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Doctoral thesis

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Meat oxidative stability as affected by animal feeding system and dietary phenolic compounds

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Summary of the research activities

Complete list of publications

Publications in peer-reviewed ISI journals:

- Luciano, G., Moloney, A. P., Priolo, A., Röhrle, F. T., Vasta, V., Biondi, L., López-Andrés, P., Grasso, S. and Monahan, F. J. Pro-oxidant and antioxidant components and the oxidative stability of beef from cattle grown on forage or concentrate-based rations. Submitted for publication to *Journal of Animal Science*

- Röhrle, F. T., Moloney, A. P., Osorio, M. T., Luciano, G., Priolo, A., Caplan, P., and Monahan, F. J. Carotenoid, colour and reflectance measurements in bovine adipose tissue to discriminate between beef from different feeding systems. Accepted for publication in *Meat Science*.

- Vasta, V., Luciano, G., Di Mauro, C., Röhrle, F. T., Priolo. A., Monahan, F. J. and Moloney, A. P. (in press). The volatile profile of longissimus dorsi muscle of heifers fed grass, grass silage or cereal concentrate: implication for dietary discrimination. *Meat Science*. doi:10.1016/j.meatsci.2010.11.003.

- Luciano, G., Vasta, V., Monahan, F.J., Lòpez-Andréz, P., Biondi, L., Lanza, M. and Priolo, A. 2011. Antioxidant status, colour stability and myoglobin resistance to oxidation of longissimus dorsi muscle from lambs fed a tannin-containing diet. *Food Chemistry*, *124*, 1036-1042.

- Vasta, V., Yáñez-Ruiz, D.R., Mele M., Serra, A., Luciano, G., Lanza, M., Biondi, L. and Priolo, A. 2010. Bacterial and protozoal communities and fatty acid profile in the rumen of sheep fed a diet containing added tannins. *Applied and Environmental Microbiology*, *76*, 2549-2555.

- Vasta, V., Mele, M., Serra, A., Scerra, M., Luciano, G., Lanza, M. and Priolo, A. 2009. Metabolic fate of fatty acids involved in ruminal biohydrogenation in sheep fed concentrate or herbage with or without tannins. *Journal of Animal Science*, *87*, 2674-2684.

- Luciano, G. Monahan, F.J., Vasta, V., Pennisi, P., Bella, M. and Priolo, A. 2009. Lipid and colour stability of meat from lambs fed fresh herbage or concentrate. *Meat Science*, *82*, 193-199.

- Luciano, G., Monahan, F.J., Vasta, V., Biondi, L., Lanza, M. and Priolo, A. 2009. Dietary tannins improve lamb meat colour stability. *Meat Science*, *81*, 120-125.

- Moreno-Rojas, J.M., Vasta, V., Lanza, A., Luciano, G., Ladroue, V., Guillou, C. and Priolo A. 2008. Stable isotopes to discriminate lambs fed herbage or concentrate both obtained from C3 plants. *Rapid Communications in Mass Spectrometry*, *22*, 3701-3705.

Proceeding papers and abstracts of conferences:

- Luciano, G., Monahan, F.J., Vasta, V., Biondi, L., Lanza, M., Bella, M., Pennisi, P. and Priolo, A. 2009. Lamb meat colour stability as affected by dietary tannins. *Italian Journal of Animal Science, 8 (Suppl. 2), 507-509. Proceedings of the XVIIIth National Congress ASPA,* Palermo, Italy, June 9-12, 2009.

- Vasta, V., Mele, M., Serra, A., Luciano, G., Scerra, M., Lanza, M. and Priolo, A. 2009. Fatty acid profile in the ruminal fluid and in the m. longissimus dorsi of lambs fed herbage or concentrate with or without tannins. *Italian Journal of Animal Science, 8 (Suppl. 2*), *555-557. Proceedings of the XVIIIth National Congress ASPA*, Palermo, Italy, June 9-12, 2009.

- Vasta, V., Yáñez-Ruiz, D. R., Mele, M., Serra, A., Luciano, G., Lanza, M. & Priolo, A. (2009). Ruminal bacteria, protozoa and fatty acid profile in sheep and goats supplemented with tannins. In *Proceedings of the XIIth International Symposium on Ruminant Physiology,* Clermont-Ferrand, France, September 6-9, 2009 (pp. 386-387)

Foreigner institutions and laboratories visited

- School of Agriculture, Food Science and Veterinary Medicine, University College of Dublin, Ireland (April – July 2007; January – June 2009)

- l'Institut Agronomique et Vétérinaire Hassan II di Rabat, Morocco (April – June 2008; October – November 2009)

Participation to national and international research projects

- Research project PRIN 2007 (*Effects of tannins of some ruminant feeds on the rumen degradability of protein and fibre fractions, on the biohydrogenation of lipids and on the quality of milk and meat*).

- LowInputBreeds (LIB) project of the European 7th FP (*Development of integrated livestock breeding and management strategies to improve animal health, product quality and performance in European organic and "low input" milk, meat and egg production*)

Contents

Relationships between lipid and myoglobin oxidation in meat

Context and research issues

Meat has traditionally held a special place in the diet because of its appealing flavour and texture and its high nutritional value. However, in recent years the meat industry has come under increasing scrutiny because of concerns such as those relating to health value, safety and animal welfare. In general, there is now a greater demand than ever by consumers for foods perceived as natural, fresh-tasting, healthy and nutritious (Morrissey et al., 1998). Several meat quality attributes are considered of importance in order to satisfy the consumer's demands. Apart from sanitary issues, the relevant meat quality parameters are generally described as nutritional properties and palatability attributes (Pearson, 1994). Among the latter, colour and flavour play a major role, being sensory properties by which meat quality is readily assessed (Liu et al., 1995). Meat and meat products are normally appreciated for their distinctive flavours which can be classified into several groups related to both endogenous and exogenous factors (Gray & Pearson, 1994). It is accepted that off-flavours originating from lipid oxidation, generally described as "rancidity" flavours, result in a significant loss of quality of muscle-based foods (Buckley et al., 1995). If the importance of flavour must be recognised, the visual appearance of meat strongly influences the consumer's purchase decision, which makes colour another key quality attribute. Clydesdale (1978) argued that colour is perhaps the most important sensory attribute of a food product, whereby, if it is deemed unacceptable, the product will not purchased, the other attributes loosing every relevance. According to this opinion, Smith et al. (2000) reported that a significant amount of retail beef is discounted in price due to discolouration, which corresponds to a substantial annual revenue loss.

It is well known that, in red meats, both colour and flavour are negatively affected by oxidative processes, with lipid oxidation products being responsible for undesirable off-flavours, whereas the oxidation of myoglobin is the major cause of meat discolouration (Greene, 1969). Lipid oxidation and Mb oxidation are believed to be linked and this is supported by simple observations that both the products of lipid oxidation and metmyoglobin (the product of myoglobin oxidation) generally increase concurrently. As reviewed by Baron & Andersen (2002), there is availability of many scientific references to the role of myoglobin as catalyst of lipid peroxidation. However, vitamin E addition to muscle model systems was shown to reduce both lipid oxidation and metmyoglobin formation (Anton et al., 1991; Yin et al., 1993), as well as high levels of vitamin E in the muscle delayed beef discolouration (Faustman et al., 1989). Vitamin E has been known for years as a powerful antioxidant able to protect phospholipids against oxidation (Halliwell, 1987). Since vitamin E is a liposoluble molecule located within the cell membrane, whereas myoglobin is a water-soluble sarcoplasmic protein, the colour-sta-

bilizing effect of vitamin E is supposed to arise somehow from its ability to inhibit lipid oxidation (O'Grady et al., 2001).

On the basis of these findings, research in meat science is aiming at better understanding the relationships between lipid and myoglobin oxidation. A deeper comprehension of the mechanisms regulating the interaction between lipid and myoglobin biochemistry in meat would allow to optimize the antioxidant interventions implemented to extend meat shelf life. In this context, particular emphasis has been placed, in the last years, on the possible mechanisms by which lipid oxidation could promote the oxidation of myoglobin with consequent negative effects on meat colour stability. Therefore, the first part of the present thesis was developed during an initial period of bibliographic study in which the results of the relevant studies published on this topic in the last twenty years were connected in order to overview the research background available

1. Lipid oxidation

It is generally accepted that, apart from microbial spoilage, lipid oxidation is the primary process by which quality loss of muscle foods occurs (Buckley et al., 1995). This process, indeed, leads to drip losses, off-odour and off-flavour development, and to the production of potentially toxic compounds (Morrissey et al., 1994; Gray et al., 1996). Lipid oxidation is the process by which molecular oxygen reacts with fatty acids to form lipid peroxides. Polyunsaturated fatty acids represent preferential substrates for lipid oxidation because of the double bonds in their molecules. The reaction proceeds by a free chain reaction mechanism involving initiation, propagation and termination stages (Morrissey et al., 1998).

The reaction between lipids and ground state oxygen is thermodynamically unfavourable and proceeds very slowly without the aid of catalysts capable of initiating and propagating lipid oxidation (Frankel, 1985). Transition metal ions (such as Fe^{2+}, Cu^{2+}) are generally accepted to be pivotal in catalyzing oxidative changes in tissues. Iron, for example, initiates lipid oxidation by generating free radicals capable of abstracting a proton from unsaturated fatty acids (Kanner, 1994). Different forms of iron have been shown to be able to initiate lipid oxidation causing oxidative deterioration in muscle foods (Gray et al., 1996). However, the relative contribution of different forms of iron, whether "free" or protein bound, heme or non-heme, oxidized or reduced, in catalyzing lipid peroxidation has not been definitively assigned (Baron & Andersen, 2002). Apart from metals, internal and external stressors incessantly threaten cells and favour the oxidation of important biomolecules, including fatty acids. The most important of these are oxygen derivatives called reactive oxygen species (ROS) produced during normal aerobic metabolism in which mitochondria consume molecular oxygen and reduce

it sequentially to produce H_2O. During this process, ROS are produced, such as free radicals (HO•, O_2^-•) or H_2O_2, which are unstable compounds able to interact with several biological molecules. Fatty acids, especially polyunsaturated fatty acids, can be easily altered by ROS to form peroxyl radicals, primary product of lipid oxidation able to propagate lipid oxidation in the cell membranes. These compounds are relatively unstable and are rapidly converted into a variety of more stable secondary compounds, among which aldehydes are responsible for rancid flavour arising from lipid oxidation (Morrissey et al., 1998). Moreover, the measurement of the individual secondary compounds by gas-chromatography or their spectrophotometric determination by means of the Tiobarbituric Acid and Reactive Substances (TBARS) assay, are generally used to measure the extent of lipid oxidation in meat.

Under normal physiological conditions, several antioxidant defence mechanisms act in order to protect the integrity of the organism (Yu, 1994). For example, animals have evolved several effective enzymatic defence systems, such as superoxide dismutase, catalase and glutathione peroxidise, which continuously neutralize the ROS produced, thus acting as preventive antioxidants. Another important protective mechanism involves compounds known as chain-breaking antioxidants, capable of interrupting the free-radical chain reactions (Stocker et al., 1991). These are vitamins E and C, carotenoids and polyphenols, and all of those molecules able to donates hydrogen to a peroxyl radical, thus interrupting the propagation of lipid oxidation. These molecules are often of dietary origin and act in the animal's tissues at very low concentration. In the case of vitamin E, for example, the rate-constant (k) for its antioxidant reaction is nearly 10^4 times faster than the propagation reaction (Morrissey et al., 1998), which means that vitamin E can scavenge peroxyl radicals about 10^4 times faster than they can react with other fatty acids, so that only relatively small amounts of vitamin E are necessary for an effective antioxidant protection. The peculiar biochemical processes occurring during the conversion of muscle to meat interfere with the harmony of the antioxidant and reducing systems with a depauperation of the antioxidant enzymes activities, which gives rise to conditions whereby the oxidative stress is not as tightly controlled as in a living organism (Buckley et al., 1995). Therefore, it is likely that over extended storage at retail, antioxidant vitamins play a very important role in protecting meat against oxidation.

In recent years, the pressing recommendations to lower the consumption of saturated fats have leaded to an increasing demand for meat products containing high levels of polyunsaturated fatty acids. However, a high degree of unsaturation of intramuscular fat increases its susceptibility to lipid oxidation (Wood et al., 1999). Furthermore, the production of precooked and restructured muscle-based foods has strongly increased, which makes the extension of their shelf life a major challenge, as processing dramatically increases the susceptibility of such foods to

the oxidative deterioration (Gray et al., 1996). Therefore, the direct addiction of antioxidants to the meat or the dietary supplementation of antioxidants have been tested as strategies for improving meat shelf life. Considering the concerns related to the use of synthetic preservatives in food, there is now considerable interest in the antioxidant properties of naturally occurring substances, including vitamin E, carotenoids and phenolic compounds (Wood and Enser, 1997).

2. Myoglobin chemistry

Myoglobin (Mb) is the protein mainly responsible for meat colour. Although other heme proteins, such as hemoglobin and cytochrome C, may also play a role in the pigmentation of meat, Mb is the major pigment in beef, lamb and pork, where it represents 70-90% of the heme proteins (Fox, 1987). Myoglobin is a protein comprising of 153 amino acids (MW = 17800 Da) and containing, in its tertiary structure, 8 α-helices. Among the numerous residues, histidine (HIS) has received a special attention because of its role in the protein's structure and function. Being involved in the oxygen transport, myoglobin also contains a prosthetic group with an iron atom that can form six bonds. Four of these bonds are with pyrrole nitrogens, while the fifth coordinates with a histidine residue (HIS-63) called proximal histidine. The sixth site is available for the reversible bound with oxygen (Mancini & Hunt, 2005). On the basis of the oxidation state of the iron atom and of the presence of oxygen as ligand, myoglobin can occurr in three different forms: deoxymyoglobin, oxymyoglobin and metmyoglobin.

Deoxymyoglobin (DMb or MbFe(II)) occurs when the heme iron is in the reduced state (Fe^{2+}) and no oxygen is present at its sixth coordination site. It imparts the purplish-red colour typical of the muscle immediately after cutting. Very low oxygen tension (< 1.4 mm Hg) is required to maintain myoglobin in this deoxygenated form (Brooks, 1935). This is the reason why, DMb is found deeply a in freshly cut piece meat stored in aerobic conditions. By binding molecular oxygen (O_2), deoxymyoglobin oxygenates and Oxymyoglobin (OMb or MbFe(II)O_2) is formed. Although the diatomic oxygen occupies the available coordination site, no change in the Fe oxidation state occurs. Oxymyoglobin is responsible for the typical cherry-red colour of meat appreciated by consumers which develops after sufficient exposure to oxygen (blooming). An oxygen partial pressure of at least 80 mm Hg is necessary to maintain myoglobin in this oxygenated form (Forrest et al., 1975). Thus, in a freshly cut piece of meat, OMb occupies an outer layer exposed to oxygen, whose depth depends on several factors (pH, temperature, oxygen partial pressure and oxygen-consuming respiratory processes; Mancini & Hunt, 2005). Myoglobin is physiologically active in the DMb and OMb forms, whereby, the reversible bound with the O_2 allows its transport from haemoglobin to the termi-

nal mitochondrial oxidase (Wittenberg & Wittenberg, 1989). However, the oxidation of both ferrous myoglobin forms to the ferric state (Fe^{3+}) leads to the formation of the oxidised form of Mb known as metmyoglobin (MMb or MbFe(III) OH_2). This autoxidation causes the discoloration of meat, with the typical colour shifting from red to brown after extended storage (Livingston & Brown, 1982; Wallace et al.,1982).

Several are the factors affecting Mb oxidation, including temperature (Brown & Mebine, 1969), pH (Goto & Shikama, 1974), metal ions and a number of active oxygen species (Wallace et al.,1982; Brantley et al., 1993; Gorelik & Kanner, 2001b). *Infra vitam*, approximately neutral pH values and the integrity of the antioxidant defence systems protect DMb and OMb against the oxidation. Furthermore, a peculiar enzymatic system known as metmyoglobin reductase, contributes to the overall meat Metmyoglobin Reducing Activity (MRA) which continuously reduces the MMb formed to the native DMb. These protective mechanisms, in concert, maintain levels of MMb in the tissues. Nevertheless, if the delicate balance between the three Mb forms is maintained at physiological conditions, *post mortem* biochemical processes occurring in muscle favour the production and accumulation of metmyoglobin (Baron & Andersen, 2002). Furthermore, during the oxidation of OMb, superoxide radical ($O_2^{-}\bullet$) is also produced which undergoes the enzymatic dismutation originating H_2O_2 (Fridovich, 1975). Metmyoglobin can be activated by reacting with H_2O_2 (Davies, 1990; 1991) and this results, first, in the formation of an unstable hypervalent myoglobin form known as perferrylmyoglobin (•MbFe(IV)=O) which, then, autoreduces rapidly to the more stable ferrylmyoglobin (MbFe(IV)=O). The latter is know as a potential powerful pro-oxidant able to initiate lipid oxidation especially under the condition found in muscle foods. Indeed, Kanner and Harel (1985a; 1985b) indicated that this activation of MMb was a necessary step in its conversion to a pro-oxidant.

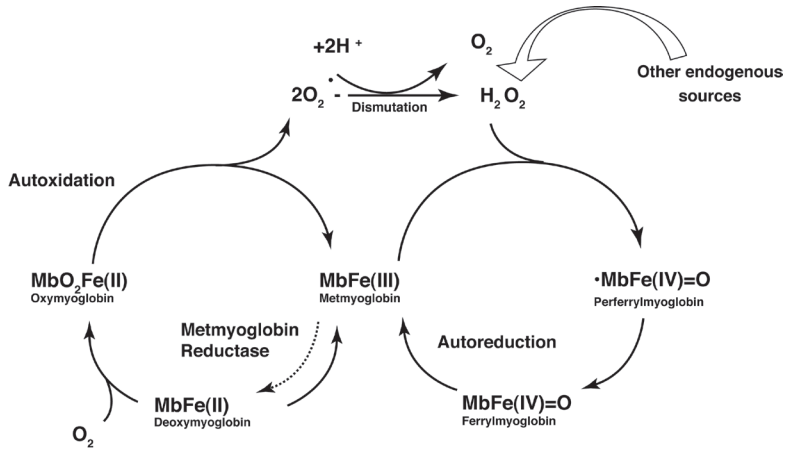

Fig. 1: Dynamic conversions between the different myoglobin forms (from Baron & Andersen, 2002).

3. Potential mechanisms by which lipid oxidation promotes myoglobin oxidation

❖ 3.1. Interaction of secondary products of lipid oxidation with myoglobin

Several studies suggest a direct role of some lipid oxidation products in favouring oxymyoglobin oxidation. Using muscle model systems, Anton et al. (1991) found a greater metmyoglobin formation in the combination of OMb with bovine microsomes than in the control system without membrane lipids. Additionally, OMb stability in microsome and liposome models was improved with elevated concentration of α-tocopherol (Yin and Faustman, 1993, 1994). The first products arising from the lipid metabolism are reactive oxygen species and lipid radicals. These primary compounds are relatively unstable and, thus, they decompose into a variety of secondary compounds (Frankel, 1987) which are more stable and polar than their parent compounds. Therefore, they can readily diffuse into the surrounding sarcoplasm where they can react with important biomolecules (Esterbauer et al., 1991). It has been known for years that lipid oxidation products can exert a deleterious effect toward heme protein (Haurowitz et al., 1941). Moreover, heme proteins are able to bind to cell membranes and fatty acids (Szebeni et al., 1988), increasing the likelihood of interacting with lipid oxidation products. Among these compounds, α,β-unsaturated aldehydes are very reactive toward proteins (Witz, 1989) and several of these are produced from fatty acids tipically found in meat.

❖ 3.1.1 Bovine myoglobin

Chan et al. (1997b) were among the first to study the potential basis of the prooxidant effect of lipid oxidation products toward myoglobin. They incubated horse OMb solution in dialysis sacs (MW cutoff 500 Da) placed in solutions of fresh or oxidised liposomes. Lipid oxidation, measured as Tiobarbituric Acid and Reactive Substances (TBARS), was detected in the sacs after 5 hours of incubation at 30°C. This demonstrated that low-molecular-weight (< 500 Da) lipid oxidation products can diffuse from the membranes and contaminate the surrounding environment. Moreover the concentration of TBARS increased with the extent of oxidation of the lipid solution and the metmyoglobin formation showed a similar trend. These authors also compared the effects of selected lipid oxidation products upon OMb oxidation and found the α,β-unsaturated aldehydes nonenale and heptenale to be very reactive. These compounds are secondary products derived from the oxidation of ω-6 polyunsaturated fatty acids (Pryor & Porter, 1990). Among the unsaturated aldehydes, 4-hydroxy-2-nonenal (HNE) has also received a special attention (Esterbauer et al., 1991; Shneider et al., 2001). Sakai et al. (1995)

documented its presence and average levels in beef and pork. The covalent attachment of HNE to glucose-6-phosphate dehydrogenase, hemoglobine and LDL has been demonstrated and has been reported to alter the tertiary structure of these molecules (Esterbauer et al., 1991; Szweda et al., 1993; Bruenner et al, 1995; Uchida et al, 1994). Faustman et al. (1999), incubated HNE with horse OMb at 37°C and pH 7.4. During 2 hours of incubation, Electrospray Ionization Mass Spectrometry (ESI-MS) revealed that HNE became adducted to OMb by a reaction, known as Michael addition. Bruenner et al. (1995) had already reported HNE adduction to both hemoglobin and β-lactoglobulin. In their experiment Faustman et al. (1999) did not investigate the site of HNE adduction on OMb. However, they hypothesized that the HNE attachment could be at the histidine residues. Indeed, among the different amino acids, the best candidates for Michael addition are cysteine, lysine and histidine (Uchida et al., 1994), but mammalian myoglobins lack cysteine residues (Livingston & Brown, 1982). Furthermore, Bolgar and Gaskell (1996) incubated HNE with apomyoglobin and used ESI-MS and tandem MS to show that HNE adduction was solely to the histidine residues.

In an *in vitro* experiment, Lynch and Faustman (2000) showed that, the incubation of horse OMb with HNE at 37°C and pH 7.2 increased MMb formation. At the same time, the concentration of HNE decreased. This was consistent with previous findings suggesting a pro-oxidant activity of HNE toward OMb by its adduction with the protein (Chan et al., 1997b; Faustman et al., 1999). Interestingly the authors also showed that preincubation of MMb with aldehydes decreased its ability to be enzymatically reduced, with the unsaturated aldehyes being more effective. Moreover, MMb preincubated with HNE showed a greater ability to catalyze lipid oxidation when incubated in the presence of liposomes and microsomes. It was suggested that the covalent attachment of HNE to MMb may alter its tertiary structure. This may lead to a difficulty to interact with the reductase, on one hand. On the other hand, the structural modification may expose the heme group to the surrounding environment, enabling it to catalyze lipid oxidation. Alderton et al. (2003) prepared OMb from bovine Mb and incubated it with HNE at different temperatures (4, 25 and 37°C) and pH (7.4 and 5.6). As expected, MMb formation was greatest at 37°C and pH 5.6, as relatively high temperatures and low pH values strongly promote the autoxidation of myoglobin. However, at 37°C, the pro-oxidant effect of HNE was not significant at pH 5.6. A clear effect of HNE at both pH values was achieved only at lower temperatures, but MMb formation was less rapid. Liquid Chromatography-Mass Spectrometry analysis (LC-MS) of OMb samples incubated with HNE at 37°C and pH 7.4 and 5.6 was performed. It showed a greater HNE adduction at pH 7.4. Interestingly, Western Blot analysis indicated the adduction to histidine residues only. Moreover, Liquid Chromatography-Tandem-Mass Spectrometry analysis (LC-MS-MS) identified six histidine residues adducted with HNE (HIS-24, -64, -93, -116, -119 and

-152; Figure 2). This result had been already achieved by Phillips et al. (2001). Among them, HIS-93 appeared to be more readily adducted. This is interesting because HIS-93 is the proximal residue coordinated with the heme group. Thus, because of the proximity of this residue to the heme group, its modification by HNE could alter the protein structure and its redox stability.

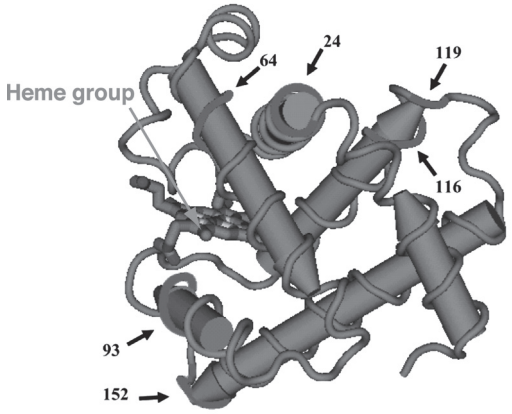

Fig. 2. The structure of bovine myoglobin with the most readily adducted residues (from Alderton et al., 2003)

❖ *3.1.2. Porcine myoglobin*

It is accepted that, in beef, relevant concentrations of vitamin E delay both lipid and myoglobin oxidation (Faustman et al. 1998). However, several authors reported that pork meat from animals fed diets rich in α-tocopherole did not show such improvement (Lanari et al., 1995; Cannon et al., 1996; Jensen et al., 1997; Houben et al., 1998; Phillips et al., 2001). Pork generally contains a greater amount of ω-6 polyunsaturated fatty acids than beef (Enser et al., 1996). Therefore, it is plausible to suppose that it can undergo lipid oxidation and produce secondary products more readily than beef. The fact that, on the contrary, vitamin E supplementation does not affect pork colour stability leaded some researchers to suppose that differences could exist between porcine and bovine Mb in their responses to lipid oxidation. Lee et al. (2003) incubated porcine OMb with microsomes from the livers of pigs fed either a vitamin E supplemented diet or a control diet. The pro-oxidant activity of HNE toward OMb and its adduction to histidine residues were confirmed, as previously observed for bovine OMb. However, while vitamin E supplementation lowered the TBARS values compared to the control, OMb oxidation was not affected by the treatment. The authors supposed that a species effect, due to differences between porcine and bovine Mb, can be the reason for their different stability to the oxidation induced by lipid oxidation products. Indeed, the amino acid sequence determines the tertiary structure of a protein, which, in turn, affects its interaction with other ligands. Differences between porcine and bovine Mb in their primary

structure have been confirmed. Porcine myoglobin, contains 9 histidine residues, while bovine myoglobin contains 13 residues (Lee et al., 2003). Moreover, porcine Mb lack HIS-116 and HIS-152, two residues reported by Alderton et al. (2003) as readily adducted to HNE.

In their experiment, Lee et al. (2003) used a temperature of 37°C and a pH 7.4. In a subsequent work, Suman et al., (2006) compared the destabilizing effect of HNE on both porcine and bovine myoglobin, adopting conditions typical of meat during storage (4°C and pH 5.6). At these conditions, it was found that the incubation of with HNE resulted in a greater formation of MetMb in bovine than in porcine OMb. This preliminary screening suggested that bovine was more prone to HNE-induced oxidation. Moreover, after 72 hours of incubation, porcine OMb formed only monoadducts with HNE, while in bovine Mb also diadducts were present. This supports the hypothesis that the lower amount of HIS residues in porcine Mb makes it less susceptible to the lipid oxidation products adduction. Finally, it was shown that, while four HIS residues resulted adducted with HNE in bovine OMb, only two residues were adducted in porcine OMb. Suman et al. (2007) adopted a proteomic approach to study the preferential HNE adduction to porcine and bovine OMb. Noticeably, incubation of both OMb with HNE at 4°C and pH 5.6 did not show any pro-oxidant effect of the aldehyde. Thus, 37°C and pH 7.4 were chosen as experimental condition for the subsequent experiments. After incubation with HNE, only monoadducted were identified in porcine OMb, confirming previous findings that porcine OMb was less prone to HNE-induced oxidation. It was also found that three HIS residues were readily adducted in porcine OMb (Figure 3), while seven adducted residues were found in bovine Mb. The HNE adduction to bovine HIS-93 confirms the results of Alderton et al. (2003) and can explain the destabilising effect of lipid oxidation products toward myoglobin in beef. The finding that in porcine Mb HIS-36 is the preferential site for HNE adduction compared to HIS-93 may explain why bovine OMb appears to be more sensitive to lipid oxidation than porcine OMb.

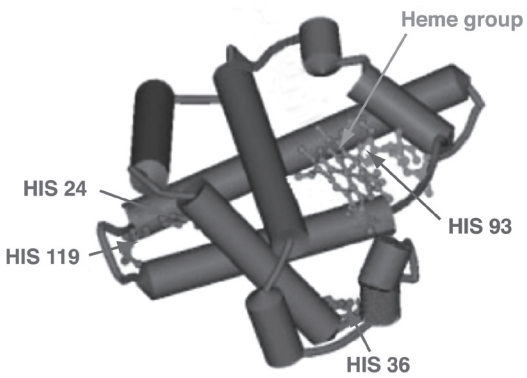

Fig. 3. The structure of the porcine myoglobin with the most readily adducted sites (from Suman et al., 2007).

❖ *3.2. Lipid oxidation-induced oxygen depletion.*

Meat is a complex system and *in vitro* model approaches have been used to study the possible mechanisms by which specific factors can affect oxidative processes (Chan et al., 1997a). Decker et al. (2005) reviewed the most common among these model systems, suggesting some factors to consider when antioxidant effectiveness in muscle foods is assessed. Many studies of lipid and myoglobin oxidation have used oxymyoglobin solutions in combination with isolated membranes in more or less complex systems. While useful, these systems do not contain important components of the muscle able to strongly affect oxidative processes. In determining the relative importance and the possible mechanisms of action of different factors involved in meat deterioration, it is important to use experimental conditions that mimic those in *post mortem* muscle.

O'Grady et al. (2001) used bovine muscle homogenates, containing components native to the muscle, to study OMb oxidation and lipid oxidation. They performed the analysis at 4°C and pH 5.5 to represent the storage condition of meat. The addition of both vitamin E and a soluble antioxidant delayed both lipid OMb oxidation. This confirmed that, even under those conditions, OMb stability may have been affected by lipid oxidation. However, interestingly, when OMb was incubated with lipid oxidation products from an oxidized muscle homogenate, there were no differences in OMb oxidation compared to the control. This is in agreement with previous findings (Lin & Hultin, 1977). Measurement of lipid oxidation products showed that their amount in the homogenate was consistently lower than that used in the experiments mentioned above (Chan et al., 1997b; Faustman et al., 1999; Lynch and Faustman, 2000; Alderton et al., 2003; Lee et al., 2003; Suman et al., 2006; 2007). These results seem to suggest that, under experimental conditions closer to those occurring in meat during storage, oxymyoglobin oxidation is unaffected by the direct interaction with lipid oxidation products. The authors also measured the dissolved oxygen in their homogenates. It was found that, increases in lipid oxidation and metmyoglobin were accompanied by consistent decreases in the dissolved oxygen in the controls. Antioxidants addition to the system lowered both lipid oxidation and OMb oxidation and maintained higher oxygen levels than the control. It is known that autoxidation of OMb to MMb is a process strongly affected by the oxygen partial pressure (Ledward, 1970). Relatively high oxygen levels are required to maintain Mb in its oxygenated form. In a typical Po_2-dependence curve (Figure 4), it is possible to notice that, at lower Po_2 values, OMb autoxidates rapidly, while the oxidation rate decreases consistently at increasing levels of oxygen pressure. (George & Stratmann, 1952).

Monahan et al. (2005) used muscle homogenates to investigate the hypothesis that the depletion of oxygen due to lipid oxidation may be responsible for myoglobin oxidation. Bovine muscles homogenates were held at 4°C and pH 5.7 and

subjected to three different treatments. One sample was stirred and bubbled with oxygen. The second sample was stirred but not bubbled. The third sample, the control, was neither stirred nor bubbled. Lipid oxidation, OMb oxidation, free radical formation and oxygen level were measured. The highest lipid oxidation and free radical formation occurred in the stirred and bubbled sample, while the lowest were measured in the control. On the contrary, OMb oxidation occurred with the greatest extent in the control sample. In this experiment, thus, it was possible to separate the rate of lipid oxidation from that of oxymyoglobin oxidation. The oxygen level was maintained high in the samples showing the lowest OMb oxidation and the highest lipid oxidation (the stirred and the stirred and bubbled samples) and decreased in the control sample, which showed the greatest OMb oxidation and the lowest lipid oxidation. Considering that lipid oxidation is an oxygen-consuming process, the authors concluded that, under conditions typical of meat storage, the lipid oxidation-induced oxygen depletion, is a possible mechanism by which lipid oxidation affects meat discoloration.

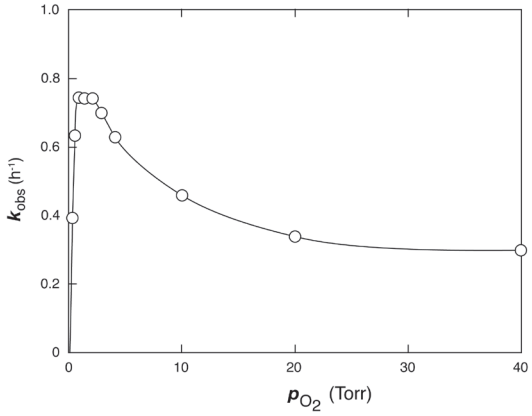

Fig. 4. The typical Po_2-dependence curve of oxymyoglobin oxidation (from Shikama 1998)

4. Conclusions

Oxidative processes represent a major cause of quality deterioration in meat and meat products. A better comprehension of the mechanisms leading to meat rancidity and discolouration would allow to calibrate the antioxidant interventions, both *infra vitam* and *post mortem*. Several studies have shown that a relation between lipid oxidation and meat discoloration exists. The pro-oxidant activity of oxidised myoglobin species toward lipids is well known and described. However, findings that high levels of antioxidants in the muscle are able to delay meat discoloration, inspired a number of researchers to study the possibility that lipid oxidation can promote myoglobin oxidation. The direct interaction of some lipid oxidation products with myoglobin and the depletion of the dissolved oxygen due to the high rate of lipid oxidation in the muscle, seem to be the most likely mechanisms by which lipid oxidation can affect colour stability. However, it should be considered that meat is a complex matrix to analyse and, often, model

systems used to study these relations, do not consider a number of important factors native to the muscle, such as the antioxidant and reducing enzymatic system, which control oxidative processes. Further work in this field is still needed to evaluate the relative contribution of each factor in controlling these deteriorative processes. Moreover, it will be important to perform the experiments using experimental conditions close to those occurring in the meat during storage.

Experiments

Preface

For the meat industry, the possibility of extending meat shelf life by delaying its oxidative deterioration represents a significant objective. The shelf life of fresh meat and of processed and precooked muscle-based foods is, indeed, a main constraint for all those involved in the meat production chain, including the primary producers, processors, distributors, and retailers. The complex biochemical mechanisms behind lipid and myoglobin oxidation allow to face the problem of meat oxidative stability with several strategies and at different levels of the production chain, involving both animal and food scientists. For example, antioxidant molecules can be directly added to the meat before packaging and display at retail. Alternatively, more or less innovative packaging systems, involving the use of modified atmospheres, can be adopted to exploit the dependence of lipid and myoglobin oxidation on the levels of oxygen and of other gases such as carbon monoxide (CO). However, such approaches need to be applied to a rather large-scale production to overcome the costs related to their implementation. Moreover, specific national regulations may strongly limit the use of additives or gases, such as CO, for food preservation.

It has been widely recognised that the diet of the animals can have a strong impact on meat quality, with remarkable effects on important carachteristics such as colour, flavour, tenderness and fatty acid composition. Therefore, the interest in the study of dietary strategies able to manipulate meat quality holds a special place in meat science. In general, the production systems mostly adopted for ruminants vary from extensive pasture-based feeding systems to intensive feeding systems in which animals are mainly raised indoors on grain-based concentrate feeds. Moreover, a number of intermediate solutions between the extreme pasrture- or concentrate-based feeding systems may consist, for example, in a growing period at pasture followed by a finishing diet based on concentrates, or in mixed diets in which animals raised at pasture are given supplemental concentrates, or in feeding systems in which herbage at pasture is replaced with silages given indoors as forage rations. Given the rather recent attention of consumers to food safety and helth issues related to the animals' management, extensive feeding systems are being revalued as considered respectful of animal welfare and for their potentially beneficial effects on product quality. The first part of the present thesis was, therefore, aimed at evaluating the effects of different feeding systems and diet compositions on meat oxidative stability and was developed with two experiments (*Experiments One* and *Two*).

The dietary administration of antioxidants can represent and interesting strategy to improve meat shelf life as, for instance, the supplementation of the diets with vitamin E has been shown to efficiously enhance the resistance of meat to

oxidative deterioration. Several compounds occurring in plants possess antioxidant properties and, therefore, their use as natural antioxidants in animal feeding could be promoted. Phenolic compounds represent an interesting example of plant-derived antioxidant molecules with promising possibilities of application in animal feeding. Phenolic compounds, such as tannins, have been so far considered for their antinutritional effects in ruminants when present at high levels in the diet. Hovewer, phenolics can greatly vary in their chemical structure and properties. Therefore, because of the large variability in the levels and proportions of the different classes of phenolic compounds, different plants may have different effects in animal nutrition. Moreover, several plants so far neglected in Mediterranean and tropical environments as well as many agri-industrial by-products representing wastes to dispose, being rich sources of phenolic compounds, could find a valuable application in ruminant feeding. The second part of the present thesis was, therefore devoted to the study of the effects of dietary phenolic compounds on meat oxidative stability and was developed with two experiments (*Experiments Three* and *Four*).

Experiments One and Two

Effect of concentrate- or herbage-based feeding systems on meat oxidative stability

Introduction

The susceptibility of meat to oxidative processes depends on several factors, one of the most important being the level of highly oxidizable substrates, such as polyunsaturated fatty acids (PUFA) in the phospholipid fraction of cell membranes, where lipid oxidation is initiated (Buckley et al., 1995). Therefore, animal feeding strategies aiming at enhancing the levels of health-promoting PUFA in meat could impair its oxidative stability. However, the stability of the meat to the oxidation is the result of the balance between pro-oxidants and antioxidants in muscle (for a review see Morrissey et al., 1998). Dietary antioxidants can be delivered to the muscle where, together with the native defence systems, they counteract the action of pro-oxidants (Descalzo & Sancho, 2008). Ruminant diets supplemented with antioxidants, such as vitamin E, have been extensively studied as means of improving meat oxidative stability. However, only recently, experiments have been clearly designed to study the effects of ruminant basal diet on the oxidative stability of meat.

It is widely accepted that livestock feeding systems can affect meat quality traits. French et al. (2000), for instance, found higher levels of PUFA in meat from steers offered grazed grass than in meat from those given concentrates. Fresh herbage is also a rich source of dietary antioxidants such as tocopherols, carotenoids, ascorbic acid and phenolic compounds (Wood & Enser, 1997), which can counteract the increased susceptibility of meat to oxidative deterioration arising from increased levels of PUFA in the intramuscular fat. Therefore, some studies have tested the hypothesis that grass-based feeding systems can improve meat oxidative stability. However, normally, animals on concentrate-based diets have higher average daily gains than those on pasture and, thus, slaughtering at constant age results in different weights and carcass compositions, while animals slaughtered at similar weights are of different ages (Priolo et al., 2001). For this reason, in the trials comparing oxidative stability of meat from animals on concentrates or pasture, animals often had different slaughter weights (Yang et al., 2002; Santé-Lhoutellier et al., 2008), or different slaughter ages (Soon Rhee et al., 2003; Realini et al., 2004; Descalzo et al., 2005; Insani et al., 2008) or different weights at the beginning of the experiment (Turner et al., 2002). Since age and growth rate affect carcass and meat quality, it may therefore be difficult to discriminate between direct and indirect effects of the diet on single meat quality traits if animals are raised at different growth rates (Priolo et al., 2002). Moreover, most of the preceeding studies compared animals grazing at pasture with animals fed indoors and environmental differences, such as space allowance and physical activity could confound the interpretation of the results (Dunne et al., 2005a; 2005b).

The comparison between the effects of feeding systems based exclusively on herbage at pasture or on concentrate feeds on meat quality may be of practical interest for lamb meat which, in many Mediterranean areas, is produced by exploiting low-input feeding systems with a large or exclusive use of pasture. However, in the case of beef production, cattle are rarely raised exclusively on pasture and frequently receive supplemental concentrates. In other instances, silages can be adopted in stall as forage rations (O'Sullivan et al., 2002). Such differences in the composition of the diet can alter the balance between pro- and antioxidants in muscle. Therefore, further investigation should clarify the impact of varying the composition of the diet on meat shelf life. No studies have focused on the effects of mixed feeding systems involving a season of silage feeding followed by a period of grazed grass with or without supplemental concentrates on the oxidative stability of beef. Furthermore, little information on the effect of long term consumption of different diets on beef oxidative stability has been provided (Mahecha et al., 2009; Warren et al., 2008). As the turnover of antioxidant vitamins in muscle is rather slow (Arnold et al., 1993), short periods of experimental feeding may be not sufficient to eliminate possible effects of the pre-experimental diets (Yang et al., 2002), considering also that long growing and finishing periods are generally adopted for cattle.

The first part of the present thesis focused on the effects of feeding systems and of the composition of the diets for ruminants on meat shelf life and was developed with two experiments dealing with the effects of herbage- or concentrate- based diets on lamb meat oxidative stability (*Experiment One*) and with the effects of varying the proportion of forages and concentrates in the diets for cattle on beef oxidative stability (*Experiment Two*).

Experiment One[1,2]

1.1. Aim.

The objective of this research was to test the effect of feeding lambs exclusively fresh herbage or concentrates on meat lipid and colour stability. To achieve a reliable comparison and to avoid interferences related to different environmental factors or different growth rates, here, for the first time, lambs were all individually penned with groups maintained at similar growth rates.

1.2. Materials and Methods

❖ *1.2.1. Animals and diets*

Fourteen male weaned Comisana lambs (average initial bodyweight 20.31 kg ± S.D 2.9 kg) were reared in individual pens at an experimental farm of the University of Catania (Italy). At 45 days of age, lambs were blocked in groups of two on a descending bodyweight basis and, within block, were randomly assigned to one of two treatments. Over seven days, sheep were adapted to the experimental diets. During this period they received the same commercial starter concentrate used for weaning, with gradual increases in the amount of experimental feeds. Over the 60-d experimental period, seven lambs (C) received a barley-based concentrate comprising, on an as fed basis, of 551 g/kg of barley, 300 g/kg of alfalfa hay, 130 g/kg of soybean meal and 19 g/kg of mineral and vitamin premix. The concentration of tocopheryl acetate in the premix was 35 mg/kg. The remaining seven lambs (H) were fed vetch (*Vicia sativa*) *ad libitum*; vetch was harvested each day and given fresh to the animals. Each morning, before supplying new feeds, refusals of both concentrate and herbage were weighed for individual daily dry matter intake determination. Lambs were weighed once a week at 0900 before morning feeding and the amount of concentrate for C-fed lambs was consequently adjusted in order to achieve similar growth rates between C- and H-fed lambs. The lambs had *ad libitum* access to water.

❖ *1.2.2. Slaughter procedures and muscle sampling*

Lambs were slaughtered at 105 d of age at a commercial abattoir. Until approximately 15 min before slaughter, animals had access to their experimental feeds. They were stunned by captive bolt. Within 20 min of slaughter, *semimem-*

1 Published as: Luciano, G., Monahan, F. J., Vasta, V., Pennisi, P., Bella, M. & Priolo, A. (2009). *Lipid and colour stability of meat from lambs fed fresh herbage or concentrate*. **Meat Science**, 82, 193-199.

2 Citations to this experiment in the present thesis are reported as: "Luciano et al. (2009b – *Exp. 1*)"

branosus (SM) muscle was excised from the left hind leg, wrapped in aluminium foil, vacuum-packaged and frozen at -20°C for subsequent analyses.

❖ *1.2.3. Feed analyses*

Fiber fractions (NDF, ADF and ADL; Van Soest et al., 1991), crude protein (method 984.13; AOAC, 1995) and crude fat (method 920.39; AOAC, 1995) of the experimental feeds were determined in triplicate.

❖ *1.2.4. Preparation of raw and cooked minced meat*

Muscle samples were removed from the freezer and, while still partially frozen, were trimmed of the outer fat and minced finely using a knife. Part of the minced was used to monitor lipid oxidation, colour stability and myoglobin oxidation of raw meat over a 2-week period of refrigerated storage in a high oxygen modified atmosphere (MAP), whereas the remaining minced meat was used to monitor lipid oxidation in cooked meat over 4 days of refrigerated aerobic storage.

For the storage study on raw meat, five subsamples of SM from each lamb were prepared by placing 12 g of minced lamb in individual polyamide/polyethylene Exovac 73 bags (McDonnells Queen St., Dublin) using one bag for each of five days of analysis. A Webomatic vacuum packaging system equipped with a gas mixer (Witt-Gase Technik KM-100-M (3), Witt Gas Techniques Ltd., Warrington, England) was used to package the samples in a high oxygen modified atmosphere (MAP; 80% O_2 : 20% CO_2). The O_2, N_2 and CO_2 permeabilities of the bags at 23°C and 0% RH were 30, 10 and 105 $cm^3/m^2/24h/bar$, respectively. The MAP meat was stored in the dark at 4°C and lipid oxidation, meat colour and haem pigments were analysed 2 h after packaging (day 0) and after 4, 7, 11 and 14 days of storage.

To monitor lipid oxidation in cooked meat, 30g of minced meat from each lamb were placed in polyamide/polyethylene Exovac 73 bags (McDonnells Queen St., Dublin), formed into patties (15 x 5 x 0.2 cm) at the bottom of the bags and vacuum-packaged. Meat was cooked by immersion of the bags in a water bath at 70°C for 30 min. The cooked samples were cooled for 15 min in a water/ice bath after which the bags were opened and lipid oxidation was measured immediately (day 0) and after 1, 2 and 4 days of aerobic storage at 4°C.

❖ *1.2.5. Measurement of meat colour*

A Minolta colour meter (model CR300, Minolta Camera Co. Ltd., Osaka, Japan) was used to measure colour coordinates L^* (lightness), a^* (redness) and b^* (yellowness) of raw minced lamb. Measurements were made using the D65 illuminant and 10° standard observer. The measurements were performed through the Exovac bag which was allowed to touch the meat surface during measurements. Triplicate readings were recorded on non-overlapping zones of the sample and average values were calculated. Hue angle (H^*) was calculated as $\tan^{-1}$ (b^*/a^*) ×

(180/π). Overall colour variation between each day of storage and the day 0 of measurement was calculated as:

$$\Delta E_{0\text{-}n} = (\Delta L^*_{0\text{-}n}{}^2 + \Delta a^*_{0\text{-}n}{}^2 + \Delta b^*_{0\text{-}n}{}^2)^{½}$$

Where, $\Delta L^*_{0\text{-}n}$, $\Delta a^*_{0\text{-}n}$ and $\Delta b^*_{0\text{-}n}$ are the differences between L^*, a^* and b^* measured at a day n of storage and their values at day 0.

❖ *1.2.6. Extraction and measurement of haem pigments.*

Haem pigments were extracted from raw meat as described by Warriss (1979). Two grams of chopped meat were placed into 50 ml centrifuge tubes and homogenized at 9500 rpm with 25 ml of 0.04M sodium phosphate buffer (pH 5.6; temperature: 4°C) for 1 min using a Heidolph Diax 900 tissue homogenizer (Heidolph Elektro GmbH & Co. KG, Kelheim, Germany). The tubes were constantly kept in a water/ice bath during homogenization. Homogenates were centrifuged at 6500 × *g* for 10 min at 4°C and, then, filtered through Whatman No 541 filter papers. The aqueous filtrate, containing the haem pigments, was kept at room temperature for 15 min. The absorbances (A) of the extract at 525 nm, 545 nm, 565 nm, 572 nm and 730 nm wavelength were recorded with Shimadzu double-beam spectrophotometer (model UV-1601; Shimadzu Corporation, Milan, Italy) against phosphate buffer used as blank. Total haem pigment (Mb) concentration - expressed as mg/g of fresh tissue - and the percentages of metmyoglobin (MMb) were calculated using the equations of Krzywicki (1982), where:

$$[\text{Mb}]\ (\text{mM}) = 0.132 \times A_{525\text{nm}}$$

$$\text{MMb \%} = [-2.514\ (A_{572}/A_{525}) + 0.777\ (A_{565}/A_{525}) + 0.800\ (A_{545}/A_{525}) + 1.098]$$

❖ *1.2.7. Measurement of lipid oxidation*

The extent of lipid oxidation in both raw and cooked meat was assessed by measuring 2-thiobarbituric acid reactive substances (TBARS), following the distillation method described by Tarladgis et al. (1960). Five grams of meat were homogenised with 97.5 ml of distilled water for 2 min. using a Heidolph Diax 900 tissue homogenizer (Heidolph Elektro GmbH & Co. KG, Kelheim, Germany). Samples were constantly kept in a water/ice bath during homogenization. The homogenate was transferred into a distillation flask containing 2.5 ml of 4N HCl. The distillation was started and an exact volume of 50 ml of distillate was collected in a graduate cylinder. A 3 ml volume of distillate was transferred into a 10 ml screw-cap pyrex tube and mixed with 3 ml of TBA reagent (0.02M thiobarbituric acid in 90% glacial acetic acid). Samples were thoroughly mixed and incubated in a water bath at 100°C for 35 min. Samples were cooled in a water/ice bath and the absorbance at 538nm wavelength was measured using a UV-VIS spectro-

photometer (Shimadzu UV Mini 1240). Standard solutions of known concentrations of 1,1,3,3,-tetra-ethoxypropane in distilled water (ranging from 0 to 65 nmoles / 3 ml) were used to calibrate the assay. An average percentage of recovery of malonaldehyde (MDA) in the distillate of 72% was found for the 6 distillators used. As described by Tarladgis et al. (1960) a K coefficient was calculated and results were expressed as TBARS values (mg of MDA / kg of meat).

❖ *1.2.8. Statistical analysis*

Data were analysed with a GLM procedure with repeated measures, using the statistical software Minitab (14, 1995). The model included diet (H *vs* C) and time (days 0, 4, 7, 11, 14 for raw meat and days 0, 1, 2, 4 for cooked meat) and their interaction as fixed effects and individual lamb as a random effect. Tukey's test was used for comparing mean values.

1.3. Results and discussion

❖ *1.3.1. Chemical composition of herbage and concentrate.*

As shown in Table 1, herbage had higher percentages of fiber fractions (NDF, ADF and ADL) and crude protein as compared to the concentrate. Vetch is a legume and therefore its protein content is normally higher compared to other plants. Petron et al. (2007) found lower growth performances in lambs grazing a botanically diverse pasture as compared to those grazing a legume-rich pasture and this was attributed to the higher protein content in the latter pasture.

Table 1
Chemical composition of the feeds offered.

	Vetch	Concentrates
Dry matter (%)	18.2	88.5
Crude protein[a]	21.0	16.3
Crude fat[a]	1.3	1.4
NDF[a]	41.4	38.8
ADF[a]	20.9	15.7
ADL[a]	10.7	7.9

[a] Expressed as % on dry matter.

❖ *1.3.2. Lambs growth performances.*

As shown in Table 2, there were no differences in body weight at slaughter, average daily gain or carcass weight between C-fed and H-fed lambs. Thus our objective of having comparable growth performances between the treatments, in order to better study the effects of the experimental diets on meat oxidative stability, was achieved.

Table 2
Effect of feeding system on lamb growth performances.

	Vetch	Concentrates	SEM	P value
Body weight at 45 days (kg)	20.13	20.5	0.77	NS[b]
Body weight at slaughter (kg)	28.63	30.29	0.82	NS[b]
ADG[a] (g)	140.64	159.75	7.69	NS[b]
Carcass weight (kg)	13.19	15.07	0.54	NS[b]

[a] ADG, mean average daily gain from 45 to 105 days.
[b] NS, not significant (P > 0.05).

❖ *1.3.3. Lipid oxidation of raw and cooked meat*

The main effects of diet and time and their interaction are reported in Table 3.

Table 3
Main effects of diet and storage time and their interaction on indices of oxidative stability in lamb.

	P values		
	Diet[a]	Time[b]	Diet x time
Raw meat			
Lipid oxidation (mg of MDA/kg of meat)	0.006	<0.0005	0.004
Lightness (L* value)	0.02	<0.0005	0.006
Redness (a* value)	0.006	<0.0005	0.004
Yellowness (b* value)	0.02	<0.0005	0.007
Hue angle (H* value)	0.005	<0.0005	0.006
Colour variation (ΔE value)	0.005	<0.0005	0.08
MMb formation (% of total myoglobin)	0.006	<0.0005	0.03
Haem pigment concentration (mg/g of meat)	0.1	<0.0005	0.01
Cooked meat			
Lipid oxidation (mg MDA/kg of meat)	0.001	<0.0005	0.005

[a] Diet: herbage (H) or concentrates (C).
[b] Time of storage: days 0, 4, 7, 11 and 14 for raw meat. Days 0, 1, 2 and 4 for cooked meat.

Regardless of dietary treatment, lipid oxidation increased in raw meat ($P < 0.0005$; Figure 1a) across the 14-day of MAP storage period. However, diet strongly affected lipid oxidation extent over time ($P = 0.006$; Table 3). TBARS values measured in meat from lambs fed herbage were lower, compared to those found in meat from animals given concentrates, after 4, 7 and 14 days of storage ($P = 0.03$; $P = 0.02$; $P = 0.05$, respectively). There was a tendency toward significance after 11 days ($P = 0.1$). A similar trend was observed in cooked meat, with a general increase in the extent of lipid oxidation ($P < 0.0005$; Figure 1b) over the 4-days of aerobic storage, but with lower TBARS values after 1, 2 and 4 days of storage ($P <$

0.001) in meat from lambs fed vetch compared to those given concentrate. Mincing represents a significant deteriorative stress for meat, disrupting cellular compartmentalization and resulting in the release and diffusion of prooxidants (Gray et al., 1996). Also high oxygen partial pressure (like that found in high oxygen MAP) can promote oxidation processes by enabling oxygen to react with muscle components (O'Grady et al., 2000). Moreover cooking deactivates the inherent antioxidant defence systems in muscle and results in the denaturation of proteins and disruption of membranes which, in turn, release pro-oxidant metal ions (Igene et al., 1979). Considering that meat was minced and either cooked or packaged in a high oxygen atmosphere, the observed increases of TBARS values with increasing time of storage were expected.

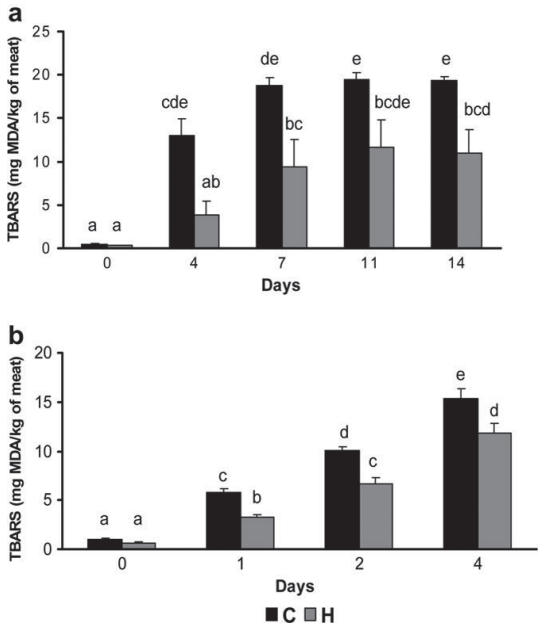

Fig. 1. (a) Effect of treatment (H or C) and time of storage (days 0, 4, 7, 11 and 14) on TBARS values of raw minced meat stored in 80% O_2:20% CO_2 over 14 days at 4 °C. (b) Effect of treatment (H or C) and time of storage (days 0, 1, 2 and 4) on TBARS values of cooked minced meat stored aerobically over 4 days at 4 °C. Values with different superscripts are significantly different ($P \leq 0.05$). Values are means plus standard errors.

The lower TBARS values measured in meat from H-fed lambs than in meat from animals given concentrate are in agreement with several studies showing the protective effect against lipid oxidation of grass-based feeding systems as compared to concentrate-based diets (Soon Rhee et al., 2003; Descalzo et al., 2005; Gatellier et al., 2005; Santé-Lhoutellier et al., 2008; Warren et al., 2008). Antioxidants, such as vitamin E, ascorbic acid and carotenoids, can be found at higher concentrations in muscles from animals fed grass than in muscles from animals raised on concentrates (Yang et al., 2002; Descalzo et al., 2005; Insani et al., 2008). This antioxidant pool can, thus, counteract the increased susceptibility of meat to lipid oxidation due to the high concentrations of PUFA usually associated with a grass-based diet. Realini et al. (2004) found improved lipid stability in *longissimus dorsi* steaks from steers raised on pasture compared to steaks from concentrate-fed animals. However, when measured on ground muscle, TBARS values were higher in meat from pasture-fed animals. The authors hypothesized that mincing could have challenged the meat from grass-fed animals more than

that from concentrate-fed steers, due to its higher content of PUFA. Our results confirm that, compared to a concentrate-based diet, a grass-based feeding system can effectively improve the stability of meat to lipid oxidation even under highly prooxidative conditions like mincing, cooking and high oxygen packaging.

❖ *1.3.4. Meat colour descriptors*

Meat from animals raised on pasture has been often reported to be darker in colour compared to that from animals fed concentrates in stalls (Muir et al, 1998; Vestergaard et al., 2000; Priolo et al., 2001). In the present study, meat lightness (*L** values, Figure 2a) did not differ between sheep fed herbage or concentrate after 2 hours of blooming (day 0 of measurement). French et al. (2000, 2001) showed that, when adult cattle are slaughtered at similar ages and levels of fatness, meat lightness did not differ between animals fed grass or concentrates. Therefore, the lack of difference in *L** values between our experimental treatments could be partly attributed to the fact that lambs were allowed to grow at similar growth rates. Moreover, this experiment is not fully comparable with most of the studies dealing with the effects of diet on meat colour, since we measured colour coordinates on minced meat, whereas colour has usually been measured on steaks. For instance, Realini et al. (2004) found that a pasture-based diet resulted in a darker beef as compared to a concentrates-based diet when colour was measured on the ribeye roll, but differences disappeared when colour measurements were performed on ground meat. We observed a general increase in *L** values over the 14-days period of refrigerated storage ($P < 0.0005$). However, lightness was affected by the diet ($P = 0.02$) and was also subjected to a Diet × Time interaction ($P < 0.01$). While *L** values increased after 7 days of storage in meat from H-fed sheep ($P = 0.05$), meat from C-fed lambs became lighter after only 4 days ($P < 0.01$) and, after 14 days, meat from C-fed animals was lighter than that from H-fed animals ($P = 0.01$).

While lightness is generally not considered an appropriate index of meat discoloration (Sapp et al., 1999; Dunne et al., 2005a), the loss of redness (*a**) and the changes in yellowness (*b**) over a period of display have been used to describe meat browning (Morrissey et al., 1994; Mancini & Hunt, 2005). The relationship between sensory evaluation of meat colour deterioration and its instrumental measurement has been recently studied (Insausti et al., 2008) and it has been shown that, over time of storage, while *b** values were positively related to sensory appreciation of meat colour degradation, *a** values were negatively correlated to the sensory evaluation of discolouration. In the present study, redness (Figure 2b) decreased ($P < 0.0005$) in meat from both C- and H-fed lambs. However, diet affected meat redness and a Diet × Time interaction was found (Table 3). After 7 days of storage, meat from animals fed herbage showed higher *a** values than that from C-fed lambs ($P < 0.01$) and still retained redness close to that measured at day 0. Our results are in agreement with other studies showing an improved stabil-

ity of redness in meat from animals fed grass-based diets as compared to concentrate-based diets (Soon Rhee et al., 2003; Gatellier et al., 2005; Insani et al., 2008). However, other authors did not find differences in meat redness stability between pasture-based and concentrates-based feeding systems (Yang et al., 2002; Santé-Lhoutéllier et al., 2008). Yellowness increased ($P < 0.0005$) across the 14 days of storage in meat from animals fed both C and H diets (Figure 2c). However diet affected this parameter ($P = 0.02$) and b^* values were also subjected to a Diet × Time interaction ($P < 0.01$).

In meat from lambs fed herbage, yellowness increased after 7 days of storage ($P < 0.01$) and remained stable thereafter. Conversely, b^* values of lamb from C-fed sheep increased after only 4 days ($P < 0.01$) and, again, after 11 days of storage ($P = 0.01$). Moreover, meat from H-fed lambs had lower b^* values than those measured in meat from C-fed animals at 14 days of measurements ($P = 0.006$). It appears, thus, that feeding lambs herbage delayed meat discolouration as compared to the concentrate-based treatment.

Other descriptors allow an appropriate evaluation of meat colour deterioration. Among them, hue angle (H^*), being a function of a^* and b^*, gives a more realistic perspective on meat browning than single colour coordinates. Therefore, increases in H^* values over time, resulting from the decreases in a^* relative to b^*, have often been used to follow meat discoloration (Young et al., 1999; Renerre, 2000; Lee et al., 2005). Regardless of dietary treatment, H^* values (Figure 3a) increased (shifted from red to yellow) over storage duration ($P < 0.0005$). However, diet affected hue angle values and a significant Diet × Time interaction was also found (Table 3); between 0 and 4 d of

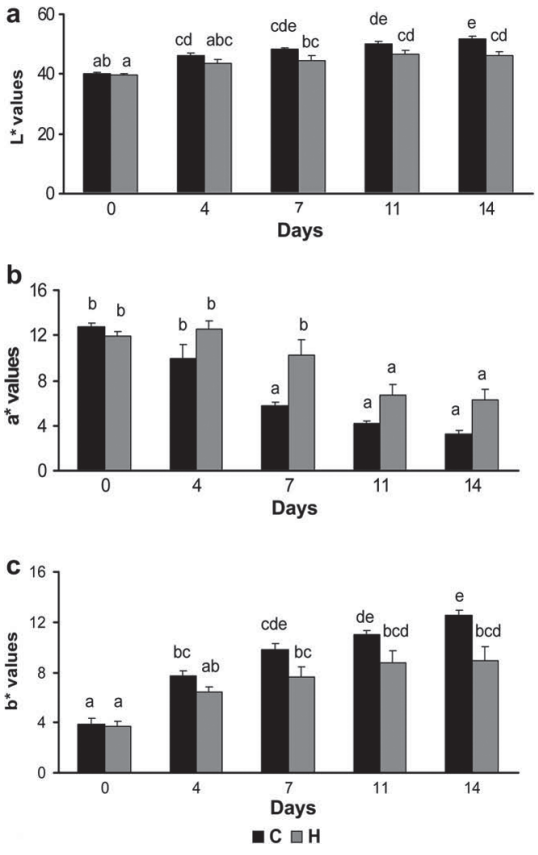

Fig. 2. Effect of treatment (H or C) and time of storage (days 0, 4, 7, 11 and 14) on (a) L* values, (b) a* values and (c) b* values of raw minced meat stored in 80% O_2:20% CO_2 over 14 days at 4 °C. Values with different superscripts are significantly different ($P \leq 0.05$). Values are means plus standard errors.

storage, *H** values increased in meat from C-fed lambs but did not change in meat from H-fed animals. Moreover, *H** values measured in meat from lambs fed vetch were lower after 7 and 14 days ($P = 0.01$) and tended to be lower after 11 days ($P = 0.1$) than those found in meat from C-fed lambs.

One variable that has received little attention in meat colour studies but may be useful is ΔE, which measures total colour distance by accounting for combined changes in *L**, *a**, and *b** (Mancini & Hunt, 2005). Therefore, ΔE values calculated between the day 0 measurement and each subsequent measurement can give a measure of the overall colour variation of meat with time (Mancini et al., 2008). In this study, diet affected overall colour variation (Table 3), whereby ΔE values of meat from H-fed lambs tended to be lower at 7 and 11 days of storage ($P = 0.1$) and were lower ($P < 0.01$) after 14 days than those of meat from C-fed animals (Figure 3b). Taken together, these results confirm the protective effect of the herbage-based diet against meat discolouration.

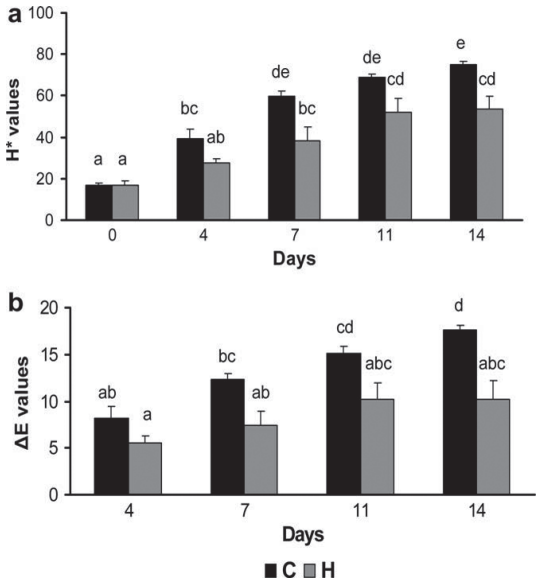

Fig. 3. Effect of treatment (H or C) and time of storage (days 0, 4, 7, 11 and 14) on (a) H* values and effect of treatment (H or C) and time of storage (days 4, 7, 11 and 14) on (b) overall colour variation (DE values) of raw minced meat stored in 80% O_2:20% CO_2 over 14 days at 4 °C. Values with different superscripts are significantly different ($P \leq 0.05$). Values are means plus standard errors.

❖ *1.3.5. Muscle haem pigments*

Regardless of dietary treatment, we found increased metmyoglobin formation (Figure 4a) over time of storage ($P < 0.0005$). However, diet affected this parameter and there was also a significant Diet × Time interaction (Table 3). After only 4 days of storage, meat from C-fed animals reached higher MMb percentages than those measured at day 0. Conversely, metmyoglobin levels increased later, after 7 days, in meat from herbage-fed animals. Moreover, lower MMb percentages were measured in lamb from H-fed lambs than in meat from C-fed lambs after 4 and 7 days ($P = 0.05$; $P = 0.03$, respectively). The changes in meat colour coordinates with time are closely related to myoglobin chemistry, whereby the oxidation of myoglobin, with the consequent accumulation of metmyoglobin, is primarily re-

sponsible for meat browning (Mancini & Hunt, 2005). Lindahl et al. (2001) reported that metmyoglobin levels were among the most important factors contributing to meat redness and yellowness in pork. Therefore, in meat colour studies, metmyoglobin determination can be useful for a better comprehension of meat discolouration. However, in some studies assessing the effects of grass- or concentrate-based diets on meat oxidative stability, metmyoglobin formation was not measured (Yang et al., 2002; Soon Rhee et al., 2003; Realini et al., 2004; Santé-Lhoutellier et al., 2008; Warren et al., 2008). On the other hand, Insani et al. (2008) found higher *a** values over time of storage in beef from animals raised on pasture than in meat from grain-fed cows, but no differences in MMb% were observed between the dietary treatments. The observed increases in metmyoglobin percentages over the 14 days of storage, may explain, in the present study, the general loss of redness and the increase in yellowness observed with time, as well as the observed increases in hue angles with increasing storage duration.

Moreover, the reduced metmyoglobin formation found in meat from lambs fed herbage diet compared to those fed concentrate could be the reason for the lower hue angle values observed in meat from H-fed sheep. As previously described, lipid and myoglobin oxidation in meat are believed to be linked. Several studies suggest a direct role of some secondary compounds arising from lipid oxidation, such as α,β-unsaturated aldehydes, in favouring myoglobin oxidation (Chan et al., 1997; Lynch & Faustman, 2000; Suman et al., 2007). Other authors proposed that the depletion of oxygen due to lipid oxidation may be responsible for myoglobin oxidation (O'Grady et al., 2001; Monahan et al., 2005). Therefore, the greater extent of lipid oxidation found, in our study, in meat from lambs given concentrates as compared to that from H-fed animals could have contributed to the greater myoglobin oxidation observed in lamb from C-fed lambs compared to meat from animals fed herbage.

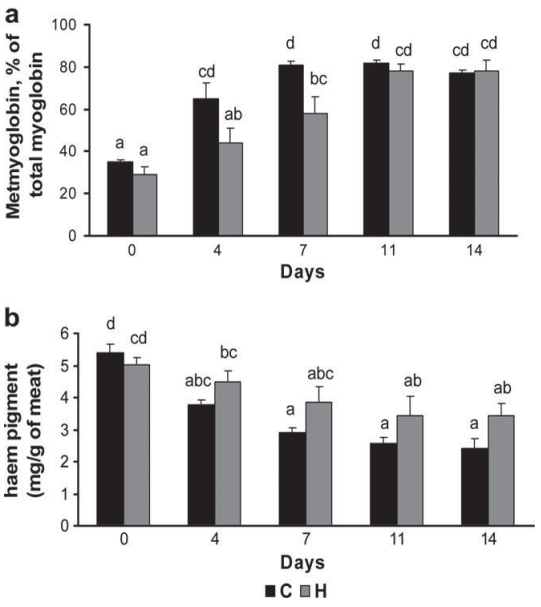

Fig. 4. Effect of treatment (H or C) and time of storage (days 0, 4, 7, 11 and 14) on metmyoglobin percentages (a) and haem pigment concentration (b) of raw minced meat stored in 80% O_2:20% CO_2 over 14 days at 4 °C. Values with different superscripts are significantly different ($P \leq 0.05$). Values are means plus standard errors.

Muscle colour is also affected

by haem pigment concentration. For instance, negative correlations between muscle haem pigments and lightness were reported (Gil et al., 2001; Dunne et al., 2006). Furthermore, Gil et al. (2001) found positive correlations between haem pigment concentration and muscle redness, while Tam et al. (1998) and Lindahl et al. (2001) demonstrated a negative correlation between the hue angle values and the pigment concentration in meat. In this study we found that haem pigment concentration decreased ($P < 0.0005$; Figure 4b) with increasing storage time in meat from both C- and H-fed lambs. Diet tended to affect this variable ($P = 0.1$) and a significant Diet × Time interaction was found (Table 3). No significant differences in haem concentration were found between C and H treatments at each day of storage. However, while haem concentration remained stable from 0 to 7 days in meat from H lambs, its decrease was faster in meat from C lambs, with lower haem concentration after 4 days. The observed general decreases in haem concentration over the 14 days of storage may explain the increases in L^* values measured over time and, coupled with the increases in metmyoglobin percentages, may also explain, both the loss of redness and the observed increases in hue angles with increasing storage duration. The reason for this decrease in haem concentration with time is not clear. Lipolysis occurring in meat over time of storage increases the levels of free fatty acids (Currie & Wolfe, 1977; Sklan et al., 1983; Lambert et al., 2001) and, in a highly lipophilic environment, metmyoglobin can undergo denaturation, with consequent exposure or release of the haem group (Baron & Andersen, 2002; Baron et al., 2002). Moreover, oxidising conditions, like high oxygen partial pressures, can promote protein oxidation (Lund et al., 2007). Since, in our experiment, meat was both minced and packaged in 80% oxygen, the observed decreases in myoglobin concentrations with time of storage may have been caused by a loss of integrity of the haem pigment due to the highly oxidising conditions occurring.

1.4. Conclusions

The results of the present study showed that feeding lambs fresh herbage resulted in a general improvement of meat oxidative stability as compared to feeding a concentrate-based diet. Specifically, the extent of lipid oxidation during storage was lower in both raw and cooked minced meat from lambs fed vetch as compared to those given concentrates. This could partly explain the lower metmyoglobin formation observed in meat from H-fed lambs than in meat from C-fed animals. Meat colour deterioration, measured as changes of colour descriptors over time, was less pronounced in meat from animals fed herbage than in meat from concentrates-fed lambs and lower hue angle values were observed in meat from H-fed lambs as compared to C-fed animals. Considering that animals were allowed to

grow at similar growth rates, were slaughtered at the same age and were all individually penned, we can conclude that, a grass-based diet can improve the oxidative stability of meat as compared to a concentrate-based feeding system. Moreover, it was possible to observe the improved oxidative stability of meat from animals fed herbage even under prooxidative conditions induced by mincing, cooking and packaging in a high oxygen atmosphere.

Experiment Two [1]

2.1. Aim

In the present study, cattle were assigned to different dietary treatments involving exclusive feeding of grazed grass or concentrates or a sequence of grass silage feeding and grazed grass with or without supplementation with concentrates. The objective was to study the effect of such differences in the feeding systems on the balance between pro- and antioxidants in muscle and on the oxidative stability of the resultant meat. Here, animals were fed the experimental diets for 11 months in order to assess the effect of long term experimental feeding and to overcome possible interferences arising from the slow turnover of antioxidant and pro-oxidant components in muscle.

2.2. Materials and methods

❖ *2.2.1. Animals and diets*

One-hundred Charolais × Limousin crossbred weanling heifers (average BW 275 ± SD 27.0 kg and age 252 ± SD 28 d) were sourced from Irish farms through a livestock market and brought to Teagasc Grange, County Meath, Ireland. The heifers were blocked in groups of 4 on a descending BW basis and, within block, assigned randomly to one of four dietary treatments, with 25 animals per treatment. Treatments included grazed pasture (P) or a barley-based concentrate and straw offered indoors (C) over the entire 11-month experimental period; pasture silage offered *ad libitum* indoors during a 5-month winter period followed by either grazed pasture (SiP) or grazed pasture plus 50% of the diet DM of a barley-based concentrate (SiPC) for the remaining 6-month summer period.

From mid-November 2006 to mid-April 2007, heifers in the P group grazed together on a 25 ha pasture (mainly *Lolium perenne* L., *Poa spp.* and *Trifolium repens* L.) with a herbage allowance of 2% of live weight per heifer. Heifers were offered the daily ration by grazing within 1.5-2.5 ha paddocks sub-divided sequentially into daily "breaks" using portable electric fences. Heifers in the SiP and SiPC treatments were housed in groups of 5 in a slatted floor shed and were offered grass silage (predominantly *Lolium perenne L.*) *ad libitum* once daily. During the summer period, from April to October 2007, heifers on treatments P and SiP were set stocked and the area of the paddock adjusted to ensure that the required allowance of pasture DM was available. Heifers on the SiPC treatment were offered a

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restricted allowance of pasture and, once daily, an increasing amount of concentrates until the allowance reached 50% of estimated total daily DM intake. Over the whole 11-month experimental period, heifers on C treatment were fed a barley-based concentrate with supplemental straw, with the ration being offered once daily to groups of 5 heifers accommodated in the same shed. The composition of the concentrate offered to animals in both C and SiPC treatments was: 430 g kg^{-1} rolled barley, 430 g kg^{-1} molassed beet pulp, 80 g kg^{-1} soybean meal, 35 g kg^{-1} molasses, 20 g kg^{-1} mineral/vitamin premix containing 2.5 g kg^{-1} α-tocopheryl acetate (Lutavit E50, BASF). Animals were weighed once per month and feed allowances for the C and SiPC groups were adjusted to maintain similar rates of growth to those of the heifers at pasture (P).

❖ *2.2.2. Slaughter procedures and muscle sampling*

One animal from the P group and one from the SiP group died during the experiment for reasons not related to the dietary treatments. The remaining 98 animals were slaughtered at Meadow Meats Limited, Rathdowney, Co Laois following EU animal welfare guidelines on the 23rd October (23 cattle) and on the 7th (28 cattle), 13th (24 cattle) and 21st (23 cattle) November 2007. Following overnight chilling of carcasses at 4°C, the entire *longissimus dorsi* muscle (LM) was excised from the right side of each carcass. Muscles were vacuum packaged and stored overnight at 4°C after which subsamples (2.5 cm thickness) were excised between the 9th and 10th rib, vacuum packaged and stored at -20°C prior to analyses.

❖ *2.2.3. Measurements of muscle vitamin E concentration and fatty acid composition*

❖ *2.2.3.1. Vitamin E.*

Total vitamin E (α-tocopherol) concentration in LM from the heifers used in the present study was measured by Röhrle et al. (2011). Briefly, muscle α-tocopherol was extracted using the method of Buttriss and Diplock (1984) as modified by Dunne et al. (2005c) and was measured by HPLC using an Agilent 1200 series (Agilent Technologies 171 Inc. Santa Clara, California, USA) equipped with a variable loop injector and a Synergi Hydro–RP 80A (250 mm × 4.6 mm) 4 µm particle size column with a corresponding guard column (Phenomenex UK, Macclesfield Cheshire). The mobile phase was methanol: water (99:1, v/v) at a flow rate of 2 ml/min. Detection was achieved using a diode array detector (Agilent Technologies, 1200 series, DAD) set at 292 nm. Percentage recovery was 72.4%.

❖ *2.2.3.2. Fatty acid composition.*

Extraction and analysis of muscle fatty acids were performed as described by Noci et al. (2005). Samples of the LM were defrosted and homogenized with a

Robot Coupe R301 Ultra food processor (Robot Coupe S.N.C., Vincennes, France). Muscle (2 g) was homogenized with 20 ml of 2:1 (v/v) chloroform/ methanol mixture containing 0.05% (w/v) butylated hydroxytoluene as antioxidant using an Ultra Turrax T25 homogenizer (Janke and Kunkel, IKA Labortechnik, Staufen, Germany). The homogenizer was rinsed with 16 ml of the solvent solution, and this volume was added to the previous 20 ml. The tubes were stored overnight at 4°C in darkness. The tube contents were filtered through Whatman No. 4 filter paper (Whatman, Ltd., Maidstone, U.K.). The tubes were rinsed with 5 ml of solvent solution, and the filter cake was rinsed with a further 5 ml of solvent. A volume of 0.02% $CaCl_2$ solution in distilled water (w/w) equivalent to 25% of the filtrate was added to the test tubes, which were shaken and left to separate overnight at 4°C. The bottom layer was poured through a funnel containing Whatman No. 4 filter paper and approximately 5 g of anhydrous Na_2SO_4. The filtrate containing the extracted lipid was collected into 50-ml screw-cap glass bottles and stored overnight at -30°C. The lipid extract was dried to a constant weight under a stream of N_2, and redissolved in 300 ml of toluene for preparation of fatty acid methyl esters (FAME). Following the method described by Kramer and Zhou (2001), the methylation procedure involved a combination of alkaline and acidic transesterification. The extracted lipid fractions were initially methylated with $NaOCH_3$, which was followed with a 4% solution of HCl in methanol. Both methylation procedures were carried out at 50°C for 20 min. Tricosanoic acid (C23:0) methyl ester was used as an internal standard for fatty acid quantification. Deionized water (2 ml) saturated with hexane (95:5 water-hexane; v/v) was added to the tube containing the FAME, followed by 2 ml of hexane. The tubes were centrifuged (800 × g) for 5 min, and the top layer containing FAME in hexane was removed and transferred to glass tubes. This step was repeated with a further 2 ml of deionized water saturated with hexane. The top layers were transferred to tubes containing 0.75 g of Na_2SO_4, and centrifuged (800 × g for 5 min). An aliquot of the supernatant (500 µl) containing FAME was transferred into a 2-ml glass vial and fur- ther diluted with 500 µl of hexane before injection.

The FAME were separated by gas chromatography using a Varian 3800 GC (Varian Instruments) equipped with a CP-Sil 88 capillary column (100 m × 0.25 mm i.d., 0.2-µm film thickness; Chrompack, The Netherlands) and a Varian 8400 autosampler. The injector and the flame ionization detector were kept at constant temperatures of 250 and 260°C, respectively. The column oven temperature was held at 40°C for 2 min, increased at 20°C/min to 80°C and held for 2 min, increased to 160°C at 20°C/min, to 220°C at 4°C/min, and to 240°C at 2°C/min and held for 8 min. The total run time was 43 min, and the carrier gas used was H_2. For peak identification, a standard mix of 37 FAME (Supelco Inc., Bellefonte, PA) was used, and individual standards from Matreya (Matreya Inc., Pleasant

Gap, PA) were used for identification of those FAME not contained in the standard mix.

The proportions of SFA, MUFA and PUFA were obtained from the sum of all the identified saturated, monounsaturated and polyunsaturated fatty acids, respectively, expressed as percentage of the total fatty acids. The concentration of the highly peroxidisable PUFA (HP-PUFA) was calculated as sum of PUFA with three or more double bonds (Yang et al., 2002) expressed as absolute concentration (mg/g of fresh muscle).

❖ *2.2.4. Meat oxidative stability measurements*

Meat oxidative stability was measured in minced LM under refrigerated storage after packaging in a high oxygen modified atmosphere (MAP). Over 11 days of storage in the dark, meat color descriptors and the extent of both myoglobin and lipid oxidation were measured as follows.

❖ *2.2.4.1. Meat samples preparation and packaging.*

Samples of LM were removed from the freezer and, while still partially frozen, were trimmed of the outer fat, chopped into small cubes and finely blended using an Osterizer BY/PAR food processor (Oster®, Sunbeam Products, Inc., Overland Park, Kansas, USA). Four sub-samples from each heifer were prepared by placing 12 g of minced beef in individual polyamide/polyethylene Exovac 73 bags (McDonnells Queen St., Dublin) using one bag for each of four days of analysis. A Webomatic vacuum packaging system equipped with a gas mixer (Witt-Gase Technik KM-100-M (3) Witt Gas Techniques Ltd., Warrington, England) was used to package the samples in a high oxygen modified atmosphere (MAP; 80% O_2 : 20% CO_2). The O_2, N_2 and CO_2 permeabilities of the bags at 23°C and 0% RH were 30, 10 and 105 $cm^3/m^2/24h/bar$, respectively. Samples were stored in the dark at 4°C and meat colour, lipid oxidation and myoglobin oxidation were analysed after 2 h of blooming (day 0) and, subsequently, after 4, 7 and 11 days of refrigerated storage. Four animals (two from the C group, one from the P group and one from the SiP group) were not considered in the study due to loss of the modified atmosphere from the bags during storage. Thus LM samples from 25 animals in the SiPC group were used, while samples from 23 animals in each of the P, SiP and C groups were used.

❖ *2.2.4.2. Lipid oxidation measurements.*

Lipid oxidation in minced beef was assessed by measuring 2-thiobarbituric acid reactive substances (TBARS), following the filtration method described by Siu and Draper (1978) with minor modifications. Briefly, 2.5 g of minced meat were homogenized with 12.5 ml of distilled water using an Ultra Turrax T25 homogenizer (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) operating at 9500

rpm. Samples were maintained in a water/ice bath during homogenization. Then, 12.5 ml of 10% (w/v) trichloroacetic acid were added to precipitate proteins and samples were vigorously vortexed. Homogenates were then filtered through Whatman No.1 filter paper. In 15-ml pyrex tubes, 4 ml of clear filtrate were mixed with 1 ml of 0.06 M aqueous thiobarbituric acid and samples were incubated in a water bath at 80°C for 90 min. The absorbance of the samples at 532 nm was measured using a Shimadzu UV Mini 1240 spectrophotometer. The assay was calibrated using solutions of known concentrations TEP (1,1,3,3,-tetra-ethoxypropane) in distilled water. Results were expressed as mg of malonaldehyde (MDA)/kg of meat.

❖ *2.2.4.3. Color stability and myoglobin oxidation measurements.*

The meat color descriptors a* (redness) and H* (hue angle) together with the reflectance (R) spectra from 400 nm to 700 nm wavelength were measured using a Minolta CM-2022 spectrophotometer (d/8° geometry; Minolta Co., Ltd. Osaka, Japan). Diffuse reflection was measured in SCE (Specular Component Excluded) mode, using illuminant A and 10° standard observer.

The measurements were made through the Exovac bag which was allowed to touch the meat surface during measurements. Triplicate readings were made on non-overlapping zones of the sample and average values were calculated. Myoglobin oxidation extent was measured by monitoring the formation of metmyoglobin (MMb) across the 11-day storage period using the equation described by Krzywicki (1979):

$$MMb\% = \{1.395 - [(Dr_{572nm} - Dr_{730nm}) / (Dr_{525nm} - Dr_{730nm})]\} \times 100$$

Where:

$$Dr_{\lambda} = \log_{10} (1/R)$$

❖ *2.2.5. Statistical analysis.*

Lipid oxidation (TBARS values), color stability parameters (a* and H* values) and metmyoglobin percentages over the 11-day of MAP storage were analysed as a repeated measures design using the statistical software Minitab 15 (Minitab Inc., State College, PA). The model included the days of storage (Time; 0, 4, 7 and 11), the dietary treatment (Diet; P, SiP, SiPC and C) and their interaction (Time × Diet) as fixed effects, while the individual animal was included in the model as random effect. Tukey's test was used to compare mean values.

A one-way ANOVA was performed to test the effect of the dietary treatment (P, SiP, SiPC and C) on the muscle fatty acid composition and vitamin E concentration. Mean values were compared by means of the Tukey's test.

2.3. Results

❖ 2.3.1. Balance between muscle antioxidant and pro-oxidant components.

Four animals were not considered in the present study; therefore, slightly different mean values for α-tocopherol concentration are reported in Table 1 compared to those published by Röhrle et al. (2011). Vitamin E concentration in muscle from heifer in the P and SiP treatments was more than two-fold higher than that measured in LM from cattle in the C group ($P < 0.0005$) and was also higher than that found in beef from animals in the SiPC treatment ($P < 0.01$). Furthermore, mean α-tocopherol concentration in LM from heifers in the SiPC treatment was significantly higher than in meat from heifers fed exclusively concentrates (C; $P < 0.01$).

There was a higher intramuscular fat percentage in the LM from animals receiving concentrates (C and SiPC groups) compared to LM from heifers in the SiP treatment ($P < 0.0005$ and $P = 0.02$, respectively; Table 1). The percentage of intramuscular fat in muscle from heifers fed exclusively grass at pasture (P group) was intermediate between that measured in LM from animals in the SiP and SiPC treatments and was lower compared to that found in muscle from animals in the C group ($P = 0.007$). The effect of diet on the percentage of intramuscular fat was reflected in the effect of the dietary treatment on the total concentration of fatty acids found ($P < 0.0005$; Table 1). Dietary treatment did not affect the percentage of SFA and MUFA in the intramuscular fat. However, the proportion of PUFA was strongly affected by the diet ($P < 0.0005$; Table 1), with a decreasing percentage as the proportion of concentrates in the diet increased. Specifically, a lower percentage of PUFA was found in muscle from animals fed exclusively concentrates (C) compared to that found in LM from cattle in the P and SiP treatments ($P < 0.001$). The proportion of PUFA in muscle from heifers in the SiP treatment was higher than that found in LM from animals in the SiPC group ($P = 0.007$) which, in turn, had a greater percentage of PUFA than that found in muscle from animals fed concentrates only (C; $P = 0.01$).

The ratio between the concentration of vitamin E and the absolute concentration of PUFA in muscle from heifers in both P and SiP treatments was more than two-fold higher than in beef from animals in the C group ($P < 0.0005$; Table 1). Conversely, the ratio between vitamin E and PUFA concentration in muscle from cattle finished at pasture with supplemental concentrates (SiPC group) did not differ significantly from that found in LM from heifers fed exclusively concentrates (C group) and was significantly lower than in beef from animals in the P ($P < 0.0005$) and SiP ($P < 0.001$) treatments. The absolute concentration of highly peroxidisable PUFA (HP-PUFA) in muscle was higher for animals from P and SiP groups ($P = 0.05$) and for those in the SiPC treatment ($P = 0.02$) compared to beef

from animals in the C group, while beef from animals in in all of the grass-based dietary treatments (P, SiP and SiPC) did not differ in the absolute concentration of HP-PUFA (Table 1).

Table 1.
Effect of the dietary treatments on muscle vitamin E concentration and fatty acid composition.

	Dietary treatment					
Item	P	SiP	SiPC	C	SEM	*P*-value
No. of heifers	23	23	25	23		
Vitamin E, µg/g of muscle	2.59 ^{a}	2.45 ^{a}	1.76 ^{b}	1.15 ^{c}	0.086	<0.0005
Total intramuscular fat, g/100g of muscle	3.09 ^{bc}	2.67 ^{c}	3.60 ^{ab}	4.12 ^{a}	1.160	<0.0005
Total fatty acids, mg/g of muscle	19.79 ^{bc}	17.23 ^{c}	25.62 ^{ab}	29.65^{a}	1.140	<0.0005
SFA, % of total fatty acids [1]	41.30	41.06	40.11	42.70	0.572	0.450
MUFA, % of total fatty acids [2]	48.89	47.69	50.77	50.22	0.575	0.227
PUFA, % of total fatty acids [3]	9.62 ^{ab}	11.04 ^{a}	8.96 ^{b}	6.94 ^{c}	0.268	<0.0005
Vitamin E ÷ PUFA [4]	$16.15 \times 10^{-4\,a}$	$14.51 \times 10^{-4\,a}$	$9.14 \times 10^{-4\,b}$	$6.93 \times 10^{-4\,b}$	6.1×10^{-5}	<0.0005
HP-PUFA, mg/g of muscle [5]	0.84 ^{a}	0.85 ^{a}	0.87 ^{a}	0.65 ^{b}	0.171	0.02

[1] Sum of: C14:0; iso C15:0; anteiso C15:0; C15:0; iso C16:0; C17:0; C18:0; C20:0; C21:0; C22:0; C23:0; C24:0; C26:0; C27:0.

[2] Sum of: C14:1; C15:1; trans-6 to trans-13 C16:1; cis-9 C17:1; trans-4 C18:1; trans-9 to trans-13 C18:1; cis-9 C18:1; cis-11 to cis-13 C18:1; trans-16 C18:1; trans-9 C20:1; cis-11 C22:1.

[3] Sum of: cis-9, cis-12 C16:2; cis-11, trans-15 C18:2; trans-10, cis15 C18:2; cis-9, cis-12 C18:2; cis-9, trans-11 C18:2; trans-10, cis-12 C18:2; cis-11, cis-14 + trans-13, cis-17 C20:2; cis-13, cis-16 C22:2; cis-9, trans-11, cis-15 C18:3; cis-9, cis-12, cis-15 C18:3; cis-8, cis-11, cis-14 C20:3; cis-5, cis-8, cis-11, cis-14 C20:4; cis-5, cis-8, cis-11, cis-14, cis-17 C20:5; cis-7, cis-10, cis-13, cis-16, cis-19 C22:5 ; cis-4, cis-7, cis-10, cis-13, cis-16, cis-19 C22:6

[4] Ratio between the concentration of vitamin E and the concentration of PUFA, both expressed as mg/g of muscle.

[5] Highly peroxidisable (HP) PUFA. Calculated as the sum of PUFA with three or more double bonds.

[a, b, c] Within a row, means without a common superscript differ ($P < 0.05$)

❖ *2.3.2. Meat oxidative stability*

❖ *2.3.2.1. Lipid oxidation.*

Lipid oxidation increased as storage time progressed regardless of the dietary treatment ($P < 0.0005$; Figure 1). Lipid oxidation increased rapidly within the first 4 days in meat from heifers in all the treatments ($P < 0.0005$), followed by slower changes thereafter. However, a significant Time × Diet interaction ($P = 0.003$) revealed different rates of development of lipid oxidation between the dietary treatments across the 11 days of storage duration. After 11 days of storage, TBARS values in meat from heifers in the P group were similar to those reached in meat from SiPC group after only 4 days. Lipid oxidation measured in beef from animals in the P, SiP and C treatments after 11 days were similar to that found in beef from heifers in the SiPC group after 7 days. A strong effect of the diet on lipid oxidation ($P < 0.0005$) accounted for the differences in TBARS values found at the different storage times. After 4 days, meat from animals in the P treatment had lower TBARS values than beef from cattle in the SiPC group ($P = 0.002$). After 7 days, lipid oxidation in meat from heifers in the SiPC treatment was higher than in meat from animals in both the P and the SiP groups ($P = 0.001$ and $P = 0.05$, respectively). Lastly, after 11 days, beef from heifers in the SiPC group reached higher TBARS values compared to those found in meat from animals in both P and C treatment ($P = 0.002$ and $P < 0.05$, respectively). Significant differences in TBARS values between beef from animals in the P, SiP and C treatments were never detected at any of the 4 days of measurement.

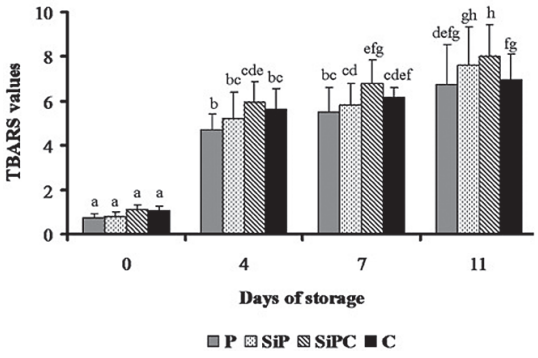

Fig. 1. Effect of the dietary treatment (P, SiP, SiPC or C) and of the time of storage (days 0, 4, 7, 11) on TBARS values of raw minced meat stored in 80% O_2 : 20% CO_2 over 11 days at 4°C. Means without a common superscript differ ($P \leq 0.05$). Values are means plus standard deviation (SD).

❖ *2.3.2.2. Color stability and myoglobin oxidation.*

A general decrease in meat redness (a* values) across the 11 days of storage was observed regardless of the dietary treatment ($P < 0.0005$; Figure 2a). However, the repeated measures ANOVA revealed a strong Time × Diet interaction ($P < 0.0005$) suggesting different rates of decrease in redness among the experimental treatments. After 11 days of storage, meat from cattle in the P treatment had similar a*

values to those measured in meat from all of the other groups at day 7 (Figure 2a).Moreover, a tendency for dietary treatment to affect meat redness was found (P = 0.1) and accounted for the higher a* values measured after 11 days in meat from heifers fed exclusively grass at pasture (P) compared to those found in meat from animals in the SiPC and C treatments (P < 0.001 and P < 0.0005, respectively). A general increase in hue angle (H*) values over the storage period was observed (P < 0.0005; Figure 2b). However, in contrast to the lipid oxidation data, the changes in H* values were slower and, compared to those measured at the start of the storage, H* values increased significantly after 7 days (P < 0.05). As for a* values, a highly significant Time × Diet interaction (P < 0.0005) indicated different rates of increase in H* values between the dietary treatments. Indeed, hue angle values measured after 11 days in meat from cattle in both P and SiP groups were similar to those reached after 7 days in beef from animals in SiPC and C treatments. The dietary treatment strongly affected this color descriptor (P < 0.0005) and, together with the differences in the rate of change in H* values between the dietary treatments, accounted for the lower H* values found in meat from animals in the P treatment than those reached in beef from heifer in both SiPC and C groups after 7 days (P = 0.013 and P = 0.001, respectively) and after 11 days (P < 0.0005) of storage. Hue angle values of meat from animals in the SiP treatment were lower than those reached in meat from animals in the C group after 7 and 11 days of storage (P = 0.045 and P <

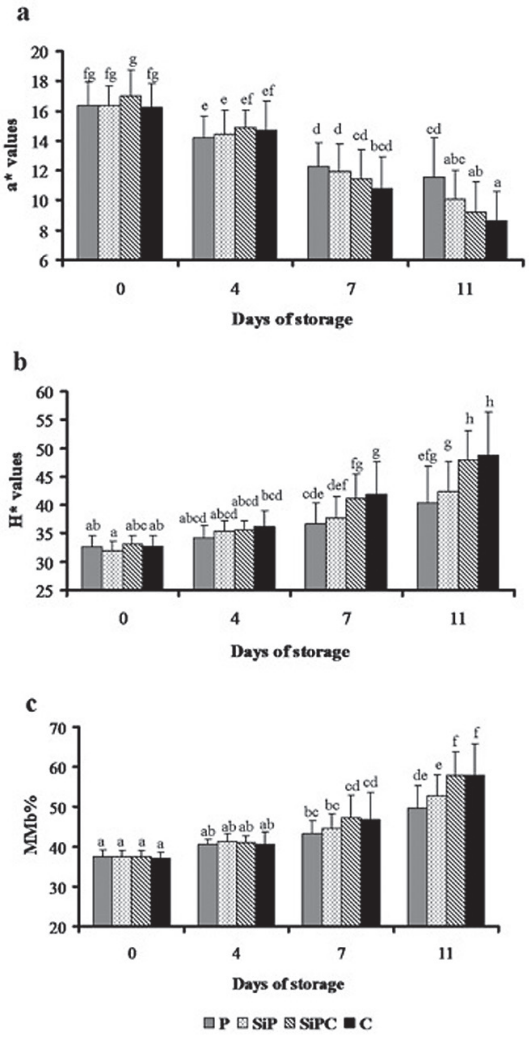

Fig. 2. Effect of the dietary treatment (P, SiP, SiPC or C) and of the time of storage (days 0, 4, 7, 11) on a* values (a), H* values (b) and metmyoglobin (MMb) percentages (c) of raw minced meat stored in 80% O_2 : 20% CO_2 over 11 d at 4°C. Means without a common superscript differ (P ≤ 0.05). Values are means plus standard deviation.

0.0005, respectively) and in meat from cattle in the SiPC treatment after 11 days ($P < 0.001$).

Myoglobin oxidation progressed over storage duration regardless of dietary treatment ($P < 0.0005$; Figure 2c). As observed for H* values, the increase in MMb% across the storage period was gradual in meat from animals in all the treatments, with significant changes observed after 7 days of storage ($P < 0.001$). However, a strong Time × Diet interaction ($P < 0.0005$) indicated that MMb accumulated differently at the meat surface depending on the dietary background of the animals. Indeed, while from 4 to 7 days of storage MMb% increased in meat from animals in both SiPC and C treatments ($P < 0.001$), they remained stable in beef from cattle in the P and SiP groups. Additionally, after 11 days of storage, the proportion of MMb in meat from heifers in the P treatment was comparable to that reached in beef from cattle in both SiPC and C groups after 7 days. Coupled with an effect of the diet on MMb % ($P = 0.002$), this accounted for the lower MMb% measured, after 11 days of storage, in meat from heifers in the P and SiP groups compared animals in both SiPC and C treatments ($P < 0.001$).

2.4. Discussion

Comparisons of the effect of concentrate- or herbage-based diets on meat shelf life have frequently shown that feeding systems based on green forages confer on meat a superior resistance to oxidative deterioration, due to the high concentrations of antioxidant molecules in green herbage (Wood and Enser, 1997). These compounds, delivered to animal tissues, can counteract the increased susceptibility of meat to oxidation arising from the high levels of PUFA in muscle of animals fed fresh herbage (Descalzo and Sancho, 2008). Among these antioxidant molecules the key role played by vitamin E has been widely recognized (Morrissey et al., 1994; Buckley et al., 1995). In the present study, greater vitamin E concentration was found in LM from animals allowed to graze at pasture (P, SiP and SiPC groups) compared to beef from heifers fed exclusively concentrates (C group). However, despite these differences, none of the dietary treatments resulted in vitamin E concentration in LM reaching 3.0-3.5 µg/g considered the threshold level able to effectively extend beef oxidative stability (Arnold et al., 1993; Liu et al., 1995). This could partially explain the lack of difference in lipid oxidation observed, across the storage period, in meat from cattle in the P and SiP treatments compared to animals fed exclusively concentrates (C).

Intramuscular fat content and fatty acid composition are key factors to consider when the effect of diet on meat oxidative stability is investigated. Higher fat content is generally found in muscle from animals raised on concentrates compared to animals allowed to graze herbage at pasture, due to the higher energy

intakes or to the lower energy expenditures associated with concentrate-based feeding systems (Aurousseau et al., 2004). Although, in the present study, the rations were regulated in order to achieve comparable energy intakes between treatments, a higher proportion of fat and a consequent greater concentration of fatty acids was found in LM from heifers fed concentrates only (C) compared that measured in muscle from cattle in both P and SiP treatments, which could be partially attributed to the higher physical activity of grazing animals compared to animals raised on concentrates indoors. The relative amount of triacylglycercols over that of phospholipids tends to increase as the fat content in muscle increases, while the proportion of phospholipids remains rather stable (Aurousseau et al., 2004). While PUFA are preferentially incorporated in phospholipids, SFA and MUFA tend to be incorporated in the triacylglycercols (Wood et al., 2003). Consequently, concentrate-fed animals often have higher proportion of SFA and MUFA in muscle than grass-fed animals if the concentrate-based diet results in a higher deposition of intramuscular fat (Yang et al., 2002; Realini et al., 2004; Santé-Lhoutellier et al., 2008). However, although statistically significant, the differences in fat content found here between concentrate- and grass-fed animals (C and P groups), were numerically not as large as those reported by other authors (Yang et al., 2002; Realini et al., 2004) and differences in final BW and carcass weight were not observed between animals in the C and P treatments (data not shown). Therefore, our results are in agreement with other studies showing that differences in SFA and MUFA percentages between muscles from animals fed concentrates or herbage are not evident with animals being allowed to grow at comparable rates (French et al., 2000; Varela et al., 2004; Descalzo et al., 2005; Vasta et al., 2009b). With regard to the susceptibility of meat to oxidative deterioration, the levels of SFA and MUFA in muscle are less influential compared to the concentrations of PUFA. Indeed, PUFA in the phospholipid fraction of cell membranes are highly oxidisable substrates in which lipid oxidation is initiated and, then, propagated in other muscle components (Morrissey et al, 1998). The lower percentages of PUFA found here in beef from animals fed exclusively concentrates (C) compared to that from grass-fed animals (P, SiP and SiPC diets) were expected, as several reports have shown that herbage-based diets produce significant increases in the proportion of PUFA compared to concentrate-based diets (Wood et al., 2008).

When the diet affects meat fat content, expressing the classes of fatty acids as percentages of total fatty acids may be misleading if muscle's susceptibility to lipid oxidation is investigated. Indeed, the susceptibility of meat to oxidative deterioration depends on the absolute concentration of PUFA and on the balance between the concentration of antioxidants (such as vitamin E) and that of the highly oxidisable substrates (Descalzo and Sancho, 2008). Although, in the present study, the proportion of PUFA was lower in the intramuscular fat of animals in the C group compared to muscles from animals fed grass (P, SiP and SiPC treatments), differ-

ences were not evident when PUFA were expressed in terms of absolute concentration (e.g. mg/g of meat; data not shown). A lack of difference in the absolute concentration of PUFA between meat from lambs fed either grass or concentrates was also reported by Ponnampalam et al. (2010). In the present study, feeding cattle exclusively with concentrates (C) produced higher fat content in muscle compared to grass-based feeding systems (P and SiP). Therefore, it is likely that concentrate feeding, by enhancing the concentration of fatty acids in LM, brought the absolute concentration of PUFA in beef from heifers in the C group close to that found in meat from cattle in the P and SiP treatments. Indeed, although PUFA are preferentially incorporated into the phospholipids, some are found in triglycerides which increase with increasing muscle fatness (Aurousseau et al., 2004).

As shown by Röhrle et al. (2011), the inclusion of concentrates in the diet for animals in the C and SiPC treatments impaired the deposition of vitamin E in muscle compared to the grass-based feeding systems (P and SiP) despite the fact that the vitamin E intake of the former groups was higher. This might be explained by the greater proportion of the readily bioavailable stereoisomer RRR α-tocopherol in the grass and silage compared to the concentrates (Röhrle et al., 2011). Consequently, beef from animals in the C and SiPC treatments had similar absolute concentration of PUFA but lower concentration of vitamin E compared to meat from cattle in the P and SiP treatments. Indeed we found a higher ratio between the concentration of vitamin E and the absolute concentration of PUFA in beef from animals in the P and SiP groups than in meat from heifers in the C and SiPC treatments. These results would suggest that meat from animals in the P and SiP treatments possesses a higher inherent resistance to lipid oxidation compared to meat from cattle in both SiPC and C groups. Our results showed that the inclusion of supplemental concentrates in the diet for animals in the SiPC treatment impaired meat lipid stability compared to beef produced by finishing heifers at pasture (P and SiP treatments). However, no measurable differences in lipid oxidation were observed between meat from animals fed concentrates only (C treatment) and that from heifers in the P and SiP treatments. Furthermore, meat from animals in the SiPC group displayed higher TBARS values than beef from concentrate-fed animals (C group) after 11 days of storage.

The susceptibility of PUFA to oxidation increases with increasing degree of unsaturation (Yang et al., 2002; Scislowski et al., 2005). For example, compared to linoleic acid (LA, cis-9, cis-12 C18:2), the susceptibility to peroxidation of linolenic acid (LNA, cis-9, cis-12, cis-15 C18:3) is more than two-fold higher (Shahidi, 1992). Although these fatty acids represent quantitatively minor components in the intramuscular fat, small changes in their concentration can produce significant effects on meat oxidative stability (Yang et al., 2002). For this reason, the overall concentration of PUFA in muscle may not be a reliable indicator of meat lipid stability and researchers have sometimes focused on the concentration of the highly

oxidisable fatty acids with more than two double bonds (Yang et al., 2002; Descalzo et al., 2005) or have considered the degree of unsaturation of the different PUFA for the estimation of their propension toward peroxidation (Scislowski et al., 2005). In the present study, greater absolute concentration of highly peroxidisable PUFA (HP-PUFA) were found in meat from animals fed grass (P, SiP and SiPC) compared to beef from heifers given exclusively concentrates (C). From this standpoint, meat from grass-fed cattle is more prone to lipid oxidation compared to that from animals raised on concentrates only. However, the rather high vitamin E levels found in meat from cattle in both P and SiP groups could have counterbalanced the increased concentration of HP-PUFA, while the lower concentration of vitamin E in meat from animals in the C group was probably sufficient to overcome the reduced concentration of pro-oxidants. This could partially explain the similar lipid stability observed in beef from animals in the P, SiP and C treatments. For example, Descalzo et al. (2005) found higher lipid stability in meat from cattle raised on pasture compared to animals fed concentrates. However, in that study, while the dietary treatments did not produce differences in the concentration of highly unsaturated fatty acids, the higher vitamin E concentration found in muscle from animals raised on pasture compared to that from concentrate-fed animals could have increased meat lipid stability of the former. Evidence for a protective effect of herbage-based diets on meat lipid oxidation has been provided using minced meat stored in high oxygen atmosphere conditions (Luciano et al. 2009b-Exp. 1). However, mincing coupled with high oxygen atmosphere represent a significant deteriorative stress for meat, disrupting cellular compartmentalization and enabling oxygen to react with muscle components (Gray et al., 1996; O'Grady, Monahan, Burke & Allen, 2000). This could be another reason contributing to explain to the lack of difference in lipid oxidation observed in the present study, in meat from animals fed exclusively grass (P and SiP) or concentrates (C). For example, Realini et al. (2004) reported improved lipid stability in muscle steaks from steers raised on pasture compared to steaks from concentrate-fed animals. However, an opposite trend was found when lipid oxidation was measured on ground muscle, probably because mincing could have challenged the meat from grass-fed animals more than that from concentrate-fed steers, due to its higher content of PUFA. In the present study, only vitamin E was measured as antioxidant component in meat. However, it is likely that feeding concentrates reduced the concentration of other antioxidant molecules in muscle from animals in the SiPC and C treatments compared to muscle from animals in the P and SiP groups. For example, β-carotene was found at higher concentration in muscle from cattle fed herbage compared to muscle from grain-fed animals and it was shown that TBARS values correlated inversely with both vitamin E and β-carotene concentration in muscle, which suggests an additive role of both vitamins in enhancing meat lipid stability (Descalzo et al., 2005; Insani et al., 2008). Based on the lipid oxida-

tion results, the balance between antioxidant and pro-oxidant components found here in beef from heifers in the SiPC group seems definitely unfavorable compared to that found in meat from animals fed exclusively grass or concentrates. A reduction in muscle vitamin E and other possible antioxidant molecules consequent to the partial replacement of pasture with concentrates may have a detrimental effect on meat oxidative stability when HP-PUFA remain high (SiPC group). Conversely, the balance between pro- and antioxidants would be more favorable when the reduction in muscle antioxidants is accompanied by a reduction in HP-PUFA (C group), or when the high concentration of HP-PUFA is counterbalanced by high levels of antioxidants (P and SiP groups). Coupled with the highly pro-oxidant storage conditions adopted in this study, this may contribute to an explanation of the higher susceptibility to lipid oxidation observed in meat from SiPC-fed animals compared to the other dietary treatments.

Feeding strategies aimed at improving meat lipid stability - such as vitamin E supplementation or herbage-based diets - have often been reported to also improve color stability due to a delay in myoglobin oxidation (Faustman et al., 2010). In agreement, here we have found that, compared to meat from cattle in the P and SiP treatment, the higher susceptibility to lipid oxidation observed in meat from cattle in the SiPC group corresponded to a higher extent of myoglobin oxidation and to a consequent reduction in color stability, with a faster decrease in a* values and a higher rate of increase in H* values. However, although lipid oxidation developed at a similar extent in beef from heifers fed exclusively grass (P and SiP treatments) or concentrates (C treatments), the latter displayed a lower myoglobin stability to oxidation which resulted in more pronounced changes in meat color stability parameters (a* and H* values). This is an interesting result, as positive effects of the diet on lipid oxidation with no effect on color stability have been frequently observed (Yang et al., 2002; Gatellier et al., 2005; Santé-Lhoutellier et al., 2008), but positive effects on color stability without a corresponding improvement in lipid stability have been seldom reported (Wiklund et al., 2005). The lack of correspondence between lipid and myoglobin oxidation complicates the comprehension of the interactions between the processes. Monahan et al. (2005) showed that high levels of oxygen provide an example of a condition in which the correlation between lipid and myoglobin oxidation is weakened. Due to the important role played by oxygen partial pressure on myoglobin chemistry (Shikama, 1998), high oxygen atmosphere packaging extends meat colour stability, by maintaining myoglobin in its oxygenated form (oxymyoglobin; OMb), but promotes lipid oxidation (Monahan et al., 2005; McMillin, 2008). This could help explain the different trends observed here, over storage duration, between the color stability parameters and lipid oxidation. The pro-oxidant action of the high level of oxygen in the bags could have promoted lipid oxidation, which may explain the pronounced increase in TBARS values observed within the first four days of storage.

Conversely, the high oxygen partial pressures could have slowed the oxidation of OMb, thus explaining the gradual increase in MMb% across the storage period and the consequent slow rates of change in a* and H* values. However, the reason for the improved color stability observed in meat from grass-fed animals (P and SiP treatments) compared to meat from concentrate-fed animals (C treatment) remains unclear, considering that the treatments did not produce differences in lipid oxidation.

The specific role of vitamin E in improving meat color stability has been extensively confirmed (Faustman et al, 1998). Several mechanisms of action have been proposed to explain the protective action of the liposoluble vitamin E against the oxidation of the water-soluble myoglobin and all of them attribute an indirect role to vitamin E mediated by its direct activity on lipid oxidation (Faustman et al., 2010). The positive effect on meat color stability of the grass-based feeding systems (P and SiP) compared to the concentrate-based diet (C) observed in the present experiment seems to be unrelated with vitamin E, as the treatments did not produce measurable differences in lipid oxidation. A possible effect of the diet on antioxidant components other than tocopherols could explain the differences in meat color stability observed. Compared to concentrate-based diets, pasture-based diets, for example, have been reported to improve the activity of important endogenous antioxidant enzymes in muscle, such as superoxide dismutase (SOD) and could result in higher activities of catalase (CAT) due to their higher contents of n-3 PUFA (Renerre et al., 1999; Gatellier et al., 2004; Descalzo et al., 2007; Insani et al., 2008). In muscle model systems, both SOD and CAT have been shown to prevent OMb from auto-oxidation without any appreciable effect on lipid oxidation (Groelik and Kanner, 2001a). Although, in the present study, the activity of antioxidant enzymes was likely impaired as meat had been previously frozen, a possible residual activity could have contributed to the overall antioxidant status of muscle, with a specific effect on myoglobin chemistry. Moreover, in addition to liposoluble antioxidants, other water-soluble molecules with strong antioxidant activity are present at higher concentration in fresh herbage than in concentrate feeds and can contribute to the antioxidant pool in muscle from pasture-fed animals (Wood and Enser, 1997). For example, ascorbic acid (vitamin C) exerted a specific protection against myoglobin oxidation without any involvement in lipid oxidation when present in muscle model systems or when added to ground meat (Groelik and Kanner, 2001b; Realini et al., 2004). Although vitamin C in muscle has been rarely measured, Descalzo et al. (2005) reported higher concentration of ascorbic acid in muscle from cattle raised on pasture compared to beef from grain-fed animals. Besides vitamin C, phenolic compounds represent an important group of hydrophilic antioxidants that are found in higher concentrations in fresh herbage than in concentrates (Hollman & Katan, 1998; Vasta et al., 2009b). Recent reports have shown that feeding ruminants with polyphenol-rich diets improved

the antioxidant status of muscle with a specific effect against myoglobin oxidation and meat discoloration, but with no impact on lipid oxidation (Luciano et al., 2009a-Exp. 3; 2011-Exp. 4).

2.5. Conclusions

The results of this study highlight that the concentration of muscle antioxidants is not the only factor explaining meat oxidative stability, as a clear interaction with the dietary background of the animals exists. Indeed, here we have shown that meat with largely different vitamin E levels (from cattle in the P, SiP and C treatments) displayed similar lipid stability, probably because of the different levels of pro-oxidant (highly peroxidisable PUFA) components arising from the diet. The interaction between antioxidants and pro-oxidants was very evident in the case of meat from the animals fed grass with supplemental concentrates (SiPC) in which the unbalanced ratio between antioxidants and highly oxidisable PUFA may have increased meat susceptibility to lipid oxidation. Also, our results confirmed the positive effect of grass-based feeding systems (P and SiP) on meat color stability compared to feeding strategies based exclusively on concentrates (C) or including high supplementation of concentrates in grass-based diets (SiPC). Moreover, the improvement in meat color stability in beef from animals fed exclusively grass at pasture compared to meat from animals raised exclusively on concentrates did not correspond to differences in lipid oxidation. This result provides an interesting evidence for a clear effect of the diet on meat color stability which seems unrelated to lipid oxidation. From a practical standpoint, it appears from the results of this study that adequate amounts of antioxidants should be provided in feeding systems including herbage-based diets supplemented with concnetrates in order to avoid detrimental effects on meat shelf life.

Experiments Three and Four

Effect of dietary phenolic compounds on meat oxidative stability

Introduction

Several compounds are able to exert antioxidant effects in biological substrates and animal tissues. Among the most known natural antioxidants, the interest in phenolic compounds has been growing in recent years. Phenolic compounds form one of the most numerous and chemically heterogeneous groups of plant secondary compounds, ranging from simple molecules - such as phenolic acids and flavonoids - to the highly polymerized tannins (Hagerman et al., 1998). The latter are oligomers and polymers of (epi)catechin or (epi)gallocatechin with widely varying degrees of polymerization (Manach & Donovan, 2004). The antioxidant properties of small phenolics have been extensively studied (Scalbert et al., 2002). Although the influence of the molecule's complexity on the antioxidant ability of polyphenols is not fully clear, in some instances, the free radical scavenging activity of polyphenols was shown to be positively related to the number of hydroxyl groups in their molecules and to their polymerization degree, that making tannins potentially important antioxidants (Hagerman et al., 1998). Besides in *vitro* assays suggesting their antioxidant activities, the direct addiction of purified phenolics and of polyphenol-rich plant extracts to muscle model systems was shown to delay lipid and myoglobin oxidation (Hayes et al., 2009).

Due to their almost ubiquitous distribution in the plant kingdom, the contribution of phenolic compounds to the dietary intake of antioxidants, which is estimated to be higher than that of vitamin E, is of great interest (Hollman & Katan, 1998). Moreover, tannins, in particular, are present in several feed resources used for livestock feeding and have been shown to affect several aspects of ruminants' nutrition and products quality (Vasta et al., 2008). The dietary administration of polyphenol- and tannin-rich plants and plant extracts has been shown to improve the oxidative stability of meat from different animals (Tang et al., 2001; Larraín et al., 2008; Nieto et al., 2010). The direct antioxidant activity of a dietary compound would assume its absorption along the gastrointestinal tract and its deposition in the tissues. If monomeric phenolics can be absorbed through the intestine, no studies have detected the presence of tannins with degrees of polymerization greater than dimers in plasma (Scalbert et al., 2002; Manach & Donovan, 2004; Manach et al., 2005). Controversial results have been provided by in vivo studies using 14C-labelled tannins to follow their metabolism in the gastrointestinal tract of rats and ruminants (Terrill et al., 1994; Perez-Maldonado & Norton, 1996; Abia & Fry, 2001). Ruminal microbial population can adapt to tannins, protecting the animals from their antinutritional effects (Smith et al., 2005). However, Makkar et al. (1995) demonstrated that rumen microbes do not hydrolyse condensed tannins. Nevertheless, *in vivo* and *in vitro* studies provided evidence for an active metabolism of condensed tannins carried out by the intestinal microflora of

both humans and rats (Déprez et al., 2000; Abia and Fry, 2001; Rios et al., 2003). These results suggest that tannins do not remain inert along the whole gastrointestinal tract; rather, tannins could undergo chemical modifications in the gut leading to their depolymerization and catabolism into smaller molecules which, in turn, could be absorbed. Therefore, the fate of phenolic compounds in the animal organisms, their rate of absorption and their antioxidant activities in the tissues are still under debate. Furthermore, considering other biological effects of dietary phenolics, such as their ability to modify muscle's fatty acid composition in ruminants (Vasta et al., 2009b), or to increase the levels of antioxidant enzymes in rat tissues (Anila & Vijayalakshmi, 2003), it may be difficult to distinguish between their direct and indirect effects on the resistance of meat to oxidative damages.

The increasing interest in the biological effects of dietary polyphenols in animals and in their potential use as natural antioxidants in livestock feeding motivated the second part of the present thesis. Here the effects of feeding lambs with polyphenol-enriched diets on meat shelf life were investigated with two experiments *(Experiments Three and Four)*.

Experiment Three [1 2]

3.1. Aim.

The objective of this novel research was to test the effect of feeding lambs with a tannin-enriched diet on the basic parameters of meat oxidative stability across 14 days of refrigerated storage in a high oxygen atmosphere. Because of the relationship between lipid oxidation and colour deterioration generally observed in meat, these two deteriorative processes were analyzed simultaneously.

3.2. Materials and methods

❖ *3.2.1. Animals and diets*

Fourteen male Comisana weaned lambs born in mid January 2007 (45 d of age and average initial body weight of 21.3 kg ± 2.8 kg) were blocked in groups of two on a descending bodyweight basis and, within block, were randomly assigned to one of two experimental dietary treatments. Lambs were individually penned for the duration of the trial. Over a 7-day period of adaptation to the experimental diets, animals received the same commercial starter concentrate used for weaning, with gradual increases in the amount of experimental feeds. For 60 days, seven lambs (Control group; C) were fed a barley-based concentrate. The remaining 7 lambs (Tannin group; T) received the same concentrate with the addition of a commercially available tannin-rich quebracho (*Schinopsis lorentzii*) extract (Figli di Guido Lapi S.p.a., Castelfranco di Sotto, Pisa, Italy). The inclusion of the extract was formulated to obtain a percentage of quebracho powder into the concentrate of 8.96% (dry matter basis). Table 1 reports the ingredients of the concentrates used for both C and T groups. Concentrates were pelleted and the quebracho powder was added before pelleting at a temperature of 40°C. The concentration of vitamin E (tocopheryl acetate) in the vitamin-mineral premix was 35 mg/kg. Individual dry matter intakes were recorded each day before morning feeding. Diets were provided once daily, whereas water was available *ad libitum*. Lambs were weighed once a week before morning feeding and the daily rations were consequently modulated in order to achieve comparable growth rates between C and T groups.

1 Published as: Luciano, G., Monahan, F.J., Vasta, V., Biondi, L., Lanza, M. and Priolo, A. (2009). *Dietary Tannins Improve Lamb Meat Colour Stability.* **Meat Science**, 81, 120-125.

2 Citations to this experiment in the present thesis are reported as "Luciano et al. (2009a – *Exp. 3*)".

Table 1
Ingredients and chemical composition of concentrates offered to lambs

	Concentrate	Concentrate + Tannins
Ingredients (g/kg as fed)		
Barley	551	496
Alfalfa hay	300	270
Soyabean meal	130	117
Mineral premix	19	17
Quebracho extract	0	100
Chemical composition (% on dry matter)		
Dry matter (%)	88.5	89.2
Crude protein	16.3	14.6
Crude fat	1.4	1.7
NDF	38.8	36.8
ADF	15.7	11.7
ADL	7.9	9.7
Ash	6.1	7.2
Total phenols[a]	0.84	59.08
Total tannins[a]	–	40.35

[a] Expressed as g of tannic acid equivalents per kg of dry matter.

❖ *3.2.2. Slaughter procedures and muscle sampling*

Sheep were slaughtered at 105 d of age at a commercial abattoir. Until approximately 15 min before slaughter, lambs had access to their experimental feeds. They were stunned by captive bolt. Immediately after slaughter, at least 100g of *semimembranosus* (SM) muscle were excised from the left hind leg, wrapped in aluminium foil, vacuum-packaged and frozen at -20°C for subsequent analyses (performed one month later).

❖ *3.2.3. Feed analyses*

Fiber fractions (Van Soest et al., 1991), crude protein (method 984.13; AOAC, 1995) crude fat (method 920.39; AOAC, 1995) and ash (method 942.05; AOAC, 1995) of the experimental concentrates were determined in triplicate. Samples of the feed offered were extracted overnight at 4°C in an aqueous acetone solution (70% v/v). After centrifugation (3000 g for 15 min at 4°C), total phenols and total tannins were determined by Folin-Ciocalteu reagent with tannic acid as standard (Makkar et al., 1993), and expressed as tannic acid equivalents per kg of dry matter.

❖ *3.2.4. Preparation of raw minced meat*

Muscle samples were prepared for measurements of lipid oxidation and colour stability on raw minced meat stored in high oxygen modified atmosphere as previously described in *Experiment One*. Muscle samples were removed from the freez-

er and, while still partially frozen, were trimmed of the outer fat. Five subsamples of SM from each lamb were prepared by placing 12 g of minced lamb in individual polyamide/polyethylene Exovac 73 bags (McDonnells Queen St., Dublin) using one bag for each of five days of analysis. A Webomatic vacuum packaging system equipped with a gas mixer (Witt-Gase Technik KM-100-M (3), Witt Gas Techniques Ltd., Warrington, England) was used to package the samples in a high oxygen modified atmosphere (MAP; 80% O_2 : 20% CO_2). The O_2, N_2 and CO_2 permeabilities of the bags at 23°C and 0% RH were 30, 10 and 105 $cm^3/m^2/24h/bar$, respectively. The MAP meat was stored in the dark at 4°C and lipid oxidation, meat colour and haem pigments were analysed 2 h after packaging (day 0) and after 4, 7, 11 and 14 days of storage.

❖ *3.2.5. Measurement of meat colour*

A Minolta colour meter (model CR300, Minolta Camera Co. Ltd., Osaka, Japan) was used to measure meat colour coordinates L^* (lightness), a^* (redness) and b^* (yellowness). As previously described in *Experiment One*, measurements were made using the D65 illuminant and 10° standard observer. The measurements were performed through the Exovac bag which was allowed to touch the meat surface during measurements. Triplicate readings were recorded on non-overlapping zones of the sample and average values were calculated. Chroma (C^*) was calculated as $(a^{*2} + b^{*2})^{1/2}$ and hue angle (H^*) as $\tan^{-1}(b^*/a^*) \times (180/\pi)$.

❖ *3.2.6. Extraction and measurement of haem pigments*

Muscle haem pigments were analysed as previously described in *Experiment One*. The extraction of haem pigments was performed as described by Warriss (1979), while total haem pigment concentration (expressed as mg/g of fresh tissue) and the percentages of metmyoglobin (MMb) were determined following the method of Krzywicki (1982). A UV-VIS spectrophotometer (Shimadzu UV-1201) was used for measuring the absorbances of the muscle extracts.

❖ *3.2.7. Measurement of lipid oxidation*

As described in *Experiment One,* the extent of lipid oxidation in meat was assessed by measuring 2-thiobarbituric acid reactive substances (TBARS), following the distillation method of Tarladgis et al. (1960). Five grams of meat were used instead of 10 g. A UV-VIS spectrophotometer (Shimadzu UV Mini 1240) was used. The K coefficient was calculated as described by Tarladgis et al. (1960).

❖ *3.2.8. Statistical analysis*

Data were analysed with a GLM procedure (Minitab 14, 1995) with a mixed model that included diet (T *vs* C) and time (days 0, 4, 7, 11, 14) and their interaction as fixed effects and individual lamb as random effect. The interaction was

removed from the model when it was not significant (P > 0.05). Tukey's test was used for comparing mean values.

3.3. Results and discussion

The effects of dietary tannins on meat quality have been so far assessed in different ways. Some studies have tested plants naturally containing tannins at different levels, such as cultivars with high or low tannin content (Cherian et al., 2002; Du et al., 2002; Larraín et al., 2007b). In other instances, polyethylene glycol (PEG), a compound able to bind to and deactivate tannins, has been added to tanniniferous feeds in order to isolate and study the effect of their tannins (Priolo et al., 2000; 2005). To our knowledge, this is the first study designed to assess the direct impact of dietary tannins on the stability of lamb quality.

❖ *3.3.1. Chemical composition of the concentrates.*

As compared to the tannin-free diet (C), the inclusion of quebracho powder in the concentrate (T) leaded to slightly higher level of acid detergent lignin (ADL; + 21%) and of ash (+ 18%) and lower level of acid detergent fibre (ADF; - 26%; Table 1).

❖ *3.3.2. Colour coordinates*

Regardless of dietary treatment, lightness (L^* values) increased ($P < 0.001$) with increasing duration of refrigerated storage (Figure 1a), and minced lamb from T-fed sheep was darker (lower L^* values) than that from C-fed sheep after 14 days of storage (time × treatment, $P < 0.001$; Figure 1a). Priolo et al. (2000) found higher L^* values in lamb from sheep fed a diet containing carob pulp (a tannin-containing feed), compared to lamb from sheep fed the same diet supplemented with PEG. The authors suggested that lower levels of pigments (haemoglobin and myoglobin) in lamb from T-fed sheep could have been responsible for the differences in colour. On the contrary, no difference in the lightness of minced SM was observed, in the present study, between the T-fed and C-fed sheep at day 0 of analysis. However, the studies are not fully comparable since *longissimus dorsi* muscle slices were used in the study of Priolo et al. (2000) while we measured meat colour on minced *semimembranosus* muscle.

Changes in a^* (redness) and b^* (yellowness) values over a period of display describe meat colour deterioration from red to brown, and reflect the myoglobin concentration and its redox state in meat (Mancini & Hunt, 2005). It has been recently shown (Insausti et al., 2008) that, over time of storage, while b^* values were positively related to sensory appreciation of meat colour degradation, a^* values were negatively correlated to the sensory degradation of colour. In the present study, redness (a^* values) decreased ($P < 0.001$) over 11 days of storage and were

greater ($P = 0.008$) in minced lamb from T-fed than C-fed sheep over the storage period (Figure 1b). Yellowness (b^*) values increased ($P < 0.001$) over the 14 days of storage in lamb from sheep fed both C and T (Figure 1c). The values of b^* were subjected to a time × treatment interaction ($P < 0.01$). Minced lamb from sheep fed the T-supplemented diet was less ($P < 0.05$) yellow than that from the control group at day 7 ($P < 0.05$) and at days 11 and 14 of storage ($P < 0.01$).

Hue angle is a good descriptor of meat browning (Young et al., 1999; Lee et al., 2005). Regardless of dietary treatment, hue angle (H^* values) increased (shifted from red to yellow) over storage duration ($P < 0.001$; Figure 2a); however, between 4 and 7 d of storage, hue angles increased in lamb from C-fed sheep but did not change in lamb from T-fed sheep. Moreover, after 7 and 11 days of storage,

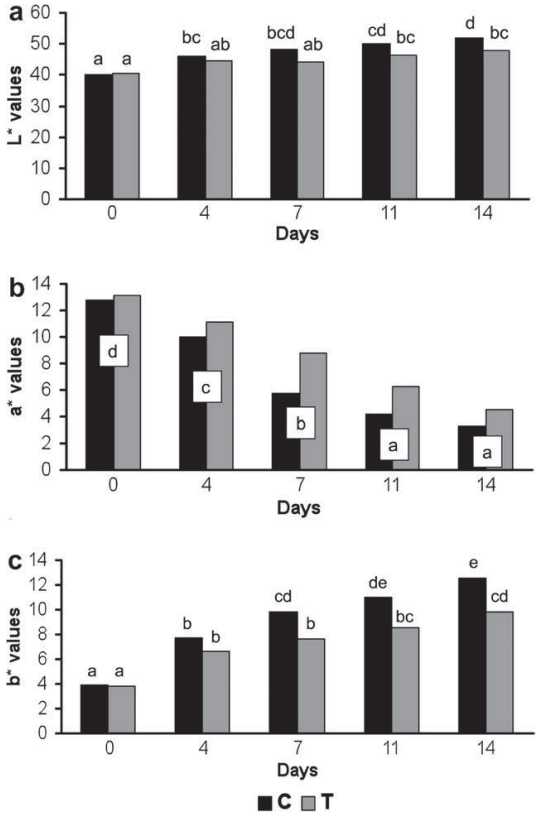

Fig. 1. Interactive effect of treatment (C or T) and time of storage (days 0, 4, 7, 11 and 14) on mean L* values (a); main effect of time of storage on a* values (b) and interactive effect of treatment and time of storage on b* values (c) of raw minced lamb from animals fed a concentrate ration with (T) or without (C) tannins, stored in 80% O_2: 20% CO_2 over 14 days at 4 °C. Values with different superscripts are significantly different ($P < 0.05$).

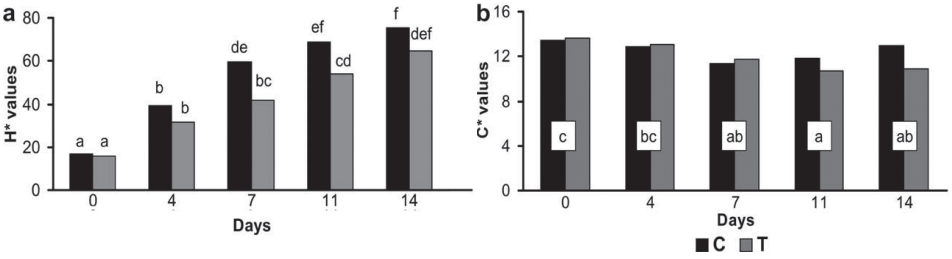

Fig. 2. Interactive effect of treatment (C or T) and time of storage (days 0, 4, 7, 11 and 14) on mean Hue angle values (a) and main effect of time of storage on mean Chroma values (b) of raw minced lamb from animals fed a concentrate ration with (T) or without (C) tannins, stored in 80% O_2: 20% CO_2 over 14 days at 4 °C. Values with different superscripts are significantly different ($P < 0.05$).

minced lamb from sheep fed T had lower ($P < 0.001$ and $P < 0.01$, respectively) H^* values than minced lamb from C-fed sheep. Thus, dietary tannins appeared to delay meat colour deterioration. Moreover, even though diet had no effect on chroma (C^*) values (Figure 2b), minced lamb stored 11 days had lower ($P < 0.01$) C^* values when compared to minced lamb stored 0 and 4 days. Minced lamb stored 0 days also had a more ($P < 0.01$) vivid colour (greater C^* values) than lamb stored 7 to 14 days.

❖ *3.3.3. Muscle haem pigments and lipid oxidation*

Percentages of metmyoglobin increased ($P < 0.001$) during the first 7 days of storage, and the average percentage of metmyoglobin tended to be lower ($P = 0.07$) in minced lamb from T-fed than C-fed sheep (Figure 3a). The haem pigment concentration decreased ($P < 0.001$) with increasing storage time. However, minced lamb from sheep fed the T-supplemented diet had greater ($P < 0.01$) haem pigment concentrations than that of sheep fed C (Figure 3b) after 11 days of storage and tended ($P = 0.10$) to have higher haem pigment concentration than C-fed sheep after 7 and 14 days of refrigerated storage. Lindahl et al. (2001) reported that both haem pigment concentration and metmyoglobin fraction were among the most important factors contributing to meat redness and yellowness in pork. Furthermore, Tam et al. (1998) and Lindahl et al. (2001) demonstrated a negative correlation between the hue angle values and the pigment concentration in meat. Thus, the observed decreases in haem concentration over the 14 days of storage coupled with the increases in metmyoglobin percentages, may explain, in the present study, both the loss of redness and the increase in yellowness observed with time, as well as the observed increases in hue angle values with increasing storage duration. Moreover, both the reduced metmyoglobin formation and the lower decreases in haem concentration found in lamb from sheep fed T diet than those fed C could be the reason for the lower hue angle values observed in lamb from T-fed sheep after 7 and 11 days of refrigerated storage.

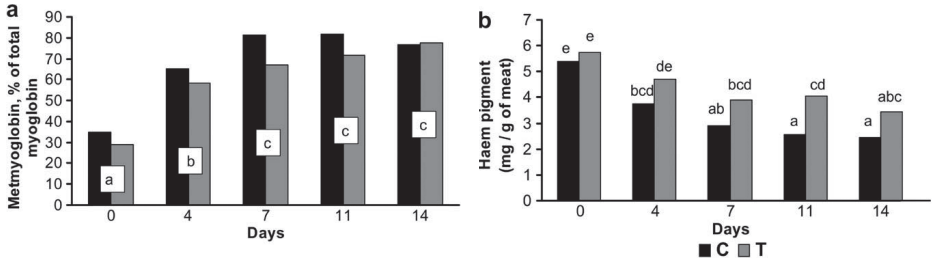

Fig. 3. Main effect of time of storage (days 0, 4, 7, 11 and 14) on mean metmyoglobin percentages (a) and interactive effect of treatment (C or T) and time of storage on haem concentrations (b) of raw minced lamb from animals fed a concentrate ration with (T) or without (C) tannins, stored in 80% O_2: 20% CO_2 over 14 days at 4 C. Values with different superscripts are significantly different ($P < 0.05$).

Decreases in myoglobin concentrations with increasing time of storage have also been described by McKenna et al. (2005). The authors hypothesized a loss of the water-soluble pigment with sarcoplasmic fluid due to the purge. Thus, although they were not measured, possible purge losses occurring during storage could explain the decreases in haem pigment concentration observed, in our study, over the 14 days of storage. However, this hypothesis does not explain the lower decreases in myoglobin concentration observed, over time of storage, in lamb from T-fed sheep as compared to that from sheep fed C. Proteins are a major target for oxidants and oxidation can occur at both the protein's backbone and on the amino acid side chains, bringing about several different protein modifications (Davies, 2005). Moreover, lipolysis occurring in meat over time of storage increases the levels of free fatty acids (Currie & Wolfe, 1977; Sklan et al., 1983; Lambert et al., 2001) and, in a highly lipophilic environment, metmyoglobin can undergo denaturation, with consequent exposure or release of the haem group (Baron & Andersen, 2002). Mincing represents a significant deteriorative stress for meat, disrupting cellular compartmentalization and resulting in the release and diffusion of prooxidants (Gray et al., 1996). Also high oxygen partial pressure (like that found in high oxygen MAP) can promote oxidation processes by enabling oxygen to react with muscle components (O'Grady et al., 2000). Lund et al., (2007) found increased lipid and protein oxidation at high levels of oxygen in the packaging atmosphere. Considering that, in our experiment, meat was both minced and packaged in 80% oxygen, the observed decreases in myoglobin concentrations with time of storage may have been caused by a loss of integrity of the haem pigment due to the highly oxidising conditions occurring. From this standpoint, the higher concentration of haem pigment found in the meat from the animals given tannins compared to that from the control group, may have been due to a protective effect of tannins against myoglobin damage. Larrain et al. (2007a) found lower protein oxidation in aged muscles from rats fed diets containing high-tannin sorghum.

An issue which should be further investigated is the effect of dietary tannins on meat fatty acid composition. Generally, increasing the degree of unsaturation of the muscle reduces its oxidative stability (Morrissey et al., 1998). However, it has been shown that some fatty acids, such as conjugated linoleic acid (CLA), can exert antioxidant activity in meat by reducing lipid oxidation and improving colour stability (Du et al., 2000; Joo et al., 2002). It has been recently reported both *in vivo* (Vasta et al., 2007; 2009b) and *in vitro* (Vasta et al., 2009a) that dietary tannins strongly interfere with fatty acids metabolism in ruminants. It could be speculated that dietary quebracho tannins, in the present study, may have played a role on lipid metabolism, that resulting in an indirect improvement of lamb oxidative stability.

Lipid oxidation increased ($P < 0.001$) across the 14-day storage period, but supplementing sheep diets with quebracho tannins had no ($P = 0.28$) appreciable effect

on TBARS values during storage at 4°C (Figure 4). The increasing TBARS values with increasing time of storage were expected. As previously mentioned, mincing, together with high-oxygen MAP, increases the susceptibility of the meat to lipid oxidation. No differences in TBARS accumulation were observed between the experimental groups over the 14 days of refrigerated storage. Considering the protective effect of dietary tannins on the colour stability parameters, and the well established relationship between lipid and myoglobin oxidation, a similar effect of the diet on TBARS accumulation over the period of analysis might have been expected. On the basis of the results obtained it seems, thus, that the improved colour stability observed in lamb from sheep fed the tanniniferous concentrate does not depend on an improved lipid stability. It is possible however that differences in susceptibility to lipid oxidation, if present, were not detected by the TBARS test used.

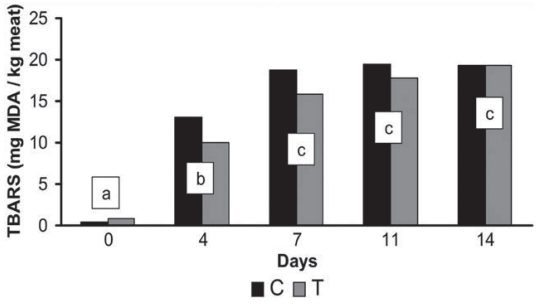

Fig. 4. Main effect of time of storage (days 0, 4, 7, 11 and 14) on mean TBARS values (expressed as mg of MDA/kg of meat) of raw minced lamb stored in 80% O_2: 20% CO_2 over 14 days at 4°C from animals fed a concentrate ration with (T) or without (C) tannins. Values with different superscripts are significantly different ($P < 0.05$).

3.4. Conclusions

The inclusion of quebracho tannins in the concentrate offered to sheep improved the colour stability of fresh lamb. Specifically, increases in hue angle values were less pronounced in lamb from sheep fed the tannin-supplemented diet compared to sheep fed the control diet. These findings could be attributed to the positive effects of dietary tannins on haem pigment concentration and metmyoglobin formation during the 14-day refrigerated storage period. Further research is needed to better understand the effect of dietary tannins on the stability of meat. The mechanism by which dietary tannins can affect the myoglobin concentration and its redox state is unclear. A direct antioxidant effect of tannins in tissue would implicate their absorption and deposition in the muscle; however, to date, the fate of dietary tannins in the organism is not clearly understood. A possible indirect effect of dietary tannins on lamb oxidative stability could involve the modification of muscle fatty acid composition due to the strong effects of tannins on lipid metabolism in ruminants. The improvement in meat colour stability observed in meat from the tannin-supplemented lambs did not involve an improved stability to lipid oxidation.

Experiment Four [1] [2]

4.1. Aim.

The results obtained with the *Experiment Three* showed a specific protective effect against myoglobin oxidation, with a consequent positive impact on meat colour stability, which appeared to be unrelated with lipid oxidation. However, in that study, it was not possible to further investigate possible mechanisms explaining the observed effects. Furthermore, highly pro-oxidants experimental conditions were adopted (freezing, mincing and high oxygen atmosphere packaging) and a muscle with a rather low inherent resistance to oxidation (*semimembranosus*) was used. Here meat colour stability was measured on fresh *longisimus dorsi* muscle steaks, under aerobic storage conditions, from the same animals used in *Experiment Three*. Furthermore, a series of assays were performed to measure the muscle's overall antioxidant status and the resistance of myoglobin to oxidation. The hypothesis was that dietary polyphenols might enhance both muscle's overall antioxidant capacity and myoglobin resistance to oxidation, leading to an improvement in meat colour stability.

4.2. Materials and methods

❖ 4.2.1. Dietary treatments and muscle sampling

Muscles from the same lambs used in *Experiment Three* were analysed in the present experiment; therefore, the detailed description of the animals' management and of the dietary treatments has been provided above. Within 20 min of slaughtering, the left *longissimus dorsi* muscle (LM) was excised from the 6th to the 13th rib. One portion was immediately vacuum-packaged and stored at 4°C for 24 hours for subsequent measurement of colour stability and metmyoglobin development of fresh LM steaks over aerobic storage. The remaining portion was immediately frozen in liquid nitrogen for 90 seconds, then wrapped in aluminium foil, vacuum-packaged and stored at -30°C until required for measurement of muscle antioxidant status and myoglobin resistance to induced oxidation.

1 Published as: Luciano, G., Vasta, V., Monahan, F.J., López-Andrés, P., Biondi, L., Lanza, M. and Priolo, A. (2011). *Antioxidant Status, Colour Stability and Myoglobin Resistance to Oxidation of Longissimus Dorsi Muscle from Lambs Fed a Tannin-Containing Diet*. **Food Chemistry**, 124, 1036-1042

2 Citations to this experiment in the present thesis are reported as "Luciano et al. (2011 – Exp. 4)".

❖ *4.2.2. Colour stability and metmyoglobin formation over aerobic storage.*

After 24 hours of anaerobic storage at 4°C, fresh LM were removed from the bags. Muscle slices (thickness: 3 cm) were placed on polystyrene trays, overwrapped with a gas oxygen-permeable PVC film and stored in the dark at 4°C. Hue angle (H^*) values and the reflectance (R) spectra were measured at the meat surface after 2 hours of blooming and, then, after 24, 72, 96 and 168 hours of refrigerated storage, using a Minolta CM-2022 spectrophotometer (d/8° geometry; Minolta Co., Ltd. Osaka, Japan). Diffuse reflection was measured in SCE (Specular Component Excluded) mode, using illuminant A and 10° standard observer. For each sample, average values were calculated from triplicate readings made on non-overlapping zones of the steak. As described on *Experiment Two,* metmyoglobin (MMb) percentages were estimated following the method of Krzywicki (1979). Furthermore, MMb accumulation at the meat surface, across the 7-day storage period, was followed by calculating the $(K/S)_{572} \div (K/S)_{525}$ ratio according to Stewart et al. (1965), where:

$$(K/S)_{\lambda} = (1 - R_{\lambda})^2 / 2R_{\lambda}$$

❖ *4.2.3. Antioxidant status of longissimus dorsi muscle*

The muscle overall antioxidant status was measured by the determination of its total phenolic compounds concentration, of its ferric reducing antioxidant power (FRAP assay) and of its radical scavenging ability (TEAC assay). All measurements were performed on the portion of the LM previously frozen in liquid nitrogen and stored, vacuum-packaged, at -30°C.

❖ *4.2.3.1. Preparation of muscle extract for antioxidant status measurements*

Aqueous muscle extracts were prepared according to the procedure described by Estévez et al. (2007) for the determination of the concentration of phenolics in meat with some modifications. Muscles were chopped into small cubes and, while still frozen, were minced using a blender. For each muscle, triplicate sub-samples (500 mg) of minced meat were placed into 50 ml centrifuge tubes and 10 ml of distilled water were added. Samples were then homogenized for 60 sec at 9500 rpm using a Heidolph Diax 900 tissue homogenizer (Heidolph Elektro GmbH & Co. KG, Kelheim, Germany) and the centrifuge tubes were constantly kept in a water/ice bath during the homogenization. Samples were then subjected to sonication (cicle: 4 × 10%; power %: 0.31) for a total duration of the sonication of 6 min (with a break of 2 min after the first 3 min of sonication) using a Bandelin Sonoplus HD2070 sonicator (Bandelin Electronic GmbH & Co. KG, Berlin, Germany). The tubes were kept in a water/ice bath during the sonication. The sonicated ho-

mogenates were centrifuged at 3000 × *g* for 15 min at 4°C (Thermo, model IEC CL31R; Thermo Fisher Scientific, Milan, Italy). Then, the supernatant was filtered through Whatman 541 filter paper.

❖ *4.2.3.2. Total phenolic compounds concentration in muscle*

The concentration of total phenolics in LM was measured using the Folin-Ciocalteau reagent as described by Makkar et al. (1993). Aliquots (500 µl) of the muscle extract were transferred into 15 ml centrifuge tubes and 500 µl of distilled water were added. The Folin–Ciocalteau reagent was diluted to a concentration of 1N with distilled water and 500 µl were added to the test tubes. After 1 min, 2.5 ml of 20% (w/v) sodium carbonate were added and the reaction mixture was mixed thoroughly for 30 sec. Following an incubation of 40 min in the dark at 25°C, the samples were centrifuged at 2700 × *g* for 10 min at 4°C to remove possible sodium carbonate precipitate. The absorbance of the samples was read at 725 nm wavelength using a Shimadzu double-beam spectrophotometer (model UV-1601; Shimadzu Corporation, Milan, Italy) against a blank containing all of the reagents except the muscle extract. For each batch of samples, aqueous solutions of tannic acid were used to calibrate the assay (concentration ranging from 0 µg to 50 µg / ml; average R^2 of the calibration curves: 0.993) and the results were expressed as mg of tannic acid equivalents (TAE) / g of muscle.

❖ *4.2.3.3. Ferric reducing antioxidant power (FRAP assay)*

Following the method described by Benzie and Strain (1996), the FRAP assay was performed on the musce extracts prepared as described in section 2.4.1. The FRAP reagent was prepared freshly by mixing 10 volumes of acetate buffer (300 mM, pH 3.6) with 1 volume of 10 mmol TPTZ solution (2,4,6-tripyridyl-s-triazine in 40 mM HCl) and with 1 volume of 20 mM aqueous ferric chloride. A blank reading at 593 nm wavelength was taken immediately after mixing 400 µl of distilled water with 3.0 ml of FRAP reagent (temperature of the reaction mixture 37°C). Then, in a glass test tube, 300 µl of distilled water were mixed with 100 µl of muscle extract and 3.0 ml of warm FRAP reagent (37°C) were added (final dilution of the sample in the mixture, 1:34). The content of the tube was mixed and the absorbance at 593 nm wavelength was recorded after exactly 4 min of incubation in a water bath at 37°C. The change in absorbance ($\Delta A_{593\ nm}$) between the final reading and the blank reading was related to that obtained with solutions of Fe^{2+} of known concentrations used for each batch of samples (aqueous $FeSO_4 \cdot 7H_2O$ ranging from 0 µM to 1000 µM; average R^2 of the calibration curves: 0.999). Results were, therefore, expressed as µmol of Fe^{2+} equivalents / g of muscle.

❖ *4.2.3.4. Trolox equivalent antioxidant capacity (TEAC assay)*

The decolorization assay described by Re et al. (1999) was performed to meas-

ure the radical scavenging ability of the muscle extracts prepared as described in section 2.4.1. Briefly, the $ABTS^{\bullet+}$ radical cation was produced by reacting 25 ml of 14 mM aqueous ABTS [(2,2-azinobis-(3-ethylbenzothiazoline 6-sulfonate)] with an equal volume of 4.9 mM potassium persulphate (final concentration: 7 mM ABTS in 2.45 mM potassium persulphate). The mixture was incubated in the dark for 16 h to allow the complete radicalization of the ABTS. The $ABTS^{\bullet+}$ radical solution was diluted with PBS (phosphate buffered saline; pH 7.4) to reach an absorbance of 0.750 ± 0.020 at 734 nm and was equilibrated at 30°C. A blank reading at 734 nm wavelength was taken immediately after mixing 4.0 ml of diluted $ABTS^{\bullet+}$ solution with 40 µl of distilled water ($A_{0\text{-}Bl}$). To take into account possible spontaneous decolorization of the radical solution during the assay, another blank reading was taken after 6 min of incubation at 30°C. For the samples, 40 µl of meat extract were mixed with 4.0 ml of diluted $ABTS^{\bullet+}$ solution and the absorbance at 734 nm was recorded after exactly 6 min of incubation at 30°C. The percentage of inhibition (Inhib. %) of the $ABTS^{\bullet+}$ radical was calculated as:

$$\text{Inhib. \%} = [(A_{0\text{-}Bl} - A_6) / A_{0\text{-}Bl}] \times 100$$

Where: A_6 is the absorbance measured after 6 min of incubation in either the blank (used for measuring spontaneous decolorization of the radical solution) or in the sample (used for measuring the Inhib. % in the sample reaction mixture).

The true percentages of inhibition were obtained by subtracting the spontaneous inhibition % of the radical solution from those calculated for each sample and were related to the Inhib. % produced by solutions of known concentration of trolox in PBS used for each batch of samples (concentration ranging from 0 mM to 2 mM; average R^2 of the calibration curves: 0.999). The results were, then, expressed as µmol of trolox equivalents / g of muscle.

❖ *4.2.4. Resistance to nitrite-induced metmyoglobin formation (IMF)*

The resistance of myoglobin to induced metmyoglobin formation was tested using two methods. The first (surface reflectance method) was the method described by Sammel et al. (2002). The second procedure was a modified extraction method, performed to measure the resistance of myoglobin to induced oxidation in an aqueous extract similar to that used for the quantification of total phenols and for FRAP and TEAC assays. Measurements were performed on the portion of LM previously frozen in liquid nitrogen and stored at -30°C.

❖ *4.2.4.1. Surface reflectance method (IMFs).*

Slices (4 x 3 x 2 cm) were cut from the frozen LM, vacuum-packaged and allowed to thaw at 4°C. Haem pigments were oxidized by submerging the samples with 50 ml of 0.3% (w/v) aqueous sodium nitrite (temperature of the solution:

25°C). After 20 min of incubation at 25°C, the oxidizing solution was removed and the LM slices were immediately blotted dry. Reflectance spectra at the meat surface were recorded, using a Minolta CM 2022 spectrophotometer, by scanning each sample in triplicate on non overlapping areas and average values were calculated. Metmyoglobin percentages were calculated according to Krzywicki (1979).

❖ *4.2.4.2. Extraction method (IMFe).*

Frozen *longissimus dorsi* muscle was finely chopped using a blender. Two grams of chopped meat were homogenized at 9500 rpm with 25 ml of 0.04M sodium phosphate buffer (pH 5.6; temperature: 4°C) for 1 min using an Ultra Turrax tissue homogenizer. Samples were kept in a water/ice bath during homogenization. Homogenates were centrifuged at 6500 × *g* for 10 min at 4°C and, then, filtered through Whatman 541 filter papers. The aqueous filtrate, containing the haem pigments, was kept at room temperature for 15 min. Haem pigments were oxidized by mixing 4.5 ml of filtrate with 500 µl of 0.3% aqueous sodium nitrite. After 20 min of incubation at 25°C, the absorbances of the extract at 525 nm, 545 nm, 565 nm, 572 nm and 730 nm wavelength were recorded using phosphate buffer as a blank. As described in *Experiment One,* percentages of metmyoglobin were, then, calculated according to the method of Krzywicki (1982).

In both the reflectance and the exraction method, the resistance to induced metmyoglobin formation (IMF) was expressed as the MMb% measured after the oxidation with sodium nitrite (Mancini et al., 2008). Samples with a high resistance to induced MMb formation are characterized by low IMF values.

❖ *4.2.5. Statistical analysis*

Colour stability (H^* values), metmyoglobin percentages and $(K/S)_{572} \div (K/S)_{525}$ values over the 7-day aerobic storage were analysed with a GLM procedure with repeated measures. The model included the hours of storage (Time; 2, 24, 72, 120 and 168 hours) and the dietary treatment (Diet; C or T) and their interaction (Time × Diet) as fixed effects, while the individual lamb was included in the model as random effect. A one-way ANOVA was used to test the effect of the dietary treatment on H^* values, MMb % and $(K/S)_{572} \div (K/S)_{525}$ values, within each time of measurement. For each meat sample, H^* values, MMb% and $(K/S)_{572} \div (K/S)_{525}$ values measured over time of storage were fitted to a linear model. The slope of the linear equations (representing the rate of change in H^* values and MMb%) was, then, correlated with the antioxidant status parameters and was subjected to a one-way ANOVA in order to test the effect of the dietary treatment on the rate of change in H^* and MMb%.

A one-way ANOVA was performed to test the effect of the tannin supplementation (C vs T) on the muscle's total antioxidant status parameters (concentration of total phenolic compounds, FRAP and TEAC values).

To test the effects of the method used and of the tannin supplementation, the data of the resistance to nitrite-induced metmyoglobin formation (IMF), were analysed with a GLM procedure with repeated measures, with a model that included the method used (Method; surface reflectance or extraction), the dietary treatment (Diet; C or T) and their interaction (Method × Diet) as fixed factors, while individual lamb was considered as random factor. The interaction was removed from the model when not significant.

4.3. Results and discussion

❖ 4.3.1. Colour stability and metmyoglobin formation over aerobic storage

Regardless of the dietary treatments, a significant increase in H^* values over the 7-day aerobic storage was observed ($P < 0.0005$; Fig. 1). Hue angle values increased following a logarithmic trend with a pronounced increase from 2 h to 24 h of storage and with slighter changes thereafter. Data were, therefore, fitted to a linear model using a semi-logarithmic transformation in which the hours of storage (2, 24, 72, 120 and 168) were expressed as their natural logarithms (ln). Linearized curves are presented in Fig. 1 (significance of the linear regression: $P = 0.001$ and $P < 0.0005$, for C and T, respectively). The slope of the linear equation represents the rate of change in H^* over time. Although the effect of the dietary treatment was not detected in the repeated measures ANOVA, a significant Time × Diet interaction ($P = 0.004$) revealed a different rate of change in H^* between the meat from C and T groups. Indeed, although significant differences in H^* values between treatments were not detected at any storage time, as compared to the meat from the tannin group, a higher rate of change (the slope of the curve) in H^* was found in meat from the control group (2.29 vs 3.74, respectively; $P = 0.032$; Fig. 1). This result is consistent with that found in the *semimembranosus* muscle (SM) from the same animals used here (Luciano et al., 2009a – *Exp. 3*). In that study, the SM was previously frozen, minced and stored in high oxygen MAP. In the present study, the slower increase in H^* in the meat from the tannin-fed animals, compared to that observed in the C-fed ones, confirms the protective role of dietary polyphenols on meat colour stability even under less stressful storage conditions (aerobic storage vs MAP) and using intact slices of fresh LM, with a higher expected inherent resistance to oxidative deterioration and discolouration than minced and previously frozen SM. Larraín et al. (2008) reported slower increases in H^* over time of storage in bacon from pigs fed a phenol-rich cranberry extract as compared to that from animals fed a control diet. Furthermore, Nieto et al. (2010) reported lower increases in yellowness (b^*) and lower decreases in redness (a^*), the colour coordinates contributing to the calculation of H^*, in meat from

lambs fed a phenol-rich rosemary extract compared to meat from lambs fed a control diet.

As for H^* values, MMb% increased over storage duration ($P < 0.0005$; Fig. 2a) in meat from both C-and T-fed lambs. The same logarithmic trend of increase found for H^* values was observed and data were, thus, linearized using the same semi-logarithmic transformation adopted for H^* values. Linearized curves are, thus, presented in Fig. 2a (significance of the linear regression: $P = 0.001$ and $P = 0.002$, for C and T, respectively) and the slope of the curve was used to express the rate of MMb accumulation. Dietary treatment affected in tendency ($P = 0.087$) MMb% and a significant Time × Diet interaction ($P = 0.045$) suggested differing rates of change in MMb accumulation between the C and T groups. Indeed, the slope of the curve, for the T-fed lambs, tended to be lower than that found for the lambs fed the control diet (2.61 vs 3.96; $P = 0.09$; Fig. 2a). Moreover, as compared to the meat from the C-fed lambs, MMb percentages in meat from the T-fed ones were lower after 72 h of storage ($P = 0.05$) and tended be lower at 24 h ($P = 0.082$) and at 120 h and 168 h ($P = 0.084$). The observed increases in metmyoglobin accumulation across the 7 days of storage could explain the increases in H^* values measured in parallel. This is supported by a strong positive correlation found between the rate of increase in MMb % and the rate of change in H^* (r = 0.931; $P < 0.0005$), which confirms that the increases in H^* values over time of storage or display may be usefully considered as an indicator of meat browning (Lee et al. 2005; Luciano et al., 2009b – *Exp. 1*). Normally, the values of the $(K/S)_{572} \div (K/S)_{525}$ ratio decrease, over time of storage or display, as metmyoglobin accumulates at the meat surface (Stewart et al. 1965; McKenna et al., 2005). In the present study the average MMb% measured after 2 h of blooming was 28% and corresponded to average values of the $(K/S)_{572} \div (K/S)_{525}$ ratio of 1.08, in agreement with the linear relationship between MMb% and the $(K/S)_{572} \div (K/S)_{525}$ values reported by Stewart et al. (1965). In parallel with the increases in MMb %, the $(K/S)_{572} \div (K/S)_{525}$ values decreased thereafter ($P < 0.0005$; Fig. 2b) following a logarithmic trend. In Fig. 2b, the data are presented with the same semi-logarithmic transformation used above (significance of the linear regression: $P = 0.001$ and $P = 0.006$, for C and T, respectively). As expected, a strong negative correlation was

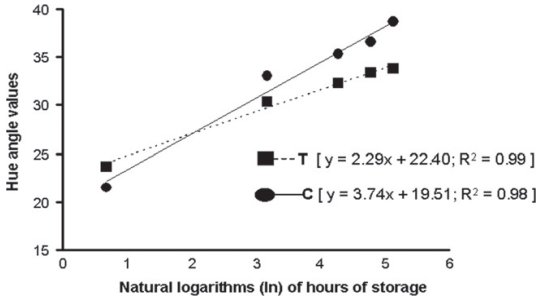

Fig. 1. Effect of the dietary treatment (C or T) and time of storage (hours 2, 24, 72, 120 and 168) on hue angle (H*) values of fresh LM steaks stored aerobically at 4 °C. The curves presented in the figure were linearised using a semi-logarithmic transformation in which the hours of storage are expressed as their natural logarithms (ln). The slope of the linear equations, presented in figure, were used to express the rate of change in H* values over time.

found between $(K/S)_{572} \div (K/S)_{525}$ values and MMb % (r = -0.991; $P < 0.0005$). As for metmyoglobin %, the values of $(K/S)_{572} \div (K/S)_{525}$ ratio showed a tendency to be affected by the dietary treatment ($P = 0.092$) and were subjected to a Time × Diet interaction ($P = 0.035$). The rate of change in the values of the ratio – the slope of the linear equation – tended to be less pronounced in meat from the tannin group compared to that observed in meat from the control group ($P = 0.07$; Fig. 2b). Moreover, as compared to the LM from C-fed animals, the $(K/S)_{572} \div (K/S)_{525}$ values in the muscle from T-fed ones was higher after 72 h ($P = 0.05$) and tended to be higher thereafter ($P = 0.1$). Finally, a strong negative correlation was found between the rate of change in $(K/S)_{572} \div (K/S)_{525}$ and the rate of change in H^* (r = -0.933; $P < 0.0005$).

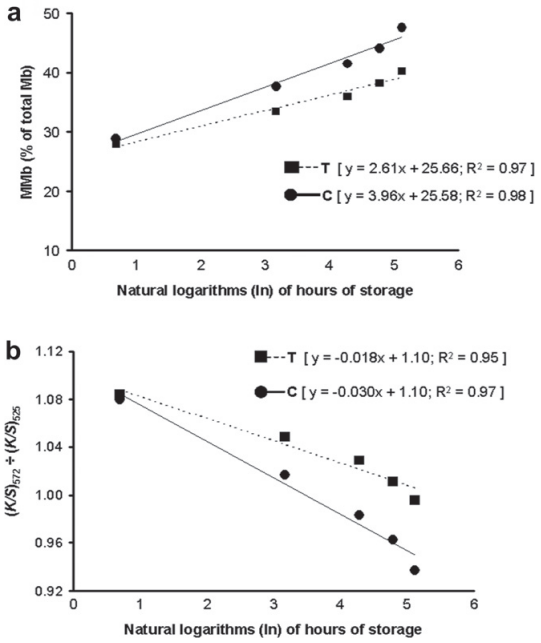

Fig. 2. Effect of the dietary treatment (C or T) and time of storage (hours 2, 24, 72, 120 and 168) on metmyoglobin (MMb) percentages (a) and on $(K/S)_{572} \div (K/S)_{525}$ values (b) of fresh LM steaks stored aerobically at 4 °C. The curves presented in the figure were linearised using a semi-logarithmic transformation in which the hours of storage are expressed as their natural logarithms (ln). The slope of the linear equations, presented in figure, were used to express the rate of accumulation of MMb and the rate of change in $(K/S)_{572} \div (K/S)_{525}$ values over time.

❖ *4.3.2. Overall antioxidant status of muscle*

❖ *4.3.2.1. Total phenolic compounds content in muscle.*

Information on the occurrence of phenolic compounds in muscle is limited, especially with respect to the effect of the dietary administration of phenolics on the presence and concentration of phenolic compounds in animal tissues. Recently, Moñino et al. (2008) showed the transmission of dietary phenolic compounds to lamb meat by detecting the presence of polyphenols in a muscle extract by HPLC analysis. In other instances, the overall phenolic compounds content in muscle has been measured using an adaptation of the assay for the determination of phenolics in plants by means of the Folin-Ciocalteau reagent (Jang et al., 2008; Estévez et al., 2007). For example, Jang et al. (2008) found a higher concentration of total phenols in meat from chicken fed a medicinal herb extract as compared to birds fed a control diet, while Estévez et al. (2007) reported a higher content of phe-

nolic compounds in frankfurters from free-range-reared pigs than those from animals fed concentrates.

In the present study, we found significantly higher concentrations of total phenols in LM from lambs fed the tannin-enriched diet as compared to that from animals fed the control diet (+31.29%; $P = 0.001$; Table 1).

Table 1

Effect of dietary treatment (C or T) on LM antioxidant status parameters.

	C	T	SEM[a]	P value
No.[b]	7	7		
Total phenols[c]	0.90	1.31	0.072	0.001
FRAP[d]	2.03	2.44	0.096	0.026
TEAC[e]	5.06	6.73	0.305	0.001

[a] SEM: standard error of the means.

[b] Number of lambs in each dietary treatment.

[c] Expressed as mg of tannic acid equivalents (TAE)/g of muscle.

[d] Expressed as μmol of Fe^{2+} equivalents/g of muscle.

[e] Expressed as μmol of trolox equivalents/g of muscle.

This result may be attributed to a transmission of polyphenols from the diet to the muscle. The concentration of total phenols and condensed tannins in the diets provided to the animals used here were published by Vasta et al. (2009b). Expressed on a dry matter basis, the control diet had 0.08% of total phenols with no detectable condensed tannins, while the tannin-supplemented concentrate had a total phenols content of 5.91%, with condensed tannins representing 4.04%. Therefore, although quebracho extract contains a high proportion of condensed tannins, it might have provided some smaller phenolic compounds that could have been directly absorbed in the gastrointestinal tract of the animals and transferred to the muscle tissue. Moreover, as already mentioned, an active metabolism of condensed tannins, carried out by intestinal microflora and leading to their depolymerization into smaller molecules, has been described (Déprez et al., 2000). Therefore, we speculated that dietary tannins, in the present study, could have undergone modifications in the intestine allowing their absorption and deposition in tissues. The average concentration of total phenols in muscles found here was lower than that reported by Estévez et al. (2007) in pork frankfurters using the Folin-Ciocalteau reagent. However, it should be noted that, although extensively and successfully used for the overall determination of total phenols in plants and plant extracts, the Folin-Ciocalteau reagent is not exclusively specific for phenolics and other reducing agents, such as ascorbic acid, can reduce it, producing interferences with the assay (Georgé et al., 2005). Besides the differences between the studies concerning the animal species and

dietary treatments, ascorbic acid was added to the meat during the frankfurter manufacture in the study of Estévez et al. (2007). This could have produced interferences with the total phenols determination giving higher concentrations than those found in the present study.

❖ *4.3.2.2. Ferric reducing antioxidant power (FRAP) and radical scavenging ability (TEAC).*

Several methods have been developed to assess the antioxidant capacity of biological samples, including animal tissue extracts. Most procedures are rapid and do not require sophisticated equipments. These methods are, generally, not specific and give a perspective of the overall antioxidant capacity of a sample without taking into account the contribution of each antioxidant component and the relationships among them (Ghiselli et al., 2000). In many cases, the antioxidant capacity of a compound involves a redox reaction. For this reason, Benzie and Strain (1996) developed a method, known as ferric reducing antioxidant power (FRAP) assay, for determining the antioxidant power of plasma as a measure of its reducing ability. The FRAP assay has been also applied to measure, in vitro, the antioxidant ability of selected polyphenols (Pulido et al., 2000). In the present study, higher FRAP values were measured in meat from T-fed lambs as compared to meat from C-fed animals (+16.81%; $P = 0.026$; Table 1). This result agrees with that reported by Moñino et al. (2008) who found enhanced FRAP values in lamb muscle as a consequence of consumption of milk from ewes fed a rosemary extract. Higher FRAP values, together with increased concentrations of antioxidant compounds, have also been reported in beef from pasture-fed animals as compared to that from concentrate-fed ones (Descalzo et al., 2007). Although other reducing compounds and metals in muscle – able to affect FRAP values - were not measured in our study, it could be speculated that the higher concentration of phenolic compounds measured in meat from the tannin-fed animals might have contributed to the greater FRAP values found in the muscle homogenates from T-fed lambs. This is supported by a positive correlation found between the concentration of phenolics and the FRAP values in muscles ($r = 0.710$; $P = 0.004$).

The antioxidant ability of a compound does not necessarily match its reducing ability and different methods for measuring the overall antioxidant capacity of a sample often use different approaches. The trolox equivalent antioxidant capacity (TEAC) assay does not measure the reducing power of an antioxidant but its radical scavenging ability, by measuring the inhibition of a pre-formed radical by antioxidant compounds (Descalzo et al., 2007). Different assays may give different information and their integrated use could be helpful for a better comprehension of the effects and mechanisms of action of antioxidants (Ghiselli et al., 2000). For example, Descalzo et al. (2007) reported higher FRAP values

in meat from pasture-fed cattle compared to concentrate-fed animals, but no differences in muscle TEAC values were found between the dietary treatments. As for the FRAP assay, the TEAC assay has been used for measuring, in vitro, the antioxidant activity of selected polyphenols (Hagerman et al., 1998). In the present study, significantly higher TEAC values were measured in muscles from T-fed lambs compared to C-fed ones (+24.81; $P = 0.001$; Table 1). In contrast with this result, Gladine et al. (2007) found no effect of dietary polyphenols on the radical scavenging ability of rat liver and muscle. However, our findings are in agreement with other studies reporting an increased radical scavenging ability of muscles from animals fed polyphenol-rich diets (Moñino et al., 2008; Jang et al., 2008). In the present study, as speculated for the results of FRAP, the higher content of total phenols in muscles from tannin-fed lambs than in muscle from C-fed animals might have contributed to enhance the radical scavenging ability measured in meat from the T group, although other antioxidants which can account for differences in TEAC values were not measured. Again, a strong positive correlation was found between TEAC values and phenols concentration in muscle ($r = 0.833$; $P < 0.0005$). Moreover, it seems that the supplementation of tannins in the diet affected the muscle's antioxidant status parameters at a similar extent as suggested by a significant positive correlation between FRAP and TEAC values ($r = 0.617$; $P = 0.019$).

❖ *4.3.3. Resistance to nitrite-induced metmyoglobin formation (IMF)*

Several methods adopted to measure the ability of the meat to reduce MMb involve a preliminary step, in which the oxidation of myoglobin is induced by the action of pro-oxidant conditions, followed by a second step in which MMb reduction is promoted: the extent of the reduction of metmyoglobin is, then, used to measure metmyoglobin reducing ability (Mancini et al., 2008). However, the rate of reduction of metmyoglobin depends on the initial level of MMb reached after the oxidation step. Different samples may differ in the inherent resistance to induced metmyoglobin formation and, therefore, the initial levels of MMb after the induced oxidation have been proposed as a measure of the resistance of the sample to induced myoglobin formation (IMF) and have been correlated to other parameters of meat discolouration (McKenna et al., 2005).

In the present study, LM steaks were treated with sodium nitrite to promote the oxidation of haem pigments and the percentages of metmyoglobin measured at the meat surface after the oxidation were taken as a measure of the sample's resistance to myoglobin oxidation (IMFs). Moreover, using a modification of the surface reflectance method, we also measured IMF in an aqueous meat extract (IMFe), in order to correlate more directly this parameter with the other antioxidant status parameters (concentration of total phenols, FRAP and TEAC), which were measured in a meat aqueous extract. The results showed an effect of the method used

(surface reflectance or extraction) on IMF values, with higher MMb % in the extraction method (average MMb%: 43.13% and 64.97% for IMFs and IMFe, respectively; $P < 0.0005$; Fig. 3). It could be speculated that sodium nitrite exerted a milder pro-oxidant effect in the intact steaks than in the aqueous extract, due to the lack of tissue structures and of some reducing compounds in the latter. Moreover, the oxidation of myoglobin is favoured by low pH values (Krzywicki, 1982) and, in the present study, in order to simulate the normal pH conditions of meat, the aqueous meat extract was buffered at pH 5.6. Conversely, LM steaks used for measuring IMFs were prepared from muscles which were excised from the carcasses immediately after slaughtering and frozen and, although pH was not measured, it is likely that, in these conditions, the LM had not attained the normal final pH of 5.5 – 5.6. Therefore, possible differences in pH between the steaks and the extracts might have contributed to the differing IMF values measured with the two procedures.

The dietary treatment (control diet or tannin-supplemented diet) strongly affected IMF values ($P < 0.0005$) but the interaction between the analytical method used (surface reflectance or extraction) and the dietary treatment was not significant. Indeed, IMF values in meat from the tannin-fed lambs were lower than those measured in meat from C-fed ones with both the surface reflectance (-10%, $P = 0.012$; Fig. 3) and the extraction method (-8.22%, $P = 0.004$; Fig. 3). This result suggests a role of dietary polyphenols in enhancing the resistance of myoglobin to oxidation and this is supported by a significant negative correlation found between IMFe and the concentration of total phenols in muscle (r = -0.710; $P = 0.004$). A negative correlation was also found between IMFs and the content of phenolic compounds in muscles (r = -0.512; $P = 0.06$). Moreover, the radical scavenging ability (TEAC) of the muscle extracts were negatively correlated with both IMFs (r = -0.514; $P = 0.05$) and IMFe (r = -0.603; $P = 0.023$). The mechanisms explaining the possible protective effect of dietary polyphenols against myoglobin oxidation are not clear. Normally, the rate of metmyoglobin accumulation in meat over time of storage seems to be related to other oxidative processes, such as lipid oxidation. However, in the *semimembranosus* muscle of the same animals used here, we have found a lower metmyoglobin formation over time in meat from the T-fed animals than in meat from

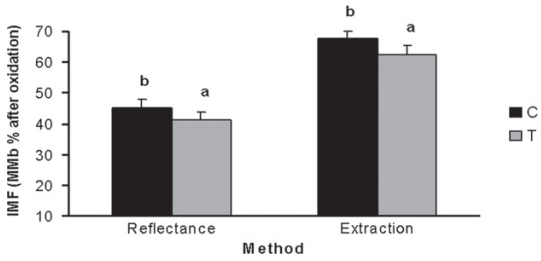

Fig. 3. Effect of method of analysis used (surface reflectance or extraction) and of the dietary treatment (C or T) on metmyoglobin percentages after nitrite-induced myoglobin oxidation (IMF). Within each analytical method, mean values bearing different superscripts are significantly different ($P \leq 0.05$). Values are means plus standard deviations.

C-fed ones, but no effect of tannins was found on lipid oxidation (Luciano et al., 2009a – *Exp. 3*). Moreover, here, the lower IMF values of meat from T-fed animals compared to C-fed lambs seem to confirm a protective role of dietary polyphenols against myoglobin oxidation that does not involve lipid oxidation. The metmyoglobin percentages after the induced oxidation of haem pigment (IMF) have been shown to correlate well with the colour stability data (Mancini et al., 2008; McKenna et al., 2005). In the present study, IMF measured with the extraction method was positively correlated with the rate of increase in hue angle values over the 7 days of storage (r = 0.631; $P = 0.015$) and also with the rate of increase in MMb % (r = 0.685; $P = 0.007$) and the rate of decrease in the $(K/S)_{572} \div (K/S)_{525}$ values (r = 0.707; $P = 0.005$) measured in parallel. The IMF values with the surface reflectance method only tended to be positively correlated with the rate of change in H^* (r = 0.487; $P = 0.078$). These results confirm the validity of the IMF as an indicator and predictor of meat colour stability. Moreover, the modified extraction procedure could help estimating the resistance of myoglobin to induced oxidation in the situations in which a spectrophotometer that measures surface reflectance is not available.

4.4. Conclusions

The results of the present study showed that the supplementation of quebracho extract in the diet of lambs produced an improvement in the overall antioxidant status parameters of *longissimus dorsi* muscle. Specifically, compared to lambs fed a control diet, higher concentrations of total phenolic compounds were measured in the muscles of the animals given tannins. In parallel, muscle from tannin-fed lambs had a higher antioxidant power, measured as both its ferric reducing ability and its radical scavenging ability. Moreover, compared to muscle from C-fed lambs, fresh LM from T-fed animals showed a greater colour stability, with lower rates of increase in hue angle and in metmyoglobin percentages over 7 days of aerobic refrigerated storage. These results confirm, under less stressful storage conditions and with a more colour-stable muscle, previous findings showing a positive effect of dietary tannins on meat colour stability in pre-frozen and minced *semimembranosus* muscles stored in high oxygen atmospheres. Finally, LM from the tannin-fed lambs showed a higher resistance to induced metmyoglobin formation than muscle from the C-fed ones. Therefore, although the possible mechanisms by which dietary polyphenols affect meat colour stability are not clear and other antioxidants and reducing compounds were not measured in this study, it seems that phenolics transferred into muscles from the diet could contribute to enhance the muscle's overall antioxidant status and to protect myoglobin against oxidation.

Concluding remarks

The diet of the animals has been shown to strongly affect meat oxidative stability by altering the balance between pro-oxidant and antioxidant components in muscle. It was demonstrated, in this thesis, that feeding systems based on the consumption of fresh herbage can efficaciously extend meat shelf life compared to feeding systems based on concentrate feeds. This encourages, therefore, the adoption of extensive pasture-based production systems which also meet the consumers' demands for husbandry practices more respectful of animal welfare. However, it also appears from the results presented here that changes in the composition of the diets in mixed feeding systems - including supplementation with concentrates to herbage-based diets - can have deleterious effects on meat shelf life if adequate amounts of antioxidants are not provided in parallel.

An interesting possibility of providing dietary antioxidants in order to improve meat shelf life appears to be the administration, in the diet, of plant secondary compounds with strong antioxidant properties, such as phenolic compounds. The results provided by the experiments previously described suggest promising applications of polyphenol-rich plant extracts in animal feeding, with important effects on meat shelf life and colour stability in particular. Considering the large occurrence of phenolic compounds in plants, neglected forage species or plant by-products rich in polyphenols could be promoted as sources of natural antioxidants. However, research should keep focusing on the comprehension of the fate of phenolic compounds in the animal organisms and on the biological properties of the different classes of these compounds, which would allow to select those sources of phenolic compounds that can be used with maximum profit.

In outline, the results presented in this thesis allow to conclude that, although meat oxidative stability is an issue which has been thoroughly investigated by meat scientists, further research is certainly needed for the comprehension of a number of key aspects that still remain unclear. If, on one hand, the strong impact of the animal feeding on meat shelf life has been proved, on the other hand a deeper comprehension of the basic biochemical mechanisms linking the oxidative deterioration of lipids and myoglobin in muscle would enable to design the most effective dietary strategies.

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