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Doctoral Thesis

LETIZIA INSERRA

Alternative Feeding Resources of Mediterranean Origin to Improve Lamb Meat and Pork Quality

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CONTENTS

Bibliographic Review

PREFACE

In the past decades, consumers showed a great interest in animal production, specifically meat and meat products. Meat is one of the most valuable foods and widely consumed animal product due to its high nutritional value. Meat has high digestibility coefficient and is considered as an important source of essential proteins and amino acids.

Meat is defined as "the edible part of the skeletal muscle of an animal that was healthy at the time of slaughter" (CFDAR, 1990). Meat is made up of the four major food components: protein, lipid, carbohydrate and water, and of the minor components: vitamins, enzymes, pigments and flavour compounds (Lambert et al., 1991). Both the minor and major constituents influence the texture, flavour, structure, colour and nutritive value of meat. The shelf-life of meat is limited by these meat components because they undergo progressive deterioration from the time of animal slaughter until the meat consumption.

The aim of this review is to demonstrate the typical mechanism of deterioration in meat resulting in the microbial spoilage, and the chemical oxidation of lipids and proteins.

MEAT QUALITY PARAMETERS

The quality of meat is combined results of the major and minor components, and their stability at handling and storage conditions. In addition meat quality could be directly related with the animal reproduction, breed, age, and sex. Furthermore, meat quality is also affected by external factors that include feeding, weather conditions, and slaughtering procedures, in which feeding plays a predominant role, and may directly or indirectly influence the colour and flavour of meat.

Colour is a fundamental to the consumer's choice of purchase. The colour measurement can either be subjective or objective. The objective measurement usually is carried out using the CIE (Commission Internationale de l'Eclairage) colour system. The CIE colour system is characterized by three fundamental colour coordinates: L* (lightness) which measures the light reflected (100 = all light reflected; 0 = all light absorbed); a* value measures redness (+ 60 = red; − 60 = green) and b* value that indicates yellowness (+ 60 = yellow; − 60 = blue) (CIE, 1986).

Priolo et al. (2001) compared the colour of meat of animals fed with 100% pasture and fed concentrate. The results showed that meat from pasture-fed animals was darker than meat from concentrate-fed animals. It was observed that in addition to animal diets, meat colour can also be affected by carcass fatness, meat ultimate pH, animal age, carcass weight and intramuscular fat content.

In another work done by Priolo et al. (2002), it was demonstrated that meat colour differences resulted in the different types of feed used, specifically the animals fed with high percentage of pasture had meat with low value of L*.

Flavour is another sensorial meat quality affected by feeding. The aromatic evaluation can be subjective and executed through the formation of a panel, or objective and done through the use of gas-chromatography. Priolo et al. (2001) have shown that flavour may be different depending on whether it is sheep meat

or cattle. Indeed the first one is characterized by branched-chain fatty acid and skatolo (3-methylindole), and also by some oxidation products derived from linoleic acid and its derivatives. In cattle, instead, it seems that the skatole is less important while the aromas arising from lipid oxidation are predominant. The flavour may also be influenced by the feeding system as demonstrated in a study conducted by Priolo et al. (2002) who found that differences in flavour exists between grass-fed and concentrate-fed lamb meat.

MEAT SAFETY

The extension of meat shelf–life is the primary aim of using conservation methods, thus retaining acceptable characteristics of flavour, colour, odour, texture, aroma, nutritional value and safety at a certain period. Consequently, during the slaughter, processing and storage of meat proper techniques are observed at each step in the production to reduce the accumulation of microorganisms in the meat and the corresponding biochemical reactions. In the microbiological point of view, muscles of healthy animals are without zoonosis, and are considered sterile, however the meat is subjected to continuous contamination from the bleeding phase until the consumption. In the abattoir, there is a high risk of contamination by microorganisms due the possible contact of muscle with animal hide and hair, or the break of gastrointestinal tract, or washing the carcass with contaminated water, or the use of non-sterilized instrument for dressing, or the contact with unhygienic personnel in the abattoir. Therefore, initial contamination depends on hygiene and sanitation in the abattoir, and the carcass or meat handling practices (Guerrero et al., 1995). Farmers, producers and also consumers have an important role in prevention and reduction of microbiological risk in meat and meat products. Many foodborne diseases are a consequence of consumption of contaminated meat (Olaoye, 2011), which could be due to poor hygiene practices during handling and food preparation. The most common pathogens found in fresh and frozen meat and also in meat products are *Campylobacter* spp., *Salmonella* spp., Enterohaemorrhagic *Escherichia coli* (EHEC) and *Listeria monocytogenes.*

Campylobacter jejuni is the most common bacterium of the *Campylobacter* spp. involved in the gastroenteritis. The main sources of *Campylobacter* food infection are raw and undercooked poultry and poultry products, but also it is typical in cattle. The most common symptoms of food infections are diarrhoea, fever, abdominal pains and other complications. The *C. jejuni* O:19 and also O:4 and O:1 serotypes are etiological agents of Guillain-Barrè and Miller Fischer

syndrome. The infective dose is around 500 organisms or less depending on host susceptibility (Godschalk et al., 2006).

Salmonella is most commonly isolated in cases of foodborne infections, both sporadic and epidemic. It is found in nature with more than 2000 serotypes but the strains most commonly spread in humans and in animal species, particularly those raised for food chain, are *S. enteritidis* and *S. typhimurium*. In Europe cases of salmonellosis are typical after the intake of eggs or egg products, but also raw and under cooked poultry, pork sausages, hams and salamis. Symptoms typical of gastroenteritis include diarrhoea, fever and abdominal pains.

Enterohaemorrhagic *E. coli* (EHEC) are the main pathogens of foodborne causing bacteria (Mor-Mur & Yuste, 2010). They are etiological agents of haemorrhagic colitis, as well as touch off complications such as thrombotic thrombocytopenic purpura or haemolytic uremic syndrome. Among these pathogenic bacteria, *E. coli* O157:H7 is the most hazardous, which is typical in swine and poultry, but can also be isolated in domestic and wild animals like sheep, goats, dogs, cats, horses and deer. The most common source of infection by *E. coli* O157:H7 are raw or uncooked meat. *E. coli* O157:H7 endures drying, chilling and fermentation storage (Mor-Mur & Yuste, 2010), and increase resistance to antibiotics, especially sulfisoxazole, tetracyclines and streptomycin.

Listeria monocytogenes is an environmental pathogen that may contaminate food in many phases of the food chain (Olaoye, 2011) and can grow in raw meat and ready-to-eat swine products. *L. monocytogenes* infections, which occurs even at low doses, are not common but poses risks for public health not due to typical stomach problems but because it causes meningitis or meningoencephalitis and abortion risking children, pregnant women, and immunocompromised occur even at low doses. *L. monocytogenes* is a facultative anaerobe thus resists vacuum packaging or modified atmosphere, and can also grow at low temperatures (Glass & Doyle, 1989). *L. monocytogenes* has been shown to be reduced by use of alginate with organic acid (Siragusa & Dickinson, 1993).

Antimicrobial packaging might be a method to increase the shelf-life and most of all to improve the safety of meat and ready-to-eat meat products by control of microbial contamination. Antimicrobial packaging is a "type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food" (Quintavalla & Vicini, 2002). Cutter (1999) noted that a 1500 ppm of TIP (triclosan-incorporated plastic) suppressed growth of *S. typhimurium*, *E. coli* O157:H7 as well as *S. aureus*, but no effect under refrigeration temperature or vacuum. Furthermore, presence of lipid or other components affiliated with adipose tissue reduced the antimicrobial effect of TIP. Edible films, on the other hand could reduce the loss of moisture during storage of meat, or reduce the rate of rancidity caused by lipid oxidation and brown coloration caused by myoglobin oxidation (Gennadios et al., 1997).

OXIDATIVE REACTIONS IN MEAT

Oxidation is defined as the introduction of electronegative atoms such as oxygen or halogens, or the removal of hydrogen in a molecule.

Oxidation is an essential process in animal metabolism. The energy released during the oxidative process, which is stored in the form of ATP, is used for many metabolic reactions of the cell. Paradoxically, the oxygen, which is essential for animal metabolic processes, becomes a threat to animal health by causing cellular damages under uncontrolled oxidation reactions (autoxidation) or in the form of reactive oxygen species (ROS). ROS are compounds with high antioxidant activity by donating oxygen to other species to other substances. ROS can be generated by ionizing radiation, metal ion-catalysed reactions, enzyme catalysed redox reactions and photochemical processes. Some important ROS in organisms are hydroxyl radical (OH^-), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Stadtman & Berlett, 1997), which can attack all cellular components. Among the important targets of ROS are the lipids of high degree of unsaturation, and more importantly the proteins leading to denaturation and consequent alterations in metabolic and structural functionalities.

Lipid peroxidation has been studied for decades making it the first focus of study in the oxidative deterioration in foods, while the protein oxidation has been ignored for a long time. Lipid oxidation is considered to be the main cause of meat and meat products quality deterioration (Morrissey et al., 1998). Lipids give flavours to cooked meat, while oxidized lipids reduce the nutritive value and sensory characteristics of the meat (Morrissey et al., 1998).

Lipid oxidation occurs as a consequence of the absorption of oxygen by the side of unsaturated, free or esterified fatty acids, and is catalysed by light, high heat or metals.

The process of lipid oxidation is divided into three phases: induction (initiation), propagation and termination phase. The induction phase (reaction 1)

consists of the detachment of a hydrogen atom from the hydrocarbon chain of a fatty acid resulting in the formation of a highly reactive alkyl radical. In the presence of oxygen this one leads to formation of peroxyl radicals, which can react with a new molecule of fatty acid resulting in a chain of reaction (propagation phase, reactions 2 and 3). The reaction between fatty acid and peroxyl radical leads to formation of lipid hydroperoxides. The termination phase (reactions 4, 5 and 6) occurs when all the radicals turn into neutral species as a result of mutual collisions (Frankel, 1984; Morrissey et al., 1998).

Induction $R-H \xrightarrow[\text{light,high heat or metals}]{} R\cdot + H\cdot$ (1)

Propagation $R\cdot + O_2 \rightarrow R + OO\cdot$ (2)

$R-OO\cdot + R-H \rightarrow R-OOH + R\cdot$ (3)

Termination $R\cdot + R \rightarrow R-R$ (4)

$R\cdot + R-OO\cdot \rightarrow R-OO-R$ (5)

$2R-OO\cdot \rightarrow R-OO-R+O_2$ (6)

Radicals and hydroperoxides (R−OOH) are the primary lipid oxidation products, and during the propagation phase new radicals are obtained: namely hydroperoxy ($ROO^{\bullet}$) and alkoxy ($RO^{\bullet}$). These new radicals give rise to the secondary lipid oxidation products such as hydrocarbons, aldehydes, ketones, lactones, esters and volatile compounds, which are responsible for the unpleasant flavour or rancidity.

Recently, the interest in the study of protein oxidation has increased because of its effects on meat quality and human nutrition (Lund et al., 2011). Early studies on protein oxidation dealt with age-related diseases such as Alzheimer (Stadtman & Berlett, 1997; Davies, 2003). The discovery that myofibrillar proteins are attacked by ROS during meat maturation and storage (Martinaud et al., 1997), which results in modification of muscle proteins and consequently loss of protein functionality and meat quality, has strengthened the interest in protein

oxidation research. Oxidation of proteins in meat products was associated with reduced texture-forming ability and water-holding capacity (WHC), reduced tenderness and juiciness of meat, as well as with the activity of muscle proteases (Carlin et al., 2006; Liu et al., 2010). Protein oxidation has been associated with physico-chemical changes in protein such as reduced solubility and functionality like gelation, emulsifying properties, or water-holding capacity, showing the possible influence of protein oxidation can influence the quality of meat (Estévez, 2011). Protein oxidation in beef has been associated to decrease in muscle tenderness (Zakrys et al., 2009) due to the protein cross-linking and the alteration of the sensitivity of proteolytic enzymes to the substrates (Estévez, 2011). The latter could lower protein digestibility and in turn decreases nutritional value. Moreover the oxidation of meat protein causes a loss of particular amino acids (Park & Xiong, 2007) so in this way nutritional value of meat decrease.

Protein oxidation caused by the interaction between proteins and ROS would seem to have the same mechanism as lipid oxidation (reactions 7-11, from Estevéz, 2011). Potential initiators of protein oxidation are ROS such as $O_2^{\cdot -}$ (superoxide radical), $HO_2^{\cdot}$ (hydroperoxyl radical) and $HO^{\cdot}$ (hydroxyl radical) and also non-radical species like H_2O_2 (hydrogen peroxide) and ROOH (hydroperoxides) (Butterfield & Stadtman, 1997). Precursors for activation of these ROS are natural components of muscle like, heme pigments, unsaturated lipids, oxidative enzymes and metals.

$$PH + HO^{\cdot} \rightarrow P^{\cdot} + H_2O \qquad (7)$$

$$P^{\cdot} + O_2 \rightarrow POO^{\cdot} \qquad (8)$$

$$POO^{\cdot} + PH \rightarrow POOH + P^{\cdot} \qquad (9)$$

$$POOH + HO_2^{\cdot} \rightarrow PO^{\cdot} + O_2 + H_2O \qquad (10)$$

$$PO^{\cdot} + HO_2^{\cdot} \rightarrow POH + O_2 \qquad (11)$$

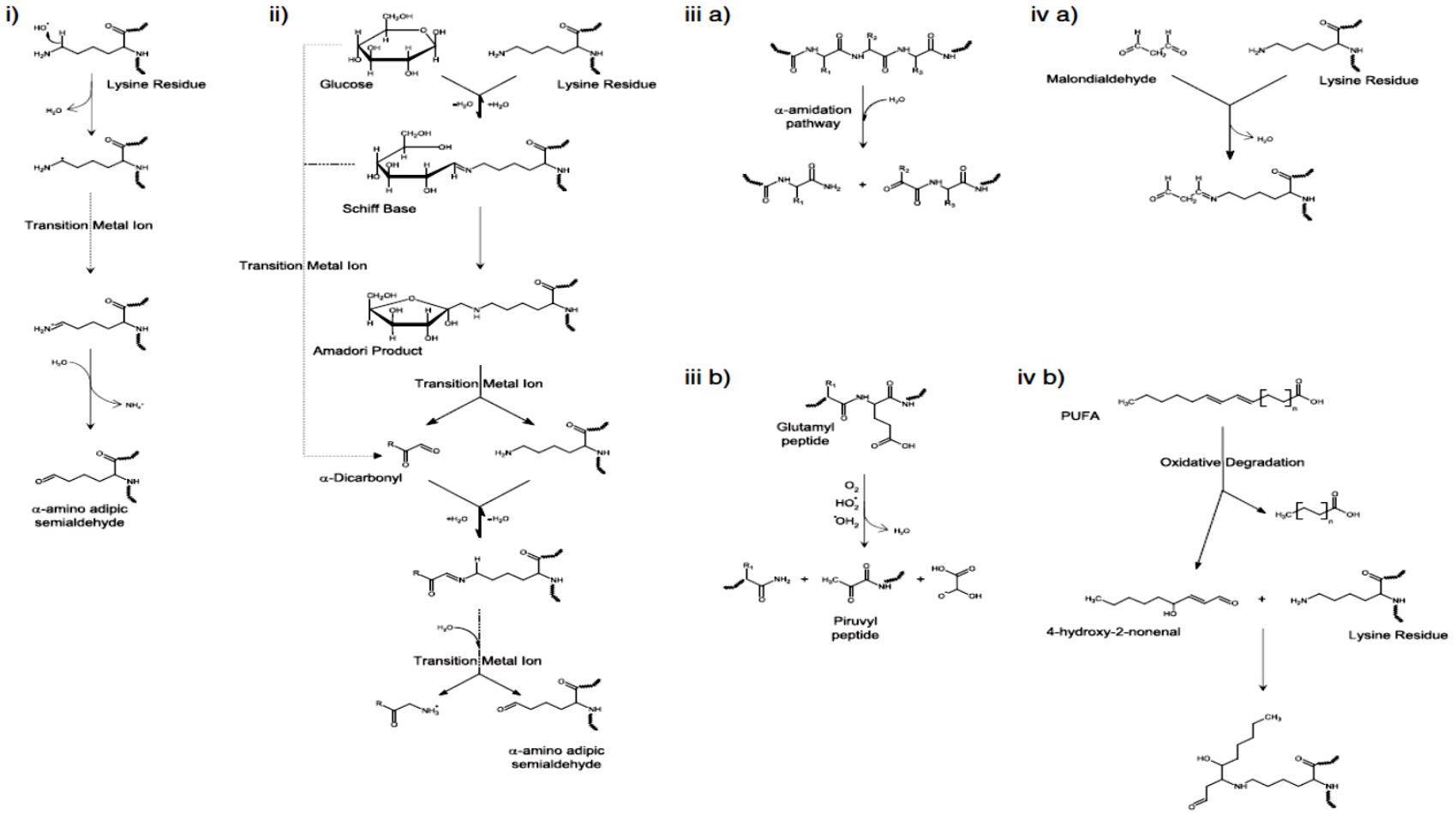
Also reactions with reduced forms of transition metals (M^{n+}) such as Fe^{2+} or Cu^{+} generate alkoxyl radical ($PO^{\bullet}$) and hydroxyl derivative (POH) (reaction 12 and 13, from Estevéz, 2011).

$$POOH + M^{n+} \rightarrow PO^{\cdot} + HO^{-} + M^{(n+1)+} \qquad (12)$$

$$PO^{\cdot} + H^{+} + M^{n+} \rightarrow POH + M^{(n+1)+} \qquad (13)$$

Among the amino acids, histidine, arginine, lysine, cysteine, tryptophan, tyrosine, phenylalanine, proline and methionine are the most susceptible to attack by ROS. In particular, cysteine and methionine are the first amino acids to be oxidized even at low concentration of ROS. For this reason they are called "sacrificial protection" because they have insignificant role in the protein functionality but have high antioxidant potential enable them to protect other amino acids against oxidation (Levine et al., 1999). On the other hand, proline, arginine and lysine are oxidized to form carbonyl compounds through metal-catalyzed reactions, while methionine or cysteine are implicated to yield sulfur-containing derivatives or in the formation of cross-links. According to Berlett and Stadtman (1997), carbonylation is an irreversible and non-enzymatic modification of proteins, which can be formed through four mechanism: i) direct oxidation of the side chains from lysine, threonine, arginine and proline (Requena et al., 2001), ii) non-enzymatic glycation in the presence of reducing sugars (Akagawa et al., 2005), iii) oxidative cleavage of the peptide backbone via the α-amidation pathway or via oxidation of glutamyl side chains (Berlett & Stadtman, 1997; Garrison, 1987) and iv) covalent binding to non-protein carbonyl compounds such 4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA) (Feeney et al., 1975) (Figure 1).

Fig. 1: (From Estevéz, 2011) Mechanisms involved in the formation of protein carbonyls. i) Metal catalyzed oxidation of basic amino acid side chains (Requena et al., 2001). ii) Non-enzymatic glycation (Akagawa et al., 2005). iii) Peptide backbone cleavage by the α-amidation pathway (a) and at glutamyl residues (Berlett & Stadtman, 1997; Garrison, 1987). iv) Binding to non-protein carbonyl compounds such as 4-hydroxy-2-nonenal (HNE) (a) or malondialdehyde (MDA) (b) (Feeney et al., 1975).

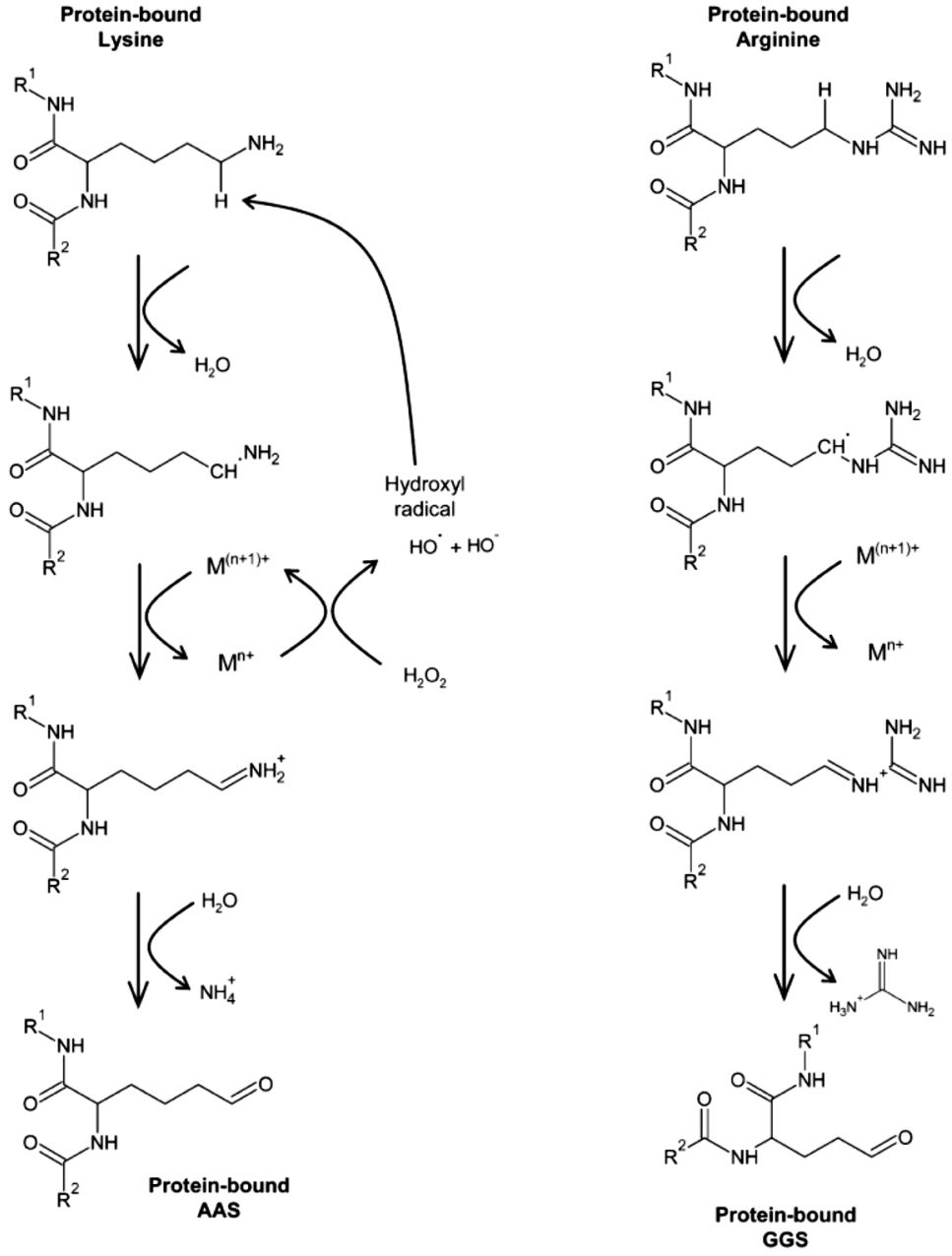


The first mechanism in the figure is the main pathway for protein oxidation (Shacter, 2000) and this has been studied for meat proteins (Estévez et al., 2009). Carbonyl residues derived from lysine, threonine, arginine and proline of myofibrillar proteins, through MCO (metal-catalyzed oxidation) system (Stadtman & Levine, 2003) follows the Fenton reaction (reaction 14) in the presence of Fe^{3+} (ferric iron) and H_2O_2:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO^- + HO^{\cdot} \quad (14)$$

The transformation of threonine in α-amino-3-keto butyric acid, lysine in α-amino adipic semialdehyde (AAS), and arginine and proline in γ-glutamic semialdehyde (GGS) occurs under MCO. Estévez et al. (2008) quantified carbonyl products in food muscle such as meat or fish through the derivatization with 2,4-dinitrophenolhydrazine (DNPH). Moreover, α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively) were identified as specific oxidation products of myofibrillar proteins using HPLC-MS. AAS and GGS have been recognized as indicators of protein oxidation in raw meat and ready-to-eat meat products (Figure 2).

Fig 2: Formation of AAS and GGS from protein-bound lysine and arginine by metal ions (M) and hydrogen peroxide. (from Estevez & Heinonen, 2010)



Thiol group of cysteine is oxidized in the presence of H_2O_2, which is formed in the cells at meat storage during post mortem. Thiol is a good marker of oxidation because they are not influenced by post-mortem aging. Oxidation of thiols leads to different reactions with the formation of various oxidation products such as sulfenic acid (RSOH), sulfinic acid (RSOOH) and disulfide cross-links (RSSR) as shown in the reactions (15-17) below (Lund et al., 2011):

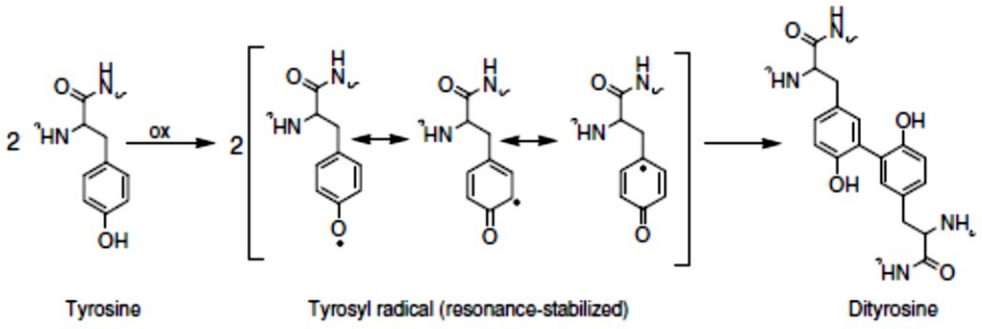
$$RSH + H_2O_2 \rightarrow RSOH + H_2O \qquad (15)$$

$$RSOH + RSH \rightarrow RSSR + H_2O \qquad (16)$$

$$2RSH + O_2 \rightarrow RSSR + H_2O_2 \qquad (17)$$

Polymerization of proteins as a result of cross-linking are the other products of oxidation of proteins, such as dityrosine, which is formed through the oxidation tyrosine residues from a radical reaction involving tyrosyl phenoxyl radical as shown below (Figure 3):

Fig. 3: from Lund et al. (2011)



Carbonyl compounds, as products of meat protein oxidation, can be generated *in vitro* using ROS-generating systems including MCO, lipid-oxidizing systems or myoglobin-mediated oxidation (Estévez, 2011). A combination of metal ions (Fe^{3+}/Cu^{2+}) with ascorbic acid also results in the formation of carbonyl residues, and found that Fe^{3+} is more effective than Cu^{2+} (Decker et al., 1993). On the one hand, Estévez and Heinonen (2010) found that Cu^{2+} is more effective than Fe^{3+} in the formation of AAS and GGS from myofibrillar protein. AAS and GGS from myofibrillar protein are formed by the presence of H_2O_2-activated system (Estévez & Heinonen, 2010). Furthermore, myoglobin can also promote protein oxidation, particularly in the protein carbonylation. In presence of H_2O_2, Mb is transformed in ferrylmyoglobin (MbFe (IV) = O), which is responsible to initiate the lipid and protein oxidation (Baron & Andersen, 2002).

The reactive oxygen species such as peroxyl radicals (ROO·) derived from lipid oxidation also promotes protein oxidation (Park et al., 2006) or possibly due to the interaction between lipid and protein oxidation (Estévez et al., 2008). However some author observed that radical such as HO· react more easily with proteins like albumin or collagen, than unsaturated fatty acids (Davies, 2005), showing that lipid and protein oxidation may occur at different rates.

Recently, Estévez et al. (2011) investigated the role of AAS and GGS on the formation of Strecker aldehydes from the amino acids leucine and isoleucine. *In vitro* the corresponding Strecker aldehyde, 3-methylbutanal and 2-methylbutanal, typical volatile compounds in meat products, were obtained (Estévez et al., 2003). This led to deduce that protein degradation products could be sources of Strecker aldehydes in the absence of oxidizing lipids and reducing sugars (Estévez et al., 2011).

The rate at which protein oxidation takes place is also influence by external factors such as pH, temperature, water activity and presence of promoters and/or inhibitors such as phenolic compounds (Estévez & Heinonen, 2010). Although the effects are unknown, it is known that the action of light and irradiation also affects protein oxidation.

The most commonly used method to measure the carbonyls produced during protein oxidation is the derivatization with 2,4-dinitrophenylhydrazine to form the 2,4-dinitrophenylhydrazone (DNPH method), which has a maximum of absorbance peak at around 370 nm. The measurement can also be carried out in HPLC, western blot or using spectrophotometer. The analysis involves the concomitant determination of carbonyl derivates and protein content in the sample (Oliver et al., 1987). Concentration of protein is determined in a control sample (without DPNH) at 280 nm using BSA as the standard. The results are expressed as nmols DNPH per mg of protein (Estévez, 2011). The DNPH method have been used in the assessment of protein oxidation in meat subjected to frozen storage such as pork (Estévez et al., 2011; Xia et al., 2009), beef (Popova et al., 2009), poultry (Rababah et al., 2010; Soyer et al., 2010), and turkey (Chan et al., 2011). The total amount of carbonyls increased after 6 month of storage at -18°C (Soyer et al., 2010). The level of carbonyl formation in meat stored under freezing conditions depends on the contemporary lipid oxidation, packaging conditions and meat processing such as pre-mincing (Estévez et al., 2011). Manufacturing and processing technologies such as γ-irradiation, which leads to the formation of ROS (Garrison, 1987), and salting, which increases the susceptibility of meat to carbonylation (Montero et al., 2005), are important areas of research but have received a little attention (Montero et al., 2005; Shimizu et al., 2009), although large amounts of carbonyl compounds have been found in cooked meat products (Estévez, 2011).

Previous studies demonstrate the importance of protein oxidation in the quality of meat and these led to looking for strategies to reduce its occurrence. One of the developments involve the use of antioxidant compounds from natural sources in animal feeding, which is a generally accepted way to improve the oxidative stability of meat (Luciano et al., 2011). Another strategy to reduce the susceptibility of meat to protein oxidation is the use of modified atmosphere or addition of potential antioxidants, such as plant phenolics in meat product formulation (Estévez et al., 2005).

CONCLUSIONS

Protein oxidation is an innovative research that calls for attention in many fields of study. There is little information about protein oxidation as compared to lipid oxidation due to some limitations such as analytical methods of assessing protein oxidation. New mechanisms to evaluate protein oxidation are necessary to understand the biochemistry, and the influence in technological processes, and to improve the meat quality which in turn affects human nutrition and health.

EXPERIMENTS

INTRODUCTION

Meat quality is an increasingly important aspect of animal production which ensures the consumers to obtain meat acceptable sensory attributes such colour and flavour among others (Liu et al., 1995) and nutritive value. The meat quality is believed to be deteriorated through oxidative reactions (Morissey et al., 1998), such as lipid oxidation, which results in the production of off-flavours in meat, while myoglobin oxidation results in discolouration of meat (McKenna et al., 2005).

Meat oxidative stability could be effectively extended with the use proper packaging systems (McMillin, 2008), by the exogenous addition of antioxidants (Zhang et al., 2010) or by adopting feeding systems that are able to improve the antioxidant status of muscle (Descalzo & Sancho, 2008). The oxidative stability of meat depends on the balance between antioxidant and pro-oxidant components in muscle (Descalzo & Sancho, 2008). The use of a diet rich in antioxidant is recommended to limit the lipid peroxidation, to protect the health of animals, to obtain high-quality products (Wood & Enser, 1997) and to extend the shelf life of muscle foods (Pokorný et al., 2001). Recently, the attention to natural antioxidant resources in meat technologies is increased (Rodríguez-Carpena et al., 2011). In particular, the latter strategies could contribute to the promotion of low-input production systems based on the use of local forages and agro-industrial by-products naturally rich in bioactive molecules, which could find valuable applications in ruminant feeding and could positively affect product quality (Vasta & Luciano, 2011).

The interest in the use of agro-industrial by-products as alternative feeding resources for ruminants is justified by the fluctuating prices and supply dynamics of conventional feeds, which lead farmers to adopt their production system accordingly. Therefore, the use of local alternative feedstuffs can offer economical advantages by reducing feeding costs and by mitigating adverse socio-environmental impacts that would otherwise arise from the disposal of several by-products (Vasta et al., 2008). Citrus fruits, mainly comprised of

oranges, lemons, grapefruits and mandarin, are widespread in the Mediterranean area. Citrus pulp are the by-products that originate from citrus fruit juice extraction, which can be dried and be used for ruminant feeding for its favourable nutrient composition. Dried citrus pulp has been used to replace high proportions of cereal concentrates in the diet with no detrimental effects on animal productivity (for a review, see Bampidis & Robinson, 2006). However, very little information has been so far provided on the effects of dietary dried citrus pulp on meat quality in general and, to the best our knowledge, no studies investigated the effects of feeding ruminants with dried citrus pulp on meat storage stability. Depending on the production and preservation procedures, citrus pulp may contain remarkable amounts of bioactive compounds naturally present in citrus fruits, including polyphenols, terpenes, carotenoids and ascorbic acid, which exhibit antioxidant properties (Balasundram et al., 2006; Abeysinghe et al., 2007; Tripoli et al., 2007). Another main component of the citrus fruits is essential oil. Essential oils have natural antimicrobial power such as citrullene and limonene (Callaway et al., 2011). Essential oils act as bacteriocidal agent when used at high concentrations, and act as bacteriostatic agent when used at low concentrations. They are effective against many microorganisms, including bacteria and some fungi (Callaway et al., 2011).

Carob tree (*Ceratonia siliqua* L.) is a Mediterranean leguminosae mainly grown in Italy, Spain, Portugal, Greece and Morocco (FAO, 2011). Traditionally, carob fruit is used in human and animal feeding. Nowadays, carob fruit has other application in food, pharmaceutical and cosmetic industries. The principal components of the carob fruit are the pods and the seeds. The seeds have high gallactomannan content and are used to produce carob gum, which is used as a natural food additive. The pods are widely used in animal feeding. They are rich in soluble sugars (about 40-50%) but they have low protein and lipid contents (Kumazawa et al., 2002). Carob fruit is characterized to contain high amount of polyphenols, but their use in animal diet is limited due to the high content of condensed tannins (3-4%; Kotrotsios et al., 2012) found to be deleterious to animal health (Priolo et al., 2000) . Condensed tannins are highly polymerized polyphenols. Polyphenols represent a heterogeneous group of plant secondary

compounds with variable chemical structure, ranging from simple molecules, such as flavonoids and phenolic acids, to the highly polymerised tannins (Hagerman et al., 1998). The antioxidant properties of phenolic compounds are well known. Kumazawa et al. (2002) reported the antioxidant activity of polyphenols contained in carob pods. In addition, Bastida et al. (2009) investigated the antioxidant activity of extracts obtained from carob fruit used as functional ingredient in preparation of pork products stored at chilling and freezing temperature.

EXPERIMENT 1[1]

1.1. Aim

The specific objective of the present research was to assess the effect of replacing cereal concentrates with high levels of dried citrus pulp in diets for growing lambs on meat lipid and colour stability.

1.2. Materials and methods

❖ *1.2.1. Animals and diets*

Twenty-nine Comisana male lambs, born in late November 2011, were weaned at 60 days of age. At 90 days of age, the lambs were weighed (average initial weight: 19.76 kg ± SD 3.84 kg) and were housed indoors in individual pens. The animals were randomly divided into three homogeneous groups and were assigned to one of the following dietary treatments: one group of 9 lambs (Control), was fed commercial concentrates including 60% of barley. One group of 10 lambs (Citrus 24%) received a mixture of the same ingredients in which the proportion of barley was reduced to 35%, while 24% dried citrus pulp was included. Another group of 10 lambs (Citrus 35%) received a diet in which the proportion of barley was further reduced to 23% and that of citrus pulp was increased up to 35%. No vitamin premix was included into the concentrates. The composition of the experimental diets is depicted in Table 1.

[1]Manuscript submitted to Meat Science Journal: Inserra, L., Priolo, A., Biondi, L., Lanza, M., Bognanno, M., Gravador, R., & Luciano, G., "*Dietary citrus pulp reduced lipid oxidation in lamb meat*".

Table 1
Ingredient and chemical composition of the experimental concentrates

	Control	Citrus 24%	Citrus 35%
Ingredients (g/100g as fed)			
Barley	60	35	23
Dried citrus pulp	0	24	35
Dehydrated alfalfa	20	19	20
Soybean meal	9	12	13
Wheat bran	11	10	9
Chemical Composition			
Dry Matter (DM)[1]	88.9	89.4	90.6
Crude Protein (CP)[2]	18.0	18.5	17.8
Neutral Detergent Fibre (NDF)[2]	34.6	31.8	33.1
Acid Detergent Fibre (ADF)[2]	13.7	16.0	18.0
Ether Extract (EE)[2]	2.2	1.6	2.2
Total Phenolic Compounds[3]	4.0	6.7	7.9

[1]Expressed as g/100g of fresh weight
[2]Expressed as g/100g of DM
[3]Expressed as g of tannic acid equivalents/kg of DM

All the ingredients composing the experimental concentrates were finely ground to pass a 5-mm screen in order to avoid selection. For 10 days before the commencement of the experimental feeding period, lambs in the three groups were adapted to the experimental diets by gradually replacing the starter concentrates with the experimental concentrates. During 56 days of experimental feeding period, the diets were offered each morning at 0900 h and the feeders were removed at 1900 h, while water was always available. The amounts of feed offered and refused were recorded every day in order to measure the daily voluntary feed intake. Samples of the feeds offered were collected 4 times during the trial, vacuum packaged and stored at -30°C for analyses. The animals were weighed weekly before the administration of the feeds. One animal from the Control group and one from the Citrus 24% group performed very poorly during the experimental period for reasons unrelated to the dietary treatments and were eliminated from the trial. Therefore, the number of lambs retained in each

treatment for the subsequent determinations was: 8 (Control group), 9 (Citrus 24% group) and 10 (Citrus 35% group).

❖ *1.2.2. Slaughter procedures and muscle sampling*

The animals were slaughtered at 158 days of age at a commercial abattoir. Lambs had access to the experimental feeds until approximately 15 min before slaughtering. Animals were stunned by captive bolt and exanguinated. The carcasses were weighed, halved and kept refrigerated at 4°C. After 24 h of refrigerated storage, the *longissimus dorsi* muscle (LM) was excised from the right half of each carcass. The pH was measured using a pH-meter Orion 9106 and the LM was vacuum packed and aged at 4°C in the dark for 4 days.

❖ *1.2.3. Laboratory analyses*

Feed samples collected during the trial were pooled and analysed for neutral detergent and acid detergent fibre fractions (NDF and ADF, respectively) according to Van Soest et al. (1991). According to AOAC (1995), feedstuffs were also analysed for crude protein (CP) and crude fat (CF) extracted with petroleum ether. As described by Makkar et al. (1993), total phenolic compounds were extracted from the feeds using aqueous acetone (70% v/v), analysed by means of the Folin-Ciocalteau reagent and expressed as tannic acid equivalents.

Regarding meat storage stability, after ageing in vacuum packaging conditions, 3 slices (2 cm thickness) were prepared from each LM using a knife. The slices were placed in polystyrene trays, covered with PVC film and stored in the dark at 4°C. Lipid and colour stability were measured after 2 h of blooming (day 0) and after 3 and 6 days of storage, using one slice of LM for each day of storage. Measurements were performed as described below.

❖ *1.2.3.1. Colour stability and myoglobin oxidation measurements*

A Minolta CM-2022 spectrophotometer (*d*/8° geometry; Minolta Co., Ltd. Osaka, Japan) was used for measuring meat colour descriptors in the CIE L*a*b* space. The following parameters were measured: Lightness (*L**), redness (*a**), yellowness (*b**), Chroma (*C**), and Hue angle (*H**).

The reflectance spectra from 400 to 700 nm wavelength were also recorded for calculation of metmyoglobin (MMb) formation as described by Krzywicki (1979).

All measurements were taken in duplicate directly on the meat surface and the mean values were computed. The spectrophotometer was set for using the illuminant A and 10° standard observer.

❖ *1.2.3.2. Lipid oxidation measurement*

Lipid oxidation was assessed by measuring 2-thiobarbituric acid reactive substances (TBARS) according to the method described by Siu and Draper (1978). Meat samples (2.5 g) were homogenised with 12.5 ml of distilled water using a Heidolph Diax 900 tissue homogenizer (Heidolph Elektro GmbH & Co. KG, Kelheim, Germany) operating at 9500 rpm. During the homogenisation, samples were put in a water/ice bath. Subsequently, 12.5 ml of 10% (w/v) trichloroacetic acid were added to precipitate proteins and then the samples were vortexed. Using a Whatman No.1 filter paper, the homogenates were filtered and 4 ml of filtrate were added to 1 ml of 0.06 M aqueous thiobarbituric acid into pyrex-glass tubes. The tubes were incubated in a water bath at 80°C for 90 min and the absorbance of each sample was read at 532 nm using a Shimadzu UV-vis spectrophotometer (UV-1601; Shimadzu Corporation, Milan, Italy). The assay was calibrated with a solution of known concentration of TEP (1,1,3,3,-tetraethoxypropane) in distilled water. Results were expressed such as mg of malonaldehyde (MDA)/kg of meat.

❖ *1.2.4. Statistical analysis*

A one-way ANOVA was used to test the effect of the dietary treatment (Control, Citrus 24% and Citrus 35%) on meat ultimate pH and colour descriptors measured after blooming.

Data of meat colour stability descriptors (a*, H* and MMb %) and lipid oxidation (TBARS values) were analysed using a mixed model. The model included the fixed effect of the dietary treatment (Diet; Control, Citrus 24%, and Citrus 35%), of the time of storage (Days; 0, 3, and 6) and their interaction (Diet × Time), while the individual animal was considered as a random effect in the model.

Multiple comparisons of the means were performed using the Tukey's adjustment. Analyses were performed using the statistical software Minitab version 16 (Minitab Inc., State College, PA).

1.3. Results and discussion

❖ *1.3.1. Muscle ultimate pH and meat colour development after blooming*

As shown in Table 2, the dietary treatment tended to affect muscle ultimate pH measured after 24 h of refrigerated storage, with values in the meat from lambs belonging to the Control group being higher in tendency compared to the Citrus 35% treatment (P = 0.052). Very little information is available on the effects of dietary citrus by-products on meat quality and it has been reported that, in lambs, muscle pH was not affected by the inclusion of dried citrus pulp in the diet (Caparra et al.; Rodrigues et al., 2008). Generally, when marked differences in muscle ultimate pH occur, a darker meat colour (lower *L** values) is associated with the highest pH values (Young et al., 1999).

Table 2
Effect of the dietary treatment on muscle pH at 24 h and on colour development after 2 hours of blooming

Item	Control	Citrus 24%	Citrus 35%	SEM	P value
pH	5.86	5.80	5.71	0.026	0.053
L* values	48.61	47.12	48.82	0.531	0.373
a* values	18.22[a]	15.60[b]	15.89[b]	0.392	0.009
b* values	12.53[a]	10.18[b]	10.77[b]	0.315	0.005
C* values	22.14[a]	18.65[b]	19.21[b]	0.482	0.005
H* values	34.46	33.01	34.09	0.417	0.364

[a, b] Within row, different superscripts indicate differences between dietary treatments ($P < 0.05$) tested using the Tukey's adjustment for multiple comparisons

However, although significant, the difference in muscle ultimate pH found in our study was not numerically large and, overall, the values found indicate a regular trend of the *post-mortem* glycolysis in muscle. This is supported by the fact that the dietary treatment did not affect the main performance parameters of lambs, such as the final weight and the daily weight gain (on average: 29.17 kg and 189.26 g/d, respectively; data not shown) which can have an impact on the muscle glycogen pool. This may partially contribute to explain the fact that meat lightness was not affected by the supplementation of the diet with citrus pulp (Table 2). The dietary treatment affected meat redness, yellowness and saturation (a*, b* and C* values, respectively; Table 2), with higher values measured in meat from lambs in the Control group compared to both the Citrus 24% and the Citrus 35% groups ($P < 0.05$). No effect of the dietary treatment was observed for hue angle (H* values). It is not easy to propose a plausible explanation for the observed effect, considering also that contrasting results have been so far provided by few studies in which different citrus by-products were supplemented in diets for different animal species. For example, the trend observed for redness and saturation is in agreement with the results found by Caparra et al. (2007) who reported a reduction in a* and C* values of lamb meat consequent to the dietary administration of dried citrus pulp. Conversely, Lanza et al. (2004) reported that meat from ostriches fed citrus pulp tended to be redder than meat from ostriches fed with conventional feedstuffs. Lastly, no effect of feeding pigs with ensiled citrus pulp was reported on meat colour (Cerisuelo et al., 2010).

❖ *1.3.2. Lipid oxidation and colour stability of meat over refrigerated storage.*

Lipid oxidation was affected by the time of storage (P< 0.0005), as TBARS values increased after 3 days, regardless of the dietary treatment (P < 0.05), followed by a stabilization thereafter (Figure 1).

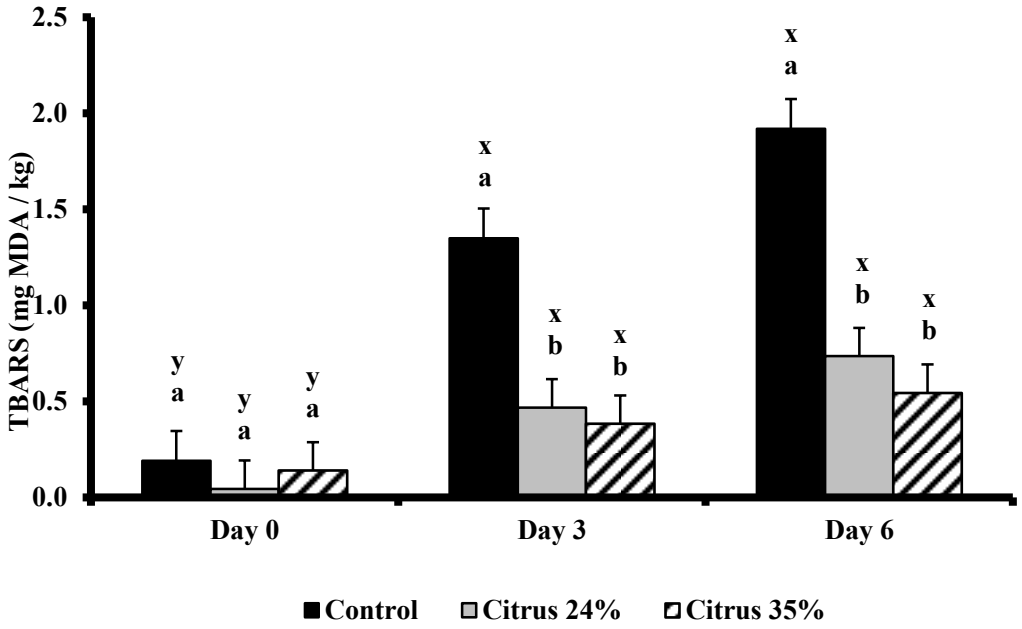


Fig 1: Interactive effect of the dietary treatment (Control, Citrus24% and Citrus35%) and time of storage (Days 0, 3 and 6) on the TBARS values measured in *longissimus dorsi* muscle slices over aerobic storage at 4°C. Values presented are the estimated least squares means and standard error bars.
[a, b] Within each day of storage, different superscripts indicate differences between dietary treatments ($P \leq 0.05$) tested using the Tukey's adjustment for multiple comparisons.
[x, y] Within each dietary treatment, different superscripts indicate differences between days of storage ($P \leq 0.05$) tested using the Tukey's adjustment for multiple comparisons.

Nevertheless, the dietary treatment as well as the Diet × Time interaction affected this parameter (P < 0.0005). After 3 and 6 days of storage, TBARS values measured in meat from lambs in the Control group were higher compared to both the Citrus 24% and Citrus 35% groups (P < 0.0005), while no difference was detected between the latter at any day of storage. Moreover, it is noteworthy that, at the end of storage, TBARS of Control group approached the value of 2 mg MDA/kg of meat which has been suggested as a threshold for the sensory detection of rancid flavours (Campo et al., 2006). Conversely, the values

measured in meat from both Citrus24% and Citrus35% were largely below this concentration, being on average 0.64 mg MDA/kg.

To our knowledge, this is the first report showing the positive effect of dietary citrus pulp in reducing lipid oxidation of meat. Generally, a higher intake of antioxidant compounds results in a deposition of these molecules in muscle with a consequent improvement of the overall muscle antioxidant capacity and stability to oxidative deterioration (Descalzo & Sancho, 2008). The protective effect of the diets containing citrus pulp against lipid peroxidation found in the present study might be explained considering the presence of antioxidant compounds in this by-product. Indeed, as discussed above, citrus by-products contain several bioactive molecules with pronounced antioxidant activity, among which phenolic compounds have been reported (Balasundram et al., 2006; Bampidis & Robinson, 2006). Similar to these reports, we found that the inclusion of dried citrus pulp among the ingredients of the diets increased their concentration of total phenols compared to the Control diet (Table 1). Some of the phenolic compounds present in citrus have been shown to be bioavailable in sheep whereby, after dietary administration of a citrus extract, naringenin was detected in plasma and was able to increase its resistance to lipid peroxidation (Gladine et al., 2007). Several studies have reported a reduction of meat oxidative deterioration in response to the administration of polyphenol-rich diets (Vasta & Luciano, 2011). Therefore, our results are in line with other studies showing the positive effects of diets rich in polyphenols against lipid oxidation.

Colour stability is mainly limited by the oxidation of myoglobin and the consequent accumulation of metmyoglobin over time (Morrissey et al., 1994). This process is responsible for meat browning and causes the progressive variation of the colour descriptors measured in meat across storage time. In particular, the decrease in meat redness and saturation (a* and C* values, respectively) and the increase in hue angle (H*) values are commonly used to describe meat colour deterioration for their positive relation with metmyoglobin concentration in meat and with the sensory evaluation of meat discolouration (Lee et al., 2005; Khliji et al., 2010; Luciano et al., 2011b). As expected, we observed

that a* and C* values decreased, while H* values and metmyoglobin % increased ($P < 0.0005$) across the 6 days of storage, regardless of the dietary treatment. Although possible mechanisms linking lipid and myoglobin oxidation are still under debate, it has been frequently reported that strategies to reduce lipid oxidation often have a positive outcome in terms of colour stability (Faustman et al., 2010). However, some studies reported a lack of correspondence between lipid and colour stability (Dunne et al., 2011; Luciano et al., 2011a; Moloney et al., 2012). In these cases, pronounced effects in lipid oxidation did not correspond to marked differences in colour stability. In the present study, while the dietary treatment clearly affected meat lipid oxidation, its effect on the colour stability descriptors was much lighter. Indeed, as previously observed for fresh meat colour measured after blooming, higher a* and C* values were overall found over 6 days of storage in meat from lambs in the Control group compared to both the Citrus 24% and the Citrus 35% treatments (Table 3). However, the Diet × Time interaction was not significant ($P > 0.05$) for any of the colour stability parameters, which indicates that the trend of variation in of the colour descriptors was not affected by the dietary treatment.

Moreover, it should be noticed that variation in the values of the colour descriptors, although significant, was not numerically large as it was particularly evident for a* and C* values. Therefore, in the experimental conditions used in the present study, colour did not appear to be a factor limiting meat storage stability. It is possible to suppose that the adoption of a modified atmosphere packaging would have allowed to extend the time of storage (McMillin, 2008) and could have better highlighted possible effects of the dietary treatment on colour stability.

Table 2
Effect of the dietary treatment and time of refrigerated storage on meat colour stability. Values presented are the least squares means with their pooled standard error

Item	Dietary treatment			Days of storage			SEM	*P* values		
	Control	Citrus 24%	Citrus 35%	0	3	6		Diet	Time	Diet × Time
a* values	15.82[a]	13.99[b]	14.34[b]	16.30[x]	14.59[y]	13.27[x]	0.361	0.056	<0.001	0.150
C* values	20.65[a]	18.21[b]	18.64[b]	19.71[x]	19.47[x]	18.31[y]	0.431	0.022	0.004	0.156
H* values	39.98	39.43	39.57	34.06[x]	41.51[y]	43.42[x]	0.468	0.837	<0.001	0.738
Metmyoglobin%	44.82	43.61	44.37	34.68[x]	47.18[y]	50.95[x]	0.749	0.756	<0.001	0.0679

[a, b] Within row, different superscripts indicate differences between dietary treatments ($P < 0.05$) tested using the Tukey's adjustment for multiple comparisons
[x, y, z] Within row, different superscripts indicate differences between days of storage ($P < 0.05$) tested using the Tukey's adjustment for multiple comparisons

4. Conclusions

The most important finding of this experiment was that increasing levels of dietary dried citrus pulp in replacement of barley made it possible to obtain a meat less prone to lipid oxidation. The effect of dietary citrus pulp on meat oxidative stability has never been investigated before and our results might be linked to the presence of antioxidant phenolic compounds in citrus pulp as confirmed by the higher overall concentration of phenolic compounds found in the diets supplemented with this by-product. With the experimental conditions adopted in this study, colour stability appeared to be not limiting the storage stability of meat, whereby slight changes in the colour descriptors were overall measured over 6 days of storage. Furthermore, changes in the colour stability parameters were not affected by the dietary treatment. It would be of interest to adopt other storage conditions, such as modified atmosphere packaging, able to extend meat storage stability in order to detect possible effects of dietary citrus pulp on meat oxidative stability over an extended storage duration. In outline, including high levels of dried citrus pulp in diets for intensively-reared lambs might represent a feasible strategy to decrease the amount of cereal concentrates without compromising animal performances and to naturally improve meat oxidative stability.

EXPERIMENT 2

2.1. Aim

This study aimed to determine the effects citrus pulp diet on the microbial spoilage in meat stored aerobically under refrigerated condition.

2.2. Materials and methods

❖ *2.2.1. Microbial Analysis*

The animal management, animal slaughter, meat sampling and storage study have been described in full detail in Experiment 1.

Meat samples from day 0 and day 6 of Control and 35% Citrus pulp groups (10 g each) were homogenized with peptone water (0.1% w/w) while in stomacher bag for 1 min. Total viable counts (TVC, log cfu g^{-1}) were determined using PCA agar incubated at 30°C for 72 h ± 3 h in a thermostat following the standard method in ISO 4833:2003. *Pseudomonas spp.* (log cfu g^{-1}) was determined using CFC agar incubated at 25°C for 44 h ± 4 h in a thermostat following the standard method in ISO 3720:2010, while *Escherichia coli* (log cfu g^{-1}) was counted on TBX agar, incubated at 44°C for 18-24 h in a thermostat following the standard method in UNI ISO 16649-2:2010.

❖ *2.2.2. Statistical analysis*

The data obtained for TVC, *Pseudomonas spp*. and *Escherichia coli* were analysed using a mixed model, in which dietary treatments (Control and Citrus 35%) and, time of storage (Days 0 and 6) and their interaction (Diet × Time) were treated as fixed effects, while the individual animal was considered as a random effect in the model.

Multiple comparisons of the means were performed using the Tukey's adjustment. Analyses were performed using the statistical software Minitab version 16 (Minitab Inc., State College, PA).

2.3. Results and discussion

❖ *2.3.1. Microbial Spoilage*

Total viable count increased significantly ($P < 0.0005$) during the six days storage period but the effect of diets was insignificant ($P > 0.05$) as well as the interaction between Diet × Time ($P > 0.05$) (Figure 1).

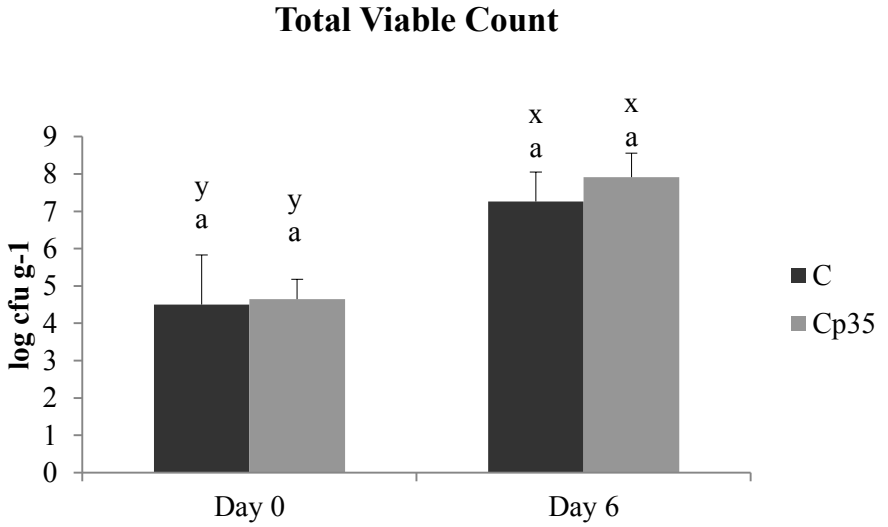


Fig 1: Interactive effect of the dietary treatment (Control and Citrus35%) and time of storage (Days 0 and 6) on the TVC determined in *longissimus dorsi* muscle slices over aerobic storage at 4°C. Values presented are the estimated least squares means and standard error bars.
[a, b] Within each day of storage, different superscripts indicate differences between dietary treatments ($P \leq 0.05$) tested using the Tukey's adjustment for multiple comparisons.
[x, y] Within each dietary treatment, different superscripts indicate differences between days of storage ($P \leq 0.05$) tested using the Tukey's adjustment for multiple comparisons.

Pseudomonas spp. increased from Day 0 to Day 6 ($P < 0.0005$) in either dietary group, while no effect of significant differences between diets was found in the same storage day. In addition, dietary treatments showed a strong tendency to be affect the *Pseudomonas* spp. counts ($P = 0.053$), while no Diet × Time interaction was observed ($P > 0.05$) (Figure 2).

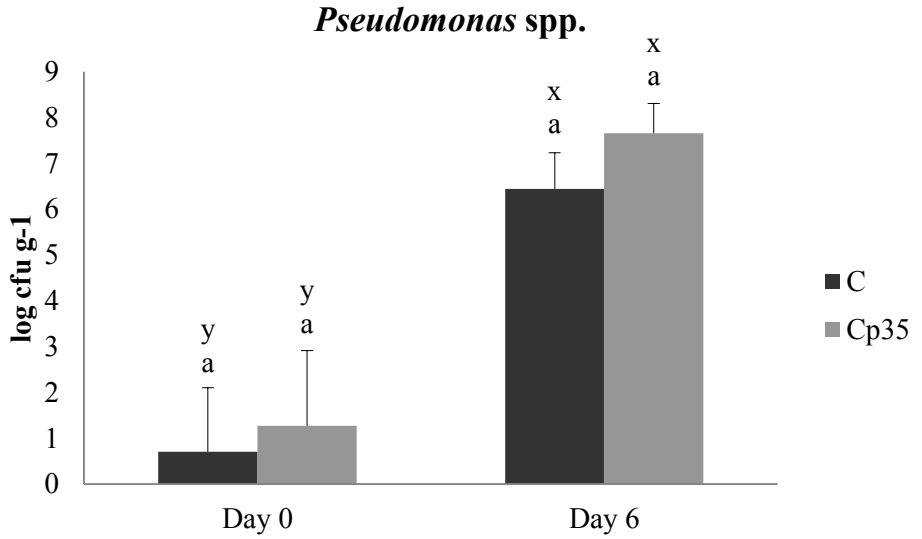


Fig 2: Interactive effect of the dietary treatment (Control and Citrus35%) and time of storage (Days 0 and 6) on the *Pseudomonas* spp. determined in *longissimus dorsi* muscle slices over aerobic storage at 4°C. Values presented are the estimated least squares means and standard error bars.
[a, b] Within each day of storage, different superscripts indicate differences between dietary treatments ($P \leq 0.05$) tested using the Tukey's adjustment for multiple comparisons.
[x, y] Within each dietary treatment, different superscripts indicate differences between days of storage ($P \leq 0.05$) tested using the Tukey's adjustment for multiple comparisons.

The population of *Escherichia coli* was not significantly affected by time of storage, dietary treatments and Diet× Time interaction ($P > 0.05$). Furthermore, no difference was observed between dietary treatments in the same day, or across time of storage for the same diet treatment (Figure 3).

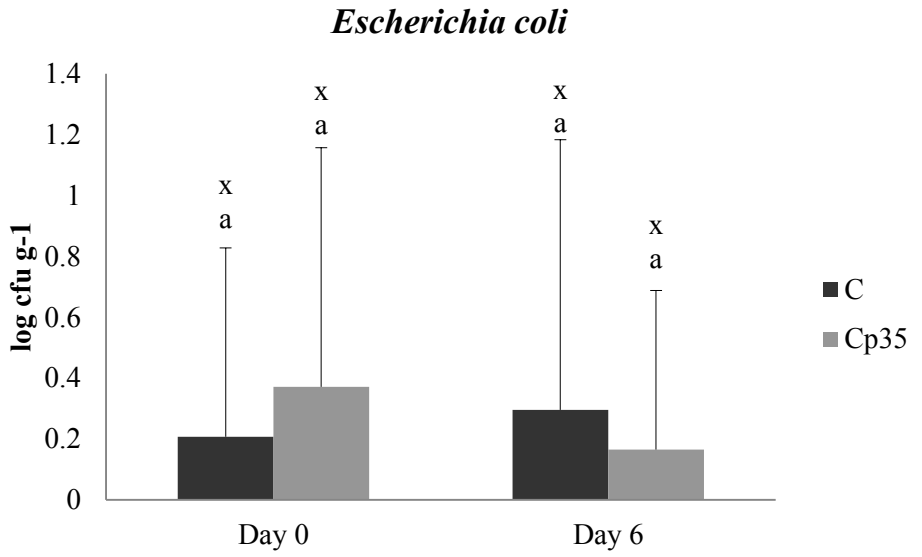


Fig 3: Interactive effect of the dietary treatment (Control and Citrus35%) and time of storage (Days 0 and 6) on the *Escherichia coli* determined in *longissimus dorsi* muscle slices over aerobic storage at 4°C. Values presented are the estimated least squares means and standard error bars.
[a, b] Within each day of storage, different superscripts indicate differences between dietary treatments ($P \leq 0.05$) tested using the Tukey's adjustment for multiple comparisons.
[x, y] Within each dietary treatment, different superscripts indicate differences between days of storage ($P \leq 0.05$) tested using the Tukey's adjustment for multiple comparisons.

The polyphenol compounds present in rosemary leaves and thyme leaves have been found to possess antimicrobial activity (Nieto et al., 2010; 2010a), while for citrus polyphenolic compounds the antimicrobial effects have not been fully investigated. The European Commission (EC) under the Regulation EC 2073/05 has established limits of (10^6 cfu / g), which was exceeded by the TVC. The *E. coli* counts, on the other hand, was lower than the EC limit and the hygienic handling procedures guaranteed value of below 1 log cfu g^{-1}. The *Pseudomonas spp.* was not significantly affected by the dietary treatments, although a significant increase in counts was observed with time of storage. Similar result was obtained by Grau (1981), in which at pH < 5.8 polyphenol compounds did not inhibit the growth of *Pseudomonas spp.* while it suppressed the growth of *Enterobacteriaceae* in meat. The *Pseudomonas spp.* are microorganisms proliferate upon storage at 4°C in aerobic conditions. These microorganisms were found to be controlled by using modified atmosphere packaging containing high CO_2 concentration as demonstrated in ostrich meat (Bigol & Ergun 2011).

4. Conclusions

The dietary citrus pulp diet did not show any effect on microbial stability of lamb meat during 6 days of storage with respect to the concentrate fed lamb meat. This could mean that proper handling procedures, and sanitation and hygienic practices were observed during the experiment. In the future study, it may be recommended to prolong the storage time to probably observe the effect of dietary treatments on the microbial spoilage of meat.

EXPERIMENT 3[1]

3.1. Aim

The objective of the present investigation was to evaluate the effect of the inclusion of two different levels of carob pulp in diets for finishing pigs on the oxidative stability of pork. Furthermore, it was studied the feeding influence of the inclusion of carob pulp in diets on the lipid composition of muscle.

3.2. Materials and methods

❖ *3.2.1. Animals and diets*

The experiment was conducted in a commercial pig farm in Sicily. Twenty-seven Large White × Pietrain pigs, born at the end of January 2012, were weaned at 30 kg bodyweight and were fed commercial concentrates until they reached 70 kg. Then, pigs were randomly assigned to one of three experimental treatments (9 animals in each group). One group (Control) was fed with commercial concentrates including 34.7% of barley. The other two groups received a mixture of the same ingredients with the inclusion of 8% and 15% of carob pulp (groups Carob 8% and Carob 15%, respectively). The composition of the diets is described in Table 1. Animals received 2 kg of concentrate/day/head and had ad libitum access to water. All animals were slaughtered in a commercial abattoir at the target live weight of 130 kg (exact mean weight reached: 131.3 ± 0.83 kg). They were electrically stunned and exsanguinated. The muscle *longissimus thoracis et lumborum* (LTL; approximately 400g) was removed from each carcass after slaughter and immediately transported, refrigerated, to the laboratory.

[1]Manuscript submitted to Meat Science Journal: Inserra, L., Bella, M., Scerra, M., Basile, P., Lanza, M., Priolo, A., & Luciano, G., "Meat quality from pigs finished on diets containing carob pulp".

Table 1: Ingredient and chemical composition of the experimental concentrates

	Control	Carob 8%	Carob 15%
Ingredients (%)			
Corn	23.5	30	36
Barley	34.7	22.5	12.5
Soya bean Meal	10	13	16
Fava bean	11	9	6.6
Wheat bran	15	11.7	8
Carob pulp	0	8	15
Soya oil	3	3	3
Minerals and vitamins[1]	2.8	2.8	2.9
Chemical Composition			
Energy (kcal /kg)	3209	3149	3127
Dry matter (DM)[2]	90.3	89.1	90.1
Ash[3]	9.9	7.2	5.5
Crude protein (CP)[3]	16.3	16.9	18.4
Neutral detergent fibre (NDF)[3]	26	20.9	19.4
Acid detergent fibre (ADF)[3]	6.69	8.41	6.72
Ether extract (EE)[3]	6.49	5.49	5.24
Total phenolic compounds[4]	2.76	2.90	3.16
Fatty acid composition (% of total extracted fatty acids)			
C12:0	0.33	0.81	0.72
C14:0	0.75	0.52	0.42
C16:0	11.17	13.64	12.50
C16:1	0.38	0.75	0.53
C18:0	2.66	3.47	3.06
trans-9 C18:1	16.26	13.27	14.75
cis-9, *cis*-12 C18:2	41.58	35.21	34.18
cis-6, cis-9, cis-12 C18:3 n-6	1.44	0.55	0.50
cis-9, cis-12, cis-15 C18:3 n-3	25.45	31.77	33.33

[1]Included: calcium carbonate, sodium chloride, total phosphorus (dicalcium phosphate, calcium phophate), premix, lysine, methionine, threonine
[2]Expressed as g/100g of fresh weight
[3]Expressed as g/100g of DM
[4]Expressed as mg of tannic acid equivalents/g of DM

❖ *3.2.2. Analyses of feedstuffs*

Experimental feed samples, collected during the trial, were analysed for neutral detergent and acid detergent fibre fractions (NDF and ADF, respectively) according to Van Soest et al. (1991). Furthermore, according to AOAC (1995), feedstuffs were also analysed for ash, crude protein and crude fat (ether extract). Total phenolic compounds were extracted from the feed samples as described by Makkar et al. (1993) using aqueous acetone (70% v/v) and analysed by means of the Folin-Ciocalteau reagent and expressed as tannic acid equivalents. The fatty acid composition of the feedstuffs was analysed by gas-chromatography using the method described by Sukhija and Palmquist (1988) and were expressed as g / 100g of total methylated fatty acids.

❖ *3.2.3. Analyses of meat samples*

Fresh LTL samples were divided into two 200-g portions. One portion was immediately vacuum-packaged and stored at -30°C pending analysis of intramuscular fatty acid composition. The remaining portion was vacuum-packaged and stored at 4°C. After 24 hours of refrigerated storage, bags were opened and the ultimate pH of LTL was measured using an Orion 9106 pH-meter equipped with a penetrating electrode (Orion Research Incorporated, Boston, MA). Then, each muscle was divided into 4 sub-samples (2 cm thickness) using a knife. The sub-samples were placed in polystyrene trays, covered with PVC film and stored in the dark at 4°C. Colour and lipid oxidation measurements were performed after 2 h of blooming (day 0) and after 5, 9 and 12 days of storage, using one sub-sample for each day of storage. All the analyses were performed as described below.

3.2.3.1. Intramuscular fatty acid composition

Intramuscular lipids were extracted according to the method used by Folch et al. (1957). Briefly, 5 g of LTL was blended with extraction solvent

chloroform/methanol (2:1, v/v) twice, filtered, placed in separator funnels and mixed with saline solution (0.88% KCl). After separation into two phases, the chloroform lipid fraction was collected and washed with distilled water/methanol (1:1, v/v). After a further filtration and evaporation by means of a rotary evaporator, lipid extracts were transferred to test tubes for subsequent gas chromatographic analysis. Duplicates of 100 mg of lipid were methylated adding 1 ml of hexane and 0.05 ml of 2 N methanolic KOH (I.U.P.A.C., 1987), and nonanoic acid (C9:0) was used as an internal standard. Gas chromatographic analysis was performed using a Varian model Star 3400 CX instrument equipped with a CP 88 capillary column (length 100 m, internal diameter 0.25 mm, film thickness 0.25 μm). Operating conditions were: a helium flow rate of 0.7 ml/min, a FID detector set at 260 °C, a split–splitless injector at 220 °C with an injection rate of 120 ml/min, an injection volume of 1 μl. The temperature program of the column was: 4 min at 140 °C and a subsequent increase to 220 °C at 4 °C/min. Retention time and area of each peak were computed using the Varian Star 3.4.1. software. The individual fatty acid peaks were identified by comparison of retention times with those of known mixtures of standard fatty acids (37 component FAME mix, 18919-1 AMP, Supelco, Bellefonte, PA) run under the same operating conditions.

Fatty acids were expressed as g /100g of total methylated fatty acids.

❖ *3.2.3.2. Colour stability and lipid oxidation measurements*

Meat colour stability was evaluated by measuring the colour descriptors *L** (lightness), *a** (redness), *b** (yellowness), *C** (saturation) and *H** (hue angle) in the CIE L*a*b* colour space. Measurements were performed using a Minolta CM 2022 spectrophotometer (*d*/8° geometry; Minolta Co. Ltd. Osaka, Japan) set to operate in the specular components excluded (SCE) mode and to measure with the illuminant A and 10° standard observer. The reflectance spectra from 400 to 700 nm wavelength were also recorded for calculation of metmyoglobin (MMb)

formation according to Krzywicki (1979). All measurements were taken in duplicate directly on the meat surface and the mean values were calculated.

Lipid oxidation was determined by measuring the 2-thiobarbituric acid reactive substances (TBARS) according to the method described by Siu and Draper (1978). Meat samples (2.5 g) were homogenized with 12.5 ml of distilled water using a Heidolph Diax 900 tissue homogenizer (Heidolph Elektro GmbH & Co. KG, Kelheim, Germany) operating at 9500 rpm. During the homogenization, samples were put in a water/ice bath. Subsequently, 12.5 ml of 10% (w/v) trichloroacetic acid were added to precipitate proteins and then the samples were vortexed. Using a Whatman No.1 filter paper, the homogenates were filtered and 4 ml of filtrate were added to 1 ml of 0.06 M aqueous thiobarbituric acid into pyrex-glass tubes. The tubes were incubated in a water bath at 80°C for 90 min and the absorbance of each sample was read at 532 nm using a Shimadzu UV/vis spectrophotometer (UV-1601; Shimadzu Corporation, Milan, Italy). The assay was calibrated with solutions of known concentration of TEP (1,1,3,3,-tetraethoxypropane) in distilled water. Results were expressed as mg of malonaldehyde (MDA)/kg of meat.

❖ *3.2.4. Statistical analyses*

Data of intramuscular fatty acids were analysed using a GLM to test the effect of the dietary treatment (Diet: Control, Carob 8% and Carob 15%). Results of meat colour stability descriptors (L*, a*, b*, C*, H* and MMb) and of lipid oxidation (TBARS values) were analysed using a GLM procedure for repeated measures. The fixed factors in the model were: the dietary treatment (Diet), the time of storage (Time; days 0, 5, 9 and 12) and their interaction (Diet × Time), while individual animal was included as a random factor.

Multiple comparisons of the means were performed using the Tukey's adjustment. The analysis was carried out using the statistical software Minitab version 16 (Minitab Inc., State College, PA).

3.3. Results and discussion

❖ *3.3.1. Muscle fatty acid composition*

The intramuscular fatty acid composition of LTL is reported in Table 2.

Table 2
Effect of the dietary treatment on the intramuscular fatty acid composition of LTL.

Item	Control	Carob 8%	Carob 15%	SEM[A]	*P* value
No. of pigs	9	9	9	-	-
Intramuscular fat (mg/100g of LTL)	2328	3283	2967	291	0.41
Individual fatty acids (g / 100g of total fatty acids)					
C12:0	0.10	0.06	0.05	0.010	0.084
C14:0	0.96[a]	0.56[b]	0.57[b]	0.071	0.023
C16:0	27.34[a]	20.42[b]	18.50[b]	1.030	< 0.001
cis-9 C16:1	3.24[a]	1.91[ab]	1.63[b]	0.271	0.028
C17:0	0.48	0.41	0.36	0.028	0.234
C18:0	9.08	10.33	11.14	0.391	0.092
cis-9 C18:1	26.82[b]	36.36[a]	38.28[a]	1.350	< 0.001
cis-9, *cis*-12 C18:2 n-6	21.49[a]	18.12[ab]	16.53[b]	0.702	0.007
cis-9, *cis*-12, *cis*-15 C18:3 n-3	0.89[c]	2.12[b]	3.18[a]	0.225	< 0.001
C20:2 n-6	0.24	0.37	0.51	0.055	0.147
C20:3 n-3 (ETA)	0.28	0.34	0.46	0.035	0.105
C20:4 n-6 (AA)	2.92	1.98	1.50	0.261	0.071
C20:5 n-3 (EPA)	0.41	0.26	0.20	0.063	0.376
C22:5 n-3 (DPA)	0.19	0.27	0.22	0.027	0.513
Classes of fatty acids (g / 100g of total fatty acids)					
SFA	37.96[a]	31.78[b]	30.61[b]	1.050	0.005
MUFA	30.06[b]	38.27[a]	39.91[a]	1.250	0.001
PUFA	26.44	23.46	22.60	0.694	0.054
n-6 PUFA	24.42[a]	20.10[b]	18.03[b]	0.807	0.001
n-3 PUFA	1.78[c]	2.99[b]	4.06[a]	0.248	< 0.001
PUFA/SFA	0.70	0.74	0.74	0.028	0.815
n-6/n-3	13.74[a]	6.72[b]	4.44[b]	1.750	< 0.001

[A] SEM: Standard error of the means.
[a,b] Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$)

The dietary treatment did not affect muscle fatness, even if the numerically highest total intramuscular fat (IMF) was found in muscle from pigs fed Carob 8% diet. Oleic acid (*cis-9* C18:1), α-linolenic acid (C18:3 n-3), n-3 poly-

unsaturated fatty acids (PUFA) and total mono-unsaturated fatty acids (MUFA) were affected by the dietary treatment ($P \leq 0.001$) and were higher in muscle from pigs fed the diets supplemented with 8% and 15% of carob pulp. Conversely, the meat from animals fed Carob 8% and Carob 15% diets contained lower percentage of palmitic acid (C16:0) as compared to the animals fed Control diet ($P < 0.001$). In the same manner meat from carob-fed animals had lower percentage of myristic acid (C14:0) and palmit-oleic acid (*cis-9* C16:1) ($P < 0.05$), linoleic acid (C18:2 n-6) and total saturated fatty acids (SFA) ($P < 0.01$), and n-6 PUFA ($P = 0.001$) with respect to meat from control-fed diet. The concentration of polyunsaturated fatty acids (PUFA) tended to be affected higher in muscle from pigs fed the Control diet compared to muscle from pigs fed the diets supplemented with 8% and 15% of carob pulp ($P = 0.055$) In outline our results showed a modification on the acid profile of meat from pigs fed diets containing either 8% and 15% of carob pulp, with an increment in n-3 PUFA rather than in total PUFA and a decrease in SFA and n-6 PUFA. Few studies evaluated the fatty acid composition of meat from animals fed with carob pulp. According to Kotrotsios et al. (2012), including carob in the diet of pigs had no significant effect on fatty acid profile of meat, except for a tendency to increase the levels of PUFA. Vasta et al. (2007) also studied the fatty acid composition of meat from lambs fed with carob-enriched diets and failed to demonstrate marked effects of the diet on the intramuscular fatty acids. However, it is not possible to directly compare studies conducted with ruminants with those using monogastric animals because, in the case of ruminants, the ruminal metabolism of fatty acids plays an important role on the fatty acid composition of the meat. Moreover, carob contains remarkable amounts of tannins which can largely affect the ruminal metabolism of fatty acids (Vasta & Luciano, 2011). In the case of monogastric animals, differences in the fatty acid composition of the diet can overall explain the fatty acid profile of the intramuscular fat. Therefore, these results observed in the present studies may be partially explained by the fatty acid composition of the experimental diets, whereby the inclusion of carob among the ingredients increased the levels of n-3 PUFA while decreasing the n-6 PUFA compared to the Control diet, as shown in Table1. This is in agreement with studies demonstrating

that carob pods contain remarkable levels of n-3 PUFA (Ayaz et al., 2009; Vekiari et al., 2011) and that the inclusion of carob pulp in the diet for livestock can increase its content of n-3 PUFA (Vasta et al., 2007).

The fact that animals fed Carob diets had lower concentration of SFA could depend on the lower content in carbohydrates (NDF) when carob was included in the diet. Indeed, carbohydrates could serve as substrate to the fat synthesis, especially for palmitic acid (Bermúdez et al., 2012). This is another important result, together with the higher concentration of MUFA resulting from feeding carob, because nutritional guidelines recommend a lower intake of SFA and a higher intake of MUFA and PUFA to prevent cardiovascular diseases (Sierra et al., 2008; Alexander, 1998). The increase in oleic acids could have also relations with important meat sensory traits, such as brightness, oiliness, juiciness, sweetness, fat hardness and cured aroma (Carrapiso et al., 2003).

In outline, from a nutritional quality perspective, the main result of this study is related to the improvement of the meat fatty acid composition consequent to the inclusion of carob in the diet. Concerning the PUFA/SFA (P/S) ratio, the UK Department of Health (1994) recommend a value above 0.4 for healthy foods and diets, while the PUFA n-6/n-3 ratio should not be higher than 4 (Simopoulos, 2002) or 6 (British Nutrition Foundation, 1992). Elevated amount of n-6 PUFA and high value of PUFA n-6/n-3 ratio promote several kind of pathogenesis such as cancer, inflammatory and autoimmune diseases, cardiovascular disease, while an opposite effect is showed with high amount of n-3 PUFA and low value of PUFA n-6/n-3 ratio (Simopoulos, 2002). In our experiment we found that the inclusion of 15% of carob in the diet strongly reduced the PUFA n-6/n-3 ratio in meat to the value of 4.44 compared to the meat from animals given the control diet in which the ratio was above 13.

❖ *3.3.2. Lipid oxidation and colour stability*

The fatty acid composition and the intramuscular fat content are key factors to explain meat lipid oxidation and provide some information about the effects of the diet on meat oxidative stability (Luciano et al., 2011a; Luciano et al., 2013). In the present study, lipid oxidation increased during the 12 days of storage period ($P < 0.001$). However, lipid oxidation was not affected by the dietary treatment or by the Diet × Time interaction (Table 3). Considering the higher percentage of n-3 PUFA in meat from carob-fed animals, a higher lipid instability could have been a drawback of including carob in the diet. Indeed, it was reported that PUFA and especially highly unsaturated PUFA in the intramuscular fat are particularly susceptible to the initiation and propagation of lipid (Morrissey et al., 1998; Wood et al., 2003). Therefore, one hand, the lack of difference in lipid oxidation between treatments could be related to the presence of phenolic compounds in the carob-supplemented diets (Table 1) that could have protected meat against oxidation despite the higher content of oxidizable substrates. On the other hand, it should be stressed that the mean values ranging from 0.060 to 0.572 mg MDA/kg of meat. These values overall indicate that meat underwent low oxidative deterioration, as the threshold TBARS value for the sensory detection of rancid flavours has been reported to be 2 mg MDA/kg of meat (Campo et al., 2006).

Table 3:
Effect of the dietary treatments and time of refrigerated storage on meat colour stability and lipid oxidation during 12 days. Values presented are the least squares means with their pooled standard error

	Dietary treatment (Diet)			Time of storage (Time)				SEM	*P* values		
Item	Control	Carob 8%	Carob 15%	0	5	9	12		Diet	Time	Diet × Time
L* values	53.36	55.08	54.56	50.60^{z}	54.08^{y}	56.83^{x}	55.82^{xy}	0.386	0.125	< 0.001	0.540
a* values	6.60	6.57	6.74	7.79^{x}	7.18^{xy}	5.59^{z}	6.00^{yz}	0.193	0.965	< 0.001	0.665
b* values	8.05	7.96	8.33	7.85	8.31	7.54	8.75	0.249	0.922	0.130	0.707
C* values	10.51	10.40	10.78	11.08	11.06	9.43	10.68	0.294	0.945	0.026	0.684
H* values	50.28	49.19	50.66	45.19^{z}	48.36^{yz}	51.55^{xy}	55.07^{x}	0.703	0.759	< 0.001	0.829
Metmyoglobin %	0.29	0.26	0.27	0.18^{z}	0.23^{yz}	0.32^{xy}	0.35^{x}	0.0145	0.770	< 0.001	0.988
TBARS (mg MDA/Kg of meat)	0.291	0.267	0.402	0.060^{z}	0.221^{y}	0.427^{x}	0.572^{x}	0.0280	0.130	< 0.001	0.504

$^{x, y, z}$ Within row, different superscripts indicate differences between days of storage ($P < 0.05$) tested using the Tukey's adjustment for multiple comparisons

Colour is the main sensory attribute for consumer's choices because they associate the red colour with freshness (Morrissey et al., 1994). Suman et al. (2006) highlighted in fresh pork the action of secondary lipid oxidation products (such as unsaturated aldehydes) on the metmyoglobin formation and accumulation on the meat surface. The oxidation of myoglobin and the consequent accumulation of metmyoglobin is the primary factor explaining changes in meat redness and yellowness in pork (Lindahl et al., 2001). In particular, the decrease in meat redness (a*) value and the increase in hue angle (H*) value are used to describe meat colour deterioration for their positive relation with metmyoglobin concentration in meat. As expected, hue angle (H*) and metmyoglobin values increased during 12 days of storage ($P < 0.001$) while the redness (a*) value decreased across the 9 days ($P < 0.001$) of storage, regardless the dietary treatment. Regarding the dietary treatment, it was not observed any effect on the colour stability descriptors (Table 3). No studies are available on the effect of feeding pigs with carob on meat colour stability. Regarding the effects of polyphenols-rich diets on pork colour stability, O'Grady et al. (2008) found no significant effects of including grape seed extract and bearberry in the diets of pigs on colour stability parameters of meat.

On one hand, a lack of effect of the diet on meat lipid oxidation could explain why differences in colour stability were not found between the experimental groups. On the other hand, as discussed above for lipid oxidation, it should be observed that in the present study the colour stability parameters were subjected to slight changes across storage duration. This is evident with regard to redness, saturation and metmyoglobin percentages which changed little over storage duration.

4. Conclusions

The results obtained with this investigation suggest the inclusion of carob pulp into concentrate-based finishing diet for pigs could be an efficient and economical feeding strategy in the Mediterranean area. These results

demonstrated that the use of carob pulp led to the increment of unsaturated fatty acids and at the same time lower percentage of saturated fatty acids, obtaining a healthier meat as request from consumers. An antioxidant effect of dietary carob pulp against meat oxidative deterioration was not observed. Nevertheless, considering that, in our experimental conditions, meat experienced slight oxidative processes, future studies should investigate the possible antioxidant effect of dietary carob pulp on the oxidative stability of pork using other storage conditions, such as modified atmosphere packaging.

CONCLUDING REMARKS

Overall, the results of this study encourage the use of citrus pulp, an agro-industrial by-product, as an alternative to cereal concentrates in lamb feeding to improve the meat quality, by delaying the oxidative phenomena in meat stored aerobically under refrigerated conditions. The lipid oxidation was suppressed in meat of lamb fed citrus pulp more than in meat of lamb fed concentrate showing the possible effects of polyphenolic compounds present in citrus pulp that could enhance the intake of natural antioxidants compared to conventional cereal-based concentrates. On the other hand, citrus pulp did not exert effects on colour stability and on microbial growth in meat over storage duration. It would be of interest to evaluate the effect of dietary citrus pulp on meat oxidative stability using different storage condition, such as modified atmosphere packaging systems.

Furthermore, the findings demonstrate the advantage of using a locally available plant material, carob pulp, in swine production for improving the polyunsaturated fatty acids content in pork, consequently producing a healthier meat compared to a conventional concentrate-based diet. However, carob pulp feeding did not improve the oxidative stability of meat as compared to the control. This could be explained by the fact that feeding carob increased the levels of readily oxidizable polyunsaturated fatty acids, which could have counterbalanced the protective effects of phenolic compounds present in carob pulp.

Therefore, the use of agro-industrial by-products rich in polyphenolic compounds could be a potential alternative to synthetic antioxidants in prolonging the shelf life of meat and could also enhance the healthy fatty acid contents in meat. Furthermore, these alternative feedstuffs could have a social and economic importance as they are cheap option for farmers in animal feeding and to reduce the production costs. Lastly, the use of these materials as feeding resources could be an efficient waste disposal management, which could result in protection from environmental pollution.

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