.1) such as superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$), hydroperoxyl radical ($HO_2^{\cdot}$) and hydrogen peroxide (H_2O_2). They can be produced intentionally or accidentally. For example, during mitochondrial respiration, ROS are produced as natural by-products: $O_2^{-\cdot}$, H_2O_2, OH are formed accidentally in low concentrations; meanwhile the phagocytes generate $O_2^{-\cdot}$, H_2O_2 to inactivate bacteria and viruses (Amaral *et al.*, 2018; Bekhit *et al.*, 2013; Papuc *et al*., 2017).

ROS not always have negative functions, indeed in small quantities they play a fundamental role in the health and functioning of the animal organism such as regulation of cell signalling pathways, the control of gene expression, modulation of skeletal muscle, defence against invading pathogens and keep antioxidant system activated (Falowo *et al.*, 2014). High levels of ROS bring to the oxidation of PUFA, oxidation of amino acids, damage of DNA and inactivation of specific enzymes. Also, ROS excess is the main cause of oxidative stress. The term "*oxidative stress*" generally refers to an imbalance between the production of ROS and the defence activity of the antioxidant systems (Fig. 3.2) (Suzen *et al.*, 2017). Oxidative stress has been linked with various diseases, including cardiovascular, inflammatory, neurological disorders and cancer (Falowo *et al.*, 2014; Papuc *et al.*, 2017).

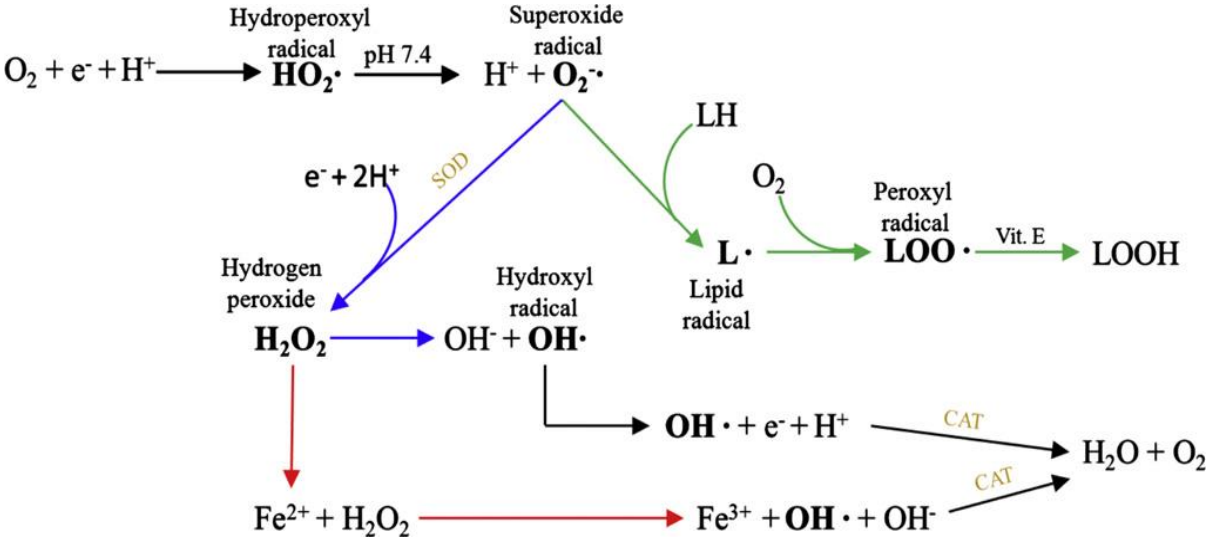

Figure 3.1. Overview of the reactions leading to the formation of ROS. Green arrows represent lipid peroxidation. Blue arrows represent the Haber–Weiss reactions and the red arrows represent the Fenton reactions. The bold letters represent radicals or molecules with the same behaviour (H_2O_2). SOD refers to the enzyme superoxide dismutase and CAT refers to the enzyme catalase. Source: Carocho & Ferreira, (2013).

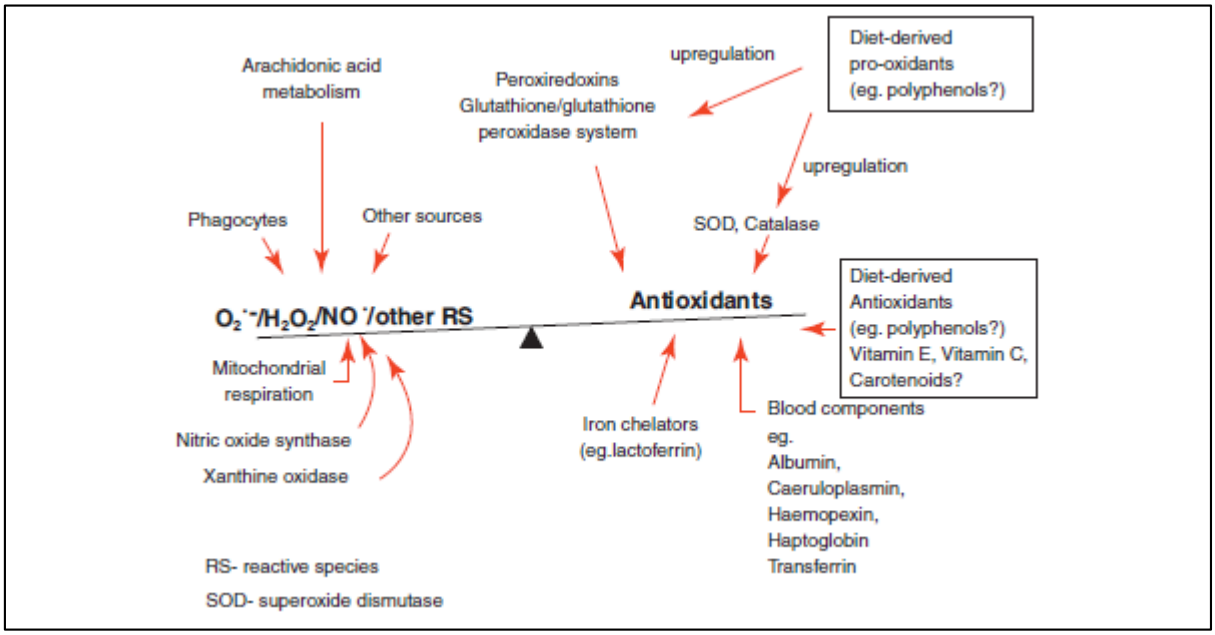

Figure 3.2. The approximate balance of antioxidants and reactive species in vivo. Source: Halliwell (2011)

In pigs, oxidative stress can occur due to several factors. First of all, during the weaning period, when piglets are separated from their mothers and replacing the milk of the sow, which is easily digestible, with a solid plant-based food. This phenomenon is known as post-weaning stress syndrome, which leads to reduced growth in piglets and higher susceptibility to diseases (Campbell *et al.*, 2013). Another factor that affect oxidative stress is the animal condition before

slaughter; including transport. Transport causes such as rough loading and unloading, distance covered, deprivation of food and water, humidity, temperature, light are common sources of oxidative stressors (Falowo *et al.*, 2014; Minka & Ayo, 2009).

The effects of oxidative stress on animals cause several changes in meat quality such as lipid and protein oxidation.

3.2. Lipid oxidation and its mechanism

The lipids are important components of the meat, that contribute to the sensory acceptability of a product, giving aroma, flavour tenderness and juiciness. Lipids are a significant energy source, but also the vehicle of essential micronutrients such as fat-soluble vitamins and long chain polyunsaturated fatty acids. The quantity and quality of lipids in meat can vary considerably depending on various factors such as the species, feeding, slaughter, carcass processing, and the way of cooking.

Lipid oxidation (Lox) mechanisms have been studied for many years. So much interest results from several undesirable changes, in particular the formation of off-flavour and off-odours, colour and texture alteration that affect food quality and safety. Lox in meat and meat products occur due to the high amount of PUFA, metal ions (copper and iron), myoglobin concentration and salt (Falowo *et al.*, 2014). Also, it may be accelerated by prooxidants, such as UV light and certain enzymes (Amaral *et al.*, 2018).

Lox occurs via two main mechanisms: enzymatic oxidation and non-enzymatic oxidation (autoxidation).

Autoxidation is the most important process involved in oxidative lipid deterioration (rancidity). This process occurs at room temperature and proceeds with a speed directly proportional to the degree of unsaturation of fatty acids; it is also accelerated by high temperatures, as happens during cooking.

Mechanism of lipid oxidation

Lox is a free radical chain reaction which occurs in three stages: initiation, propagation, and termination.

a) Initiation

The Lox reaction always starts when the reactive oxygen species attack the fatty acid (LH) at a double bond, and abstract a hydrogen atom with the consequent formation of the lipid radical ($L^{\cdot}$), called also alkyl radical. (Min & Ahn, 2005).

$$LH \rightarrow L^{\cdot} + IH \quad (1)$$

b) Propagation

The resulting lipid radical ($L^{\cdot}$) quickly reacts with a molecular oxygen, forming various radical species such as peroxyl radical ($LOO^{\cdot}$). The peroxyl radical can react with another unsaturated fatty acid by abstracting a hydrogen atom and generating a lipid hydroperoxide (LOOH) and a new alkyl radical ($L^{\cdot}$) (Frankel, 2012). The propagation reaction takes place indefinitely, until the termination products, consisting of a wide range of oxidation products, are obtained (Schaich *et al.*, 2013).

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (2)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (3)$$

The lipid hydroperoxides (LOOH) are at the same time oxidation products and substrates for subsequent reactions. In the presence of Fe^{2+}, they can undergo degradation forming the alkoxyl radical ($LO^{\cdot}$) and the hydroxyl radical ($OH^{\cdot}$). Both, as radicals, can continue the propagation phase by directly attacking a new fatty acid (Frankel, 2012).

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + OH^{\cdot} + Fe^{3+} \quad (4)$$

Alkoxyl radicals can form alcohols (LOH) or undergo a reaction known as β–scission to form volatile and non-volatile compounds, such as aldehydes. Aldehydes formation bring to the rancidity in food, responsible for off-odour, off-flavour and colour change (Falowo *et al.*, 2014).

$$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot} \quad (5)$$

$$LO^{\cdot} \rightarrow RCHO + L^{\cdot} \quad (6)$$

c) Termination

The Lox process terminates with the interaction of two free radical species, generating non-radical compounds.

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow LOOL + O_2 \quad (7)$$

$$LOO^{\cdot} + L^{\cdot} \rightarrow LOOL \quad (8)$$

$$L^{\cdot} + L^{\cdot} \rightarrow LL \quad (9)$$

Enzymatic oxidation is catalysed by lipoxygenase, which is an enzyme able to produce hydroperoxides through the oxidation of polyunsaturated fatty acids. The lipoxygenase molecule contains an iron atom (in the ferric form) responsible for the formation of the free

radical (R·) that reacts with oxygen to give a peroxyl radical (Fig. 3.3). At this point (to close the catalytic cycle) the enzyme, in the ferrous form, reacts with the peroxyl radical to form its anion and return to the ferric form (Papuc *et al.*, 2017).

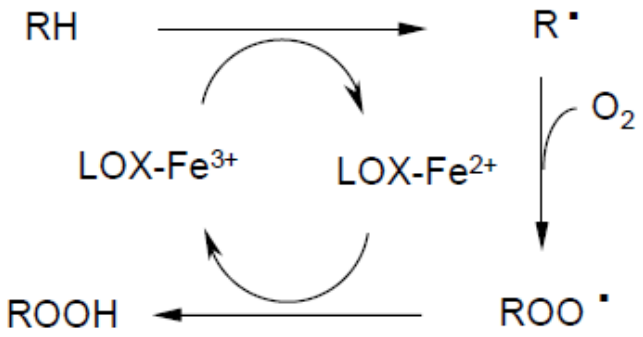

Figure 3.3. Lipoxygenase action. Source: Papuc et al. (2017)

3.3. Protein oxidation

Meat is an important source of high-quality proteins, which have an important role in nutritional and technological properties of meat and meat products. Proteins undergo several modifications during muscle conversion to meat and transformation in meat products. Major cause of protein denaturation is protein oxidation (Pox) due to *pm* changes, handling, processing and storage (Bekhit *et al.,* 2013; Estévez, 2011). Pox, respect to Lox, is an innovative issue in the study of meat quality. If Lox was studied in depth, Pox gained interest in recent decades. Pox is a free radical chain reaction similar to Lox but with greater pathway complexity and a larger variety of oxidation products (Lund *et al.,* 2011) and depends on several factors such as animal species, muscle, treatment etc. According to Estevez (2011) Pox begins with the abstraction of a hydrogen atom by ROS from protein (PH) with the formation of a carbon-centred radical (P·) (*reaction 10*), which is consecutively converted into a peroxyl radical (POO·) (*reaction 11*) in the presence of oxygen, and an alkyl peroxide (POOH) (*reaction 12*) by abstraction of a hydrogen atom from another molecule.

Further reactions with $HO_2\cdot$ or with transition metals ions (M^{n+}) such as Fe^{2+} or Cu^{+}, lead to the production of an alcoxyl radical (PO·) (*reaction 13 and 14*) and its hydroxyl derivative (POH) (*reaction 15 and 16*).

$$PH + HO\cdot \rightarrow P\cdot + H_2O \quad (10)$$

$$P\cdot + O_2 \rightarrow POO\cdot \quad (11)$$

$$POO\cdot + PH \rightarrow POOH + P \quad (12)$$

$$POOH + HO_2 \rightarrow PO^{\cdot} + O_2 + H_2O \quad (13)$$

$$POOH + M^{n+} \rightarrow PO^{\cdot} + HO^{-} + M^{n+1} \quad (14)$$

$$PO^{\cdot} + HO_2^{\cdot} \rightarrow POH + O_2 \quad (15)$$

$$PO^{\cdot} + H^{+} + M^{n+} \rightarrow POH + M^{(n+1)+} \quad (16)$$

Several alterations can occur as a result of interactions of proteins with ROS, such as the oxidation of the amino acid side chains, the cross-linking between proteins, the degradation of peptide bonds, the fragmentation and aggregation of the proteins themselves (Guyon *et al.*, 2016; Lund *et al.*, 2011; Soladoye *et al.*, 2015). Some amino acids (arginine, lysine) are oxidized through metal-catalyzed reactions generating carbonyl residues, while others such as methionine and cysteine are the most susceptible to oxidation due to their reactive sulphur atoms generating sulphur-derivatives (Lund *et al.*, 2011; Soladoye *et al.*, 2015).

Meat naturally contain heme proteins (myoglobin and haemoglobin) which are responsible for meat colour and are good pro-oxidants for Pox. In the presence of H_2O_2, myoglobin forms hypervalent species such as perferrylmyoglobin ($^{\cdot}$MbFe(IV)=O) and ferrylmyoglobin (MbFe(IV)=O) (Papuc *et al.*, 2017).

$$MbFe\ (III) + H_2O_2 \rightarrow {}^{\cdot}MbFe\ (IV) = O \rightarrow MbFe\ (IV) = O \quad (17)$$

Perferrylmyoglobin has been found to initiate lipid and protein oxidation, by abstracting a hydrogen atom from a PUFA or generating long-lived protein radicals (Estévez, 2011; Lund *et al.*, 2011; Papuc *et al.*, 2017).

$$^{\cdot}MbFe\ (IV) = O + LH \rightarrow MbFe\ (IV) = O + L^{\cdot} + H^{+} \quad (18)$$

Also Lox by-products promote Pox with the formation of carbonyl compounds. Major products formed during Pox are shown in Fig. 3.4.

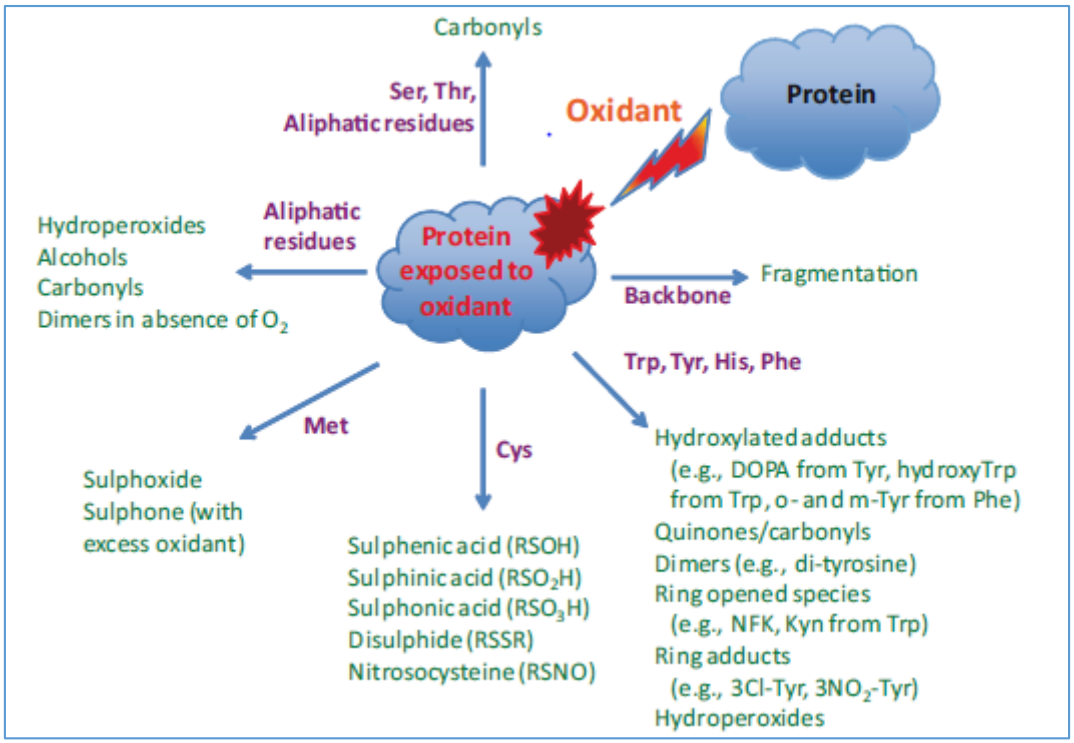

Figure 3.4. Overview of products formed during protein oxidation. Abbreviations: DOPA, 3,4-dihydroxiphenylalanine; NFK, N-formylkynurenine; Kyn, kynurenine; 3Cl-Tyr, 3-chlorotyrosine; 3-NO_2-Tyr, 3-nitrotyrosine. Source: Davies (2012).

Oxidation of meat proteins affects quality traits of meat and meat products. Several physio-chemical changes occur during Pox which bring to loss of their functionality: denaturation, including loss of their native tertiary structure, modification in texture traits, colour, flavour, reduced water holding capacity; reduced protein solubility and digestibility, loss of proteolytic activity; decrease of nutritional value due to the loss of essential amino acids (Bekhit *et al.*, 2013; Estévez, 2011; Falowo *et al.*, 2014; Lund *et al.*, 2011; Papuc *et al.*, 2017). The consumption of these denaturised proteins increase the risk of some diseases (Zhang *et al.*, 2013).

3.4. Antioxidants

The classical definition of antioxidant was proposed by Halliwell & Gutteridge (1995) as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" and was found imperfect, then in 2007 the same author proposed a simplified version "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell, 2007). Generally, antioxidants (Aox) are those compounds that inhibit, delay or control the oxidation and counteract the effect of ROS. Aox are not able to avoid completely the oxidative stress damage. Their functionality depends on various parameters such as the type of ROS, the way

and place where it was formed and the Aox concentration, otherwise they can act also as prooxidants (López-Alarcón & Denicola, 2013).

Aox are classified into two major groups: synthetic and natural.

Synthetic antioxidants

Synthetic Aox are molecules produced in the laboratory using various techniques. They are widely used in the food, cosmetic and pharmaceutical industries. Most commonly used are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are polyphenolic compounds able to inactivate free radicals and to stop the chain reaction. Although, they are efficient and relatively cheap, their use is restricted due to the potential toxicological and carcinogenic effects (Amaral *et al.,* 2018; Karre *et al.,* 2013; Kumar *et al.,* 2015). Therefore, special attention has been given to the use of Aox from natural sources.

Natural antioxidants

Natural Aox are divided in two groups, enzymatic and non-enzymatic Aox (Fig. 3.5).

Enzymatic Aox are synthesized by the human body and are divided into primary and secondary enzymes. Primary antioxidants are composed of three enzymes: superoxide dismutase, catalase and glutathione peroxidase. They prevent the formation of free radicals or convert them into non-harmful molecules (Mamta *et al.*, 2014). The secondary antioxidants include glutathione reductase and glucose-6-phosphate dehydrogenase. These enzymes do not counteract free radicals directly, but support the activity of other Aox.

The non-enzymatic Aox are not found in the body naturally, but could be supplemented with the diet. They include vitamins, minerals, carotenoids, polyphenols and other antioxidants. Dietary antioxidants have beneficial effects on human health preventing several diseases such as heart disease and cancer, and prevent aging process (Decker & Park, 2010).

Vitamins are micronutrients necessary for the good functionality of the body. Vitamin A have beneficial effects on eyes, skin and internal organs; vitamin C could prevent the DNA damage (Brar *et al.*, 2014) and exerts essential antioxidant activity in the brain (Pisoschi & Pop, 2015) and vitamin E is the best lipid-soluble antioxidant which prevent lipid oxidation.

Carotenoids are found in fruits and vegetables of reddish-orange-green colour. They are able to bind and eliminate free radicals, and are known to prevent atherosclerosis (Carocho & Ferreira, 2013; Wojcik & Wozniak, 2010).

Minerals are necessary in small proportion in the human body, but are important for the good functionality of enzymes. They include zinc, selenium, copper, calcium etc., important in preventing several diseases such as hypertension, cardiovascular disease and diabetes (Decker & Park, 2010).

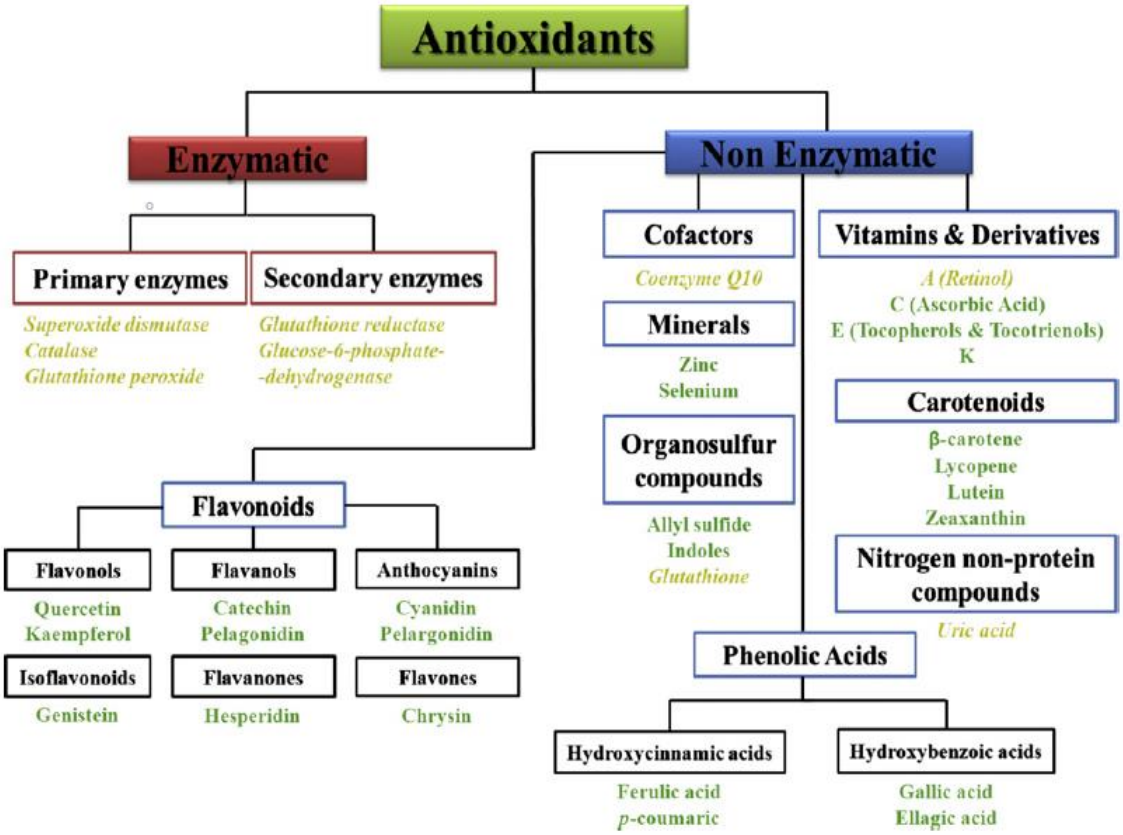

Figure 3.5. Schematic representation of antioxidant classification. Source: Carocho et al. (2013)

Polyphenols are compounds found naturally in the plant kingdom, and are involved in defence system against pathogens microorganisms, predators and UV light (Pandey & Rizvi, 2009). They are characterized by the presence of at least one aromatic ring, substituted by several hydroxyl groups. Several studies reported that these molecules possess high antioxidant activity and can remove free radicals. The main classes include phenolic acids, flavonoids, stilbenes and lignans. The differences between these classes depends on the structure, the number and the position of the hydroxyl groups in these molecules (Shahidi & Ambigaipalan, 2015).

Mechanism of antioxidant action

Antioxidants are compounds able to delay or inhibit the oxidation processes, that occur under the influence of atmospheric oxygen or reactive oxygen species ROS. There are mainly three types of mechanism action of antioxidants: chain breaking, preventive and synergetic (Brar *et al.*, 2014).

a) Chain breaking mechanism

Chain breaking mechanism is considered the classical mechanism of action of antioxidants (López-Alarcón & Denicola, 2013). They reduce the concentration of free radicals by interacting with them and inactivating them (*reaction 19*).

$$LOO^{\cdot} + AH \rightarrow LOOH + A^{\cdot} \quad (19)$$

$$A^{\cdot} + LOO^{\cdot} \rightarrow LOOA \quad (20)$$

This reaction explains the ability of antioxidants to block the propagation of radical chain reactions. They scavenge free radicals and generate antioxidant radicals which are more stable (*reaction 20*). This kind of behaviour is typical for carotenoids, tocopherols, ascorbate, ubiquinone, thiol compounds, albumin, bilirubin and uric acid (Pisoschi & Pop, 2015).

b) Preventive and scavenger mechanism

Preventive antioxidants prevent the formation of radicals by preventing their formation or converting them into non radical products. They act through various mechanisms such as chelation of transition metals, inactivation of peroxides or quenching of ROS so that radical reactions do not start. For example, the enzyme catalase (CAT) catalyses the conversion of hydrogen peroxide into oxygen and water (*reaction 21*), the enzyme superoxide dismutase (SOD) decompose the superoxide into hydrogen peroxide and oxygen (reaction 22). Proteins such as ferritin and transferrin chelate the transition metals, specifically iron, and prevent the catalytic effect they may provide to the production of free radicals (Brar *et al.,* 2014).

$$2H_2O_2 \rightarrow 2H_2O + O_{2\,(CAT)} \quad (21)$$

$$2O^{-\cdot} + 2H^{+} \rightarrow H_2O_2 + O_{2\,(SOD)} \quad (22)$$

c) Synergetic mechanism

Synergetic mechanism occurs when one antioxidant couples with another one and work in synergy (Brar *et al.,* 2014). Combining antioxidants results in major effectiveness against oxidative processes.

$$LOO^{\cdot} + AH \rightarrow LOOH + A^{\cdot} \quad (23)$$

$$A^{\cdot} + BH \rightarrow AH + B^{\cdot} \quad (24)$$

Brewer (2011) reported that in the combination between two antioxidants, one reacts with peroxyl radical and is consumed (*reaction 23*) and the second one generates the first one (*reaction 24*).

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CHAPTER 4

MANIPULATION OF PORK QUALITY BY FEEDING

4.1. Pork fatty acid composition

Pigs are monogastric animals, which means that they have a simple stomach compared to ruminants, that have four-chambered stomach. The digestive system of pig is very similar to humans. The digestion in pigs starts in the stomach with the breaking down of the dietary components and consequently absorption in the small intestine (Rosenvold & Andersen, 2003). Pig feed must contain the right nutrient requirements and these must be provided in balanced amounts, so that essential elements for growth are not lacking. This can guarantee the development of healthy, quality meat.

Fatty acid (FA) composition of pork is influenced by several factors such as genotype, breeding, gender and feeding methods (Vehovský *et al.*, 2018). However, depends also on the type of fat and muscle. In Table 4.1 is reported the FA composition in different pork tissue.

Table 4.1. Fatty acid composition in different pork tissue. Source: Vehovský et al. (2018)

Fatty acid	IMF	Backfat	Visceral tissue	Neck	Shoulder	Loin	Ham	Belly
Lauric acid	0.21	0.09						
Myristic acid	2.54	1.62	1.6	0.137	0.085	0.087	0.059	0.188
Palmitic acid	28.68	26.82	26.7	2.76	1.74	1.78	1.17	3.87
Palmitoleic acid	5.48	2.7	2.2	0.244	0.176	0.165	0.122	0.335
Stearic acid	8.67	15.94	10.2	1.85	1.08	1.15	0.721	2.53
Oleic acid	31.98	33.5	36.4	4.38	3	2.81	1.97	6.26
Linoleic acid	12.11	10.03	16.1	1.55	1.02	0.899	0.676	1.95
α-linolenic acid	4.09	5.79	1.2	0.518	0.305	0.312	0.193	0.697
Arachidic acid	2.92	0.1	0.3	0.052	0.044	0.038	0.034	0.068
Eicosenoic acid				0.109	0.07	0.073	0.046	0.163
Eicosadienoic acid	0.1	0.45						
Docosahexaenoic			1.8	0.019	0.018	0.011	0.009	0.025

Is well known that meat, when is high in fat is undoubtedly more tender, juicy and tasty. Nonetheless, international food standards recommend to reduce fat consumption. Fat contained in pig meat has decreased by 15-30% compared to the past (Wood & Enser, 2017). Today, thanks to the selection of pigs with well-developed muscle and a balanced relationship between fat cuts and lean cuts, meat quality improved. A good strategy for improving nutritional profile and pork quality could be animal nutrition.

4.2. Lipids in pig diets

Fatty acids are frequently included in animal diets, in order to meet the energy requirements (Mordenti *et al.,* 2008). During the past decades, there was an increased interest in the use of fatty acids in the diets of farm animals, especially unsaturated fatty acids (UFA), in order to optimize the UFA:SFA ratio (Doreau & Chilliard, 1997).

Fatty acids digestion is different in ruminant and non-ruminant animals. In ruminants dietary FA are bio-hydrogenated by the rumen microorganisms, with the conversion of UFA to MUFA and SFA, and only a small quantity is absorbed into tissue lipids (Nieto & Ros, 2012; Wood *et al.,* 2008). In contrast to ruminants, in monogastric animals FA are not degraded by the microbes, but are absorbed unchanged from the intestine and incorporated into tissue lipids (Nieto & Ros, 2012). The FA digestion takes place in the small intestine, where the pancreatic lipase breaks the triacylglycerols down to mainly 2-monoacylglycerols and free fatty acids and the formation of micelles aids absorption, with lipid uptake mediated by the lipoprotein lipase enzyme, which is widely distributed throughout the body (Woods & Fearon, 2009). Dietary FA in pigs are transferred from the feed directly to the muscle and fat tissue. Thus, the FA composition of the muscle and fat tissue is closely related to the FA composition of their diet (Dugan *et al.,* 2015). If pigs are fed a diet without FA inclusion they synthesize and deposit only SFA and MUFA (mainly oleic acid), PUFAs deposition occurs only if they are included in the diet (Ellis & McKeith, 1999).

The n-3 PUFAs are known to have a large variety of health benefits against cardiovascular diseases (CVDs). Various studies indicate promising antihypertensive, anticancer, antioxidant, antidepression, antiaging, and antiarthritis effects (Siriwardhana *et al.,* 2012). Feeding pigs with n-3 fatty acids can increase their contents in pork. Good amount of PUFA can be reached by dietary supplementation with fish oil/fish meal, linseed, walnuts, soybean, canola oils, which contain their precursor α-linolenic acid (ALA, 18:3n-3) for n-3, and linoleic acid (LA, 18:2n-6) for n-6. From these precursors are obtained long chain PUFAs. This occurs through different reactions involving the action of some elongating enzymes (elongase) and others that are involved in adding double bonds (desaturase) (Nieto & Ros, 2012) (Fig. 4.1). These enzymes act on both n-3 and n-6 FA, but have a preference for n-3 PUFA (Raes *et al.,* 2004).

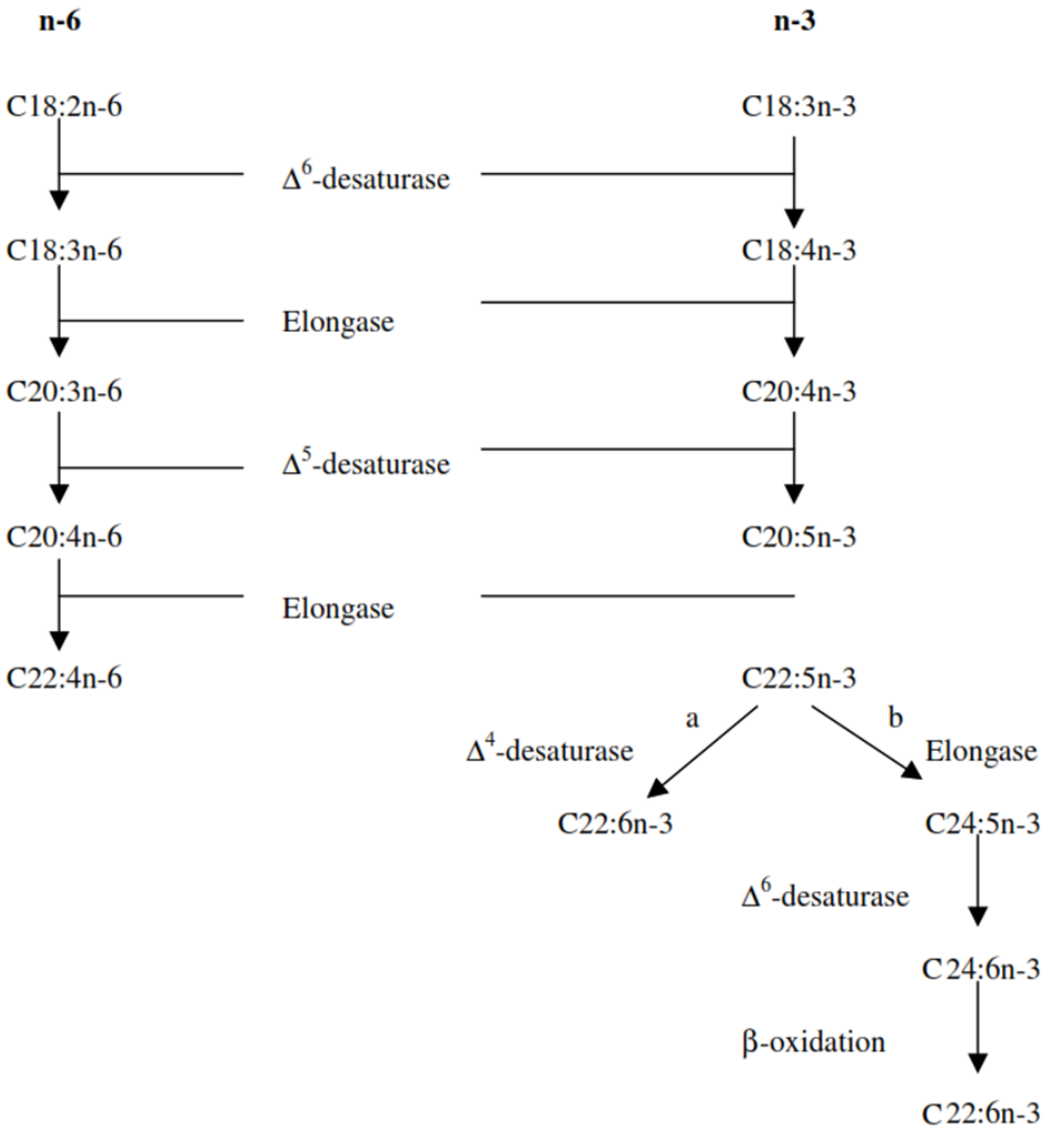

Figure 4.1. Conversion of C18:2n-6 and C18:3n-3 to their longer chain fatty acid products (a) conventional pathway (Brenner, 1989); (b) pathway proposed by Sprecher et al. (1995). Source: Raes et al. (2004)

4.2.1. Lipid sources

During the years there was a growing interest for modifying the FA composition of pork, to make it more suitable for consumers' demand. The cereal-based diet of pigs transfers to the meat a high level of n-6 PUFA and small amount of n-3 PUFA, with a high n-6/n-3 ratio (Woods & Fearon, 2009). Therefore, there was the necessity to enrich meat with n-3 FA, increasing PUFA amount and decreasing SFA. These changes make meat nutritionally more attractive, bringing health benefits to the consumers.

Several studies have shown that n-3 PUFA amount could be increased in muscle and adipose tissue of pigs by including in the diet vegetable oils, fish oil, marine algae and some seeds.

Pieszka (2007) have studied the effect of the inclusion of vegetable oils in pig diets. He reported that feeding pigs with palm oil, linseed oil, rapeseed oil and sunflower oil at 3% enriched meat with UFA.

Supplementation with sunflower oil and palm oil increased MUFA amount, linseed oil and rapeseed oil increased PUFA amount. The highest level of n-3 PUFA was found in the meat of linseed oil group. Also, higher levels of EPA and DHA were found in the groups supplemented with linseed and rapeseed oil compared to palm and sunflower oil. The n-6/n-3 PUFA ratio was lower in linseed oil group. However, the inclusion of vegetable oils in the diets decreased the oxidative stability of the meat.

Park *et al.* (2009) reported that soybean oil dietary supplementation of pigs increased PUFA content, especially ALA and DHA without significant effects on growth performance and carcass characteristics. They showed that PUFA content can be increased linearly with the feeding period. Okrouhlá *et al.* (2018) reported that dietary inclusion of rapeseed oil in pigs increased n-3 FA and decreased n-6/n-3 ratio, while soybean oil increased n-6 FA content.

Is well known that fish oil is a rich source of n-3 FA, able to decrease n-6/n-3 ratio. The incorporation of n-3 PUFA in pork by fish oil feeding have been studied by several researchers (Haak *et al.*, 2008; Irie & Sakimoto, 1992; Øverland *et al.*, 1996). They reported that n-3 PUFA amount increased with the increasing of supplemental levels of fish oil in pig diet. However, feeding pigs with fish oil (1-3%) caused off-flavour of the pork, making it not suitable for human consumption (Øverland *et al.*, 1996). Marine algae is an alternative to fish oil supplementation in pig diets (de Tonnac, *et al.*, 2018; Moroney *et al.*, 2012). De Tonnac *et al.* (2018) analysed the effect of soybean and palm oil, extruded linseed, extruded linseed and microalgae, and only microalgae inclusion in pig diets. They found higher levels of PUFA in linseed and microalgae groups, especially higher C18:3n-3 and C20:3n-3 PUFA for linseed group, and higher C20:4n-3, C20:5n-3, C22:6 n-3 in microalgae group.

In many studies linseed has been used to increase the n-3 PUFA in pork. The motivation for linseed inclusion in pig diet is the high ALA content in it, which is a precursor for the long-chain n-3 PUFAs: eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA), beneficial to the human health. According to Corino *et al.* (2008) inclusion of 5% of extruded linseed in pig diets increased n-3 PUFA content in muscle and backfat. Also decreased n-6/n-3 ratio from 12 to 4.5 in muscle, and from 11 to 3 in backfat. No adverse effects were observed on growth, carcass characteristics and meat quality. Also, Enser *et al.* (2000), Guillevic *et al.* (2009), Juárez *et al.*, (2011), Karolyi *et al.* (2012), Kouba *et al.* (2003) and Bečková & Václavková (2010) confirm that feeding linseed diet increased the

content of n-3 PUFA in all tissues, but not the DHA content, and decreased the n-6/n-3 ratio. Matthews *et al.* (2000) reported that ALA content increased in all tissues as the linseed amount were increased in pig diets. According to Corino *et al.* (2014) the use of whole linseed in pig diet could be better than linseed oil. The seed contain also natural Aox, which could slow down the oxidative processes of PUFA.

In pigs, dietary treatments based on vegetable oils did not affect growth performance and carcass characteristics, but affect FA composition of muscle and backfat (Jasińska & Kurek, 2017). Other studies reported that inclusion of vegetable oils, fish oil and marine algae increase PUFA content and decrease SFA, improving n-6/n-3 ratio (Morel *et al.,* 2006; Park *et al.,* 2012; Wojtasik-Kalinowska *et al.,* 2018).

However, increasing PUFA amount increases lipid oxidation with the production of off-flavour compounds in pork: to prevent lipid oxidation, supplementation of pig diets with antioxidants is recommended (Nieto & Ros, 2012).

4.3. Natural antioxidants in pig diet

Animal nutrition moves towards increasing n-3 PUFA amount, and lowering the n-6/n-3 ratio to meet the nutritional guidelines (de Tonnac *et al.*, 2018). This nutritional strategy decreases the oxidative stability of meat during storage, with negative effects on meat shelf-life and nutritional value (Brenes *et al.*, 2016). Vitamin E is widely used in animal feed. Supra-nutritional levels of vitamin E could improve oxidative and colour stability of meat during storage (Buckley *et al.,* 1995; de la Fuente *et al.,* 2009; Kasapidou *et al.,* 2012; Muíño *et al.,* 2014). However, due to its synthetic origin and limited efficacy when n-3 PUFA supplementation is increased (Brenes *et al.,* 2016; Gladine *et al.,* 2007), there was the necessity to include other Aox in animal diets. In recent years there was an increasing interest in the use of natural Aox from plants in livestock production. The plant kingdom is a rich source of Aox which occurs in all parts of plants, containing bioactive compounds able to minimize oxidative stress effects. Among natural Aox, polyphenols received high interest due to their presence in most plants and their antioxidant properties (Gladine *et al.,* 2007; Scalbert *et al.,* 2002).

The metabolism of polyphenols has been widely studied, but is still inconclusive. Polyphenols are generally poorly absorbed, widely metabolized and rapidly eliminated. Their metabolism occurs through a common pathway (Fig. 4.2).

Most polyphenols are present in food in the form of esters, glycosides or polymers that cannot be absorbed in their native form, but must be hydrolysed by intestinal enzymes or by colonic microflora before they can be absorbed (Brenes *et al.,* 2016; Manach *et al.,* 2004). The

microflora has a great capacity for deglycosylation which occurs very quickly, releasing aglycones. These aglycones can also be degraded by the local microbiota into simple aromatic acids. During the absorption process, in the small intestine polyphenols may be conjugated, releasing methyl, glucuronide and sulphate derivatives into circulatory system (Manach *et al.*, 2004; Scalbert *et al.*, 2002). All these phenolic metabolites may be absorbed or excreted in the faeces. Polyphenol absorption depends on their chemical structure rather than the concentration. Monomeric and dimeric polyphenols could be readily absorbed in the small intestine, while oligomeric and polymeric polyphenols could reach the colon almost unchanged (Brenes *et al.*, 2016). The amount of polyphenols absorbed by the body tissues differ from the dietary level; their concentration in plasma rarely exceeds 1 nM (Del Rio *et al.*, 2013; Surai, 2014). The unabsorbed compounds remain in the gut, where have a positive effect on the gut environment by controlling diarrhoea, intestinal cramps and other gastro-intestinal disorders in animals, improving animal performance (Kamboh *et al.*, 2015; Lipinski *et al.*, 2017).

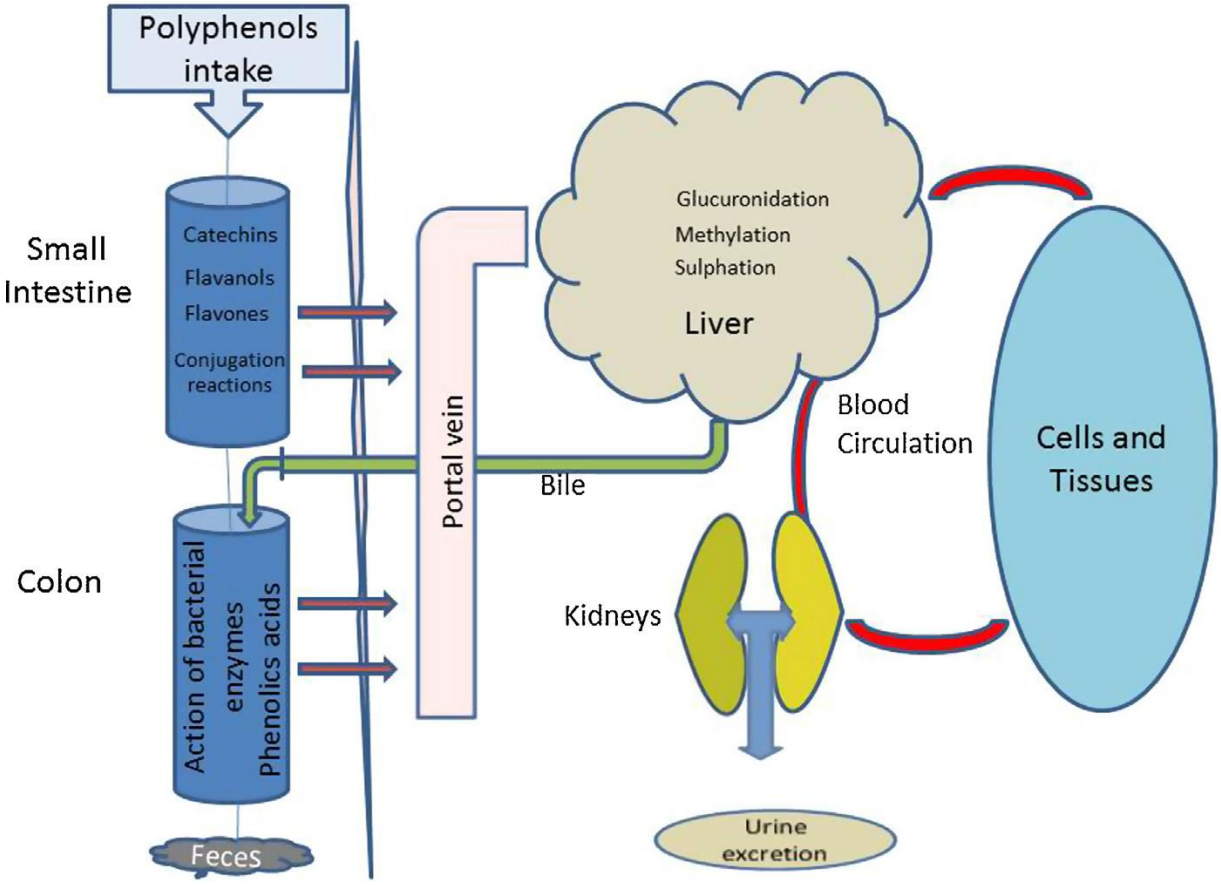

Figure 4.2. Metabolism of dietary polyphenols. Source: Brenes et al. (2016)

Several studies have reported that natural antioxidants inclusion in animal diets increases the oxidative stability and improve meat quality (Table 4.2).

Studies carried out by Ranucci *et al.* (2015) showed that pig supplementation with a plant mix derived from oregano essential oil and sweet chestnut wood extract (0.2%) decreased lipid oxidation and received higher scores for colour and taste in the consumer tests.

Rossi *et al.* (2013) observed that 5 mg/kg feed verbascoside supplementation of pigs from weaning to slaughter (166 days) increased α-tocoferol levels in LTL muscle, enhanced oxidative status and sensory attributes without affecting other meat quality parameters. In another experiment, Rossi *et al.* (2017) reported that pig supplementation with an antioxidant mixture containing 50 mg of vitamin E and 5 mg verbascoside, 45 days before slaughter, improved in vivo antioxidant status and oxidative and colour stability of LTL muscle compared to the control group.

In a study conducted by Ahmed *et al.* (2016) pig supplementation with an herb combination (pomegranate, Ginkgo biloba, liquorice) at 0.4 % in natural and fermented form resulted in an increased n-3 PUFA in LTL muscle, and reduced n-3/n-6 ratio, also reduced TBARS values of pork during storage.

In another study, Cheng *et al.* (2017) found that a reduced protein diet supplemented with 250 mg/kg feed of oregano essential oil in growing-finishing pigs enhanced the sensory attributes, increased n-3 PUFA proportion of LTL muscle and prevented lipid oxidation. In contrast, Simitzis *et al.* (2010) reported that 35-days supplementation with different levels of oregano essential oil (0.25, 0.5 and 1 ml/ kg of feed) did not affect pig meat quality parameters.

Wine industry produces a great quantity of residues in a short period of the year, whose disposal is expensive and involves pollution problems (Bustamante *et al.*, 2008; Selani *et al.*, 2011). The reuse of winemaking by-products can reduce the environmental impact. These products, such as skin and seeds, are rich in polyphenolic compounds, which are characterized by a high antioxidant activity. In recent years there was an increased interest in the inclusion of winery by-products in animal diets as an alternative to vitamin E. Zhao *et al.* (2018) found that lamb dietary supplementation with 5 or 10% of wine grape pomace for 74 days increased feed efficiency and decreased WBSF and collagen content in LTL muscle, also decreased ROS and MDA levels. In another study, Guerra-Rivas *et al.* (2016) reported that lamb supplementation with 500 mg/kg of vitamin E was more effective in preventing microbial growth, lipid oxidation and meat discoloration respect to 50 mg/kg of grape seed or 5.5% of grape pomace.

Pig supplementation with winery by-products was reported by several studies. Bertol *et al.* (2017), Ciuca *et al.* (2013), Habeanu *et al.* (2018) and O'Grady *et al.* (2008) found that winery by-products inclusion in pig diets did not affect animal performance or carcass quality. In contrast, Yan & Kim (2011) showed that 30 g of fermented grape pomace improved growth

Table 4.2. Effect of dose concentration and time of dietary natural antioxidants on meat quality

Natural source	Dose in diet	Animal	Feeding duration	Meat types	Results	References
Herbal extract mixture (sage, nettle, lemon balm and coneflower)	500 mg/kg feed	pig	From 60 ± 0.5 kg to 112 ± 2.0 kg of BW	LT muscle	Improved meat oxidative stability, lowered cholesterol and increased PUFA content in meat.	Hanczakowska, Świątkiewicz, and Grela (2015)
Verbascoside (Verbenaceae extract)	15 mg	pig	38 days before slaughter	LT muscle	Enhanced oxidative status and colour indices.	Rossi *et al.* (2014)
Sugar cane extract	5 and 25 g/kg	pig	42 days	LT muscle	Improved oxidative stability.	Xia *et al.* (2017)
Dried fruit and vegetable pomaces	From 8 to 10%	pig	From 25± 1.1 kg to 107±1.8 kg of BW	LT muscle	Improved fatty acid composition and protected from an excessive oxidation of lipids and cholesterol.	Pieszka *et al*. (2017)
Verbascoside	5 mg/kg feed	pig	166 days	LT muscle	Enhanced oxidative status and sensory attributes.	Rossi *et al.* (2013b)
Grape pomace and grape seed extract	GP-5.5%/kg GSE-50mg/kg	lamb	From 14.3±2.05 kg to 27kg LBW	LTL muscle	No significant differences between polyphenol treatments and control group.	Guerra-Rivas *et al.* (2016)
Red wine extract	900ppm	lamb	From 14.3±1.3kg to 26.6 ±1.5kg	LT muscle	Red wine extract was less efficient against lipid oxidation than vitamin E but more efficient than the control.	Rivas-Cañedo *et al.* (2013)
Grape pomace	5 and 10%	chicks	21 days	muscle	Increased oxidative stability and PUFA content.	Chamorro *et al.* (2015)
Ground linseed and quercetin	85 g linseed + 2 g/kg quercetin	lamb	5 weeks	LT muscle	Increased total n−3 fatty acid content, reduced lipid peroxidation.	Andrés *et al.* (2014)
Oregano oil	1ml/kg	pig	2 months	LTL muscle	Strong antioxidant effects retarding lipid oxidation in meat during refrigerated storage.	Simitzis *et al.* (2008)
Grape pomace fermented by Saccharomyces boulardii	30g/kg	pig	105 days	LTL muscle	Improved the growth performance, nutrients digestibility, FA composition and reduced lipid oxidation.	Yan & Kim (2011)
Grape pomace	5 and 10%	lamb	78.1 (±1.7) kg to 111.0 (±4.8) kg BW	LTL muscle	Improve feed efficiency and meat tenderness, decreased ROS and MDA levels.	Zhao *et al.* (2018)

Oregano essential oil	200 mg/kg	turkey	4 weeks before slaughter	breast	Increased lipid oxidation.	Botsoglou *et al.* (2003)
Turmeric powder	4.5 g/pig/day	pig	30 days	LTL muscle	Induced reductions in a* and b* indexes and in Chroma, lipid oxidation was lower.	Mancini *et al.* (2015)
Turmeric and Andrographis paniculata	0.5%	goat	14 weeks	Biceps femoris muscle	Increased redness colour, improved meat oxidative stability.	Karami *et al.* (2011)
Tea catechins	2-4g/kg of feed	goat	60 days	LT, GM, SM	Inhibited lipid oxidation, decreased drip loss of fresh meat and improved meat colour stability.	Zhong *et al.* (2009)
Thyme leaves (Thymus zygis ssp. gracilis)	3.7% and 7.5%	sheep	8 months	LTL muscle	Delayed colour deterioration, lipid oxidation and bacterial counts.	Nieto *et al.* (2010)
Red wine extract	900 mg/kg of feed	lamb	40 days	LTL muscle	Did not improve lipid or protein stability.	Muíño *et al.* (2014)
Grape pomace concentrate	30 and 60 mg/kg	chicken	3 weeks	breast	Inhibited lipid oxidation.	Sáyago-Ayerdi *et al.* (2009)
Grape pomace	5-10 g/kg	chicken	28 days	breast	Improved meat quality parameters.	Aditya *et al.* (2018)
Thymus vulgaris	3%	rabbit	6 weeks	LD	Higher n−3 fatty acids content and lower lipid oxidation.	dal Bosco *et al.* (2014)

performance and nutrient digestibility, and decreased TBARS values. Also, Kafantaris *et al.* (2018) found that grape pomace inclusion in piglets diets enhanced antioxidant mechanisms, prevented oxidative stress, improved growth performance and enriched meat with n-3 PUFA. Their results are confirmed by Chedea *et al.* (2019), who reported that 5% of grape pomace inclusion in piglet diets increased polyphenol content in plasma and antioxidant activity in the liver, spleen and kidney.

4.4. References

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CHAPTER 5

TESTED PRODUCTS

5.1. Linseed

Linseed is an annual plant belonging to the Linaceae family, genus Linum, of which about 200 species are part (Nykter *et al.*, 2006). *Linum usitatissimum L* is the only species cultivated for industrial purposes. The other species present a modest interest. Linseeds are oval and range in colour from dark red, almost brown to light yellow (the colour could be modified through simple plant breeding techniques). The ends are sharp, the seeds are smooth to the touch and shiny (Fig. 5.1). Linseeds are harvested between July and September by threshing, in the same way as wheat. In the last few decades there was an increasing interest in industrial utilization of linseed, thanks to its chemical composition. The growing area of linseed increased in the last years, also the production amount. In 2017 world production of linseed was 2.79 million tonnes. Major producers of linseed were Kazakhstan followed by Russia and Canada (Table 5.1).

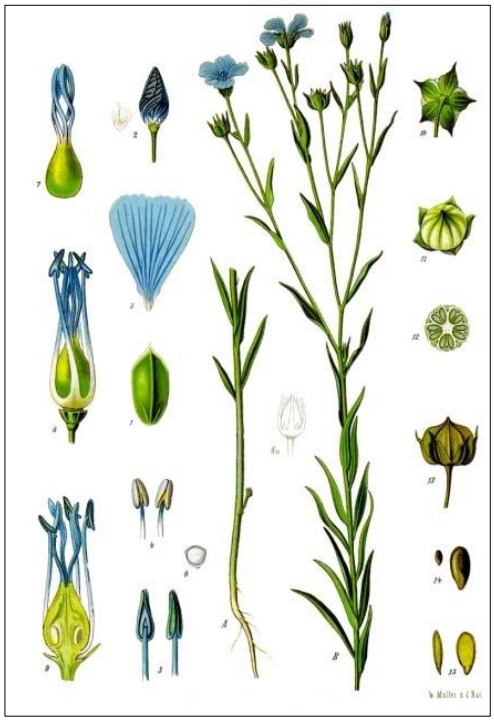

Figure 5.1. Morphology of the linseed plant

Linseed is an ancient culture that dates back to around 8,000 years ago, but still today there is an increasing interest in linseed based products. Linseeds are grown for different products (Fig. 5.2). Seeds are used in human nutrition and for healthy purpose due to the high nutritional value, linen clothes have become very popular and linseed oil has been used for centuries in painting (Karg, 2011), and also in food industry.

Table 5.1. Linseed, production quantity. Top 10 countries for 2017. Source: FAOSTAT (2017)

Country	Linseed, production quantity (tonnes)
World	2,794344
Kazakhstan	683,338
Russia	610,118
Canada	507,606
China	362,034
India	184
United States	97,59
Ethiopia	96,863
Ukraine	46,14
United Kingdom	46
France	42

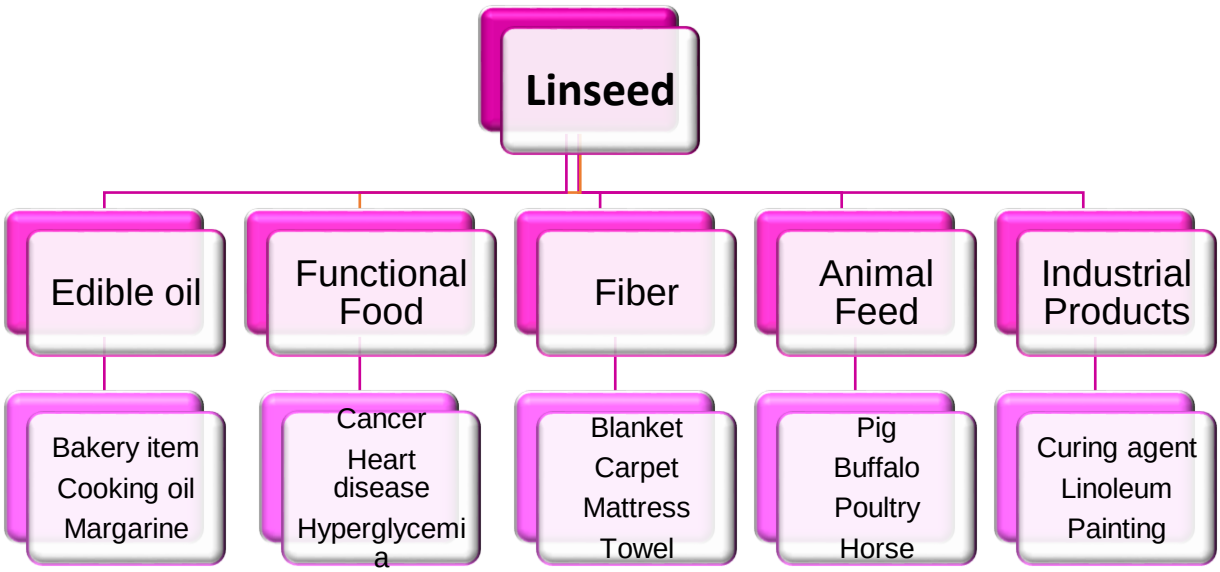

Figure 5.2. Schematic diagram of linseed use. Source: Jhala and Hall (2010)

The most interesting substances contained in linseed are represented by fatty acids, especially unsaturated, proteins of high biological value and lignans (Morriss & Vaisey-Genser, 2003). The composition of linseed can vary with genetics, growing environment, seed processing and method of analysis (Bernacchia *et al.*, 2014). Nykter *et al.* (2006) reported some advantages for growing linseed in the northern countries compared to southern countries. Linseed grown in cool weather zones improve oil content and quality with a higher amount of n-3 fatty acids. The composition of linseed is provided in Table 5.2.

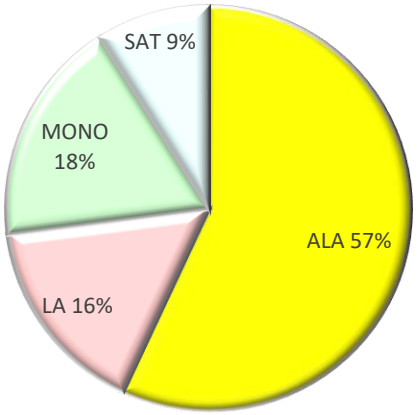

Figure 5.3. Fatty acid composition of linseed. Source: Morris et al. (2003)

Morris *et al.* (2003) reported that the average chemical composition of linseed was 41% fat, 28% total dietary fibre, 20% protein, 7.7% moisture, 3.5% ash, and 1% simple sugars. Linseeds are an important source of fat, especially unsaturated. Only 9% of total fatty acids are saturated, 18% are monounsaturated and 73% polyunsaturated (Fig. 5.3). Among the polyunsaturated fatty acids there are high percentages of essential fatty acids rich in n-3 (α-linolenic acid, ALA) and n-6 (linoleic acid, LA) that our body is not able to produce on its own, and therefore must be introduced with food. For an optimal effect of these two fatty acids the ratio between the quantity of n-3 and that of n-6 PUFA is very important, in fact in linseed the n-3/n-6 ratio is optimal, because of high content of ALA, so our body can use both as its best.

Linseeds have also a high amount of soluble and insoluble fibres. The first, which dissolve in water, help lower cholesterol levels and regulate blood sugar. The insoluble fibres are not absorbed and increase the volume of the stools, favouring intestinal transit. Linseed proteins are similar to those of soya. They are rich in essential amino acids that our body are not able to produce on its own and therefore they must be introduced with the diet. Linseed proteins have the ability to absorb high water amount, that influence positively the texture of food products (Rabetafika *et al.*, 2011).

As the demand for food of high nutritional value is continually growing, in recent years there is a growing interest for linseed, not only for the excellent nutritional characteristics, but also

for the products from this crop, including oil and lignans that have nutraceutical effects and are used as functional foods.

Table 5.2. *Chemical composition of linseed. Source: Bernacchia et al. (2014)*

Fatty Acid	**g/100g of linseed**
α–linolenic acid	22.8
Linoleic acid	5.9
Oleic acid	7.3
Stearic acid	1.3
Palmitic acid	2.1
Aminoacids	**g/100g of linseed**
Glutamic acid	19.6
Aspartic acid	9.3
Arginine	9.2
Glycine	5.8
Cysteine	1.1
Histidine	2.2
Isoleucine	4
Leucine	5.8
Lysine	4
Methionine	1.5
Proline	3.5
Serine	4.5
Threonine	3.6
Tryptophan	1.8
Tyrosine	2.3
Valine	4.6
Dietary Fibres	**g/100g of linseed**
Soluble Fibre	4.3-8.6
Insoluble Fibres	12.8-17.1
Adverse Health Compounds	
Cadmium	0.52μg/kg of linseed
Protease inhibitors	13.3mg/g crude protein
Cyanogenic compounds:	mg/100g of linseed
Limamarin	11
Linustatin	150
Neolinustatin	140

Minerals	**mg/100g of linseed**
Calcium	236
Magnesium	431
Phosphorus	622
Potassium	831
Sodium	27
Zinc	4
Copper	1
Iron	5
Manganese	3
Vitamin	**mg/100g of linseed**
γ–tocopherol	522
α–tocopherol	7
δ–tocopherol	10
Ascorbic acid/vitamin C	0.5
Thiamin/vitamin B1	0.5
Riboflavin/vitamin B2	0.2
Niacin/nicotinic acid	3.2
Pyridoxine(vitamin B6	0.6
Pantothenic acid	0.6
Carbohydrates	**mg/100g of linseed**
Neutral arabinoxylan fraction	1.2
Acidic Rhamnogalacturonan fraction	0.4
Phenolic compounds	
mg/g linseed powder	
Ferulic acid	10.9
Chlorogenic acid	7.5
Gallic acid	2.8
mg/g linseed	
Secoisolariciresinol	165
Laricinesol	1.7
Pinoresinol	0.8
Total Flavonoids	35-70

Also linseeds are used for the improvement of the nutritional characteristics of the meat obtained from animals fed with a linen-based supplement in the diet. Several authors report the enrichment of pig tissues with n-3 PUFA by incorporating linseed in pig diets.

5.2. Grapes

Grape is one of the most widely-grown fruit crops in the world. The crop has a high adaptability and resistance; its optimal environment are the temperate climates but can survive even at temperatures below 10°C. Most grapes come from cultivar *Vitis Vinifera*. Grape cultivation surface is about 7 millions of hectares, where 5 countries represent 50% of the world vineyard (Fig. 5.4) (OIV). The total production of grapes in the world in 2017 was about 74.2 million tonnes. China with 13.1 million tonnes was the biggest producers in the world of grapes (19% of the world production), followed by Italy (7.2 million tonnes), United States (6.7 million tonnes), France (5.9 million tonnes) and Spain (5.4 million tonnes) (FAO). Grapes are consumed as both fresh and processed products (Fig. 5.5). Almost 50% of the total grape production is transformed into wine, the remaining 50% is represented by fresh and processed products such as jam, juice, grape musts, dried grapes and vinegar.

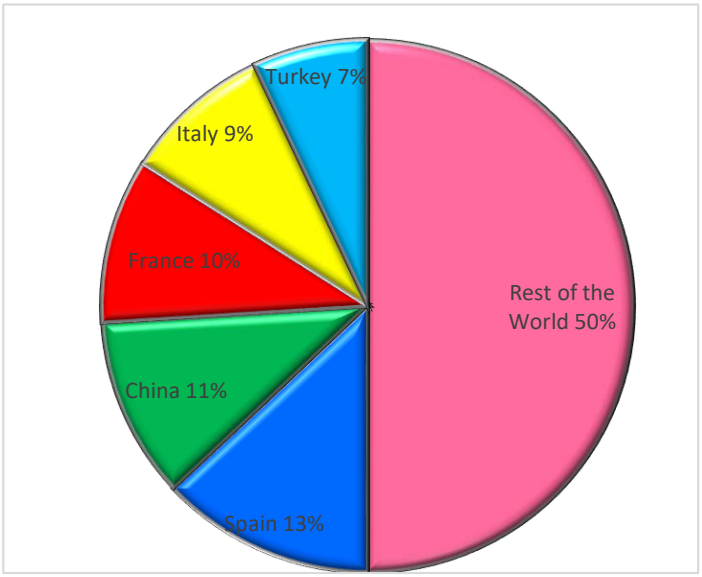

Figure 5.4. Area under vines. Source: OIV

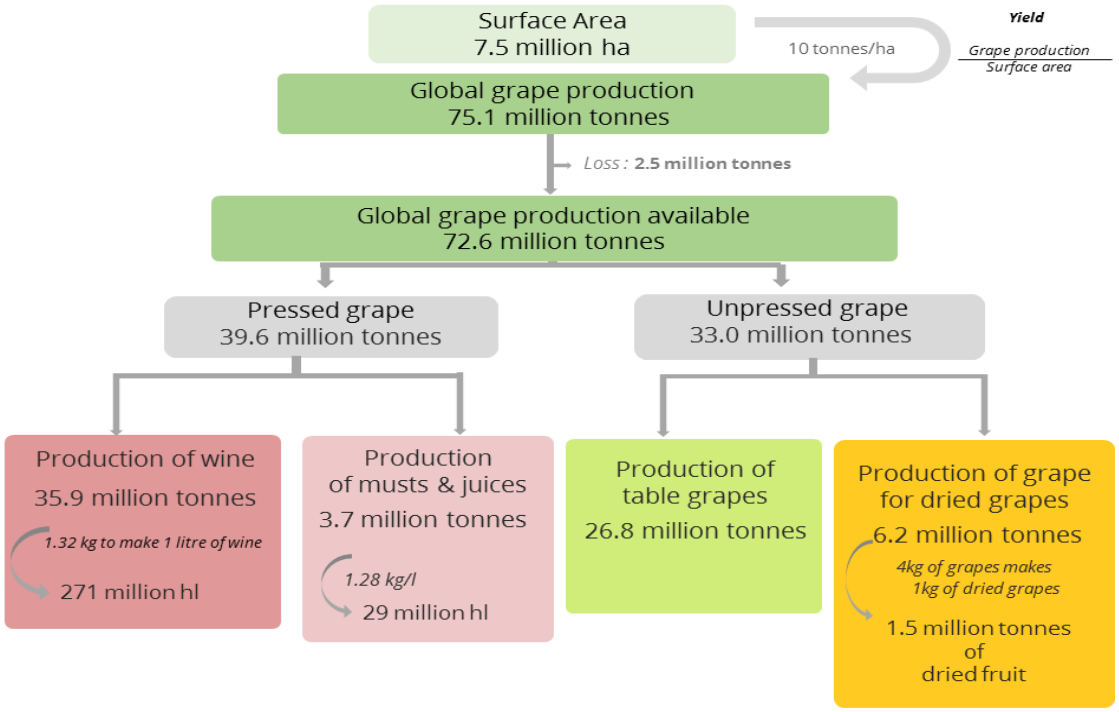

Figure 5.5. Global vitiviniculture situation 2014. Source: OVI-International organization of vine & wine

Grape and grape products represent a valuable source of bioactive compounds with many benefits for human health. As shown in Table 5.3, grapes are rich in carbohydrates that can be assimilated immediately, mineral salts, vitamins: A, of group B and C. In addition, grapes have a high amount of polyphenols which possess different biological activities such as antioxidants, anticancer, cardioprotective and anti-inflammatory (Xia *et al.*, 2010). The composition and taste of grapes depend on the production area, climatic conditions and the variety.

Wine industry produces a great quantity of residues in a short period of the year, whose disposal is expensive and involves pollution problems (Bustamante *et al.*, 2008; Selani *et al.*, 2011). With the pressing of 100 kg of grapes about 25 kg of pomace (or

Table 5.3. Nutrient value of grape. Source: https://www.gov.uk

For 100g servings	***g/100g***
Water	81.1
Protein	0.6
Total fat	0.1
Ash	0.5
Carbohydrates	17
Energy (kcal)	67
Total sugars	17
Glucose	7.7
Fructose	9.3
Micronutrients	***mg/100g***
Vitamin A	2 µg
Vitamin B1	0.09
Riboflavin	0.01
Niacin	0.2
Vitamin C	3
Vitamin E	0.2
Vitamin B6	0.04
Pantothenic acid	0.12
Sodium	1
Potassium	213
Calcium	11
Magnesium	7

marc) are produced. Fifty % of the grape pomace is made from the skins, 25% from the stalks and the remaining 25% from the grape seeds (Fig. 5.6).

These products are rich in polyphenol compounds, which are characterized by a high antioxidant activity. Winery by-products were often undervalued in the past due to the lack of information on their content. Most common use was for "wine alcohol" production, which is used to fortify wines (García-Lomillo & González-SanJosé, 2017). The grape processing residues are also disposed of by distribution on the ground, used for animal feed, destroyed or used for the production of biomass. The commercial value of grape residues depends on the humidity, the presence of the stalks and on the quantity of sugars and alcohol (Bordiga *et al.*, 2019). Therefore, several studies have been conducted on the use of these by-products during the years.

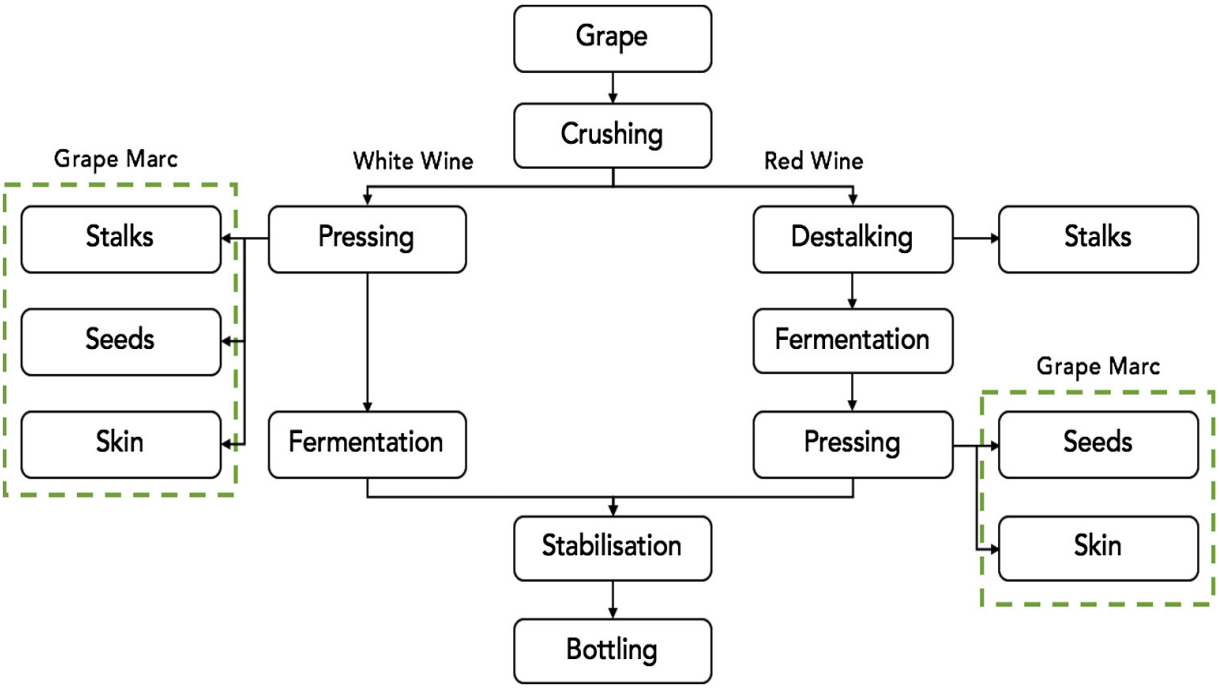

Figure 5.6. Simplified wine production diagram detailing the source and composition of grape marc waste. Source: Zhang et al., (2017)

5.2.1. Grape skin

Grape skin, as other winemaking by-products is a rich source of phenolic compounds. Polyphenols are a group of chemical substances widely distributed in plants, characterized by the presence of more than one phenolic group per molecule. Several studies have stated that polyphenols can have antioxidant characteristics with beneficial health effects, such as help to reduce the risk of cardiovascular disease and cancer (Teixeira *et al.*, 2014). Red grape skin

polyphenols contribute to the colour and stability of wine. The total extractable polyphenols in the skin is around 30%, lower than in the seeds (60%) (Bordiga *et al.*, 2019). In Table 5.4 is shown the phenolic composition of grape skin reported by Ky *et al.* (2014), who analysed the phenolic composition from six red wine cultivars. The amount of polyphenol compounds depends on different factors such as cultivar, climatic conditions, zone of production and extraction techniques. The most abundant phenolic compounds in the red grape skin are anthocyanins and anthocianidins, which have antioxidant properties, antimicrobial effect, reduce lipid peroxidation and the risk of many diseases (Khoo *et al.*, 2017). Anthocianins are the main responsible for the red colour of the grape, and are very susceptible to different agents such as light, temperature, pH etc.

Figure 5.7. Grape skin

Table 5.4. Phenolic composition of grape skin. Source: Ky et al. (2014)

Compounds	mg/g DW (dry weight)
Total phenolic content	45.2
Total tannins	73.0
Total anthocyanins	12.1
Catechin	1.4
Epicatechin	0.4

Red grape skin extract is very high in resveratrol. Salehi *et al.* (2018) reported numerous beneficial health effects, such as anti-cancer, antiviral, neuroprotective, anti-aging, anti-inflammatory and "life extension" effects. In conclusion, the use of grape processing residues as a raw material for the production of bioactive compounds may constitute an interesting alternative.

5.3. Oregano

Oregano is a perennial aromatic plant belonging to the Origanum genus of the Lamiaceae family. The species of this genus (which are about 40 - 50) come mainly from the Mediterranean basin, most common species is *Origanum vulgare* (Kotronia *et al.*, 2017). Antique oregano plant

has been widely used in Mediterranean cooking as food flavouring and for food preservation. Also it has been used in traditional medicine to treat different ailments and pains(García-Beltrán & Esteban, 2016).

In the last years this aromatic plant received more attention due to the growing interest in safer and minimally processed products, replacing synthetic antioxidants with natural.

The oregano composition depends on the specie, climate, time of recollection and the stage of growth (Tab. 5.5).

Recent studies reported that oregano has several properties: antimicrobial action, antioxidant properties, antimycotic, anti-inflammatory and insecticidal activity (Kotronia *et al.*, 2017; Singletary 2010). Their beneficial effects depend on appropriate dosages, otherwise if used in excess could be toxic.

Beneficial properties of oregano plant is due to the presence of essential oils which are products of the secondary metabolism of plants (García-Beltrán & Esteban, 2016). Several studies reported that carvacrol and thymol (Fig. 5.9) are the main components present in oregano essential oil responsible for the bioactive properties (Almeida *et al.,* 2013; García-Beltrán and Esteban 2016; Kotronia *et al.,* 2017; Singletary 2010). Due to its therapeutic properties oregano oil is used not only in food industry but also in cosmetic and pharmaceutical industry.

Figure 5.8. Origanum vulgare.

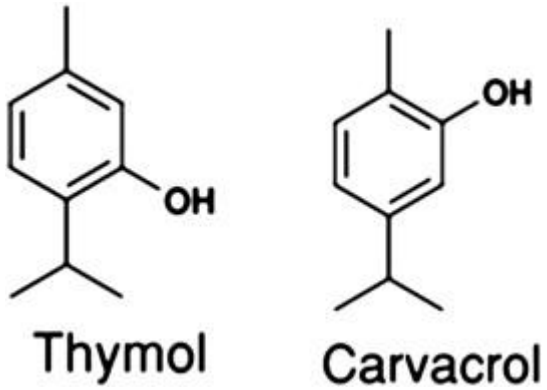

Figure 5.9. Molecular structure of two of the main components of oregano essential oil

Table 5.5. Chemical composition of oregano. Source: USDA Database

Content in 100 g of dried oregano		
Water	g	9.93
Energy	kcal	265
Protein	g	9
Fat	g	4.28
Carbohydrate	g	68.92
Fibre	g	42.5
Minerals		
Ca	mg	1597
Fe	mg	36.8
Mg	mg	270
P	mg	148
K	mg	1260
Na	mg	25
Se	µg	4.5
Vitamins		
Vitamin A	IU	1701
Thiamin B1	mg	0.177
Riboflavin B2	mg	0.528
Niacin B3	mg	4.64
Pantothenic acid	mg	0.921
B6	mg	1.044
Vitamin C	mg	2.3
Vitamin E	mg	18.26
Vitamin K	µg	621.7
Lutein+Zeaxanthin	µg	1895
β−Carotene	µg	1007
Folate	µg	237

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CHAPTER 6

AIM OF THE RESEARCH

Meat and meat products are important sources of protein, providing essential amino acids, fat, minerals, vitamins and other nutrients. However, in recent years, some epidemiological studies have associated red meat and processed meat consumption with an increased risk of cardiovascular disease and colorectal cancer. A good strategy for improving nutritional profile and pork quality could be the manipulation of animal diet.

The aim of this research was to produce "healthier" pork by feeding pigs with ingredients rich in n-3 fatty acids as extruded linseed and addition of synthetic or natural antioxidants for improving meat stability.

Two experiments were performed to investigate the potential application of extruded linseed and synthetic or natural antioxidants from oregano and grape skin in pig diets, on the carcass characteristics and pork quality. The first experiment was made as a preliminary test, for the evaluation of the tested products.

- The first experiment investigated the effect of inclusion of extruded linseed supplemented with supra-nutritional doses of vitamin E and Selenium or natural antioxidants from grape skin extract in pig diets on the carcass characteristics and lipid fatty acid composition, meat quality and shelf-life of *Longissimus thoracis et lumborum* (LTL) muscle packaged in modified atmospheres (MAP) with oxygen (70% O_2/30% CO_2) or nitrogen (70% N_2/30% CO_2), during refrigerated storage.
- In the second experiment, in comparison with the first one, a diet with only extruded linseed was included, and the diet with natural antioxidants was supplemented with oregano extract to improve the antioxidant activity. The effects of the diets on the same parameters of the previous experiment were evaluated, in

addition to the microbial growth of LTL muscle packed in high oxygen MAP (35% CO_2, 65% O_2).

CHAPTER 7

MATERIAL AND METHODS

7.1. Solvents and reagents

Trichloroacetic acid 10% (TCA), 0.06M 2-thiobarbituric acid (TBA), tetraethoxypropane (TEP) were obtained from Sigma-Aldrich, Milan, Italy; sulphuric acid 96%, hydrogen peroxide 30%, kjeltabs, Petroleum ether, Methanol, chloroform from PanReac AppliChem, Darmstadt, Germany; potassium chloride 0.88%, potassium hydroxide 2N, hexane from Carlo Erba, Milan, Italy; Tridecanoic acid from Larodan Fine Chemicals AB, Malmö, Sweden.

7.2. Tested products

Extruded linseeds were added in all experimental diets. The chemical composition of extruded linseed was characterized as follows: moisture (8%), crude fibre (25.0%), crude protein (20.2%), crude lipid (29.6%), and ashes (3.0%). The α-linolenic acid amounted to 54.7% of total fatty acids. In the diets the α-linolenic acid content (g per 100 g of total fatty acids) was 5.2% in the control and 25.4% in the experimental diets with linseed inclusion. GSE was produced by Enocianina Fornaciari s.n.c. (Reggio Emilia, Italy) and oregano extract by Phenbiox s.r.l., (Bologna, Italy). Grape skin and oregano extracts are natural products used in food industry as a supplement, included in finished products at concentrations ranging from 2 to 4 g kg-1, according to the manufacturer's suggestions, that we complied with.

7.3. Animals and diets

All the experimental procedures were in accordance to the Italian legislation (D.Lgs 4 Marzo 2014 n. 26 art. 2 punto F).

Experiment I

Twelve castrated male Italian Large White pigs, evenly divided according to weight into three groups of four subjects each, were housed in three 9 m^2 concrete floored pens. Starting from 79.4±7.4 kg live body weight (LBW) and till slaughtering, at 135.4±9.7 kg LBW, the subjects were fed restricted for 70 days on 8.5% of metabolic weight. The animals were fed with three different diets: control group (C) a barley/soybean based diet; linseed and synthetic antioxidants group (LE), 5% of extruded linseed supplemented with supra-nutritional levels of synthetic antioxidants (200 ppm of α-tocopheryl acetate and 0.21 ppm of Se); linseed and grape skin extract group (LGSE), 5% extruded linseed with 3 g/kg of red grape skin extract, providing 29.8 ppm of polyphenols (expressed as gallic acid equivalent). In LE and LGSE, 5% of barley was substituted with 5% of extruded linseed.

The diets were isoenergetic and isoproteic and with the same lysine/digestible energy ratio. Water was always available through nipple drinkers and the diet was distributed in liquid form (water : feed ratio 3:1). Grape skin extract was diluted in the water of the diet.
Composition of experimental diets and their fatty acid proportions are shown in Table 7.1.

Experiment II

Forty-eight Large White pigs, balanced for sex and weight, allotted in 16 pens of 3 animals each, were fed with four different diets (12 pigs per diet): control group (C) a barley/soybean based diet; linseed group (L), 5% extruded linseed; linseed and synthetic antioxidants group (LE), 5% extruded linseed with 200 ppm vitamin E and 0.21 ppm selenium added (supra-nutritional levels); linseed and vegetal extracts group (LVE), 5% extruded linseed with extracts from grape skin (3 g/kg feed) and oregano (2 g/kg feed) added in liquid form to the water used to dilute the feed. The total amount of polyphenols was 10.4 g/L for grape-skin extract and 3.9 g/L for oregano extract (expressed as Gallic Acid Equivalent). Linseed substituted an equal amount of 5% of barley in L, LE and LVE group. The diets were isoenergetic and isoproteic. Composition of experimental diets and their fatty acid proportions are shown in Table 7.2.

Table7.1. Experiment I: composition of the experimental diets (as fed basis)

		Diet		
		C	LE	LGSE
Ingredients				
Extruded linseed	%	0.0	5.0	5.0
Barley	%	89.5	84.4	84.5
Solvent extracted soybean meal	%	7.0	7.0	7.0
L-Lysine	%	0.3	0.3	0.3
L-Threonine	%	0.1	0.1	0.1
Calcium carbonate	%	1.2	0.8	1.2
Dicalcium phosphate	%	1.0	1.0	1.0
Sodium chloride	%	0.4	0.4	0.4
Minerals and vitamins premix[1]	%	0.5	0.5	0.5
Vitamin E + selenium[2]	%	0.0	0.5	0.0
Red grape skin extract	%	0.0	0.0	0.3
Calculated nutrient composition				
Digestible energy (DE)	Kcal/kg	3082	3151	3154
Chemical composition				
Calcium	%	0.78	0.76	0.80
Phosphorus	%	0.52	0.53	0.53
Digestible phosphorus	%	0.24	0.25	0.25
Lysine	%	0.85	0.87	0.87
Digestible lysine	%	0.73	0.75	0.75
Lysine/DE ratio	g/Mcal	2.75	2.75	2.75
Dry matter	%	90.5	90.6	90.7
Crude protein	% as fed	13.2	13.7	13.6
Crude fat	"	1.6	3.5	3.5
Crude fiber	"	5.0	5.0	4.9
ADF	"	7.0	6.8	7.0
NDF	"	20.6	19.6	20.8
ADL	"	1.3	1.2	1.4
Crude ash	"	5.2	5.1	5.0
Fatty acids composition	(% of total FAs)			
C 14:0 (myristic)		0.59	0.26	0.32
C 16:0 (palmitic)		27.53	15.94	16.70
C 16:1 (palmitoleic)		0.08	0.02	0.02
C 18:0 (stearic)		1.30	2.86	2.79
C 18:1n-9 (oleic)		12.47	16.10	15.96
C 18:2n-6 (linoleic)		52.16	35.50	35.44
C 18:3n-3 (α-linolenic)		5.81	29.24	28.65
C 20:1 (eicosenoic)		0.06	0.04	0.11

C, control; LE, extruded linseed, 50 g kg^{-1}, vitamin E, 200 mg and Se 0.21 mg kg^{-1}; LGSE, extruded linseed, 50 g kg^{-1}, red grape skin extract 3 g kg^{-1} (four pigs per group).

[1]Providing following nutrients (per kg diet as fed) : vitamin A, 15,000 UI; vitamin D3, 2000 UI; vitamin E (α-tocopheryl acetate), 50 mg; vitamin K, 2.5 mg; vitamin B1, 2.0 mg; vitamin B2, 5.0 mg; calcium D-pantothenate, 15.0 mg; niacin,

25.0 mg; vitamin B12, 0.036 mg; vitamin B6, 4.0 mg; folic acid, 1.0 mg; biotin, 0.15 mg; choline chloride, 346.0 mg; Zn (ZnO), 100.0 mg; Cu ($CuSO_4$), 15.0 mg; Mn (MnO), 25.0 mg; Fe ($FeSO_4$), 150.0 mg; I ($Ca(IO_3)_2$), 1.5 mg; Co ($CoCO_3$), 0.4 mg; Se (Na_2SeO_3), 0.1 mg. [2]Providing vitamin E (α-tocopheryl acetate) 200 mg and Se 0.21 mg kg^{-1} as fed.

Table 7.2. Experiment II: composition of the experimental diets (as fed basis)

		C		L		LE		LVE	
		1st	2nd	1st	2nd	1st	2nd	1st	2nd
Ingredients									
Extruded linseed	%	0.00	0.00	5.00	5.00	5.00	5.00	5.00	5.00
Barley meal	%	85.50	91.00	80.50	86.60	80.30	86.40	80.50	86.60
Soybean meal	%	11.00	5.50	11.00	5.00	11.00	5.00	11.00	5.00
L-Lysine	%	0.31	0.29	0.30	0.29	0.30	0.29	0.30	0.29
DL-Methionine	%	0.06	0.04	0.06	0.03	0.06	0.03	0.06	0.03
L-Threonine	%	0.05	0.04	0.05	0.03	0.05	0.03	0.05	0.03
Calcium carbonate	%	1.18	1.13	1.19	1.15	0.89	0.85	1.19	1.15
Dicalcium phosphate	%	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Salt (NaCl)	%	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
vitamin/mineral pre-mix[1]	%	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin E and Selenium pre-mix[2]	%	0.00	0.00	0.00	0.00	0.50	0.50	0.00	0.00
Vegetal extract (Grape skin + oregano)	g *per* kg of feed	0.00	0.00	0.00	0.00	0.00	0.00	3.00+2.00	3.00+2.00
Calculated nutrient composition									
Digestibile energy	kcal/kg	3189	3168	3255	3235	3248	3228	3255	3235
Chemical composition									
Crude protein	%	14.89	11.31	15.39	11.73	15.37	11.71	15.39	11.73
Crude fat	%	1.75	1.74	3.58	3.58	3.58	3.58	3.58	3.58
Crude fiber	%	4.33	4.2	4.62	4.48	4.61	4.47	4.62	4.48
Ca	%	0.80	0.79	0.82	0.79	0.82	0.79	0.82	0.79
P	%	0.54	0.54	0.55	0.53	0.55	0.53	0.55	0.53
Fatty acid composition	% (of total FAs)								
C14:0	%	0.47	0.39	0.25	0.21	0.25	0.22	0.26	0.22
C16:0	%	29.01	24.25	18.13	15.20	17.78	15.59	18.80	15.31
C16:1	%	0.49	0.34	0.17	0.15	0.17	0.17	0.02	0.15
C18:0	%	2.03	1.51	4.00	3.18	3.88	3.34	4.16	3.23
C18:1 n-9	%	14.92	13.50	20.60	18.12	20.24	18.45	21.29	18.26
C18:2 n-6	%	47.55	53.67	33.50	34.69	33.91	34.09	32.52	34.47
C18:3 n-3	%	4.77	5.70	22.83	28.02	23.25	27.73	22.38	27.95
C20:1	%	0.74	0.64	0.53	0.41	0.52	0.42	0.57	0.41

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed) (twelve pigs per group).

1st = feed administered from an average weight of 80 kg to 120 kg (growing period); 2nd= feed administered from an average weight of 120 kg to slaughter (finishing period).

[1]Providing the following nutrients (per kg diet as-fed): Vitamin A 15,000 IU; Vitamin D3 2,000 IU; Vitamin E (alpha-tocopheryl-acetate), 50 mg; Vitamin K, 2.5 mg; Vitamin B1, 2 mg; Vitamin B2 5 mg; Vitamin B5, 15 mg; Vitamin B6, 4 mg; Vitamin B12, 0.036 mg; Niacin, 25 mg; Folic acid, 1 mg; Biotin, 0.15 mg; Choline, 346 mg; Cu, 15 mg; Fe, 150 mg; Mn, 25 mg; Co, 0.4 mg; I, 1.5 mg; Zn, 100 mg; Se 0.1 mg.

[2]Providing the following nutrients (per kg diet as fed): Vitamin E (alpha-tocopheryl-acetate), 200 mg and Se, 0.21 mg.

The trial started from 79 ± 5.8 kg LBW till slaughtering, at 150.5 ± 9.9 kg LBW and was divided into two feeding periods: growing and finishing period. During the first period, from an average weight of 79.9 ± 5.8 kg to 113.4 ± 10.6 kg, the amount of the supplied feed was calculated as 7.5% of the metabolic weight (1st on Table 7.2). During the finishing period, from 113.4 ± 10.6

kg to the slaughter at an average weight of 150.5 ± 9.9 kg, the amount of the supplied feed was calculated as 8.5% of the metabolic weight (2nd on Table 7.2). Before the end of the experiment one pig from the LVE group died by natural causes.

7.4. Growth performance and carcass measurements

The animals were weighed individually at the beginning and at the end of the experiment. The following parameters were determined: body weight, average daily gain and the feed conversion ratio.

The pigs were slaughtered in a commercial abattoir by exsanguination after electrical stunning, in agreement with the Council Regulation (EC) No 1099/2009 on the protection of animals at the time of the killing.

After slaughter, individual hot carcass weight was recorded, and backfat and muscle thicknesses were measured by Fat-o-Meat'er at level of 3/4 last rib, at 8 cm from the splitting line of the carcass (D.M. N. 15163, 2012, October 12). Cold carcass weight was calculated as hot carcass weight / 0.98, and dressing percentage (hot and cold) as carcass weight (hot and cold)/slaughter weight x 100.

7.5. Sampling

After slaughtering, each carcass was graded using Fat-o-Meat'er and dissected in commercial cuts. At dissection the whole left LTL muscle (Fig.7.1) and a sample of subcutaneous adipose tissue at the last rib level, were removed from each carcass. The samples were stored at +4°C and sent to the laboratory for subsequent analyses.

Figure 7.1. Longissimus thoracis et lumborum muscle

At 24 h *pm*, individual LTL muscles were sliced into two sections, one intended for the shelf-life study (slices were packed in modified atmosphere (MAP)) and the other one destined to chemical analyses, was vacuum packed (Elegen, Scandiano, Reggio Emilia, Italy) and stored at -20°C. Individual backfat samples were vacuum packed and stored at -20°C for the subsequent analyses.

7.6. Packaging and shelf-life study

For the shelf-life study colour, pH, microbiology and thiobarbituric acid reactive substances (TBARS) analyses were performed. The procedures are described below.

Experiment I

From each loin, 6 slices (approximately 2-2.5 cm thick) (Fig.7.2), designated for the evaluation of the shelf-life in two different modified atmospheres, were individually weighed and packed in a total of 72 high barrier trays lidded with a PET/EVOH/PE film (AERPACK System, kindly supplied by Coopbox Group, Italy). The whole package oxygen transmission rate was < 0.1 cm^3 day^{-1} (air, 25 °C). MAP was performed using a semiautomatic vacuum compensation thermosealing machine (Ca.Ve.Co, Italy). Two different gaseous mixtures were used: 70%N_2/30%CO_2 (N_2) and 70%O_2/30%CO_2 (O_2). Gas composition of the headspace was analysed before opening packages using a HWD-GC equipped with a concentric CRT I column (6ft x 1/4”; outer column: activated molecular sieve; inner column: porous polymer mixture) (Alltech Italia, S.r.l, Italy). Gas chromatographic conditions: gas carrier Helium (65 mL min^{-1}); temperature 55 °C; analysis time 5 min. A septum was glued onto the surface of the lid and a 50 mL gas aliquot was withdrawn with a gastight syringe and injected in the gas chromatograph. The calibration was performed by injecting separately pure gases as external standard (supplied by Sapio; pureness 99.9) and calculating the response factor for each one.

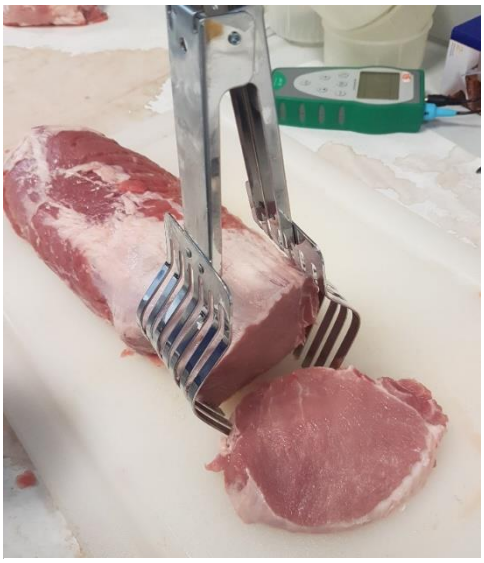

Figure 7.2. Samples preparation for MAP

All samples packaged in modified atmosphere were stored in the dark at 3±1 °C for a maximum of 8 days.

Experiment II

At 24 h pm, a section of LTL muscles of each pig, were sliced in 5 subsamples (approximately 2-2.5 cm thick), which were placed in trays and then packed under modified atmosphere (35% CO_2, 65% O_2). Twelve loins (11 loins for LVE group) were analysed for each

treatment group. The packages were randomly assigned to different storage periods (0, 4, 6, 8 and 12 days) and stored at 2±1°C in darkness until analysis. MAP was performed using a semiautomatic vacuum compensation thermosealing machine (Ca.Ve.Co, Italy). Gas composition of the headspace was analysed as described above.

7.7. Physical parameters of raw and cooked muscle

The values of pH were measured at 24 h pm using a portable Crison pH-meter (Fig. 7.3) equipped with a Xerolite electrode (Crison Instruments, Alella, Spain). The pH probe was calibrated using two buffers (pH 4.0 and 7.0).

The sample surface colour was determined using a Minolta CM-600d spectrophotometer (Konica Minolta Holdings, Inc, Osaka, Japan) (Fig. 7.4). The illuminant used was D65 with 8mm diameter aperture. The instrument was calibrated using a white calibration plate before analysis. The samples were analysed in three different points, avoiding the area of visible fat, and the measurements were averaged. Colour measurements complied the CIELAB colour system, where the three fundamental coordinates are L*- "lightness", a*- "redness", b*- "yellowness" values. Further, Chroma (C*), referred to as saturation index and colour intensity, was calculated as: $\sqrt{(a^{*2} + b^{*2})}$, and hue angle (H*), which defines colour, was calculated as: arctan (b*/a*). Moreover, for the shelf-life study, colour variation (ΔE) was calculated as $\Delta E=\sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2}$ where ΔL*, Δa*and Δb* are the difference between time 0 (at 24 h *pm*) and the values L*, a* and b* respectively at different times of refrigeration.

Figure 7.3. pH-meter

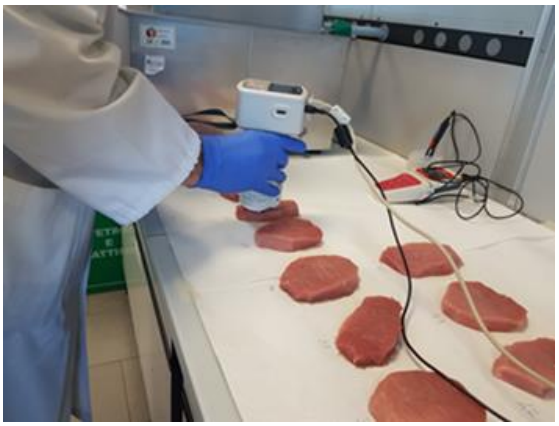

Figure 7.4. CIE-LAB colour measurement

Drip loss was determined according to Honikel (1998), slightly modified. A slice of fresh muscle (about 100 g) was hooked and then suspended in an inflated bag, ensuring that the sample did not make contact with the bag (Fig. 7.5). The weight loss percentage after 48 h of storage at 4°C was calculated.

Cooking loss was calculated as weight difference between raw and cooked samples. In brief, slices of about 2.5 cm thickness, weighing approximately 100 g, placed in vacuum plastic bags, were put in water-bath till the core temperature reached 70 °C (Fig. 7.6). The internal temperature of samples was controlled during cooking with a temperature probe. The samples were weighed after cooling. Cooking losses were expressed as a percentage of the initial sample weight.

Figure 7.5. Drip loss analyses

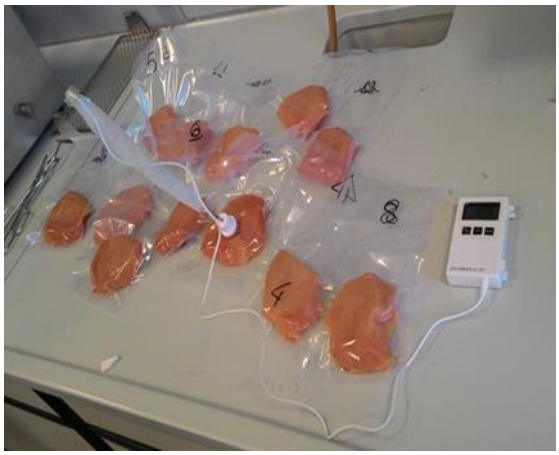

Figure 7. 6. Sample preparation for Cooking loss analyses

The Warner–Bratzler shear force (WBSF) was determined on the cooked samples. The samples were cut, parallel to the longitudinal orientation of the muscle fibres according to Honikel (1998) method, into six cylindrical cores (Ø 1.50 cm). Each core was sheared with a WBSF device attached to a Zwick Z50 kN Testing Machine (model BT1-FB050TN, Zwick Roell, Kennesaw, GA USA) (Fig. 7.7) having a 1kN load cell equipped with the V-shaped blade that have a triangular hole of 60° at a speed of 250 mm/min. The peak force (average value of 5 measurements for each sample) was expressed as kg.

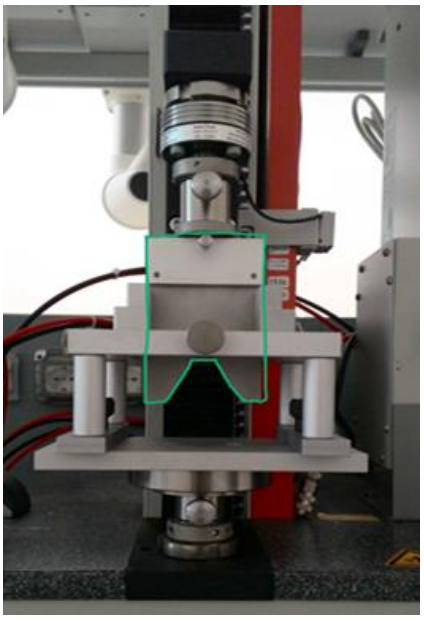

Figure 7.7. Dynamometer

7.8. Microbiological analysis

Experiment II

Ten grams of meat were taken aseptically from each tray and homogenised with 90 ml of sterile saline solution (0.9% NaCl) inside a sterile blender bag in a Stomacher 400 (Instruments Lab Control, Reggio Emilia, Italy) for 60 s at room temperature. Serial decimal dilutions were made in sterile saline solution. One ml of appropriate dilutions was dispersed in Petri dishes with brain heart infusion agar (BHIA) for the enumeration of total mesophilic counts (TMC) and violet red bile glucose agar (VRBGA) for the *Enterobacteriaceae* (Fig. 7.8). Each sampling was replicated twice. All plates were examined visually for typical colony types that were associated with each growth medium, after incubation at 30ºC for 24 h. Microbial counts were expressed as base-10 logarithms colony forming units per grams ($\log_{10}$ CFU/g).

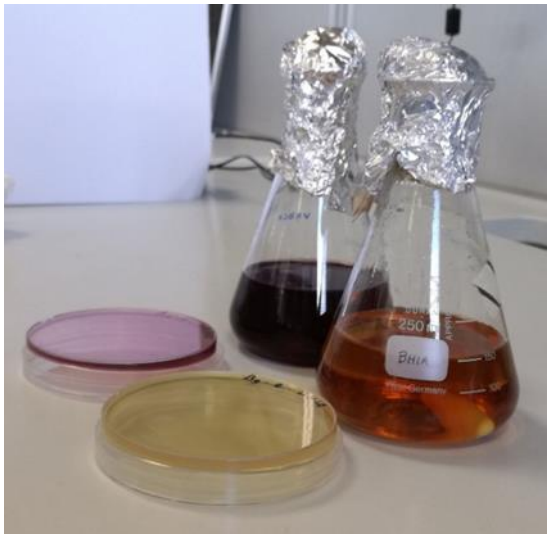

Figure 7.8. Microbiological analysis

7.9. TBARS determination in raw and cooked muscle

Experiment I and II

Oxidative stability was evaluated by the 2-thiobarbituric acid reactive substances (TBARS) measurements according to Siu & Draper (1978) method. In detail, each sample of muscle was minced and an aliquot of 2.5 g was homogenized in 12.5 mL of distilled water at 9500 rpm for 2 min, using an Ultra-Turrax tissue homogenizer (IKA, Germany), and then vortexed for 1 min at high speed. Samples were centrifuged for 20 min at 2000 rpm at 4°C with 12.5 mL of 10% trichloroacetic acid (TCA) (Sigma-Aldrich, Milan, Italy) and the supernatant decanted through a paper filter (Whatman 541). Four mL of clear filtrate were transferred into 15 mL pyrex screw cap test tubes and added of 1 mL of 0.06M 2-thiobarbituric acid (TBA). A distilled water-TCA-TBA reagent blank was prepared and treated like the samples. The samples were heated in a water bath at 80 °C for 90 min and then cooled. Absorbance at 532 nm was measured against a blank sample on two replicates of each sample on a Jasco spectrophotometer (Model V550, UV/VIS, Tokyo, Japan) immediately after cooling (Fig. 7.9). TBARS were expressed as mg of malondialdehyde (MDA) per kg of muscle using tetraethoxypropane (TEP) (Sigma-Aldrich, Milan, Italy) as a standard.

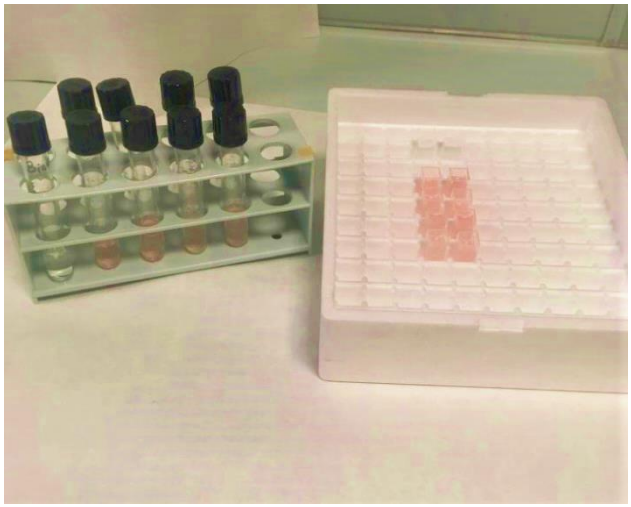

Figure 7.9. TBARS analysis

7.10. Chemical composition of muscle

Experiment I and II

After thawing, samples of LTL muscle were analysed in duplicate to determine the moisture, crude fat and crude protein, according to the methods of the Association of Analytical Chemists (AOAC, 1995). Crude fat was determined by extraction in petroleum ether after acidification with 3 N HCl solution. Moisture and crude fat were determined also for backfat. Results were expressed as percentage of wet matter.

Total lipids were extracted according to Folch *et al.* (1957) for LTL muscle and according to IUPAC (1979) for the backfat and perirenal fat.

7.11. Fatty acid profile of LTL muscle, backfat and perirenal fat

Experiment I and II

Fatty acid profile of lipids was determined using a TRACE™GC Ultra (Thermo Electron Corporation, Rodano, Milano, Italy) equipped with a Flame Ionization Detector, a PTV injector, and a TR-FAME Column (Thermo Scientific, Rodano, Milano), 30 m long, 0.25 mm i.d., 0.2 μm film thickness (Fig. 7.10). An aliquot of 25 mg of lipids (extracted as describe above) was subjected to methylation by means of a methanolic solution of potassium hydroxide (KOH 2N) according to Ficarra *et al.* (2010), using tridecanoic acid (C13:0) (Larodan Fine Chemicals AB, Malmö, Sweden) as internal standard. The injection of the fatty acid methyl ester sample (1 μL) was performed in split mode with a split flow of 10 mL/min, operating at a constant flow of 1 mL/min of Helium as carrier gas. The temperature of injector and detector was kept at 240 °C. The temperature program used for the analysis started from 140 °C, was maintained for 2 min, then increased to 250 °C, at a rate of 4 °C min^{-1}, and kept at this temperature for 5 min. The peaks of the fatty acid methyl esters (FAMEs) were recorded and integrated using Chrom-Card software (vers. 2.3.3, Thermo Electron Corporation, Rodano, Milano, Italy) and identified by comparison with the retention times of standard solutions with known quantities of various methyl esters (Supelco® 37 Component FAME mix, PUFA standard n.2, Animal Source, Supelco, Bellafonte, PA, USA and single FAMEs standard, Larodan Fine Chemicals AB, Malmö, Sweden). For quantification purposes, the response factor was calculated, and the method of the internal standard was used. The amount of each FAME in the sample was expressed as FAME relative percentage with respect to the total amount of FAMEs.

Iodine value (IV) of backfat was calculated adopting the equation proposed by Lo Fiego *et al.* (2016): IV = 85.703 + [C14:0] x 2.740 - [C16:0] x 1.085 - [C18:0] x 0.710 + [C18:2n-6] x 0.986.

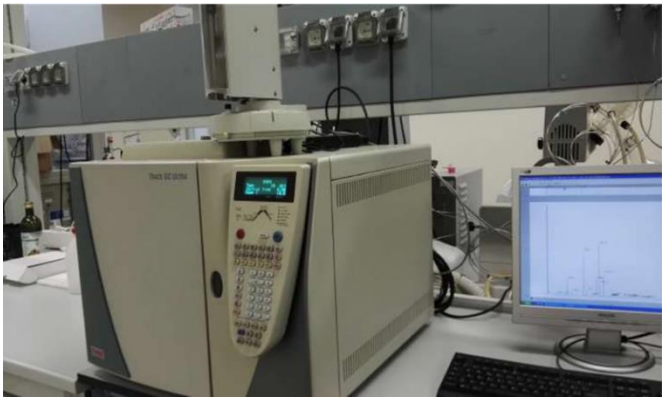

Figure 7.10. Gas chromatography

7.12. Statistical analysis

Statistical analysis for the two experiments was performed by means of analysis of variance using the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA). The statistical model for performance (only for the Experiment II), carcass characteristics, qualitative characteristics and chemical composition of fresh LTL muscle, fatty acid composition of subcutaneous adipose tissue, perirenal tissue and LTL muscle, used dietary treatments (C, LE and LGSE- Experiment I; C, L, LE, LVE – Experiment II) as independent variables. The pen is considered as experimental unit for feed conversion calculation. For the performance data of the subjects from Experiment I, only the arithmetic means were calculated.

Experiment I

The data from samples stored in MAP were statistically analysed within storage day including in the model also the packaging atmosphere (O_2 or N_2) and the interaction between dietary treatments and packaging atmosphere. The interaction was never statistically significant ($P>0.05$) and was thus removed from the model.

The two degrees of freedom of dietary treatments were *a priori* splitted into two orthogonal contrasts comparing, respectively, control group *vs* average of extruded linseed groups (C *vs* (LE+LGSE)/2) and antioxidant supplemented groups between them (LE *vs* LGSE).

Experiment II

The shelf-life data were analysed by repeated measures analysis using the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA), including in the statistical model the dietary

treatments of animals (C, L, LE and LVE), the times of storage of meat samples (0, 4, 6, 8, 12 days) and the relative interaction, as fixed effects, and the pig as random effect.

7.13. References

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CHAPTER 8. EXPERIMENT I

Effects of dietary linseed and synthetic or natural antioxidants on shelf-life and quality of pork

RESULTS AND DISCUSSION

8.1. Growth performance and carcass characteristics

The data of growth performance and carcass characteristics in pigs are reported in Table 8.1 and 8.2. There were no consistent differences for the growth performance between groups (pen or dietary treatment). Moreover, no significant effect (P>0.05) of dietary treatments were detected on carcass characteristics. The final body weight reached by the pigs at the end of the experiment was similar for the three groups. Guillevic *et al.*(2009) and Haak *et al.* (2008), feeding linseed to finishing light weight pigs, could not detect either any difference in the growth performances and carcass characteristics.

Table 8.1. Growth performance of pigs fed with the experimental diets

Items	Dietary treatments					
	C	Std Dev	LE	Std Dev	LGSE	Std Dev
Initial body weight (kg)	79.3	9.67	80.1	6.52	78.9	7.88
Final body weight (kg)	137	14.36	133.2	6.56	136.1	9.29
Average daily gain (g)	824	0.09	759	0.13	816	0.08
Feed conversion ratio	3.25	*	3.47	*	3.22	*

C, control; LE, extruded linseed, 50 g kg^{-1}, vitamin E, 200 mg and Se 0.21 mg kg^{-1}; LGSE, extruded linseed, 50 g kg^{-1}, red grape skin extract 3 g kg^{-1} (four pigs per group).
R-MSE: Root Mean Square Error.
* average per pen

Table 8.2. Carcass characteristics of pigs fed with the experimental diets

Items	Dietary treatment			R-MSE
	C	LE	LGSE	
Carcass weight(kg)	115.1	112.8	113.1	9.074
Cold carcass weight (kg)	112.8	110.5	110.8	8.891
Dressing Percentage (%)	83.9	84.8	83.1	1.046
Muscle thickness (mm)	65.5	63.3	63.5	4.907
Backfat thickness (mm)	22.3	25.3	25.5	3.274
Lean meat content (%)	55.6	53.6	53.8	2.053

C, control; LE, extruded linseed, 50 g kg^{-1}, vitamin E, 200 mg and Se 0.21 mg kg^{-1}; LGSE, extruded linseed, 50 g kg^{-1}, red grape skin extract 3 g kg^{-1} (four pigs per group).
R-MSE: Root Mean Square Error.

8.2. Fresh meat quality

The effect of dietary treatments on qualitative characteristics of meat is reported in Table 8.3. According to Corino *et al.* (2014), muscle pH is not affected by linseed feeding. Our results confirm the inferences of their meta-analysis. The control group showed a slightly higher b* and Chroma values ($P<0.05$), denoting a greater yellowness and colour intensity than linseed groups. No difference was found between LE and LGSE groups. Further, drip and cooking losses, oxidative stability in raw and cooked muscle, shear force and chemical composition (moisture, crude protein and lipid contents of LTL muscle) were affected neither by linseed nor by the antioxidants. Corino *et al.*(2008) and Haak *et al.* (2008) found that linseed inclusion did not influence these qualitative traits in meat of light pigs either. Moreover, Boler *et al.* (2009) reported that vitamin E reduces lipid oxidation but has no effect on any carcass characteristics and loin quality. Eventually, the lack of effects of linseed feeding on chemical parameters of the muscle (moisture, crude protein and lipid contents) confirms the findings of Hoz *et al.* (2003) on tenderloin.

Table 8.3. Qualitative characteristics and chemical composition of longissimus thoracis et lumborum muscle from pigs fed with the experimental diets

Items	Dietary treatment			P-value		R-MSE
	C	LE	LGSE	C vs (LE+LGSE)/2	LE vs LGSE	
pH *post mortem* (24h)	5.58	5.53	5.59	0.679	0.236	0.067
L*	55.83	56.26	55.70	0.878	0.616	1.526
a*	1.97	0.74	0.56	0.127	0.847	1.284
b*	12.88	12.08	11.18	0.044	0.177	0.870
Chroma	13.13	12.15	11.20	0.049	0.232	1.049
Hue angle	81.98	86.75	87.17	0.142	0.908	5.047
Drip loss (%)	3.46	3.13	3.39	0.763	0.731	1.032
Cooking loss (%)	17.05	19.78	16.98	0.358	0.110	2.236
TBARS (raw muscle)	0.05	0.07	0.09	0.134	0.396	0.034
TBARS (cooked muscle)	0.39	0.41	0.28	0.698	0.341	0.178
Shear force (Kg)	4.99	4.87	4.85	0.669	0.957	0.471
Chemical composition (%)						
Moisture	72.30	71.96	72.04	0.544	0.896	0.785
Crude Protein	23.39	23.16	23.44	0.845	0.575	0.688
Lipids	1.36	1.74	1.27	0.571	0.145	0.418

C, control; LE, extruded linseed, 50 g kg^{-1}, vitamin E, 200 mg and Se 0.21 mg kg^{-1}; LGSE, extruded linseed, 50 g kg^{-1}, red grape skin extract 3 g kg^{-1} (four pigs per group). R-MSE: Root Mean Square Error.

8.3. Quality characteristics of meat stored in MAP

The analysis of the inner gaseous atmosphere composition of the experimental samples showed that the relative percentages of the gases have not changed throughout the storage time. This result is not unexpected, because of the refrigerated (3±1°C) and short storage time length (8 days), the high barrier materials used, which strongly limits the gas transfers in and out the packages, and the bacteriostatic activity of CO_2 that, slowing the microbial growth, avoids oxygen consumption and as a consequence its decrease. In these conditions, all the modification registered on the meat samples can be attributed only to the presence or absence of oxygen in the atmosphere surrounding the product and to the meat composition.

The effects of dietary treatments and of gaseous mixtures in packaging during the 8 days of refrigerated storage on various physio-chemical characteristics of LTL muscle are shown in Table 8.4. The dietary treatments influenced most of the parameters studied. Linseed groups

exhibited lower weight losses; however, the reduction was significant (P<0.05) only on days 4 and 6. The comparison between the antioxidants added to the linseed diets did not reveal significant differences (P>0.05) in this parameter.

In any time-lapse interval considered, the weight loss was unaffected by the packaging atmosphere. The pH of LTL muscle ranged from 5.46 to 5.55 during the 8 days of storage. These values are quite common in medium-heavy pigs. Dietary linseed inclusion did not affect the pH values, whereas grape skin extract addition yielded higher pH values than synthetic antioxidant at day 6 (P = 0.01): the difference (5.46 *vs* 5.50) is anyway negligible from a realistic point of view. Our results conflict with the findings of Lorenzo *et al.* (2014), who found that the addition of natural antioxidants, derived from grape seed extract, lowered the value of pH throughout the storage. This difference is likely to be ascribable to the different origin of the grape extracts utilized (seed or skin) and, especially, to the fact that in the present work the extract was added to the diet of the pigs and not directly into the meat.

Table 8.4. Effect of the diet and packaging on the quality parameters of longissimus thoracis et lumborum muscle, refrigerated 8 days (3 ± 1°C)

Items	Dietary treatment			Packaging		P-value		R-MSE
	C	LE	LGSE	O_2	N_2	C vs (LE+LGSE)/2	LE vs LGSE	
Day 4								
Weight loss %	3.67	2.70	2.84	2.86	3.28	0.021	0.737	0.834
pH	5.52	5.54	5.54	5.55**	5.51**	0.112	0.636	0.031
TBARS	0.14	0.14	0.13	0.16**	0.12**	0.932	0.186	0.016
ΔE0_4	3.58	3.83	3.94	5.30**	2.27**	0.724	0.911	1.978
Day 6								
Weight loss %	4.57	3.07	3.87	3.39	4.29	0.042	0.190	1.178
pH	5.46	5.46	5.50	5.47	5.49	0.093	0.010	0.026
TBARS	0.08	0.03	0.04	0.10**	0.001**	0.015	0.497	0.035
ΔE0_6	3.81	4.23	3.30	5.12**	2.44**	0.953	0.271	1.644
Day 8								
Weight loss %	5.13	4.22	4.78	4.44	4.98	0.272	0.396	1.286
pH	5.51	5.51	5.53	5.53**	5.50**	0.309	0.230	0.026
TBARS	0.16	0.09	0.06	0.18*	0.03*	0.208	0.656	0.147
ΔE0_8	3.88	4.17	3.75	5.33**	2.53**	0.922	0.641	1.761

C, control; LE, extruded linseed, 50 g kg^{-1}, vitamin E, 200 mg and Se 0.21 mg kg^{-1}; LGSE, extruded linseed, 50 g kg^{-1}, red grape skin extract 3 g kg^{-1} (four pigs per group). R-MSE: Root Mean Square Error. *=P<0.05; **=P<0.01

In O_2 packaged samples, significant, though negligible, increases ($P<0.01$) in pH values were recorded at 4 and 8 days of storage. This agrees with Muhlisin *et al.* (2014), who showed that, at increasing oxygen content in the gas mixture, pH of pork was higher than in samples stored at higher levels of nitrogen. However, Viana *et al.* (2005) found that MAP did not exert a strong effect on pH of fresh pork loin.

The colour evolution, expressed by ΔE, during refrigerated storage time, did not differ among dietary treatments. The ΔE values were all above the value of 2, which is considered the threshold to appreciate visual changes of the colour (Lorenzo *et al.*, 2014). With regard to the gas mixtures, the ΔE in the samples stored in oxygen were significantly higher ($P<0.01$) in any time-lapse interval considered. Over storage, the ΔE values remained almost constant in the two gas mixtures, higher than 5 in O_2 and roughly half in N_2.

Figure 8.1 and 8.2 reports LTL muscle colour parameters evolution. In detail, as shown in Figure 8.1, the a* value, which is an index of redness, was consistently higher ($P<0.05$) in high oxygen MAP over storage, regardless of the dietary treatment. The values observed in nitrogen MAP are perceived as grey colour, as the values ranged from 3.2 and 4.6 (de Santos *et al.*, 2007). The same pattern was observed for the b* value (Fig. 8.2).

As concerns the oxidative stability, in the time-lapses considered, TBARS values were unaffected by the dietary treatment on day 4 and 8 (Table 8.4). Only on day 6, meat from the control showed a greater oxidation ($P<0.05$) than in antioxidants groups. No difference was found between LE *vs* LGSE.

The different modified atmospheres affected the oxidative stability of the muscle, that was always lower in samples packaged in high O_2 MAP, which yielded to a significantly higher TBARS values on day 4 and 6 ($P<0.01$), and on day 8 ($P<0.05$) of storage. When oxygen is readily available, a substrate such as meat is more prone to oxidation (Smiddy *et al.*, 2002) and, in agreement with our results, Spanos *et al.* (2016) observed that samples of LTL muscle stored in MAP with an oxygen concentration of 50% or higher showed a significantly lower oxidative stability compared to samples stored under 0% of oxygen.

However, even the highest determined value of TBARS was lower than 0.18 mg MDA/kg meat, far below the threshold value of 0.5 mg MDA/kg muscle for organoleptic detection of rancidity as suggested by Lanari *et al.* (1995). All our samples, regardless of the different MAPs, could be classified as fresh meat, through the 8 days of storage.

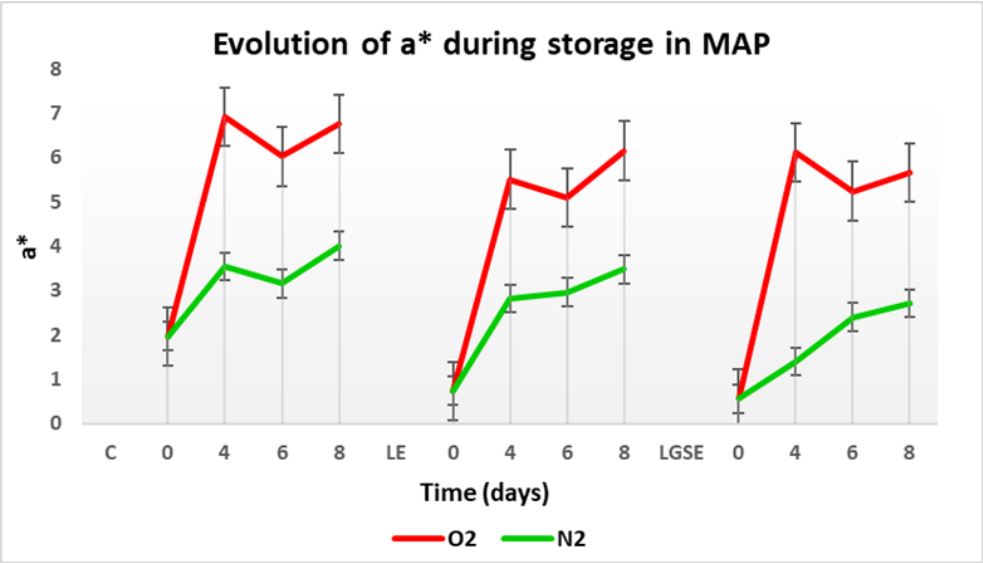

Figure 8.1. Redness, "a" values in relation to storage time in MAP: oxygen (O2) or nitrogen (N2)*

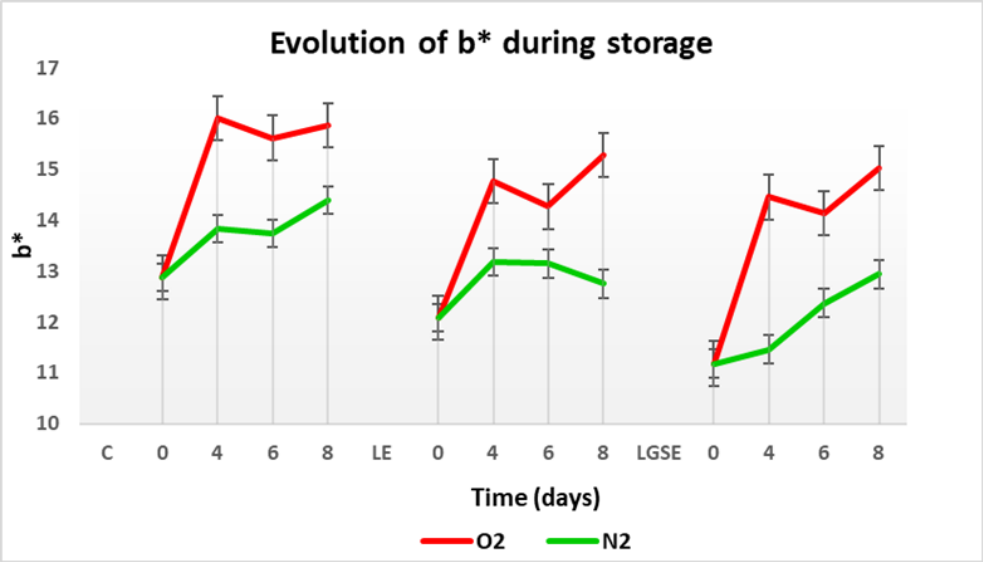

Figure 8.2. Yellowness, "b" values in relation to storage time in MAP: oxygen (O2) or nitrogen (N2)*

8.4. Adipose tissue and intramuscular fatty acid composition

Table 8.5 shows lipid content and fatty acid (FA) composition of subcutaneous adipose tissue.

No differences attributable to extruded linseed dietary inclusion or type of antioxidant were found for lipid content of backfat. The proportion of total saturated (SFA), monounsaturated

(MUFA) and polyunsaturated (PUFA) fatty acids was not affected by the inclusion of linseed in the diet, in agreement with Musella *et al.* (2009) who detected no difference in the percentage of main FA classes in ham covering trimmed fat between control and linseed-fed pigs.

The total content of n-6 PUFA, although tendentially lower in linseed fed subjects, was not significantly influenced by dietary treatments ($P>0.05$) either. Overall, among n-6 PUFA and MUFA, only γ-linolenic (C18:3n-6) and heptadecenoic acids (C17:1) were affected by dietary linseed. Conversely, n-3 PUFA increased significantly ($P<0.01$) with linseed dietary inclusion.

This confirms the findings of Riley *et al*. (2000). The increase of the proportion of the total n-3 PUFA is ascribable to the higher proportions ($P<0.01$) of α-linolenic (ALA, C18:3n-3) and eicosatrienoic (C20:3n-3) acids which trebled and docosapentaenoic acid (DPA, C22:5n-3) that doubled, whereas eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) remained unchanged. Guillevic *et al.* (2009) could not find any correlation between ALA intake and the proportion of DHA in adipose tissues either. Riley *et al.* (2000) hypothesized that the limited accumulation of longer chain PUFA in adipose tissues could be due to scant capacity for the synthesis of these products starting from dietary ALA.

On the whole, these trends led to a significant ($P<0.01$) reduction in the n-6/n-3 PUFA ratio, as was previously observed also by Musella *et al*. (2009) and Riley *et al.* (2000). In our study, the n-6/n-3 PUFA ratio dropped from 12.6 in control pigs to less than 3.5 in linseed fed pigs. Thus, the inclusion of 5% extruded linseed in the finishing diet enabled to bring this ratio far below the threshold indicated by Simopoulos (2008) to avoid adverse health consequences, without impairing technological parameters of subcutaneous adipose tissue. In fact, Lo Fiego *et al.* (2005) indicate, as a guarantee of good preservation aptitude, contents of stearic acid (C18:0) and linoleic acid (C18:2n-6) higher than 12 and lower than 15%, respectively, and an Iodine value minor than 70.

As showed in Table 8.5, and as expected, no difference was found between the linseed fed groups (LE *vs* LG) for any of the parameters taken into account.

Table 8.6 shows the fatty acid composition of LTL muscle. In general, the variations observed in the FA compositions resembled what already seen in the backfat tissue. In fact, total SFA, MUFA and PUFA percentages did not differ among dietary treatments ($P>0.05$). Compared to C, linseed groups showed a higher content of lauric acid (C12:0; $P<0.01$) and lower in vaccenic acid (C18:1 n-7; $P<0.05$). No hypothesis could be put forward to explain these variations that, though trivial, were statistically significant.

Table 8.5. Lipid content (%) and fatty acid profile (% of total fatty acids) of backfat from pigs fed with the experimental diets

Items	Dietary treatment			P-value		R-MSE
	C	LE	LGSE	C vs (LE+LGSE)/2	LE vs LGSE	
Lipids	86.14	86.56	87.55	0.752	0.765	4.560
Fatty acids:						
C 10:0 (capric)	0.13	0.10	0.10	0.407	0.988	0.053
C 12:0 (lauric)	0.07	0.05	0.07	0.591	0.328	0.027
C 14:0 (myristic)	1.16	1.15	1.18	0.975	0.659	0.093
C 16:0 (palmitic)	24.66	24.59	24.39	0.735	0.731	0.785
C 17:0 (heptadecanoic)	0.35	0.32	0.30	0.338	0.673	0.055
C 18:0 (stearic)	15.32	14.95	15.47	0.843	0.437	0.896
C 20:0 (eicosanoic)	0.15	0.26	0.22	0.103	0.501	0.076
C 16:1 (palmitoleic)	1.62	1.51	1.57	0.553	0.674	0.208
C 17:1 (heptadecenoic)	0.32	0.26	0.24	0.016	0.517	0.040
C 18:1n-7 (vaccenic)	1.51	1.39	1.65	0.547	0.420	0.281
C 18:1n-9 (oleic)	39.47	38.08	39.30	0.956	0.220	2.040
C 20:1 (eicosenoic)	0.67	0.83	0.81	0.075	0.828	0.126
C 18:2n-6 (linoleic)	12.64	11.60	10.46	0.236	0.458	2.070
C 18:3n-3 (α-linolenic)	0.84	3.26	2.74	0.000	0.103	0.406
C 18:3n-6 (γ-linolenic)	0.09	0.05	0.07	0.026	0.152	0.022
C 20:2n-6 (eicosadienoic)	0.42	0.54	0.50	0.196	0.605	0.116
C 20:3n-3 (eicosatrienoic)	0.12	0.43	0.43	0.000	0.906	0.089
C 20:4n-6 (arachidonic)	0.30	0.25	0.26	0.323	0.859	0.070
C 20:5n-3 (eicosapentaenoic)	0.00	0.15	0.00	0.486	0.254	0.170
C 22:2n-6 (docosadienoic)	0.00	0.00	0.00	0.377	0.466	0.004
C 22:4n-6 (docosatetraenoic)	0.07	0.05	0.04	0.127	0.434	0.027
C 22:5n-3 (docosapentaenoic)	0.09	0.18	0.20	0.000	0.583	0.028
C 22:6n-3 (docosahexaenoic)	0.02	0.02	0.02	0.864	0.510	0.016
Total Saturated	41.83	41.40	41.72	0.719	0.717	1.185
Total Monounsaturated	43.58	42.06	43.57	0.587	0.363	2.224
Total Polyunsaturated	14.60	16.54	14.72	0.523	0.338	2.546
Total n-6	13.53	12.49	11.33	0.257	0.470	2.183
Total n-3	1.07	4.05	3.39	0.000	0.088	0.484
n-6/n-3 fatty acid ratio	12.64	3.09	3.37	0.000	0.622	0.780
Iodine Value	63.72	62.98	61.79	0.410	0.520	2.511

C, control; LE, extruded linseed, 50 g kg^{-1}, vitamin E, 200 mg and Se 0.21 mg kg^{-1}; LGSE, extruded linseed, 50 g kg^{-1}, red grape skin extract 3 g kg^{-1} (four pigs per group). R-MSE: Root Mean Square Error.

Table 8.6. Fatty acid profile (% of total fatty acids) of Longissimus thoracis et lumborum muscle from pigs fed with the experimental diets

Items	Dietary treatment			P-value		R-MSE
	C	LE	LGSE	C vs (LE+LGSE)/2	LE vs LGSE	
C 10:0 (capric)	0.11	0.29	0.30	0.355	0.993	0.306
C 12:0 (lauric)	0.05	0.16	0.11	0.000	0.019	0.024
C 14:0 (myristic)	1.07	1.18	1.13	0.343	0.646	0.140
C 16:0 (palmitic)	23.11	23.85	23.48	0.442	0.656	1.117
C 17:0 (heptadecanoic)	0.12	0.14	0.21	0.303	0.185	0.076
C 18:0 (stearic)	13.41	13.50	13.10	0.780	0.381	0.616
C 20:0 (eicosanoic)	0.12	0.12	0.13	0.871	0.822	0.020
C 16:1 (palmitoleic)	2.81	2.99	2.73	0.801	0.261	0.304
C 17:1 (heptadecenoic)	0.07	0.15	0.18	0.062	0.560	0.075
C 18:1n-7 (vaccenic)	3.30	2.84	2.74	0.038	0.686	0.345
C 18:1n-9 (oleic)	39.39	39.79	40.68	0.612	0.646	2.639
C 20:1 (eicosenoic)	0.59	0.52	0.55	0.249	0.618	0.071
C 18:2n-6 (linoleic)	10.52	9.07	9.03	0.276	0.976	2.069
C 18:3n-3 (α-linolenic)	0.40	1.27	1.37	0.000	0.540	0.218
C 18:3n-6 (γ-linolenic)	0.08	0.09	0.13	0.350	0.352	0.057
C 20:2n-6 (eicosadienoic)	0.21	0.19	0.19	0.380	0.971	0.040
C 20:3n-3 (eicosatrienoic)	0.05	0.16	0.18	0.001	0.481	0.043
C 20:4n-6 (arachidonic)	3.53	2.49	2.50	0.093	0.991	0.897
C 20:5n-3 (eicosapentaenoic)	0.07	0.35	0.36	0.004	0.954	0.120
C 22:2n-6 (docosadienoic)	0.01	0.01	0.01	0.167	0.600	0.004
C 22:4n-6 (docosatetraenoic)	0.51	0.19	0.26	0.006	0.497	0.132
C 22:5n-3 (docosapentaenoic)	0.39	0.52	0.60	0.227	0.599	0.214
C 22:6n-3 (docosahexaenoic)	0.08	0.12	0.04	0.992	0.255	0.095
Total Saturated	38.00	39.23	38.46	0.364	0.469	1.452
Total Monounsaturated	46.16	46.29	46.88	0.811	0.776	2.824
Total Polyunsaturated	15.85	14.47	14.67	0.574	0.941	3.574
Total n-6	14.85	12.04	12.11	0.173	0.977	3.064
Total n-3	1.00	2.43	2.56	0.002	0.755	0.551
n-6/n-3 fatty acid ratio	15.33	4.99	4.76	0.000	0.827	1.438

C, control; LE, extruded linseed, 50 g kg^{-1}, vitamin E, 200 mg and Se 0.21 mg kg^{-1}; LGSE, extruded linseed, 50 g kg^{-1}, red grape skin extract 3 g kg^{-1} (four pigs per group).
R-MSE: Root Mean Square Error.

Total n-6 PUFA content was not affected by the diet, and the only significant variation was shown by the docosatetraenoic acid (C22:4n-6; $P<0.01$), which resulted higher in the C group. The same trend was observed by Riley *et al.* (2000). The total n-3 PUFA proportion, as seen in the

backfat, was significantly higher (P<0.01) in the linseed groups. In detail, ALA, eicosatrienoic acid and EPA more than tripled (P<0.01).

Thus, EPA that did not change with diet in adipose tissue, increased in LTL muscle. This agrees with the results of Corino *et al.* (2008), who observed that EPA is preferentially stored in the muscle rather than in the adipose tissue, and Riley *et al.* (2000), who inferred that α-linolenic acid intake elicits eicosapentaenoic acid increments more in muscle than in adipose tissue. Hence, also in the muscle, the n-6/n-3 PUFA ratio was significantly reduced (P<0.01) to one-third in linseed fed pigs. However, the n-3/n-6 value was lower in the backfat respect to the LTL muscle (Fig. 8.3).

Not even in this tissue, except for the proportion of lauric acid, any variation was found between the two different dietary antioxidants, for any of the parameters taken into account.

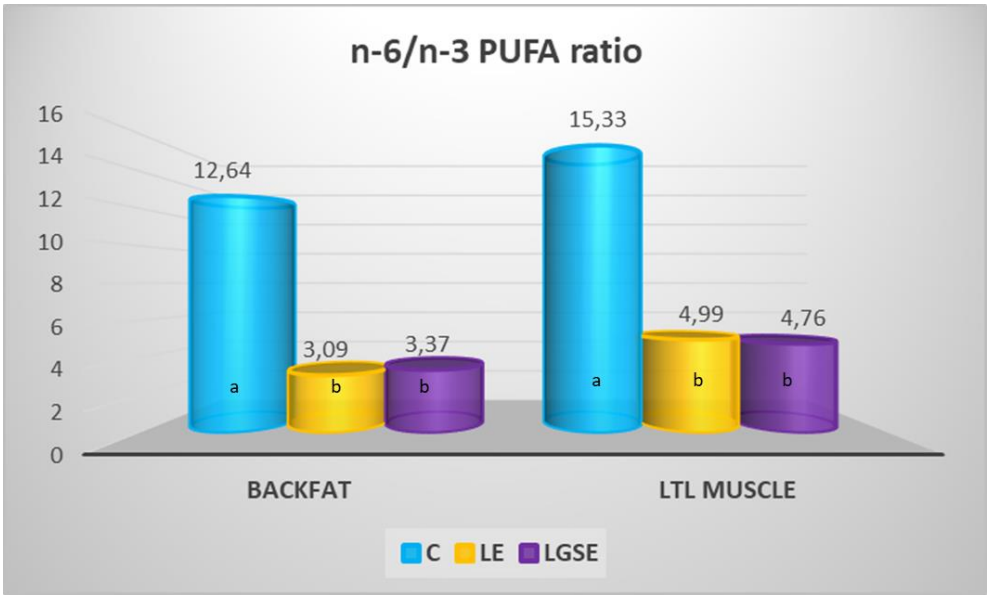

Figure 8.3. The n-6/n-3 PUFA ratio in LTL muscle and backfat
Different letters indicate statistically significant differences (P<0.05) among treatments

8.5. Conclusions

Our results confirm that 5% of dietary extruded linseed included in the pig finishing diet is a suitable means to increase n-3 PUFA content and to reduce the n-6/n-3 PUFA ratio in pig tissues without affecting growth performance, carcass characteristics and impairing technological characteristics of adipose depots. In general, under the point of view of human nutrition, it ameliorates the fatty acid profiles in both backfat and LTL muscle.

Also, qualitative characteristics and chemical composition of muscle are not affected by dietary linseed inclusion associated with either synthetic or natural antioxidants.

In this research linseed feeding, supplemented with supra-nutritional levels of antioxidants, did not impaired oxidative stability compared to a standard diet and reduced weight losses during chilled storage.

As expected, high concentration of oxygen in MAP brought about an increase in oxidative products and yielded redder meat, irrespective of the dietary treatment.

In linseed fed pigs, dietary red grape skin extract was as effective as synthetic antioxidant in maintaining quality characteristics of pork during storage, even in high oxygen MAP.

8.6. References

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CHAPTER 9. EXPERIMENT II

Effect of dietary vitamin E and selenium or vegetal extracts on the quality and shelf-life of pork from medium-heavy pigs fed with extruded linseed

RESULTS AND DISCUSSION

9.1. Growth performance and carcass characteristics

The effect of dietary treatments containing extruded linseed and vitamin E or vegetal extracts on growth performance and carcass characteristics in pigs are reported in Table 9.1 and 9.2. Experimental diets did not influence growth performance of pigs and their carcass characteristics. Our results are in agreement with Kouba *et al.* (2003) and Karolyi *et al.* (2012), who reported that feeding pigs with linseed had no effect on pig performance and carcass traits. Kafantaris *et al.* (2018) and Yan and Kim (2011) showed that pigs fed with grape pomace increased significantly the average daily gain and decreased the feed conversion ratio. Also in our experiment was observed a similar trend, however the differences were not significant. Rossi *et al.* (2013) and Ranucci *et al.* (2015) reported no effect on carcass characteristics from pigs fed with natural antioxidants.

Table 9.1. Growth performance of pigs fed with the experimental diets

Items	Dietary treatment				R-MSE
	C	L	LE	LVE	
Initial body weight (kg)	77.4	82.3	80.1	79.7	5.716
Final body weight (kg)	146.7	153.0	150.8	151.5	13.645
Average daily gain (g)	671	687	684	701	0.117
Feed conversion ratio*	3.7	3.6	3.6	3.5	0.201

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed) (twelve pigs per group).
R-MSE: Root Mean Square Error.
*Considering pen as experimental unit.

Table 9.2. Carcass characteristics of pigs fed with the experimental diets

Items	Dietary treatment				R-MSE
	C	L	LE	LVE	
Hot carcass weight (kg)	124.8	129.3	127	127.3	6.631
Cold carcass weight (kg)	122.3	126.7	124.5	124.7	6.488
Hot Dressing Percentage (%)	84.9	84.7	84.3	84.2	1.733
Cold Dressing percentage (%)	83.3	82.9	82.6	82.5	1.769
Muscle thickness (mm)	57.3	60.9	58.4	61.4	4.701
Backfat thickness (mm)	32.8	31.9	31.2	30.6	5.127
Lean meat content (%)	50.5	50.9	51.1	51.6	2.433

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed) (twelve pigs per group).
R-MSE: Root Mean Square Error.

9.2. Fresh meat quality

The effects of dietary treatments on meat quality parameters are reported in Table 9.3. The data showed that none of the measured quality parameters (pH, colour, drip and cooking loss, TBARS in raw and cooked muscle, shear force, moisture, crude protein and lipids) were significantly different among dietary treatments. Our results are in agreement with Haak *et al.* (2008) who reported that feeding pigs with 3% of linseed did not affect meat quality parameters (pH, drip loss, colour, lipid oxidation) and also, did not induce negative effects on meat quality. Also, O'Grady *et al.* (2008), found no effect on lipid oxidation and quality of raw and cooked meat from pigs fed with different amount of grape seed extract. In contrast with our results, Guillevic *et al.* (2009) and Kouba *et al.* (2003) found higher TBARS values for pigs fed with 4.2% of extruded linseed and 3% of linseed respectively, respect to the control diet. Moreover, Kouba *et al.* (2003) reported that pork from pigs fed with linseed was tougher than pork from pigs fed the control diet. In a study conducted by Rivas-Cañedo *et al.* (2013), lambs were fed with a rich source of n-3, supplemented with either vitamin E or red wine extract: they demonstrated that the red wine extract was less effective in preventing lipid oxidation than vitamin E, but more effective than the control group. Zhao *et al.* (2018) observed that 5 and 10% of wine grape pomace supplementation in lamb diet did not affect pH, meat colour and cooking loss, but decreased shear force. Also, 10% of wine grape pomace decreased TBARS values. In another study, Ahmed *et al.* (2016) reported that natural herb combination supplemented in pig diet significantly reduced TBARS values and crude fat with increasing amounts of moisture, but no effect on pH was observed.

In a study similar to ours, conducted by Bertol *et al.* (2017), pigs were fed with 3% oil blend containing 50% of canola oil and 50% of linseed oil supplemented with sequential levels of 3-5% and 6-10% of grape pomace: the authors demonstrated that TBARS values were not affected neither by the inclusion of the oil blend nor by the grape pomace in the pig diet. Also, they reported a higher ($P<0.06$) pH for oil blend dietary treatment. The a* values were lower ($P<0.10$) in the meat from pigs fed with oil blend and higher ($P<0.10$) in the meat from pigs fed with higher levels of grape pomace, which might be due to the higher amount of tocopherols in the tissues, that could prevent myoglobin oxidation.

Table 9.3. Qualitative characteristics and chemical composition of longissimus thoracis et lumborum muscle from pigs fed with the experimental diets

Items	Dietary treatment				R-MSE
	C	L	LE	LVE	
pH (24h *post mortem*)	5.61	5.54	5.58	5.54	0.069
L*	52.67	52.80	53.34	53.04	2.386
a*	3.23	2.64	1.88	2.12	1.348
b*	12.41	11.92	11.59	11.91	0.909
C*	12.91	12.32	11.79	12.16	1.061
H*	75.73	78.12	81.01	80.25	5.805
Drip loss	2.42	3.41	2.89	2.60	1.604
Cooking Loss	21.96	23.15	21.15	20.94	3.360
TBARS raw muscle	0.11	0.11	0.08	0.16	0.121
TBARS cooked muscle	0.40	0.49	0.40	0.37	0.130
Shear Force	6.58	6.40	6.33	5.99	1.092
Chemical composition (%)					
Moisture	68.96	68.27	67.98	68.72	2.102
Crude protein	23.40	23.62	23.81	22.93	1.041
Lipids	1.65	1.55	1.39	1.76	0.417

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed) (twelve pigs per group).
R-MSE: Root Mean Square Error.

9.3. Shelf-life study

Results are shown considering that ANOVA revealed statistically significant interaction "diet x time" for pH and microbiology, and no interaction for colour and lipid oxidation parameters.

As reported in the first experiment, during the refrigerated storage, the inner gaseous atmosphere composition of the experimental samples did not change. This result is not unexpected, because of the refrigeration (2±1°C) and the high barrier materials used, which strongly limit the gas transfers in and out the packages, and the bacteriostatic activity of CO_2 that, slowing the microbial growth, avoids oxygen consumption and as a consequence its decrease.

9.3.1. Weight loss and pH of LTL muscle

Mean pH value of LTL muscle ranged from 5.47 to 5.62 during the storage period. This pH range is in agreement with other findings (Holmer *et al.,* 2009; O'Grady *et al.,* 2008). The control group showed higher pH values ($P<0.05$) at day 0 compared with L and LVE (Fig. 9.1). The LTL muscle pH values of all groups subsequently dropped until day 6, when the control group showed the lowest values, significantly different from LE ($P<0.05$). For the remaining storage period the pH values were not significantly affected by the addition of linseed, vitamin E and selenium and vegetal extracts. Our results contrast with O'Grady *et al.* (2008), who found no differences for pH during 16 days of storage of meat from pigs supplemented with grape seed extract and bearberry. Also Rossi *et al.* (2017) reported no differences for pH values of meat from pigs fed with an antioxidant mixture. The differences we found, although statistically significant, are anyway negligible from a biological point of view, and the recorded pH values are within a range of normality for pig meat.

Even if the pH values were slightly different, the weight loss was not affected ($P>0.05$) by the dietary treatments, neither by the storage time (Fig. 9.2).

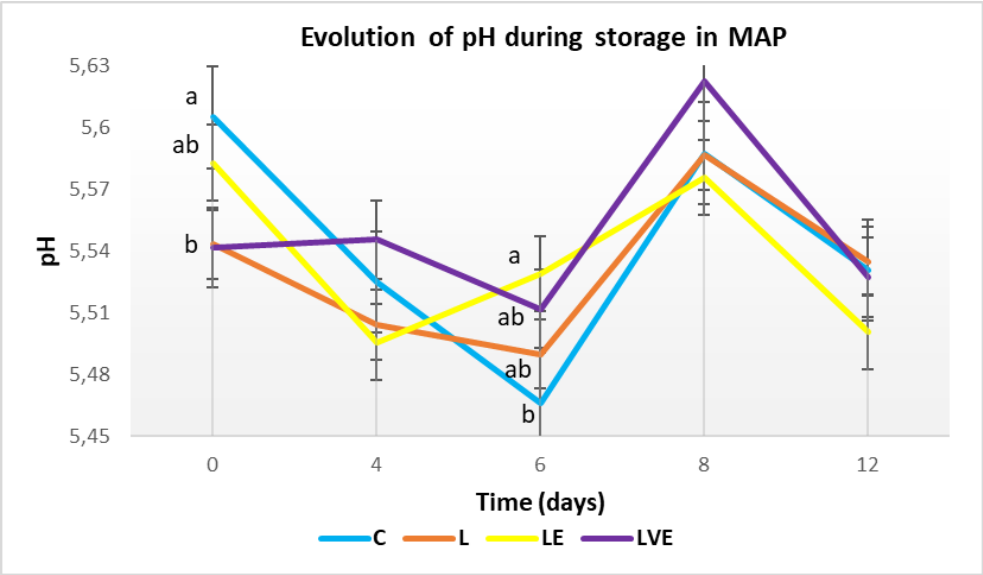

Figure 9.1. Effect of experimental pig diets on pH values of longissimus thoracis et lumborum muscle during refrigerated storage.

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed).

Different letters indicate significant differences (P<0.05) among treatments within time.

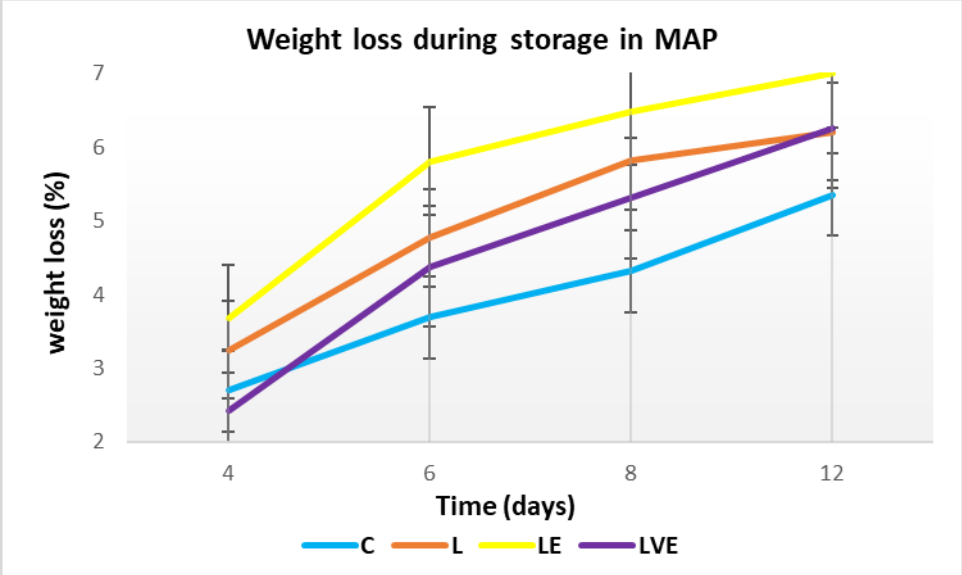

Figure 9.2. Effect of experimental pig diets on weight loss of longissimus thoracis et lumborum muscle during refrigerated storage.

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed).

9.3.2. Colour parameters of LTL muscle

Table 9.4 shows the changes in colour parameters values during refrigerated storage. No statistical differences were found among the dietary treatments. Similar results were reported by Ranucci *et al.* (2015); Rossi *et al.* (2013); Simitzis *et al.* (2010) who found no differences for colour parameters of meat from pigs fed with plant antioxidants. During refrigerated storage the L* value was lower (P<0.05) for day 0, increased until day 6 and subsequently remained constant throughout the storage, resulting in lighter meat. The a*, b* and C* values increased (P<0.05) during the first 4 days of storage and decreased between 8 and 12 days. The H* value decreased (P<0.05) during the first 4 days of storage and slightly increased between 8 and 12 days. The increase of L*, a* and b* during the refrigerated storage in high oxygen MAP is attributable to the meat oxygenation, with the production of oxymyoglobin (Lindahl *et al.*, 2006).

Table 9.4. Effect of experimental pig diets on colour parameters of longissimus thoracis et lumborum muscle during refrigerated storage

	Dietary treatment					Storage time					
	C	**L**	**LE**	**LVE**	**SE**	**0**	**4**	**6**	**8**	**12**	**SE**
L*	55.68	54.97	55.23	54.23	0.57	52.95^{c}	54.72^{b}	55.87^{a}	55.72^{a}	55.89^{a}	0.37
a*	6.24	6.22	5.87	6.21	0.42	2.47^{b}	7.34^{a}	7.31^{a}	7.16^{a}	6.4^{c}	0.25
b*	14.96	14.81	14.71	14.76	0.19	11.96^{d}	15.45^{bc}	15.81^{a}	15.65^{ab}	15.17^{c}	0.14
C*	16.31	16.2	15.96	16.14	0.33	12.3^{b}	17.17^{a}	17.48^{a}	17.28^{a}	16.53^{c}	0.21
H*	68.16	68.34	69.44	68.3	1.14	78.82^{a}	65.05^{c}	65.57^{c}	65.87^{c}	67.49^{b}	0.68

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed) (twelve pigs per group).
Different letters in the same line indicate statistically different means (P<0.05). SE: standard error.

9.3.3. Microbiological counts

Mean TMC ranged from 0.87 to 2.91 log_{10} CFU/g on day 0 and increased until a maximum of 3.79 log_{10} CFU/g on day 12 of storage (Fig. 9.3). Initially (day 0) C group had higher (P<0.05) TMC than other dietary treatments. Significant differences in TMC occurred among dietary treatments during storage: group C tended to have higher values in comparison with the other groups, and the differences became significant at day 8 and 12 (P<0.05). In all dietary groups the *Enterobacteriaceae* (Fig. 9.4) were lower than 1 log_{10} CFU/g at 0 day of storage, and this may be attributed to good hygienic practices. The C group showed significantly higher values (P<0.05) than the other groups until 6 days of storage; then the mean values of the groups tended to become

similar, and the only significant differences (P<0.05) could be detected between LVE (lower count) and C (day 8) or LE (day 12). Overall, the samples from LVE group showed at every storage time the lowest *Enterobacteriaceae* count.

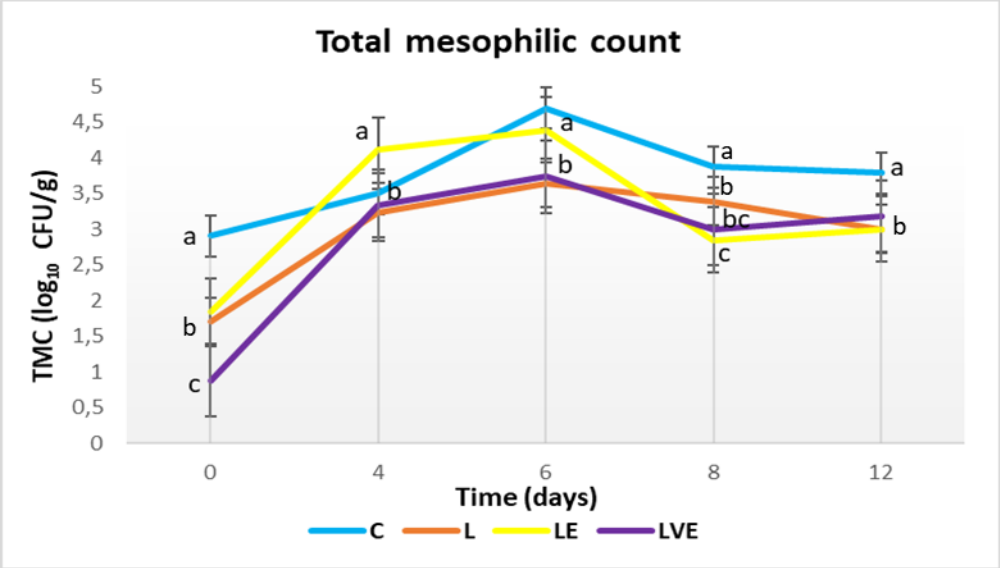

Figure 9.3. Total mesophilic counts

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed).

Different letters indicate statistically significant differences (P<0.05) among treatments within time.

Our data seem to indicate that linseed and vegetal extracts were effective in preventing microbial growth respect to the C group. These results are in agreement with Lorenzo *et al.* (2014) who observed lower TMC for pork patties with the addition of grape seed extract. Also, Reddy *et al.* (2013) reported improved microbiological quality in restructured mutton slices treated with grape seed extract. Our results contrast with Guerra-Rivas *et al.* (2016) who reported that vitamin E was more effective in preventing microbial growth than grape seed extract in lamb diet. Also, Carpenter *et al.* (2007) reported no antimicrobial effect for raw pork patties with grape seed extract addition. Throughout the experiment all the microbiological counts, also in C group, were under the microbiological selling threshold (6 $\log_{10}$ CFU/g) of raw meat established by European Food Safety Agency (EFSA). This may be partly due to the gas composition of packaging, since the presence of carbon dioxide has antimicrobial effect and also restricts microbial growth of the meat (Jeremiah, 2001). Also other studies confirm the antimicrobial ability of grape skin extract and

oregano extracts against food spoilage microorganisms (Beres *et al.*, 2017; Tournour *et al.*, 2015; Tuttolomondo *et al.*, 2013).

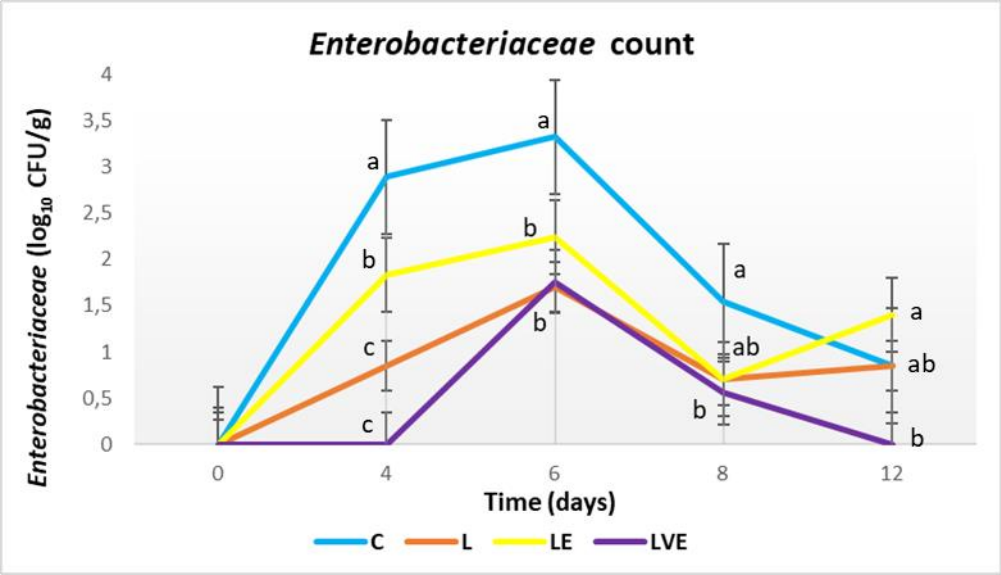

Figure 9.4. Enterobacteriaceae counts

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed).
Different letters indicate statistically significant differences (P<0.05) among treatments within time.

9.3.4. Lipid oxidation of LTL muscle

Figure 9.5 shows the effect of dietary treatments on the lipid oxidation during the refrigerated storage. The TBARS mean values ranged from 0.09 to 0.28 mg MDA/kg of meat over 12 days of storage period for all dietary treatments, and were lower than the threshold level for raw meat of 0.5 mg MDA/kg of meat suggested by Lanari *et al.* (1995). Lipid oxidation in LTL muscle increased with storage time for all dietary treatments, however no significant differences were found among the groups. Slight and no statistically significant (P>0.05) differences were observed for the L group, with higher values for lipid oxidation from day 6 onwards, this could be due to the absence of antioxidants and high amount of PUFA in this diet (Corino *et al.*, 2008). Our results seem to indicate that addition of GSE and oregano did not exert a relevant antioxidant effect on the LTL muscle. It is interesting to note that vegetal extracts were as effective as vitamin E. Similar results were reported by *O'Grady et al.* (2008) who showed no antioxidant effect of grape seed extract and bearberry addition in the raw meat and also Bertol *et al.* (2017) reported that lipid oxidation in the loin samples was not affected by the supplementation with grape pomace in the

pig diet. In contrast to our results, several studies reported that dietary supplementation with plant extracts or essential oils improved lipid stability in pigs (Ahmed *et al.*, 2016; Rossi *et al.*, 2013), lambs (Guerra-Rivas *et al.*, 2016; Zhao *et al.*, 2018) or poultry (Botsoglou *et al.*, 2003; Brenes *et al.*, 2008). The lack of significant effects in our study could be due to low dietary levels of total polyphenols added.

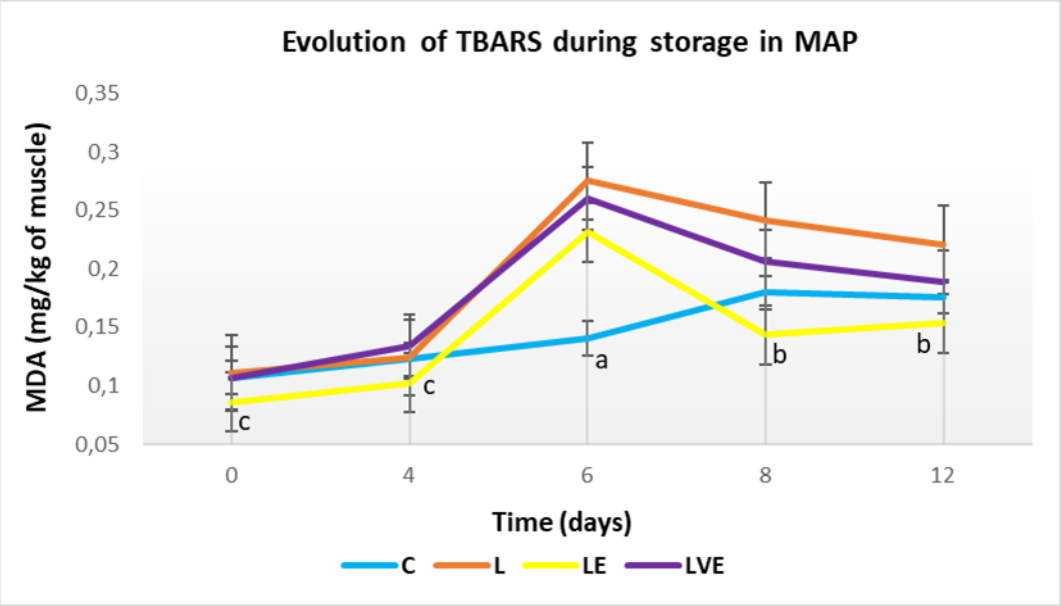

Figure 9.5. Effect of dietary treatment and storage time on evolution of TBARS (mg MDA/kg of meat) of longissimus thoracis et lumborum muscle during refrigerated storage.

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed).

Different letters indicate significant differences (P<0.05) among times.

9.4. Fatty acid composition of LTL muscle, backfat and perirenal fat

The effects of dietary treatments on the fatty acid composition of LTL muscle of pigs are shown in Table 9.5. The SFAs were not affected (P>0.05) by the dietary supplementation with extruded linseed and antioxidants. The amount of heptadecenoic acid (C17:1) was significantly lower (P<0.05) in the LTL muscle of pigs fed with diet containing extruded linseed without antioxidants respect to the C group, however the total MUFA amount was not affected. Our results are in agreement with Enser *et al.* (2000) and Haak *et al.* (2008).

The total n-6 PUFA amount was similar among dietary treatments. The C diet generated a higher (P<0.05) percentage of γ–linolenic acid (C18:3n-6), arachidonic acid (C20:4n-6) and

docosatetraenoic acid (C22:4n-6) compared to LE and LVE diets. In contrast with our results Kouba *et al.* (2003) found lower ($P<0.05$) amount of arachidonic acid in the LTL muscle from pigs fed with linseed respect to the control diet.

In comparison with the C diet, the diets supplemented with linseed resulted in significantly higher ($P<0.05$) amounts of the n-3 PUFA, including ALA, EPA and DPA. The concentrations of these FAs were thrice greater in the experimental diets respect to the C diet, however there was no effect ($P>0.05$) on the amount of DHA. Similar results were obtained by Haak *et al.* (2008) and Kouba *et al.* (2003) in pigs fed with linseed diets.

The values of FAs reported in the present study showed that dietary supplementation with antioxidants in pig diets did not influence the FA composition of LTL muscle. Yan & Kim (2011) showed that 30 g/kg of fermented grape pomace product supplementation in pigs for 105 days decreased only the eicosanoic acid (C20:0) amount in the LTL muscle; the total SFA, MUFA, PUFA and PUFA/SFA ratio were not affected by the dietary treatment. In contrast to our data, Cheng *et al.* (2017) reported that oregano oil supplementation in pig diets from growing period to slaughter, decreased MUFA and SFA content and enhanced the deposition of n-3 PUFA. Also, Kafantaris *et al.* (2018) demonstrated that grape pomace inclusion in the diets of 20-days old piglets, for 30 days, increased significantly n-3 PUFA (EPA, DHA, ALA) and decreased significantly n-6/n-3 ratio compared to the control diet in the quadriceps muscle.

More differences in the FA profile occurred in the backfat than in the LTL muscle (Table 9.6). In the case of the subcutaneous fat, the proportions of SFA and MUFA were higher ($P<0.05$) in the C diet than in L, LE and LVE diets. Especially, lauric (C12:0), palmitic (C16:0) and vaccenic (C18:1n-7) acid concentration were higher in C diet respect lo LE diet; heptadecanoic (C17:0) and heptadecenoic (C17:1) acid concentration were lower in L and LVE diets; and oleic (C18:1n-9) acid was higher in C group respect to L, LE and LVE diets.

Backfat of pigs fed experimental diets showed a higher content of total PUFA ($P<0.05$). The C diet had a higher ($P<0.05$) amount of arachidonic (C20:4n-6) and docosatetraenoic acid (C22:4n-6) but lower proportion of total PUFA compared to L, LE and LVE diets. Also, the n-3 PUFA increased significantly ($P<0.05$) in backfat of pigs fed the experimental diets; the ALA resulted in a 5-fold increase, eicosatrienoic (C20:3n-3) in a 4-fold increase and DPA in a 2-fold increase respect to the C diet, however, the DHA content remained unchanged.

Table 9.5. Fatty acid profile (% of total fatty acids) of longissimus thoracis et lumborum muscle from pigs fed with the experimental diets

Items	Dietary treatments				R-MSE
	C	L	LE	LVE	
C 10:0 (capric)	0.12	0.11	0.11	0.11	0.019
C 12:0 (lauric)	0.07	0.08	0.08	0.08	0.011
C 14:0 (myristic)	1.19	1.22	1.27	1.29	0.136
C 16:0 (palmitic)	23.25	23.29	24.04	24.13	1.232
C 17:0 (heptadecanoic)	0.23	0.21	0.25	0.23	0.048
C 18:0 (stearic)	12.64	12.64	12.88	13.10	1.226
C 20:0 (eicosanoic)	0.14	0.14	0.14	0.14	0.021
C 16:1 (palmitoleic)	3.23	3.03	3.06	3.02	0.444
C 17:1 (heptadecenoic)	0.30^{a}	0.25^{b}	0.26^{ab}	0.28^{ab}	0.039
C 18:1n-7 (vaccenic)	4.18	4.03	4.05	3.95	0.356
C 18:1n-9 (oleic)	38.98	37.09	38.79	37.76	2.333
C 20:1 (eicosenoic)	0.63	0.64	0.67	0.66	0.088
C 18:2n-6 (linoleic)	9.36	9.92	8.65	9.08	2.052
C 18:3n-3 (α-linolenic)	0.48^{b}	1.88^{a}	1.67^{a}	1.78^{a}	0.335
C 18:3n-6 (γ-linolenic)	0.22^{a}	0.19^{ab}	0.16^{b}	0.19^{ab}	0.045
C 20:2n-6 (eicosadienoic)	0.23	0.24	0.22	0.23	0.038
C 20:3n-3 (eicosatrienoic)	0.07^{b}	0.22^{a}	0.20^{a}	0.22^{a}	0.036
C 20:4n-6 (arachidonic)	3.49^{a}	2.98^{ab}	2.15^{b}	2.27^{b}	0.975
C 20:5n-3 (eicosapentaenoic)	0.14^{b}	0.59^{a}	0.41^{a}	0.47^{a}	0.170
C 22:2n-6 (docosadienoic)	0.004	0.005	0.005	0.004	0.002
C 22:4n-6 (docosatetraenoic)	3.49^{a}	2.98^{ab}	2.15^{b}	2.27^{b}	0.975
C 22:5n-3 (docosapentaenoic)	0.14^{b}	0.59^{a}	0.41^{a}	0.47^{a}	0.170
C 22:6n-3 (docosahexaenoic)	0.09	0.10	0.07	0.08	0.036
Total Saturated	37.65	37.69	38.77	39.08	2.377
Total Monounsaturated	47.31	45.03	46.82	45.66	2.841
Total Polyunsaturated	15.04	17.28	14.41	15.26	3.861
Total n-6	13.83	13.65	11.43	12.05	3.143
Total n-3	1.22^{b}	3.63^{a}	2.98^{a}	3.21^{a}	0.778
n-6/n-3 fatty acid ratio	11.39^{a}	3.75^{b}	3.95^{b}	3.80^{b}	0.453

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed) (twelve pigs per group).
Different letters indicate significant differences ($P<0.05$) among dietary treatments.
R-MSE: Root Mean Square Error

Table 9.6. Lipid content (%) and fatty acid profile (% of total fatty acids) of backfat from pigs fed with the experimental diets

Items	Dietary treatments				R-MSE
	C	L	LE	LVE	
Moisture (%)	8.85	8.83	8.63	8.23	1.331
Lipids (%)	85.35	83.75	86.77	86.02	5.914
Fatty acids:					
C 10:0 (capric)	0.074	0.074	0.070	0.075	0.009
C 12:0 (lauric)	0.086^{a}	0.082^{ab}	0.077^{b}	0.085^{ab}	0.007
C 14:0 (myristic)	1.369	1.351	1.304	1.377	0.066
C 16:0 (palmitic)	25.68^{a}	24.90^{ab}	24.54^{b}	24.90^{ab}	0.722
C 17:0 (heptadecanoic)	0.38^{a}	0.30^{b}	0.35^{ab}	0.32^{b}	0.053
C 18:0 (stearic)	16.30	15.16	15.31	15.35	1.347
C 20:0 (eicosanoic)	0.21	0.20	0.21	0.20	0.024
C 16:1 (palmitoleic)	1.86	1.79	1.68	1.73	0.251
C 17:1 (heptadecenoic)	0.36^{a}	0.29^{b}	0.32^{ab}	0.30^{b}	0.045
C 18:1n-7 (vaccenic)	2.43^{a}	2.71^{ab}	2.20^{b}	2.23^{ab}	0.201
C 18:1n-9 (oleic)	39.30^{a}	37.31^{b}	37.29^{b}	36.82^{b}	1.550
C 20:1 (eicosenoic)	0.90	0.82	0.84	0.83	0.128
C 18:2n-6 (linoleic)	9.04	9.81	9.88	10.01	1.127
C 18:3n-3 (α-linolenic)	0.80^{b}	3.90^{a}	4.13^{a}	3.97^{a}	0.412
C 18:3n-6 (γ-linolenic)	0.18^{a}	0.15^{b}	0.17^{ab}	0.16^{ab}	0.019
C 20:2n-6 (eicosadienoic)	0.43	0.45	0.46	0.47	0.060
C 20:3n-3 (eicosatrienoic)	0.13^{b}	0.56^{a}	0.59^{a}	0.60^{a}	0.073
C 20:4n-6 (arachidonic)	0.24^{a}	0.19^{b}	0.19^{b}	0.19^{b}	0.028
C 20:5n-3 (eicosapentaenoic)	0.01^{b}	0.05^{a}	0.05^{a}	0.05^{a}	0.008
C 22:2n-6 (docosadienoic)	0.01	0.01	0.01	0.01	0.001
C 22:4n-6 (docosatetraenoic)	0.10^{a}	0.08^{b}	0.08^{b}	0.08^{b}	0.012
C 22:5n-3 (docosapentaenoic)	0.09^{b}	0.21^{a}	0.23^{a}	0.23^{a}	0.020
C 22:6n-3 (docosahexaenoic)	0.02	0.03	0.03	0.03	0.005
Total Saturated	44.10^{a}	42.08^{b}	41.86^{b}	42.31^{ab}	1.805
Total Monounsaturated	44.85^{a}	42.48^{b}	42.33^{b}	41.90^{b}	1.888
Total Polyunsaturated	11.05^{b}	15.45^{a}	15.81^{a}	15.79^{a}	1.572
Total n-6	9.99	10.69	10.78	10.92	1.203
Total n-3	1.06^{b}	4.76^{a}	5.03^{a}	4.87^{a}	0.459
n-6/n-3 fatty acid ratio	9.55^{a}	2.25^{b}	2.15^{b}	2.24^{b}	0.154
Iodine Value$^{(c)}$	58.93^{b}	61.29^{a}	61.52^{a}	64.43^{a}	2.198

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed) (twelve pigs per group).
Different letters indicate significant differences (P<0.05) among dietary treatments.
R-MSE: Root Mean Square Error

Similar changes in FA concentrations in the backfat were observed by Huang *et al.* (2008), Kouba *et al.* (2003) and Nuernberg *et al.* (2005). Corino *et al.* (2008) reported that 5% of extruded linseed inclusion in pig diets increased significantly ALA, EPA, DPA and also DHA. Also, they found higher amount of arachidonic acid in the backfat respect to the control diet. However, they reported no differences in the content of SFA, MUFA and PUFA in backfat. Bečková & Václavková (2010) observed that 13.4% of ground linseed inclusion in pig diets increased significantly the content of linoleic acid (LA) and ALA in the backfat and decreased the content of myristic, palmitic, oleic and arachidonic acid compared to the control group. Also, they observed lower content of total SFA and MUFA and lower n-6/n-3 PUFA ratio, which is in agreement with our results.

The iodine value was lower (P<0.05) in the C diet respect to the experimental diets, however no value exceed 70, as suggested by Lo Fiego *et al.* (2005).

FA profile of perirenal fat of pigs fed with experimental diets is shown in Table 9.7. FA analyses of this tissue showed that dietary treatments caused similar significant changes in the FA composition of perirenal fat as in the backfat. However, in perirenal fat was observed a higher amount of total SFA and lower amount of MUFA and PUFA, also lower iodine value.

As reported for the LTL muscle, antioxidant inclusion in the pig diets did not affect the backfat and perirenal fatty acid composition. Our results are in accordance with Bertol *et al.* (2017), who reported no effect on FA composition of backfat from pigs fed with 3% of the oil blend and sequential levels of 3-5% or 6-10% of grape pomace. Yan and Kim (2011) reported that fermented grape pomace inclusion in pig diets decreased total SFA and increased total PUFA, mainly linoleic acid (C18:2n6).

The results of the current experiment on the FA composition showed that extruded linseed inclusion in pig diets tend to reduce the amount of arachidonic (C20:4n-6) and docosatetraenoic acid (C22:4n-6), while it increased (P<0.05) the proportions of long chain n-3 PUFA in pig LTL muscle, backfat and perirenal fat. The proportions of ALA and eicosatrienoic acid (C20:3n-3) were

Table 9.7. Fatty acid profile (% of total fatty acids) of perirenal fat from pigs fed with the experimental diets

Items	Dietary treatments				R-MSE
	C	L	LE	LVE	
C 10:0 (capric)	0.094	0.094	0.093	0.087	0.013
C 12:0 (lauric)	0.094	0.097	0.092	0.093	0.009
C 14:0 (myristic)	1.49	1.51	1.47	1.49	0.066
C 16:0 (palmitic)	29.43^{a}	28.29^{b}	27.95^{b}	28.37^{b}	0.621
C 17:0 (heptadecanoic)	0.40	0.32	0.35	0.32	0.079
C 18:0 (stearic)	23.68^{a}	21.97^{b}	21.73^{b}	22.95^{ab}	1.407
C 20:0 (eicosanoic)	0.22	0.21	0.21	0.21	0.025
C 16:1 (palmitoleic)	1.43^{a}	1.30^{ab}	1.28^{ab}	1.19^{b}	0.186
C 17:1 (heptadecenoic)	0.26	0.20	0.23	0.21	0.05
C 18:1n-7 (vaccenic)	1.70^{a}	1.55^{ab}	1.57^{ab}	1.49^{b}	0.142
C 18:1n-9 (oleic)	31.79^{a}	29.54^{b}	30.30^{ab}	29.62^{ab}	2.041
C 20:1 (eicosenoic)	0.56	0.49	0.55	0.54	0.077
C 18:2n-6 (linoleic)	7.31	8.98	8.70	8.34	1.579
C 18:3n-3 (α-linolenic)	0.69^{b}	4.09^{a}	4.08^{a}	3.74^{a}	0.588
C 18:3n-6 (γ-linolenic)	0.13	0.12	0.13	0.12	0.018
C 20:2n-6 (eicosadienoic)	0.25	0.30	0.30	0.30	0.052
C 20:3n-3 (eicosatrienoic)	0.08^{b}	0.41^{a}	0.43^{a}	0.41^{a}	0.065
C 20:4n-6 (arachidonic)	0.21^{a}	0.17^{b}	0.16^{b}	0.17^{b}	0.034
C 20:5n-3 (eicosapentaenoic)	0.01^{b}	0.05^{a}	0.05^{a}	0.05^{a}	0.01
C 22:2n-6 (docosadienoic)	0.004	0.004	0.003	0.003	0.001
C 22:4n-6 (docosatetraenoic)	0.09^{a}	0.06^{b}	0.06^{b}	0.06^{b}	0.01
C 22:5n-3 (docosapentaenoic)	0.08^{b}	0.20^{a}	0.21^{a}	0.19^{a}	0.03
C 22:6n-3 (docosahexaenoic)	0.02	0.03	0.03	0.03	0.006
Total Saturated	55.39^{a}	52.49^{b}	51.90^{b}	53.52^{ab}	1.698
Total Monounsaturated	35.73^{a}	33.09^{b}	33.94^{ab}	33.06^{b}	2.262
Total Polyunsaturated	8.88^{b}	14.42^{a}	14.16^{a}	13.42^{a}	2.252
PUFA/SFA	0.16	0.28	0.27	0.25	0.05
Total n-6	8.00	9.64	9.37	8.99	1.673
Total n-3	0.88^{b}	4.78^{a}	4.79^{a}	4.42^{a}	0.676
Iodine value	48.26^{b}	52.38^{a}	52.54^{a}	50.94^{ab}	2.575

C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed) (twelve pigs per group).
Different letters indicate significant differences (P<0.05) among dietary treatments.
R-MSE: Root Mean Square Error.

greater in the subcutaneous fat and perirenal fat respect to LTL muscle, which is in agreement with previous findings (Corino *et al.,* 2008; Karolyi *et al.,* 2012; Kouba *et al.,* 2003). However, EPA, DPA and DHA proportions were higher in the LTL muscle, which was also observed by Corino *et al.* (2008), Enser *et al.* (2000), Karolyi *et al.* (2012) and Kouba *et al.* (2003).

Our results are in agreement with previous findings where pigs fed linseed diets changed the ALA and EPA proportions in different tissues (Corino *et al.,* 2008; Hoz *et al.,* 2003; Huang *et al.,* 2008; Nuernberg *et al.,* 2005). The n-3 PUFA content increased with the decreasing content of arachidonic acid in LTL muscle, backfat and perirenal fat. It is well known that the n-6 and n-3 PUFA compete for desaturase enzymes in order to be converted in long chain PUFA (Artemis P. Simopoulos, 2010). However, Huang *et al.* (2008) and Nuernberg *et al.* (2005) reported that in pigs fed linseed diets, the enzymes prefer n-3 to n-6 PUFA. In our study we observed that DHA content was not affected by the dietary supplementation with linseed. Our results are in accordance with previous studies that have reported no influence of linseed diets on the DHA levels in pig tissues (Guillevic *et al.,* 2009; Haak *et al.,* 2008; Juárez *et al.,* 2010; Raes *et al.,* 2004). However, some studies found a small but significant increase in DHA content in LTL muscle and backfat after feeding relatively low levels of ALA from linseed (Corino *et al.,* 2008; Enser *et al.,* 2000). Also, Huang *et al.* (2008) found an increase in DHA content in the muscle of pigs fed a high content of linseed (10%). The lack of the effect on the DHA content could be attributed to the inhibition or low activity of the desaturase enzymes, on the other hand due to the competition between ALA and DHA for the desaturase enzymes in order to be incorporated into the tissues, when the dietary ALA content is high (Juárez *et al.,* 2010; Karolyi *et al.,* 2012; Raes *et al.,* 2004).

As expected, the n-6/n-3 PUFA ratio of LTL muscle, backfat and perirenal fat of pigs fed the control diet were higher ($P<0.05$) respect to the experimental diets (Fig. 9.6). The n-6/n-3 PUFA ratio was decreased by extruded linseed inclusion in pig diets, which lead to a significant increase in ALA, EPA and DPA percentages and decrease in arachidonic acid (C20:4n-6). In the present experiment, the n-6/n-3 PUFA ratio was reduced ($P<0.05$) from 11.39 in the C diet to 3.75, 3.95 and 3.80 in L, LE and LVE respectively in LTL muscle; from 9.55 in the C diet to 2.25, 2.15 and 2.24 in L, LE and LVE respectively in the backfat; and from 9.13 in the C diet to 2.02, 1.97 and 2.03 in L, LE, LVE respectively in perirenal fat. Similar results were reached by Bečková & Václavková (2010), Huang *et al.* (2008) and Okrouhlá *et al.* (2013). The n-6/n-3 ratio obtained in

our study is in line with Nutritional Guidelines, which recommend to reduce n-6/n-3 PUFA ratio down to 4, that is beneficial for human health (Weill *et al.*, 2002).

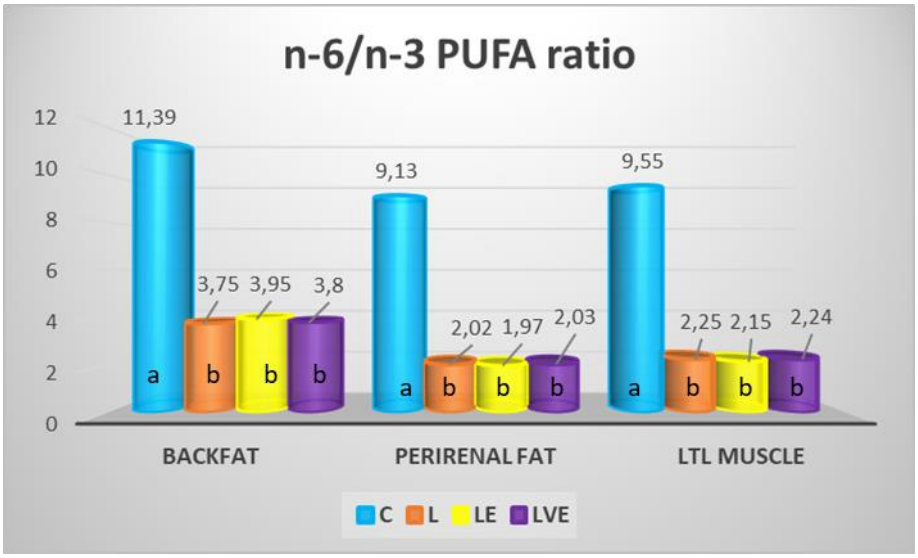

Figure 9.6. The n-6/n-3 PUFA ratio in LTL muscle and backfat from pigs fed experimental diets
C, control group; L, experimental group with 5% of extruded linseed; LE, experimental group with 5% of extruded linseed, 200 ppm vitamin E and 0.21 ppm selenium; LVE, experimental group with 5% of extruded linseed and extracts from grape skin (3 g kg^{-1} feed) and oregano (2 g kg^{-1} feed).
Different letters indicate significant differences ($P<0.05$) among dietary treatments.

9.5. Conclusion

The present study examined the effect of four dietary treatments on the quality parameters and shelf-life of pork. Our results suggest that the addition of linseed and antioxidants in the diets does not affect growth performance, carcass traits, quality parameters and chemical composition of meat. However, linseed inclusion in pig diet considerably enhanced n-3 PUFA content and reduced the n-6/n-3 PUFA ratio, obtaining pork with high nutritional value, beneficial for human health.

Linseed inclusion together with synthetic or natural antioxidants in the pig diets did not lowered the pork quality during refrigerated storage. Moreover, all experimental diets improved, in comparison with control diet, the microbial status of meat during refrigerated storage, in particular the *Enterobacteriaceae.*

9.6. References

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CHAPTER 10

GENERAL CONCLUSIONS

Nowadays, there is a growing awareness of the relationship between meat consumption and human health. Several scientific studies have been conducted to enhance the composition and nutritional value of meat and meat products. There was an increasing interest in the development of feeding systems and strategies of supplementation, able to improve animal performance and carcass traits, that led to the production of meat products of high quality.

Synthetic additives have been widely used in animal nutrition, but they have been associated with several harmful effects on humans and animals due to their toxicity. Today, consumers became more conscious of the source of their food, preferring natural additives that have functional properties, which can enhance the nutritional value of food products and increase consumers' health benefits. The inclusion of natural additives instead of synthetic additives in pig diets in order to increase the oxidative stability, seems to be an interesting nutritional strategy, that can avoid any further manipulation of the meat.

The results of this study showed that performance and carcass parameters were not affected by linseed and antioxidant supplementation in pig diets neither in the Experiment I nor in the Experiment II. Also, qualitative characteristics and chemical composition of muscle are not affected by dietary linseed inclusion associated with either synthetic or natural antioxidants.

However, 5% of extruded linseed inclusion in pig diets improved the fatty acid profiles in LTL muscle, backfat and perirenal fat increasing considerably the n-3 PUFA, that are known to provide health benefits by reducing the risks for several diseases. Due to the high content of n-3 PUFA, the n-6/n-3 PUFA ratio was reduced towards values beneficial for human health.

Linseed inclusion together with synthetic or natural antioxidants in the pig diets did not reduced the pork quality during refrigerated storage. Moreover, all experimental diets improved, in comparison with the control diet, the microbial status of meat during refrigerated storage, in particular the *Enterobacteriaceae*. In the Experiment I, high concentration of oxygen in MAP brought about an increase in oxidative products and yielded redder meat, irrespective of the dietary treatment. However, it is interesting to note that vegetal extracts were as effective as synthetic

antioxidants in preventing lipid oxidation. Furthermore, grape skin extract is a waste by-product of the wine industry and its valorisation could help to maintain an environmental balance.

Further experimental investigations are needed to explain the lack of an antioxidant activity of grape skin and oregano extract in LTL muscle and to find the optimal supplementation dose.

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