# UNIVERSITÀ DEGLI STUDI DI CATANIA

**Dipartimento di Agricoltura, Alimentazione e Ambiente**

# UNIVERSITY OF COPENHAGEN

**Department of Food Science, Food Chemistry Section**


**Agricultural Transformation by Innovation (AgTraIn)**
**Erasmus Mundus Joint Doctorate Programme (EMJD)**

PhD Thesis Title

## PLANT SECONDARY COMPOUNDS IN SMALL RUMINANT FEEDING: AN ALTERNATIVE TO SYNTHETIC COMPOUNDS FOR IMPROVING MEAT QUALITY IN LOW-INPUT FARMING SYSTEMS

**(2011-2014)**

by


**Rufielyn Sungcaya Gravador**

Supervisors:
Prof. Alessandro Priolo
Prof. Mogens Larsen Andersen
Dr. Marianne Lund
Dr. Giuseppe Luciano
Dr. Sisse Jongberg

# PREFACE

The PhD programme is called Agricultural Transformation by Innovation (AgTraIn), which is a three-year Erasmus Mundus Joint Doctoral Programme, funded by the European Commission. It generally aims to successfully develop and transform the farming systems in the developing world.

The PhD thesis is a requirement to be fulfilled by the candidate to be awarded the double degree by the two Universities involved in the programme: University of Catania, Italy and University of Copenhagen, Denmark.

Th PhD thesis deals with the utilization of naturally and locally available materials in the Mediterranean countries as alternative feedstuffs for lambs with the aim of reducing the production cost and improving the quality of meat without compromising the health and productivity of small ruminants. The feeding trials using lambs were done at the University of Catania. The meat quality was assessed in both Universities.

In addition to the AgTraIn funds, the PhD research was done in collaboration with the following (1) The European Community financial participation under the Seventh Framework Programme for Research, Technological Development and Demonstration Activities, for the Integrated Project LOW-INPUT BREEDS FP7-CP-IP 222623; (2) The Danish Council for Independent Research Technology and Production within The Danish Agency for Science Technology and Innovation for granting the project entitled: "Antioxidant mechanisms of natural phenolic compounds against protein cross-link formation in meat and meat systems" (11–117033); and (3)The University of Pisa under the research project: Progetto di Ricerca di Ateneo 'Improvement of nutritional and dietetic properties of beef and lamb meat: an interdisciplinary approach'. And so the Author would like to thank the proponents of the projects that helped fund the research activities, most especially to AgTraIn and European Commission.

Moreover, the author would like to express her sincerest gratitude to the people, who in one way or another, have contributed to the completion of this journey:

The Supervisors
Prof. Alessandro Priolo, Prof. Mogens Larsen Andersen
Dr. Marianne N. Lund, Dr. Sisse Jongberg,and Dr. Giuseppe Luciano

the Teaching, administrative and technical staffs of the two universities involved
in the PhD programme and the, AgTraIn committee and secretariat

Friends
Family, especially to Inay and Papa
& To The Creator.

**Rufielyn Sungcaya Gravador**
From Republic of the Philippines
December 10, 2014
University of Catania

# LIST OF PUBLICATIONS

## Experiment 1

**Gravador, R.S.**, Jongberg, S., Luciano, G., Andersen, M.L., Priolo, A., Lund, M. (2014). Dietary citrus pulp improves protein stability in lamb meat under aerobic conditions. *Meat Science, 97,* 231-236.

## Experiment 2

**Gravador, R.S.**, Luciano, G., Jongberg, S., Bognanno, M., Serra, M., Andersen, M.L., Lund, M., & Priolo, A. (Submitted 2014). Fatty acids and oxidative stability of meat from lambs fed carob-containing diets. *Food Chemistry*.

## Experiment 3

**Gravador, R.S.**, Serra, A., Luciano, G., Pennisi, P., Vasta, V., Pauselli, M., Mele, M., & Priolo, A. (2014). Volatiles in raw and cooked meat from lambs fed olive cake and linseed. *Animal,* doi:10.1017/S1751731114002730.

## Others

Inserra, L., Priolo, A., Biondi, L., Lanza, M., Bognanno, M., **Gravador, R.**, & Luciano, G. (2014). Dietary citrus pulp reduces lipid oxidation in lamb meat., *Meat Science, 96,* 1489-1493.

**Gravador, R.S.**, Jongberg, S., Andersen, M.L., Luciano, G., Priolo, A., Lund, M. (2013). Preliminary Investigation on the Effects of Dietary Citrus Pulp on Protein Oxidation in Lamb Meat. Book of Abstracts. (Page 59). 59th ICoMST International Congress of Meat and Technology. August 18-23, 2013, Izmir, Turkey

**Abbreviations and Symbols**

| | |
|---|---|
| a* | Redness descriptor measured in the CIELab colour space |
| ADG | Average daily gain |
| AFR | Alternative feeding resources |
| ALA | Alpha-linolenic acid |
| b* | Yellowness descriptor measured in the CIELab colour space |
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| BW | Body weight |
| C | Control |
| C* | Chroma measured in the CIELab colour space |
| Ca24 | 24% carob pulp |
| Ca35 | 35% carob pulp |
| CLA | Conjugated linoleic acid |
| Cp24 | 24% citrus pulp |
| Cp35 | 35% citrus pulp |
| H* | Hue angle measured in the CIELab colour space |
| HiOx-MAP | High oxygen modified atmosphere packaging |
| DHA | Docosahexaenoic acid |
| DM | Dry matter |
| DNPH | 2,4-dinitrophenylhydrazine |
| DTNB | 5,5'-dithiobis(2-nitrobenzoic acid) |
| DMI | Dry matter index |
| DPA | Docosapentaenoic acid |
| DTT | Dithiothreitol |
| EPA | Eicosapentaenoic acid |
| ESR | Electron spin resonance |
| EDTA | ethylenediaminetetraacetic acid |
| GC-MS | Gas chromatography spectroscopy |
| L | Linseed |
| L* | Lightness descriptor measured in the CIELab colour space |
| LA | Linoleic acid |
| LD | *Longissimus dorsi* |
| LDS | lithium dodecyl sulfate |
| LRI | Linear retention index |
| LNA | Linolenic acid |
| MDA | Malondialdehyde |
| MHC-CL | Myosin heavy chain cross-link |
| MMb | Metmyoglobin |
| MPI | Myofibrillar protein isolate |
| MUFA | Monounsaturated fatty acids |
| n-3 | Omega-3 fatty acids; group of essential fatty acids (LNA, EPA, DHA) |
| n-6 | Omega-6 fatty acids; group of essential fatty acids (LA, arachidonic acid) |
| $^{\bullet}OH$ | hydroxyl radical |
| PDMS | polydimethylsiloxane |
| PET | Polyethylene terephthalate |
| PUFA | Polyunsaturated fatty acids |
| PVB | Polyvinyl butyral |
| OC | Olive cake |
| OCL | Olive cake and linseed |

| | |
|---|---|
| PVC | Polyvinyl chloride |
| $R^{\bullet}$ | Alkyl radical |
| RI | Radical signal intensity |
| $RO^{\bullet}$ | Alkoxyl radical |
| $RO_2^{\bullet}$ | Peroxyl radical |
| ROOH | lipid hydroperoxide |
| ROS | Reactive oxygen species |
| SDS-PAGE | Sodium dodecyl polyacrylamide gel electrophoresis |
| SFA | Saturated fatty acids |
| SPME | Solid-phase microextraction |
| TBA | Thiobarbituric acid |
| TBARS | Thiobarbituric reactive substances |
| TCA | Tricholoacetic acid |
| VOC | Volatile organic compound |

# TABLE OF CONTENTS


**I. ABSTRACT** 1

**II. INTRODUCTION** 2

A. Meat in the diet 2

B. Modification of meat fatty acid composition 3

C. Oxidative stability of high-PUFA meats 4

D. Strategies to improve the oxidative stability of high PUFA meats 7

E. Alternative feeds 8

*1. Citrus and by-products* 9

*2. Carob and phenolic-rich plants* 10

*3. Olives and by-products* 11

F. Objectives 12

**III. EXPERIMENT 1** 13

Dietary citrus pulp improves protein stability in lamb meat stored under aerobic conditions

1.1. Introduction 14

1.2. Materials and Methods 15

1.3. Results and Discussion 18

**IV. EXPERIMENT 2** 23

Fatty acids and oxidative stability of meat from lambs fed carob-containing diets

2.1. Introduction 24

2.2. Materials and Methods 25

2.3. Results 28

2.4. Discussion 32

**V. EXPERIMENT 3** 37

Volatiles in raw and cooked meat from lambs fed olive cake and linseed

3.1. Introduction 38

3.2. Materials and Methods 39

3.3. Results and Discussion 40

**VI. CONCLUSIONS** 49

**VII. REFERENCE LIST** 50

# LIST OF TABLES


| Table No. | Title | Page No. |
|---|---|---|
| 1.1 | Effect of the dietary treatment and time of refrigerated storage on the free thiol content and carbonyl content in meat. | 19 |
| 2.1 | Ingredients and chemical composition of the diets. | 29 |
| 2.2 | Lamb growth rate, voluntary intake and feed efficiency, fatty acid intake, and ultimate pH of lamb *longissimus dorsi* muscle as affected by dietary treatments. | 29 |
| 2.3 | Effects of dietary treatments on the intramuscular fat content and fatty acid composition of lamb *longissimus dorsi* muscle. | 30 |
| 2.4 | Effect of the dietary treatment and time of refrigerated storage of lamb meat on lipid oxidation, protein oxidation, and colour parameters. | 33 |
| 3.1 | Volatile organic compounds detected in the raw *longissimus dorsi* muscle from lambs fed with diets containing olive cake and linseed. | 44 |
| 3.2 | Volatile organic compounds detected in the cooked *longissimus dorsi* muscle from lambs fed with diets containing olive cake and linseed. | 46 |

## LIST OF FIGURES


| Figure No. | Title | Page No. |
|---|---|---|
| 1 | Basic steps in the autoxidation of fatty acid, RH | 5 |
| 2 | Dynamic conversion between the different myoglobin pigments | 6 |
| 3 | The antioxidant action of $QH_2$ on free radical formed on substrate RH. | 8 |
| 1.1 | Electron Spin Resonance (ESR) spectra (a) and radical intensity (b) of myofibrillar protein isolates (MPI) extracted from lamb meat. The lambs were fed concentrate-based diet (Control), 24% citrus pulp (Cp24), or 35% citrus pulp (Cp35) in concentrate, and the meat was stored in the dark for 0, 3 and 6 days at 4°C on polystyrene trays overwrapped with oxygen- permeable PVC film. | 18 |
| 1.2 | A representative SDS-PAGE gel of non-reduced and reduced myofibrillar protein isolates (MPI) extracted from lamb meat of animals fed concentrate based diet (Control) or 35% citrus pulp mixed with concentrate (Cp35), and stored in the dark for 0 and 6 days at 4°C on polystyrene trays wrapped with oxygen- permeable PVC film. | 20 |
| 2.1 | Electron Spin Resonance (ESR) spectra (a) and radical signal intensity (RI) (b) of myofibrillar protein isolates (MPI) extracted from lamb meat. MPI samples were stored for 0, 3 and 6 days in the dark at 4°C on polystyrene trays wrapped with oxygen-permeable PVC film. The lambs were fed with diets consisting of cereal-based concentrates (Control), or concentrates containing 24% or 35% carob pulp (Ca24 and Ca35, respectively). Values presented are means plus standard errors. Values with different superscripts are significantly different ($P<0.05$). | 34 |

# I. ABSTRACT

Sheep meat is a source of polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids that confer beneficial effects to human health, but it also contains high concentration of saturated fatty acids (SFA) that are associated with the development of certain diseases. To attain a healthier intramuscular fatty acid composition, that is, meat with high PUFA to SFA ratio, and low in fatty acids *n-6* to *n-3* ratio, several strategies focused on animal feeding have been tested. However, a sufficient amount of antioxidants must be present in meat to counterbalance the increase in the content of readily oxidizable unsaturated fatty acids, which is vulnerable to oxidation, and from the associated deterioration of meat colour and proteins, thus antioxidants are crucial in preserving the nutritive value and extending the shelf life of the meat.

The increasing costs of feedstuffs for livestock led to the utilization of cheaper resources as alternatives to reduce the production cost. Here, locally available materials and agro-industrial by-products commonly found in the Mediterranean countries (citrus pulp, carob pulp, and olive cake) were used in feeding trials with lambs and the effects on meat quality were evaluated. These plant-derived materials contain considerable amounts of secondary bioactive metabolites, which affect the fatty acid composition and/or act as antioxidants. Hence, it was hypothesized that at appropriate levels of inclusion in to the lamb diets, these feedstuffs would not negatively affect animal health and productivity, and would represent a strategy to naturally produce a healthy and oxidatively stable meat.

In the first study (Experiment 1), Comisana male lambs were fed for 60 days: a conventional cereal-based concentrate diet, or concentrates in which 24% or 35% dried citrus pulp was included in partial replacement of barley. Following slaughter, the slices of *longissimus thoracis et lomborum* muscle were packed aerobically and stored for up to 6 days at 4°C in the dark. The inclusion of citrus pulp in the diet effectively inhibited protein oxidation in meat by reducing protein radicals, carbonyl formation, and thiol loss in comparison to the Control. The results showed the ability of citrus pulp to be conveniently included among the ingredients of a concentrate-based diet and to result in an improved oxidative stability of meat proteins. In the second study (Experiment 2), Comisana lambs were fed for 60 days: conventional concentrates or a diet in which cereal concentrates were partially replaced by 24% or 35% carob pulp. The results demonstrated that feeding carob pulp did not affect animal productivity and improved the muscle content of PUFA, among which is rumenic acid, and reduced the SFA concentration and the *n-6/n-3* PUFA ratio as compared to the control diet. Moreover, no extensive colour, lipid and protein oxidation in meat were observed over 6 days of aerobic storage. Therefore, dietary carob in lamb diet could be an effective strategy to improve the PUFA content in the meat without compromising animal growth performance and meat oxidative stability. In the third study (Experiment 3), the effect of feeding lambs with diets including linseed and olive cake on the evolution of volatile compounds (VOCs) in meat was assessed. Specifically, Appenninica lambs fed commercial concentrates or diets containing linseed, or olive cake, or combination of both. The VOC profile was determined through SPME-GC-MS analysis of raw and cooked meat The cooked meat showed higher concentrations of VOCs than raw meat and most of these were derived from fatty acid oxidation. Nevertheless, in both raw and cooked meat, none of the lipid oxidation-derived volatiles was significantly affected by the dietary treatments. Therefore, the results suggest that the replacement of cereal concentrates with linseed and/or olive cake in diets for lambs did not cause appreciable changes in VOC profile of meat.

# II. INTRODUCTION

Quality and cost are misconceived to be two inseparable issues, that is, high quality product demands for a high cost. Quality of meat as discussed here, deals with healthy meat that contains high PUFA to SFA ratio, and low *n-6* to *n-3* fatty acids ratio, with sensory attributes acceptable to consumers and stable across time of storage and/or display at retail. Apart from microbial spoilage, the maintenance of these quality characteristics equates to the resistance to oxidative damages of meat lipid and protein components.

The oxidative reaction in meat and meat products, which is considered to be the major source of product quality loss, can be slowed down by the use of chemical preservatives; however their application has been refused by some consumers due to the possible negative impacts to health. Cheaper and natural alternatives to these compounds have been found in locally available plants and plant materials, and in by-products from agricultural industries. These alternative feedstuffs contain plant secondary metabolites, like phenolic compounds, that could potentially exert comparable degree of antioxidant protection as the commercially available counterparts.

The Mediterranean countries are important producers of Carob (*Ceratonia siliqua L.*), olive oil extraction residues (olive cake), and wastes from citrus fruit processing (citrus pulp), which contain significant amount of plant bioactive compounds that can affect meat quality traits, such as the intramuscular fatty acid composition. The utilization of these resources in livestock feeding for meat production could possibly result in an improved nutritional quality and antioxidant capacity of the product. Therefore the aim of the thesis was to assess the effects of including these materials as ingredients in the diets for growing lambs.

Specifically the following describes the feeding trials undertaken. In the first feeding study (Experiment 1), the dried citrus pulp was used to replace the cereal concentrate in the diet fed to growing lambs. The meat was subjected to storage stability study, during which the progress of oxidative reactions was determined through the measurable changes in meat proteins. In the second feeding trial (Experiment 2), growing lambs were fed diets, in which cereal concentrates were partially replaced with carob pulp. The effect of dietary carob pulp on the meat fatty acid composition was studied. Moreover, the susceptibility of the meat to discoloration, and to lipid and protein oxidation during storage was determined. Finally, the third feeding study (Experiment 3) aimed at determining the effects of feeding lambs with diets including linseed and/or olive cake on the appearance of volatile organic compounds in raw and cooked meat.

The overall scope of these studies was to assess the utilization of these alternative feed resources that could result in the efficient conversion of agro-industrial wastes to a high-value meat and to the possible reduction of the production costs linked to animal feeding.

### A. Meat in the diet

The human diet provides essential nutrients that play a significant role in the maintenance of a healthy life, but it may also be a source of substances that are associated with the development, prevention or treatment of certain diseases such as cardiovascular diseases, hypertension, obesity, cancer and diabetes to mention a few. As a result, some consumers demand not only for nutritious and safe foods, but also for foods that serve to prevent and treat diseases and disorders, which are referred to as functional foods (Jiménez-Colmenero et al., 2001).

Meats have often been criticized for their high fat content and, for evidence that correlates red meat consumption with the risks of colon cancer, cardiovascular diseases (CVD), obesity and metabolic syndrome (McAfee et al., 2010; Biesalski, 2005). The high fat content of the meats is complemented by the presence of fatty acids, such as the omega-3, conjugated linoleic acid (CLA), and trans-vaccenic acid, which have shown positive impact on human health (Scollan et al., 2006). Meat is also an important source of monounsaturated fatty acids (MUFA; Woods & Fearon, 2009), which have been correlated to low rate of cardiovascular diseases and breast cancer, and higher life expectancy (Kiritsakis, 1999). Moreover, meat provides micronutrients such as iron, selenium, vitamins and minerals, as well as highly digestible proteins (Biesalski, 2005). Due to the benefits from red meat consumption and the inconsistencies in the research findings about the negative impact to health, it has been concluded that in a balanced diet, moderate consumption of lean red meat is unlikely to increase the risk for acquiring diseases; rather, in a long term, it may positively impact health through the influence on the intake of essential nutrients (McAfee et al., 2010).

The ratio of the saturated and unsaturated fatty acids is an important factor to assess the nutritional quality of meat. The pathogenesis of diseases such cardiovascular diseases, cancer, and inflammatory and autoimmune diseases is promoted by high intake of SFA and a low intake of PUFA and by a concurrent intake of high levels of *n-6* fatty acids and levels of low *n-3* fatty acids (low *n-6/n-3* ratio) (for a review Simopoulos, 2002). Therefore, the ratios of the PUFA to SFA, and of the *n-6* to *n-3* PUFA are used to evaluate the effects of meat on health. The recommended value for the PUFA:SFA ratio in the diet is 0.45 or above while for the PUFA *n-6:n-3* ratio should be below 4 (Department of Health and Social Security, 1984). The fatty acid profile of lambs and beef is characterized by a low PUFA:SFA ratio (Enser et al., 1998; Department of Health, 1984; Dawson et al. 2010). These facts leave challenge to the meat production industry to provide consumers with meat that is safe and with healthy fatty acid composition.

**B. Modification of meat fatty acid composition**

The fact that the nutritional composition of meat is influenced by the animal diet (Williams, 2007) has opened the opportunity for meat producers to take control over the properties of the products through strategies implemented *in vivo*, which include the animal nutrition and feeding management (Jiménez-Colmenero et al., 2001). The animal diets can play a role in the regulation of the biological processes in the muscle, which then affect the meat quality (Andersen et al., 2005). Nutritional strategies have been successful in the muscle enrichment with the healthy PUFA, such as the *n-3* PUFA, CLA, as well as in the reduction of the amount of SFA (Scollan et al., 2006; Wood et al., 2008), through the use of PUFA-rich lipid feedstuffs such as forage, oil-rich cereals, oilseeds, fish oil, marine algae, chia see, lupin, hemp and camelina (Woods & Fearon, 2009). According to Huang et al. (2008), the long-term dietary administration of *n-3* PUFA-rich sources to animals may stimulate the differentiation of pre-adipocytes into adipocytes, which enhances the deposition of these fatty acids in the intramuscular fat.

Linseed oil supplemented in the diet of Suffolk cross-wether lambs produced meat with the highest proportion of linolenic acid (C18:3*n-3*) and *cis*-9, *trans*-11 CLA in comparison to the dietary supplementation with fish oil, protected lipid, fish oil and marine algae (Nute et al., 2007). Likewise, dietary linseed supplemented in the diets of Merino lambs increased the *longissimus thoracis* content of the long chain *n-3* PUFA (C20:3*n-3*; C22:5*n-3*; C22:6*n-3;* Andrès et al., 2014). On the one hand the content of oleic acid (C18:1 *cis-9*) in the muscle was increased when animal diets were supplemented with either olive oil (Nuernberg et al., 2005) or olive cake (Mele et al., 2014). Furthermore, peas used as a protein source for lambs resulted in meat with higher

proportion of intramuscular linoleic acid (C18:*2n*-6) and linolenic acid, and their long chain fatty acid derivatives compared to the diets in which either faba beans or soybean meal were used as protein source (Scerra et al., 2011).

The meat from ruminant usually contain high levels of SFA, and in spite of the dietary strategies aiming to increase the PUFA:SFA ratio in the muscle, it rarely reaches the recommended level (Dawson et al., 2010). One reason for this is linked to the peculiar lipid metabolism that take place in the rumen, known as ruminal biohydrogenation. In this process, the dietary unsaturated fatty acids, such as linoleic and linolenic acids, are progressively isomerized and saturated to form several carbon 18 diene and monoene isomer intermediates, resulting in the formation of stearic acid (C18:0) as the final product (Bessa et al., 2007; Vasta et al., 2009b). The ruminal microorganisms that take part in the ruminal biohydrogenation process belong to the *Butyrivibrio* genus: (1) *Butyrivibrio fibrisolvens* converts linoleic acid to rumenic acid, and rumenic acid to vaccenic acid and (2) *Butyrivibrio proteoclasticus* converts vaccenic acid to stearic acid (Vasta et al., 2010b). It has been demonstrated *in vitro* and *in vivo* that some plant metabolites, such as phenolic compounds and tannins in particular, interfere with the biohydrogenation (Vasta et al., 2009a) and increase the $\Delta^9$-desaturase protein expression (Vasta et al., 2009c), which is the enzyme that catalyzes the conversion of trans-vaccenic acid to CLA in both the muscle and mammary gland. When fed to growing lambs, condensed tannins resulted in impairment of the conversion of vaccenic acid to stearic acid (Vasta et al., 2009b), while the population of *B. fibrisolvens* were higher, and that of *B. proteoclasticus* tended to be lower in the ruminal fluid from the tannin-fed lambs than from the control lambs (Vasta et al., 2010b). Furthermore, the concentration of total PUFA and of CLA was greater in both ruminal fluid and intramuscular fat from lambs fed a tannin-supplemented diets as compared to a tannin-free control diet. Taken together, these results support that dietary tannins interfere with biohydrogenation through the inhibition of the activity and proliferation of ruminal microorganisms.

**C. Oxidative stability of high-PUFA containing meats**

Feeding the animals with unsaturated fatty acids, particularly to increase the *n-3* fatty acids, is advantageous from the nutritional point of view, but may bring the drawback of decreasing the resistance of meat to oxidative deterioration of muscle lipids (Bremner et al., 1976; Warnants et al., 1996; Wood et al., 1999; Rhee et al., 1988). Although there is no simple correlations, studies have shown that lipid oxidation in meat may induce myoglobin oxidation (Monahan et al., 2005) and protein oxidation (see review Lund et al., 2011; Viljanen et al., 2004). Hence, to preserve the healthy fatty acid composition of the meat, the enhancement of the resistance of meat to oxidative deterioration upon storage and display must be attained.

In meat systems, the unsaturated fatty acids in the phospholipid membranes represent the preferential substrates for lipid oxidation, while the initiators are as follows: (1) decomposition of endogenous species ($H_2O_2$, ROOH) or radicals ($O^{2\bullet-}$, $ROO^\bullet$, $HO^\bullet$, $NO^\bullet$, $GS^\bullet$) (2) exogenous species ($^1O_2$, $O_3$) or radicals ($^\bullet NO_x$, $SO_3^{-\bullet}$), (3) agents (UV, ionizing radiation, heat), and (4) transition metals ($Fe^{n+}$, $Cu^{n+}$, etc.; Baron & Andersen, 2002; and others). The lipid oxidation process is divided into three parts: initiation, propagation, and termination (Figure 1; RH is the fatty acid substrate). At the initiation step, the hydrogen is abstracted from the unsaturated substrate to yield a free radical, which combines with oxygen to form a peroxy-free radical. This radical abstracts hydrogen from another unsaturated molecule to yield a peroxide and a new free radical, thus starting the propagation reaction. The hydroperoxides formed at the propagation step are the primary oxidation products. These are generally unstable and decompose into the secondary oxidation products, which include a variety of compounds such as carbonyls. The

propagation step can be followed by termination if the free radicals react with themselves to yield in non-active products (Frankel, 1980).

The lipid hydroperoxides decompose to form low molecular weight compounds like aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones, which are often volatile and responsible for the development of rancid flavours (Rivas-Cañedo et al., 2013). Hence, lipid oxidation influences the volatile profile of the meat, thus affecting the meat aroma/flavour, which is one of the main quality attributes of muscle foods. The aroma volatiles of cooked meat from lamb fed diets containing fish oil had higher concentration of unsaturated aldehydes, hydrocarbons and alkylfurans that are derived from the autoxidation of PUFA, compared to a not supplemented control diet (Elmore et al., 2000). Vitamin E supplementation in lamb diet resulted in lower levels of lipid oxidation-derived compounds, such as 2-heptanone and 1-penten-3-ol in meat than the control fed lambs (Rivas-Cañedo et al., 2013).

**Initiation:**

RH----------> $R^{\bullet}$ + $H^{\bullet}$

**Propagation:**

$R^{\bullet}$ + $O_2$ ----------> $RO_2^{\bullet}$

$RO_2^{\bullet}$ + RH ----------> ROOH + $R^{\bullet}$

**Termination:**

$R^{\bullet}$ + $R^{\bullet}$ ----------> R—R

$R^{\bullet}$ + $RO_2^{\bullet}$ ----------> $RO_2R$

$nRO_2^{\bullet}$ ----------> $(RO_2)_n$

Figure 1. Basic steps in the autoxidation of fatty acid, RH.

Another breakdown product of hydroperoxides is malondialdehyde (MDA), which is commonly used as a marker of the extent of lipid oxidation in muscle foods, and measured by the reacting with thiobarbituric acid (TBA; Fernández et al.,1997). In addition to the volatile profile of meat, lipid oxidation also affects the deterioration of colour, nutritive value, rheological properties, solubility and potential formation of toxic compounds such as 4-hydroxy-nonenal, upon storage of foods containing fats and oils (Baron & Andersen, 2002; Batifoulier et al., 2002).

The colour of fresh meat is determined by the ratios of the heme proteins: purple deoxymyoglobin, red oxymyoglobin and brown metmyoglobin and the shift in meat colour is dictated by the oxidation-reduction state of the central iron atom ($Fe^{2+}$ to $Fe^{3+}$) of the heme group, and is affected by factors such as temperature, pH, metmyoglobin reducing activity, oxygen partial pressure and lipid oxidation (Figure 2; Faustman et al., 2010; Mancini & Hunt, 2005). The brown metmyoglobin is undesirable to the consumers from the standpoint of the meat colour (Love and Pearson, 1970). Moreover, the interaction between metmyoglobin and $H_2O_2$ produces either perferryl myoglobin or ferryl myoglobin that are capable of oxidizing both lipids and proteins (Davies, 1990). Moreover, the dissociation of both heme from myoglobin, and iron from heme may also contribute to the mechanism by which myoglobin enhances lipid oxidation (Faustman et al., 2010).

Colour measurements are usually done through: (1) computer vision based on analysis of digital camera images, (2) instrumental colour measurement using colorimeters or spectrophotometers with different colour systems, illuminants, observers and aperture sizes, (3) measurement of

myoglobin redox forms, and (4) and visual colour appraisal (Mancini & Hunt, 2005). A correlation between the changes in the oxymyoglobin, redness descriptor (a* values in the CIE Lab colour space) and lipid oxidation measured through TBARS test in meat showed that lipid oxidation was able to enhance meat discoloration (Zakrys et al., 2008). This is in agreement with the observation that the dietary supplementation of livestock with PUFA-rich sources resulted in meat susceptible to both lipid oxidation and discoloration (Nute et al., 2007). On the other hand, the dietary supplementation with alpha-tocopheryl acetate reduced both discoloration and lipid oxidation of beef packaged in high-oxygen modified atmosphere (HiOx-MAP) conditions (Gatellier et al., 2001). Similarly, the meat from pasture finished cattle was protected from lipid oxidation and, to a lesser extent, from myoglobin oxidation than the meat from animals finished on a mixed diet (Gatellier et al., 2005).

In the muscle system, transition metals (both heme and non-heme iron), myoglobin and oxidizing lipids are generally accepted to play major roles in the initiation of protein oxidation (Salminen et al., 2008; Stadman & Levine, 2003). Protein oxidation results in changes in the functional properties of proteins due to modification of amino acid side chains, formation of protein polymers, loss of solubility, and change in proteolytic susceptibility (Lund et al., 2011; Lund et al., 2007b). Further, the oxidation of both PUFA and amino acids diminish the nutritional quality of meat (Lund et al., 2011; Mercier et al., 2004). The reduced level of meat lipid and protein oxidation with the use of rapeseed, camelina meal, and their combination with rosemary extract (Salminen et al., 2006) shows the possible link between these two oxidative reactions. On the other hand, fruit extracts or quercetin reduced protein oxidation, colour and texture deterioration of the cooked patties at chilled storage more than the control burger patties (Ganhão et al., 2010), while white grape extract preserved colour, inhibited TBARS formation, and reduced protein carbonyl formation in beef patties (Jongberg et al., 2011b).

For meat production and processing industries, any finding that focuses on delaying the oxidative changes in myoglobin, lipid and proteins in high PUFA-containing meats, and microbial spoilage, would be highly significant.

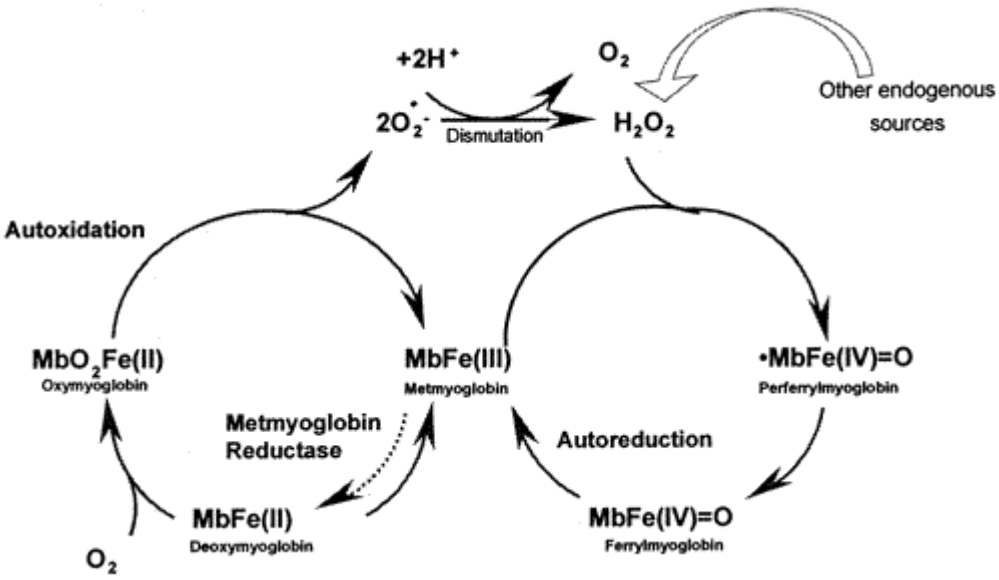


Figure 2. Dynamic conversion between the different myoglobin pigments (Baron and Andersen, 2002).

**D. Strategies to improve the oxidative stability of high PUFA meats**

The resistance of foods to oxidative changes can be increased through the use of chemically-synthesized preservatives; however, due to the associated health hazards on their use, natural substances are more preferred. Some natural antioxidants that are typically explored are the tocopherols and plant phenolics (Fernández-López et al., 2005; Govaris et al., 2004), which can be present in high levels in some agricultural by-products (Rodrígues-Carpena et al., 2011, Salminen et al., 2006), and can be delivered through the animal diet or through direct application to foods. The extracts of avocado peel and seeds used as ingredient in porcine patties inhibited discoloration, lipid and protein oxidation at chilled storage (Rodríguez-Carpena et al., 2011), similarly to when black currant (*Ribes nigrum L*) extract was added to raw pork patties (Jia et al., 2012). Other fruit extracts were also able to reduce the carbonyl formation and discoloration in cooked burger patties during chilled storage (Ganhão et al., 2010).

Dietary strategies aimed at improving oxidative stability of meat, were also found to be successful. Supplementation of the dietary fat-enriched diet of turkey with alpha-tocopheryl acetate resulted in meat with reduced levels of TBARS, slight decrease in carbonyl content but no positive effect on colour stability upon refrigerated storage (Mercier et al., 1998). Similarly, the addition of vitamin E or plant extracts rich in polyphenols (PERP), or combination in the PUFA-enriched diets for cows resulted in steaks with higher lipid stability than the un-supplemented animals (Gobert et al., 2010). In addition to the higher lipid stability, a more stable colour was observed in meat from lambs raised on milk from ewes fed diets including thyme leaves (Nieto et al., 2010). Beef from pasture fed animals had higher proportions of *n-3* PUFA but had lower TBARS, better colour (Gatellier et al., 2005), and lower levels of protein oxidation than beef from conventional diet fed animals (Insani et al., 2008). Similarly pasture feeding increased the oxidative stability of lamb meat in spite of the significant increase in the muscle PUFA content (Luciano et al., 2009b; Santé-Lhoutellier et al., 2008b).

There are several contradicting results about the relationship between lipid, protein and myoglobin oxidation, and these do not give a clear view of which reaction comes first. In spite of this, researches showed that the control over oxidative reactions in meat lipids may also result in the control of oxidative damages of both myoglobin and protein. The tocopherols act as scavengers of chain-carrying peroxyl radicals or act to diminish the formation of lipid radicals that initiate the oxidation (Botsoglou & Botsoglou, 2010). For example, olive oil contains alpha-tocopherol, which is absorbed in the intestine and distributed between the different animal tissues (Wolf, 2006). This explains why the combination of olive cake and linseed in the diet of lambs protected from lipid oxidation in meat, despite the high concentration of PUFA in the intramuscular fat (Luciano et al., 2013). Similarly, pasture feeding that increased the muscle content of PUFA did not compromise the stability of the meat, due to the antioxidant compounds present in fresh herbage that helped to meet the balance between the antioxidant and pro-oxidant muscle components (Luciano et al., 2009b; Santé-Lhoutellier et al., 2008b; Mercier et al., 2004). Indeed, antioxidant compounds that cannot be synthesized by animals can be found in higher concentrations in green herbage compared to cereals (Mercier et al., 2004). Apart from tocopherols and carotenoids, some dietary phenolic compounds may be incorporated into the tissues and can exert antioxidant activities through scavenging of free radicals or reactive oxygen species, or chelation of metal ions, thus blocking the pro-oxidant action of iron (Jia et al., 2012; Rodríguez-Carpena et al., 2011; Ganhao et al., 2010; Nieto et al., 2010; Descalzo & Sancho, 2008). The action of a phenolic compound ($QH_2$) against lipid oxidation is simplified in Figure 3 (Roginsky & Lissi, 2005). The effectiveness of phenolics against lipid and protein oxidation in meat could be due to: (1) inhibition of hexanal formation that in turn could induce protein oxidation; and (2) inhibition of the formation of lipid oxidation

products by scavenging free radicals and by metal chelation (Vuorela et al., 2005). In some cases, protein oxidation was affected by phenolic compounds to a lesser extent than lipid oxidation, which could be accounted to the widely accepted results that: (1) lipid oxidation occurs faster than protein oxidation, although in some cases it is the other way around; and (2) proteins could form covalent bond with phenolic compounds that would change their antioxidant activity on proteins, and may also result in lower sulfhydryl groups level, as sulfhydryl groups react with phenolic compounds (Jia et al., 2012; Lund et al., 2011; Jongberg et al., 2011a; Lund et al., 2007a).

In the light of the above, it can be stated that the nutritional value, oxidative stability, and safety of meat and meat products rich in PUFA can be preserved over time if adequate amounts of antioxidant compounds are simultaneously provided. Dietary antioxidants are said to be stronger than the antioxidants directly added to foods (Govaris et al., 2004), which can be attributed to a more uniform distribution of the former into the phospholipid membranes, where they can effectively inhibit oxidative reactions *in situ* (Lauridsen et al., 1997).

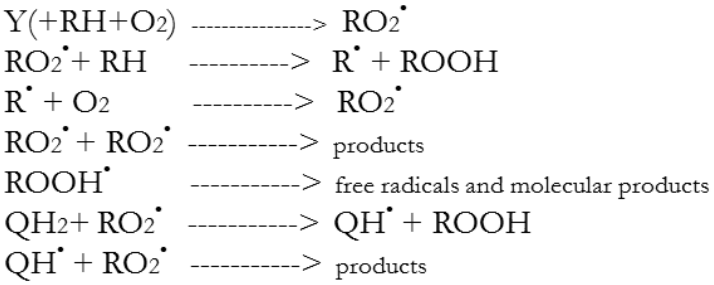


Figure 3. The antioxidant action of $QH_2$ on free radical formed on substrate RH.

**E. Alternative feeds**

The increase in the cost of feedstuffs for livestock in the recent years results in the increased product price and in the competition between the local markets and the low-cost imported products (Luciano et al., 2013). Consequently, a number of naturally and locally available materials have been explored to be used as feed resources to replace the conventional feedstuffs, which are collectively called as alternative feed resources (AFR). Besides lowering the production costs, these materials should not compromise the health and performances of the animals, and should meet the demands of the consumers for high-quality animal products; such as, meat with quality determinants acceptable to consumers (such as colour and flavor; Priolo et al., 2001) and fatty acid composition (Enser et al., 1998) as recommended by the health authorities.

In the Mediterranean countries, some AFR that showed no deleterious effects on livestock productivity and product quality are shrubs, seeds and pods of leguminous plants such as *Ceratonia siliqua,* novel pastures, and agro-industrial wastes like olive cake and citrus pulp (Vasta et al., 2008). In particular, the use of the by-products of agricultural industry could be an effective way of disposing the wastes, and more importantly, to use wastes for the production of meat, a high-value product for human consumption. Some AFR contain remarkable amounts of bioactive compounds, such as natural antioxidants, which could exert positive effects upon meat quality, and could be ideal for the production of meat and other animal products that are drug-free, healthy, oxidatively stable, and with acceptable sensory properties.

## *1. Citrus and by-products*

Citrus by-products are comprised of different fruit components such as peels and seeds. The citrus peels contain dietary fiber and ascorbic acid (Gorinstein et al., 2001), while the citrus seeds have unsaturated fatty acids (Bocco et al., 1998) and limonoids (Braddock, 1995). The major phenolic compounds present in these by-products are phenolic acids and flavonoids (Bocco et al., 1998; Marín et al., 2002), which have been found to exert protective effects against cancers, viruses and platelet aggregation (Benavente-Garcia et al., 1997). Another citrus by-product is represented by the non-marketable whole citrus fruits, which contain degradable carbohydrates and could be an alternative to cereals as energy source (Piquer et al., 2009). Citrus pulp, on the other hand, is a mixture of citrus peel, inside portions, seed and culled fruits that remain after juice extraction (Lanza, 1982; Grasser et al., 1995). This is called *pastazzo* in Italy. Dried citrus pulp contains approximately 8% crude protein, 11% crude fiber, 73% nitrogen free extract and 14% sugars on a dry matter basis and a low content of phosphorus (Bhattacharya & Harb, 1994) but has high energy, and calcium contents (Lanza, 1982; Deaville et al., 1994). Citrus pulp is traditionally used for livestock feeding (Bampidis & Robinson, 2006). Its high pectin concentration can be quickly degraded in the rumen to release energy for rapid microbial growth (Bueno et al., 2002).

Orange pulp used to replace cereal grains in feeds for fattening lambs did not cause deleterious effects on animal performance (Lanza et al., 2001). From 16% to 40% levels of inclusion in the diet for lambs, citrus pulp did not change the growth, slaughter performances and carcass quality (Caparra et al., 2007). However at 45% level of inclusion in the diet, solar dried citrus pulp used in place of concentrates for fattening lambs had a negative impact on the feed conversion efficiency, carcass weight, dressing percentage and carcass compactness (Caparra et al., 2007). The meat of lambs fed ensiled citrus pulp contained higher moisture (Scerra et al., 2001) and higher linoleic and linolenic acids contents than the meat from lambs fed the control diet (Caparra et al., 2000). Accordingly, the meat from ostrich fed citrus pulp had increased PUFA and decreased SFA and MUFA contents compared to the control diet (Lanza et al., 2004). Moreover, the sensory evaluation showed favorable results in meat from lambs fed citrus pulp-containing diets than of animals fed the control diets (Lanza et al., 2001). Considering the high level of inclusion at which citrus pulp can be potentially included in the diets of ruminants, this by-product might represent an advantageous strategy to economize production without harmful effects on animal productivity.

Furthermore, citrus by-products are potential sources of functional compounds that combined nutritional value and capacity to inhibit oxidative changes, making them interesting materials in the food industry (Fernández-López et al., 2008). A functional compound, the citrus fiber, is linked with flavonoids, polyphenols and carotenes, all with antioxidant properties (Marín et al., 2002), which could explain the low TBARS values measured in bologna sausage prepared with powder of orange dietary fiber (Fernández-López et al., 2004). In addition, high lipid stability in *mortadella* with orange dietary fiber and rosemary essential oil (Viuda-Martos et al., 2010) and in vacuum-packed sausages prepared with orange dietary fiber and oregano essential oil (Viuda-Martos et al., 2010) were found, in comparison to control. The antioxidant properties of the polyphenolic compounds in the citrus fiber are related to metal-chelation, free radical scavenging, hydrogen donation, and inhibition of enzymatic systems responsible for initiating oxidation reactions (Viuda-Martos et al., 2010). The flavonoids, although having low lipid solubility can contribute to decreasing the oxidation rate in food emulsions (Zhou et al., 2005). The flavonoids present in the orange dietary fiber include hesperidin and narirutin (Viuda-Martos et al., 2010). Dietary supplementation of hesperidin in lambs did not have negative

effects on growth or health of the animals, instead improved the meat antioxidant properties during refrigerated storage (Simitzis et al., 2013).

## 2. *Carob and phenolic rich plants*

Carob (*Ceratonia siliqua L*) is a multipurpose tree native to the Mediterranean countries that is well-adapted to the harsh climatic conditions. Carob pods in the form of dry pulp are commonly used in livestock feeding (Vasta & Luciano, 2011). The carob pods contain high amount of polyphenols such as condensed tannins (Priolo et al., 2000; 2002).

Tannins are polyphenolic plant metabolites that have varying molecular weights and complexity and are classified as hydrolysable tannins or condensed tannins (Makkar, 2003). The hydrolysable tannins contain a central core of glucose or another polyol esterified with gallic acid also called gallotannins, or with hexahydroxydiphenoic acid, also called ellagitannins (Koleckar et al., 2008). On the other hand, condensed tannins, also called as proanthocyanidins, are oligomers or polymers composed of flavan-3-ol nuclei (Koleckar et al., 2008). The presence of multiple phenolic hydroxyl groups enable tannins to complex with proteins, and to a lesser extent with metal ions, amino acids, and polysaccharides (Makkar, 2003). Moreover, tannins act as metal chelator, inhibitor of pro-oxidative enzymes, and quencher of free radicals (Koleckar et al., 2008). Upon reaction with free radicals, tannins form resonance-stabilized phenoxyl radicals (Liu et al., 2012).

Grape seed extract and *Cistus ladanifer*, both sources of condensed tannins, added to feeds for lambs protected the meat from lipid oxidation but not from discoloration (Jerónimo et al., 2012). In contrast, quebracho tannins in sheep diets did not affect the meat lipid stability but improved the colour stability during refrigerated storage (Luciano et al., 2009b). The protective effects of tannins against oxidative changes in meat, whether added directly to meat or through the diets, could be explained considering their antioxidant capacity. In fact, crude polyphenol fraction of carob showed a better inhibitory effect against the discoloration of beta-carotene than other polyphenol compounds and the effectiveness is comparable to authentic polyphenol (Kumazawa et al., 2002). Condensed tannins reduced lipid oxidation due to their capacity to scavenge free radicals (Liu et al., 2012) and to possible indirect antioxidant capacity by (a) activation of endogenous antioxidant defense system (Liu et al., 2012) or enzymes (Gladine et al., 2007a) or (b) interaction with the other antioxidant compounds or with pro-oxidant compound in meat (Jerónimo et al., 2012). However at concentration higher than necessary, the extract containing tannins added to diets of animals becomes a pro-oxidant, although the reason behind was not verified (Liu et al., 2009).

Moreover, as previously discussed, dietary quebracho tannins were able to affect ruminal biohydrogenation as manifested by the lower muscle concentration of stearic acid and SFA, and higher levels of CLA and PUFA, as compared to a tannin-free diet (Vasta et al., 2009b). Similarly, feeding lambs with sulla (*Hedysarum coronarium L.*), which is also a source of condensed tannins, increased the fat content of CLA and long chain PUFA (eicosapentaenoic) and increased meat lightness compared to a control diet (Priolo et al., 2005). When carob pulp was used to replace barley at the level of 20% in lamb diet no effect on animal performances and on the carcass and proximate meat characteristic was found. However, carob pulp cause lower dressing percentage and feed conversion efficiency, and the lean colour was lighter than the control (Priolo et al., 1998). Furthermore at the level of 45% or higher, carob pulp inclusion in lamb diet, animal growth performances were compromised (Vasta et al., 2007a; Priolo et al., 2000). Thus nutritional strategies that include tannins could potentially be a useful way to

improve some meat quality traits, provided that the correct level of inclusion into the diet is chosen to prevent deleterious effects on the animal performance parameters (Vasta et al., 2009b).

### *3. Olives and by-products*

Olive oil production industries leave residues, known as olive cake, that are made up of olive pulp, skin, stones, residual oil and water (Molina-Alcaide et al., 2008; Vargas-Bello-Pérez et al., 2013), with low crude protein but high fibre fractions and ether extracts (Abbeddou et al, 2011). Olive cake could constitute a relatively low cost ingredient in total mixed rations for livestock feeding (Molina-Alcaide et al., 2008).

Olive cake used as a substitute for concentrates in lamb diet significantly increased the MUFA concentration of the meat due to the considerable amount of oleic acid in the olive cake (Mele et al., 2014). The combination of linseed and olive cake in the diet, on the other hand, increased the PUFA content of the meat but did not compromise the oxidative stability due to the substantial amount of alpha-tocopherol in the olive cake, which can be deposited into the animal tissues and be able to protect the unsaturated fatty acids from oxidation (Luciano et al., 2013). Besides the lipophilic antioxidants (Amro et al., 2002; Martín-García et al., 2003), the presence in the olive cake of flavonoids, lignans, and secoiridoids typical of olive oil (Servili et al., 2004; 2009) could restore/regenerate the vitamin E from its oxidized form into active form or protect it from oxidation (Decker et al., 2002).

Besides olive cake, other potential feeds for livestock could originate from the olive-oil industry. For example, the supplementation of pig diets with olive leaves or alpha-tocopheryl acetate did not affect meat fatty acid composition and protein oxidation in pork, but decreased the rate of lipid oxidation (Botsoglou et al., 2012). The olive leaf extract at level of 300 mg GAE/kg (gallic acid equivalents) added to pig diet was as effective as alpha-tocopherol at the level of 50 μg/kg in the inhibition of MDA formation, alleviation of the pro-oxidant effects of the *n-3* fatty acids and NaCl, and protein carbonyl formation (Botsoglou et al., 2014). Leaves of olive tree can be regarded as a particularly rich source of phenolic compounds (Botsoglou et al., 2014), the major biologically active constituents are classified as oleuropeosides including oleuropein and verbascoside, flavonoids including luteolin, luteolin-7-O-glucoside, apigenin-7-O-glucoside, diosmetin-7-O-glucoside, diosmetin, rutin and catechin, and simple phenolics including tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid (Botsoglou et al., 2014; Botsoglou et al., 2012), as well as alpha-tocopherol. These antioxidant compounds might enter the circulatory system and can be delivered to the animal muscle (Botsoglou et al., 2012), thus increasing the resistance of the meat to oxidative degradation.

.

## F. Objectives

The PhD thesis was shaped to be a part of the development and transformation of farming systems in the developing world. In line with this, an innovation in livestock production in the Mediterranean countries was chosen. This is through the transformation of seemingly useless materials, such as wastes commonly found in local places, in the production of valuable animal products for human consumption like meat. These materials serve as alternative ingredients to the conventional concentrate diets for small ruminants. These alternative feedstuffs are plant-derived materials and/or wastes of agricultural industries, therefore are natural and drug-free. Moreover, these are cheaper materials to partially replace the highly-priced conventional feed ingredients, therefore reducing the cost of production.

Therefore the general aim of this thesis project was to use citrus pulp, carob pulp and olive cake, commonly produced in the Mediterranean countries, as alternative feedstuffs to conventional concentrate ingredients for lambs, without compromising the animal metabolic state and to deliver bioactive molecules in a chemical-free strategy.

Specifically the aim was to evaluate the effects of these feeding alternatives on the meat quality parameters (1) fatty acid composition (2) oxidative stability by monitoring the measurable changes in colour, lipids, volatile organic compounds, and proteins in meat stored under retail conditions.

# II. EXPERIMENT 1
# Dietary citrus pulp improves protein stability in lamb meat stored under aerobic conditions

# Dietary citrus pulp improves protein stability in lamb meat stored under aerobic conditions[1]

## 1.1. Introduction

The delay in the oxidation of meat lipids and proteins is important due to the widely accepted correlation with the deterioration of important sensory properties such as flavour, texture, and colour (Decker et al., 1995). The oxidation of proteins in muscle foods has been linked with solubility and functionality changes, as well as with deterioration of tenderness and juiciness (Lund et al., 2011). Further, the oxidation of polyunsaturated fatty acids (PUFA) and amino acids diminishes the nutritional quality of meat (Mercier etal., 2004; Lund et al., 2011).

The demands for healthy and safe diets are constantly growing and consequently, food scientists explore naturally occurring substances that could replace the chemically-synthesized counterparts used to extend the shelf life of foods. Natural antioxidants in various forms have been used to reduce or prevent lipid and protein oxidation in meat and meat products such as plant extracts (Jongberg et al., 2011b; Lund et al., 2007a; Fernández-López et al., 2005) and agricultural by-products (Rodrígues-Carpena et al., 2011; Salminen et al., 2006; Vasta & Luciano, 2011), and through pasture feeding (Luciano et al., 2009b; Santé-Lhoutellier et al., 2008a; Mercier et al., 2004). It was found that the dietary administration of antioxidant compounds was more effective in protecting the tissues from oxidation than the direct addition of similar antioxidant compounds to muscle foods (Govaris et al., 2004).

Citrus pulp is a by-product of citrus fruit juice extraction and consists of citrus peel, inside portion of the fruit, seed, and culled fruits (Lanza, 1982; Grasser et al. 1995). The pectin-rich solar dried citrus pulp mixed with concentrate feed creates a favorable condition for cellullolysis in the rumen of lamb (Caparra et al., 2007), and can be successfully used to replace cereal concentrates (Bampidis & Robinson, 2006). Citrus fruits contain bioactive components, such as the flavonoids hesperidin, narirutin, naringin, and eriocitrin, which in their structure have one or more phenolic groups with antioxidant properties and the ability to scavenge free radicals (Benavente-García et al., 1997). In a feeding study where hesperidin was added to the lamb diet, evidence showed that hesperidin was distributed through the circulatory system, retained in the tissues, and protected the lamb meat against oxidation for several days under refrigerated conditions (Simitzis et al., 2013). Citrus extracts increased the resistance to oxidation of the lipid in cooked meatballs (Fernández-López et al., 2005), and in cooked turkey meat when added directly or applied to PET trays (Contini et al., 2012). Also, orange dietary fiber with oregano essential oil showed protective effects by lowering the lipid oxidation levels in bologna sausages (Viuda-Martos et al., 2010). The bioactive polyphenol compounds identified were narirutin and hesperidin, and it was suggested that the protection was due to the capacity of polyphenols to act as metal-chelators, free-radical scavengers, hydrogen donors, and inhibitors of the enzymatic systems responsible for initiating oxidation reactions (Viuda-Martos et al., 2010).

[1]**Gravador, R.S.**, Jongberg, S., Luciano, G., Andersen, M.L., Priolo, A., Lund, M. (2014). Dietary citrus pulp improves protein stability in lamb meat under aerobic conditions. *Meat Science, 97,* 231-236.

Recently (Inserra et al., 2014), we have used dried citrus pulp to partially replace cereal concentrate in lamb diets, and the possible effects on the shelf life of the meat have been studied. Dried citrus pulp showed a favorable effect against meat lipid oxidation. Considering that lipid and protein oxidation are believed to be connected (Lund et al., 2011), we have hypothesized a possible effect of dietary citrus pulp on meat protein oxidation over storage duration. Therefore, here we have analyzed the same samples used by Inserra et al. (2014) to further investigate the effect of dietary dried citrus pulp on protein oxidation in meat over storage duration.

## 1.2. Materials and Methods

### *1.2.1 Animal management and dietary treatments*

Twenty-six male Comisana lambs, aged 90 days and weaned at an average weight of 19.8 (±3.5) kg, were used for the feeding trial at an experimental farm in Villarosa, Enna, Sicily. The lambs were randomly distributed into three experimental groups according to dietary treatments, and were penned individually outdoors. The three diets fed consisted of (1) commercial concentrate with 60% barley (Control, n=8), (2) concentrate with 35% barley and 24% dried citrus pulp (Cp24, n=9), or (3) concentrate with 23% barley and 35% dried citrus pulp (Cp35, n=9). No vitamin premix was included into the diets. For 10 days the lambs were adapted gradually to the experimental diets. Feeds were offered between 09.00 h and 18.00 h while water was always available. At the age of 158 days, the lambs were slaughtered in a commercial abattoir. They were stunned by captive bolt and exsanguinated. The carcasses were halved and after 24 h at 4 °C, the *longissimus thoracis et lomborum* muscle was excised from the right half, vacuum-packed, and aged for 4 days at 4 °C. From each animal, three slices of meat were sampled, individually placed on polystyrene tray, overwrapped with oxygen-permeable PVC film, and each was assigned to one of three storage periods: 0 (2 hours), 3, or 6 days in the dark at 4 °C. After end of storage time, meat slices were vacuum-packed and stored at -30 °C until analysis.

### *1.2.2 Chemicals*

Reagent grade chemicals and deionized distilled water (Milli Q) were utilized all throughout the analyses.

### *1.2.3 Isolation of Myofibrillar Protein*

The method used for the isolation of myofibrillar proteins (myofibrillar protein isolates, MPI) was adopted from Jongberg et al., (2011b) with some modifications. An aliquot of 4.0 g lamb meat trimmed of fat and connective tissues, was cut into small pieces and placed into a 50 ml capacity falcon tube with 20 ml chilled isolation buffer (10 mM $Na_3PO_4$, 1 M EDTA 0.1 M NaCl, 2 mM $MgCl_2$ and, pH 7.0). The mixture was homogenized by an Ultra Turrax T-25 homogenizer two times for 15 s with 15 s interval. The homogenate was centrifuged for 15 min at 2600g at 4°C (Sigma Laborzentrifugen 3k15, Bie and Berntsen A/S, Denmark). The supernatant was decanted and the pellet was re-suspended in 10 ml isolation buffer and the same homogenization and centrifugation was repeated twice. The pellet was then re-suspended in 10 ml chilled 0.1 M NaCl, mixed thoroughly with a glass spatula for 1 min, and centrifuged. This procedure was repeated three times. The MPI extracts were spread onto petri dishes, frozen overnight at -80 °C, and then freeze-dried (Edwards, Buch & Holm A/S) for 3 days. The lyophilized MPI was stored at -20 °C prior to analyses.

*1.2.4 Protein Radical Intensity Measurement using ESR Spectroscopy*

A quartz Electron Spin Resonance (ESR) tube (inner diameter = 4 mm, wall = 0.5 mm; Wilmad, Buena, NJ, USA) was filled with lyophilized MPI to a height of ≥5 cm and the mass was determined. The tube was placed into the cavity of the JEOL JES-FR30X ESR spectrometer (JEOL Ltd., Tokyo, Japan) and the spectrum was measured utilizing the following conditions: Microwave power, 4 mW; center field, 336 mT; sweep width, 5 mT; sweep time, 2 min; modulation width, 0.125 mT; time constant, 0.3 s; accumulations, 1. The radical intensity was measured relative to the intensity of the signal of an internal manganese, Mn(II), standard. The radical signal intensity was calculated based on the density of the sample measured as g/cm in the ESR tube [radical signal intensity = (signal area sample/signal area Mn(II))/density sample (g/cm)]. Nine replicates per dietary treatment, except for the Control group (n=8), were measured and the results were expressed as means ± SD (standard deviation).

*1.2.5 Protein Concentration Determination by BCA Method*

An aliquot of 10 mg of lyophilized MPI was dissolved in 1.0 ml 5% sodium dodecyl sulfate (SDS; AppliChem GmbH, Darmstadt, Germany) in 0.1 M tris (hydroxymethyl)-(aminomethane) (tris; Sigma-Aldrich St. Louis, MO, USA) buffer at pH 8.0 in a water bath at 80°C for 1 h with mixing using vortex mixer every 15 min. Dissolved MPI were immediately centrifuged (Microcentrifuge 154, Ole Dich Instrument makers ApS, Hvidovre, Denmark) at 4500g for 15 min. The supernatant was diluted 10 times with the same buffer, and the protein concentration was quantified using the Pierce BCA (bicinchoninic acid) Protein Kit Assay (Thermo Specific, Pierce Biotechnology Rockford, IL, USA) following the manufacturer's instructions and using bovine serum albumin (BSA; Sigma-Aldrich St. Louis, MO, USA) with concentrations ranging from 25-2000 µg/µl as standard. The absorbance was measured at 560 nm (Labsystems Multiskan Ex Version 1.1, Bie and Berntsen A/S, Denmark). The corrected absorbance ($Abs_{standard}$ - $Abs_{blank}$) were plotted against concentration and used to calculate the protein content of the MPI solution.

*1.2.6 Thiol Quantification by DTNB*

The concentration of thiol groups in MPI was quantified after derivatization with Ellman's reagent (Ellman, 1959). An aliquot of 0.5 ml of the above dissolved MPI was diluted with 2.0 ml 5% SDS in 0.1 M tris buffer at pH 8.0 in a 3.5 ml cuvette. The absorbance, $Abs_{pre}$, was measured at 412 nm (Cary UV−visible Spectrophotometer Varian, Herlev, Denmark). Then, 0.5 ml 5,5-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma-Aldrich St. Louis, MO, USA) was added and the solution was allowed to react in the dark at room temperature for 30 min after which the absorbance, $Abs_{post}$, was read at 412 nm. The same procedure was done with the blank ($Abs_{blank}$) and the standards consisting of L-cysteine (Merck, Bie and Berntsen A/S, Denmark) with concentrations that ranged from 0.4-125 µM. All absorbance were corrected using the blank as follows: $Abs_{corr} = Abs_{post} - Abs_{pre} - Abs_{blank}$. The standard concentrations were plotted against the absorbance and linear regression was performed to calculate the MPI thiol concentration. Measurements were done with nine replicates except for the Control group (n=8) and results were reported as mean nmol thiol/mg protein ± SD.

*1.2.7 Cross-link Formation by SDS-PAGE*

Based on the protein concentration of the dissolved MPI, aliquots were diluted to obtain a concentration of 2 mg protein/ml with 5% SDS in 0.1 M tris buffer at pH 8.0. An aliquot of 1.6 µl was mixed with 14.4 µl of loading solution. For the non-reduced MPI, the loading solution

was prepared as follows: 80 µl lithium dodecyl sulfate sample buffer (LDS; Invitrogen, Carlsbad, CA, USA) and 208 µl Milli Q water, while for the reduced samples the loading solution was prepared from 80 µl LDS sample buffer, 32 µl 1 M dithiothreitol (DTT; AppliChem GmbH, Darmstadt, Germany), and176 µl Milli Q water. Mixtures were heated for 10 min at 80 °C with shaking (EscoProvocell™ Shaking Micro Incubator, Buch & Holm A/S), and centrifuged thereafter for 15 s at 12100g (MiniSpin Eppendorf, Buch & Holm A/S). The gel electrophoresis was run on NuPAGE Novex 3-8% TRIS-acetate gels following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). In brief, aliquots of 10 µl of the MPI in loading solution and 3 µl of Precision Plus Protein Standard All blue marker (Bio-Rad Laboratories, Inc., Hercules, CA, USA), were loaded to the wells of the gels, and the cassette was filled with chilled SDS tris-acetate running buffer (Invitrogen). Following the electrophoresis run for 90 min at 150 V, the gels were individually placed in a tray with fixation solution (50% ethanol, 7% acetic acid, 43% Milli Q water) overnight. The gels were rinsed with MilliQ water and stained with 40 ml of SYPRO Ruby Protein Gel Stain (Invitrogen, Carlsbad, CA, USA) overnight. The gels were washed with a solution of 10% ethanol, 7% acetic acid and 83% Milli Q water before scanning (Typhoon Trio Variable Mode Imager, GE Healthcare Bio Sciences, AB, Uppsala, Sweden).

*1.2.8 Protein Carbonyls Quantification by DNPH Method*

The determination of carbonyl groups in MPI was done by derivatization with 2,4-dinitrophenylhydrazine (DNPH; Fluka Sigma-Aldrich Chemie GmbH, Steinheim, Switzerland) following the method of Levine et al. (1994) with some modifications. A 5 mg aliquot of MPI was dissolved in 0.5 ml 6 M guanidine hydrochloride (Sigma-Aldrich St. Louis, MO, USA) in 20 mM potassium dihydrogen phosphate at pH 2.3 for 1 h at 50 °C with mixing using vortex mixer every 10 min. An aliquot of 0.5 ml of 10 mM DNPH in 2 M HCl was added to the dissolved samples (S), while 0.5 ml of 2 M HCl was added to the blank samples (B). These were incubated at 37 °C water bath for 1 h with mixing every 10 min. Then, 375 µl of 50% TCA solution was added to all samples, mixed for 30 s and allowed to react for 10 min at chilling condition prior to 10 min centrifugation at 16500g. The supernatant was discarded and the pellet was washed three times with 1 ml 1:1 ethanol:ethylacetate solution containing 10 mM HCl (wash solution). At each time, the pellet in wash solution was mixed and left for 10 min before centrifugation for another 10 min at 16500g. The washed pellets were finally dissolved in 1 ml 6 M guanidine hydrochloride (Sigma-Aldrich St. Louis, MO, USA) in a water bath at 37 °C for 30 min with mixing every 10 min, and centrifuged for 10 min at 16500g. The supernatant was transferred to a quartz cuvette and the absorbance (Abs) at 280 and 370 nm were used for the determination of carbonyl content (nmol/mg protein) using an absorption coefficient of $22000 M^{-1}cm^{-1}$ for the formed hydrazones following the equation below (Levine et al., 1994). The contribution from the blank samples (B) was subtracted from the corresponding samples (S). Measurements were done in replicates of nine except for Control group (n=8) and the values were expressed as mean ± SD.

$$\text{carbonyl content}\left(\frac{\text{nmol}}{\text{mg}}\text{protein}\right)=\frac{\text{Abs}_{370}}{22000(\text{Abs}_{280}-\text{Abs}_{370}\times 0.43)}\times 10^{6}$$

*1.2.9 Statistical Analysis*

The General Linear Model with repeated measures of the Minitab Software Version 16 (Minitab Inc., State College, PA) was used to analyze the data collected. The dietary treatments (Control, Cp24, or Cp35) and storage time (0, 3, or 6 days), and their interaction were considered as fixed effects, while the individual lamb was considered as a random effect in the statistical model. The Tukey's test was used for multiple comparisons of the means.

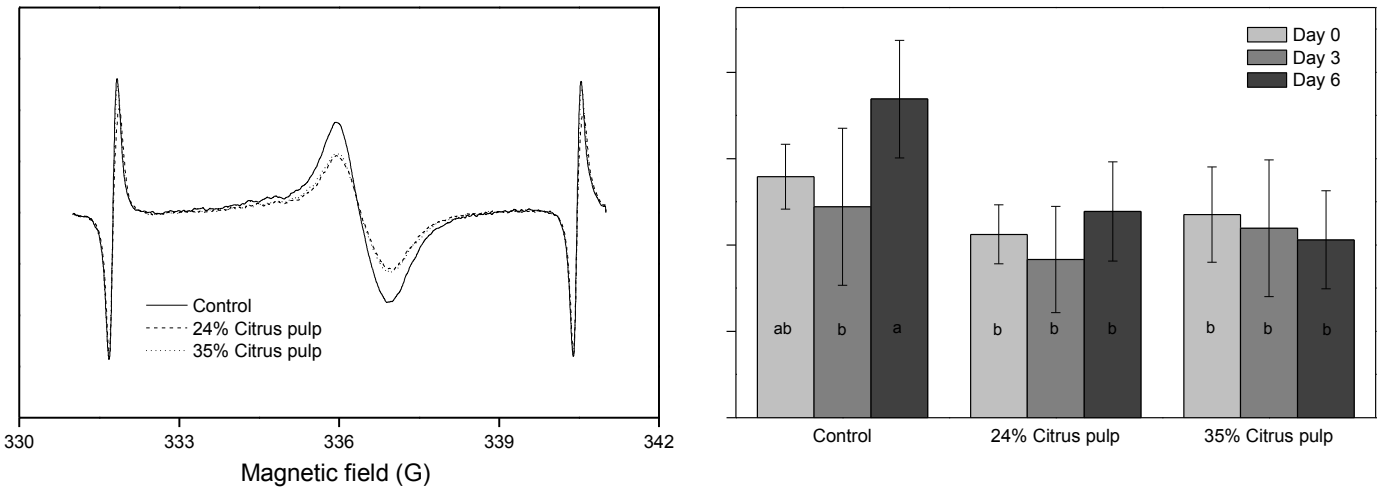


Figure 1.1. Electron Spin Resonance (ESR) spectra (a) and radical intensity (b) of myofibrillar protein isolates (MPI) extracted from lamb meat. The lambs were fed concentrate-based diet (Control), 24% citrus pulp (Cp24), or 35% citrus pulp (Cp35) in concentrate, and the meat was stored in the dark for 0, 3 and 6 days at 4°C on polystyrene trays overwrapped with oxygen- permeable PVC film.

## 1.3. Results and Discussion

In the present study, dried citrus pulp was used as a low-cost alternative to cereal concentrates in lamb feeding (24% dried citrus pulp, Cp24, and 35% dried citrus pulp, Cp35). The dietary regimes did not affect ($P > 0.05$) the main lamb performance parameters, with values of final live-weight, average daily weight gain and carcass weight being on average: 24.47 kg, 119.9 g/d and 12.39 kg, respectively. These findings confirm the results of previous studies demonstrating citrus pulp can be used to replace a high proportion of cereals in the diet with no adverse effects on animal productivity (Bampidis & Robinson, 2006).

The oxidative stability of meat proteins during storage at 4 °C were studied by following the extent of protein oxidation in myofibrillar protein isolates (MPI) from lamb meat stored aerobically in the dark for 0, 3, or 6 days. The oxidation of proteins may take place through a radical chain reaction mechanism (Lund et al., 2011), and Electron Spin Resonance (ESR) spectroscopy was applied to measure the level of protein radicals formed. Radicals are precursors of oxidation, and commonly considered as highly reactive species. However, in high molecular weight compounds, such as proteins, radicals may be stabilized and thereby accumulate during storage. The myofibrillar protein, myosin, has been shown to form long-lived radicals when oxidized with $H_2O_2$-activated myoglobin (Lund et al., 2008b). Hence, the more oxidized the meat samples are, the more protein radicals may accumulate, and a higher radical level can be detected. The change in the radical level as determined by ESR spectroscopy in the MPI of lamb meat stored up to 6 days at refrigerated conditions was found to be affected by lamb diet ($P < 0.001$), storage time ($P = 0.008$), and the interaction between diet and storage time ($P = 0.021$) (Fig. 1.1). The radical intensity increased significantly in the Control from day 3 to 6 ($P = 0.0045$), and the level of accumulated protein radicals in the Control samples at day 6 was found to be significantly higher than both Cp24 ($P = 0.0017$) and Cp35 (P = 0.0001) samples (Fig. 1.1). In contrast, no remarkable differences in radical intensity between Cp24 and Cp35 samples at any storage times were observed. These results demonstrate that over an extended refrigerated storage, the partial replacement of dietary barley with either 24% or 35% dried citrus pulp was effective in protecting meat against protein radical formation.

**Table 1.1.** Effect of the dietary treatment and time of refrigerated storage on the free thiol content and carbonyl content in meat.

| | Dietary treatment (Diet) | | | Days of storage (Time) | | | SEM | *P* values | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Control | Cp24 | Cp35 | 0 | 3 | 6 | | Diet | Time | Diet×Time |
| Free thiol[1] | 57.73[a] | 57.35[a] | 53.18[b] | 57.86[x] | 56.76[x] | 53.44[y] | 0.534 | <0.001 | <0.001 | 0.379 |
| Carbonyl[1] | 1.77[a] | 1.34[b] | 1.40[b] | 1.40[y] | -- | 1.60[x] | 0.041 | <0.001 | 0.001 | 0.719 |

[1]Expressed as nmol/mg of proteins

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

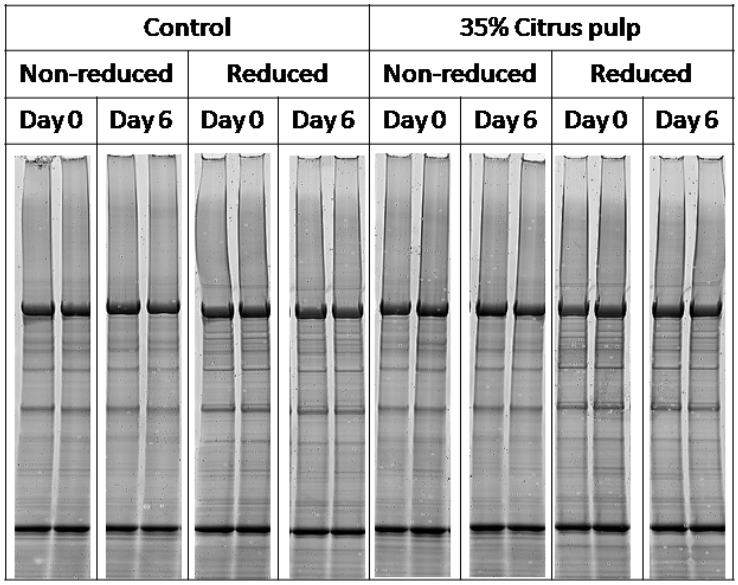


Figure 1.2. A representative SDS-PAGE gel of non-reduced and reduced myofibrillar protein isolates (MPI) extracted from lamb meat of animals fed concentrate based diet (Control) or 35% citrus pulp mixed with concentrate (Cp35), and stored in the dark for 0 and 6 days at 4°C on polystyrene trays wrapped with oxygen-permeable PVC film.

The thiol concentration in lamb meat was highly affected by the dietary treatment ($P < 0.001$) (Table 1.1). An overall decreasing trend in thiol content was observed during the six-day refrigerated storage of lamb meat ($P < 0.001$) (Table 1.1). However no significant difference of the interaction between diet and time was observed. A reduction in thiol concentration in meat proteins indicates the occurrence of thiol oxidation (Lund et al., 2011). The thiol content of meat from the Cp35 group was significantly lower than the thiol content of meat in both Control ($P = 0.0002$) and Cp24 ($P = 0.0005$) samples. However, a significant thiol loss between day 0 and day 6 was observed in all the dietary treatments, being 12.5%, 5.7% and 4.9% for Control, Cp35 and Cp24, respectively, indicating that the meat from citrus pulp-fed lambs was less susceptible to thiol oxidation than the Control-fed lambs.

In order to investigate the fate of the oxidized thiols in the meat in more detail, SDS-PAGE analysis was performed to evaluate the possible formation of protein cross-linking. The oxidation of thiols yielding cross-linked myosin heavy chain (CL-MHC) has previously been reported in pork (Lund et al., 2007b), Parma ham (Koutina et al., 2012), beef (Jongberg et al., 2011b; Zakrys-Waliwander et al., 2012), and lamb meat (Kim et al., 2012). Protein cross-linking has been associated with increased meat toughness, and has been detected by the use of gel electrophoresis (Lund et al., 2007b). The SDS-PAGE gels of MPI in non-reduced and reduced states of two dietary treatments, Control and Cp35 at days 0 and 6, did not show any CL-MHC (Fig. 1.2), which resembles results observed with meat from lamb fed concentrate or pasture stored aerobically for seven days (Santé-Lhoutellier et al., 2008a) and with meat stored without oxygen for more than seven days (Jongberg, 2011b; Lund et al., 2007b) at refrigerated condition. High-oxygen modified atmosphere packaging (HiOx-MAP) has been associated with reduced meat tenderness (Kim et al., 2010; Lund et al., 2007b; Lund et al., 2011), which has been linked to the formation of CL-MHC induced by thiol oxidation yielding protein disulfides. The CL-MHC has been observed in bovine muscle stored for nine days (Jongberg et al., 2011b) and for 14 days (Zakrys-Waliwander et al., 2012), where approximately15.6% and 30.8% nmol thiol/mg protein were oxidized, respectively, while in the present study, only 12.5% and 5.7% nmol thiol/mg protein were oxidized in Control and Cp35 samples, respectively. The absence of CL-MHC in Control and Cp35 samples may suggest that the level of CL-MHC was too low to be

detected by SDS-PAGE. The present storage study was conducted under oxygen-permeable PVC film and only for six days, whereas in the above mentioned studies, the meat was stored in HiOx-MAP for more than six days. The less harsh conditions at which the lamb meat was stored in the present study may explain the lower level of observed oxidation. The thiol concentration at day 0 in Cp35 samples was lower as compared to Control samples, but it cannot be attributed to the immediate thiol oxidation inCp35 since no CL-MHC was observed at day 0 and even at day 6 in meat from both dietary treatments. It may be speculated that the initial lower protein thiol level in Cp35 samples were not caused by oxidation, but that less thiols were available in the MPI due to reaction with phenolic compounds in the citrus pulp. Thiols can react with quinones formed by oxidation of phenols, and generate thiol-quinone adducts as identified in minced beef stored under high-oxygen atmosphere(Jongberg et al., 2011a). This hypothesis is supported by *in vivo* studies involving lambs which showed evidence of absorption of citrus flavanones naringin (Gladine et al., 2007b) and hesperidin (Simitzis et al., 2013), which in turn protected the animal tissues against lipid peroxidation.

The presence of carbonyl groups are a widely used marker of protein oxidation, which are formed by the modification of amino acid side chains of mainly threonine, proline, arginine, and lysine, and which are usually attributed to metal-catalyzed oxidation (Stadtman & Levine, 2003). Factors other than metals such as myoglobin, lipid derived reactive oxygen species, and physical factors such as water activity are also known to promote carbonylation in proteins (Estévez, 2011). Increasing levels of protein carbonyls have been detected in various stored raw or processed meats (Koutina et al., 2012; Rodrígues-Carpena et al., 2011; Lund et al., 2008a; Lund et al., 2007b; Salminen et al., 2006). The level of carbonyls in the lamb meat samples was highly affected by the dietary treatments ($P < 0.001$) and generally increased from day 0 to day 6 ($P = 0.001$), but no Diet × Time interaction was found (Table 1.1). There were more carbonyl compounds formed in the Control group than in the citrus pulp groups, Cp24 ($P < 0.001$) and Cp35 ($P < 0.001$). The protein carbonyls quantified in this study (1.3-1.9 nmol/mg proteins) is comparable with the results found in beef and pork stored for at least six days (0.6–2.0 nmol carbonyls/mg protein) (Lund et al., 2007a; Lund et al., 2007b; Rodrígues-Carpena et al., 2011). Whereas a minimal degree of oxidation took place in meat proteins from all the dietary treatments, a smaller change was observed in Cp24 and Cp35 samples indicating citrus pulp potentially delays protein carbonyl formation, and hence protects against protein oxidation in meat.

The ability of citrus pulp to delay protein oxidation as observed in the current study can be related to the bioavailability of the antioxidant components. Citrus flavanone glycosides, naringin and hesperidin, administered orally either in pure form, citrus juice or whole fruit were shown to be absorbed from the human gastrointestinal tract (Ameer et al., 1996). Citrus fruit juice consumption by rats caused a lowering of serum low density lipoprotein cholesterol, which was suggested to be due to the endogenous effects of citrus components including flavonoids (Kurowska et al., 2000). In a similar manner, citrus extracts, which contain naringin, introduced through cannula in sheep, resulted in plasma protection against lipid peroxidation (Gladine et al., 2007b). In the process, the glycosidic fraction of naringin is hydrolyzed while the flavanone fraction was not modified prior to absorption. These results show that both extracts and mixtures of citrus polyphenols can be absorbed into the tissue and be effective against oxidation. A similar effect may explain why meat proteins from lamb fed citrus pulp containing diets in the current study were protected from oxidation compared to a control dietary treatment. It is widely known that meat from concentrate fed animals are less protected against lipid oxidation than meat from pasture fed due to the natural antioxidants, such as vitamin E, ascorbic acid, and carotenoids in the plants that protect the susceptible PUFA against attack by reactive oxygen species (Luciano et al., 2009b; Santé-Lhoutellier et al., 2008a). More specifically, lower levels of

protein carbonyls were detected in meat from pasture-fed (Santé-Lhoutellier et al., 2008a) or acorn-fed animals than meat from concentrate-fed ones (Ventanas et al., 2006) demonstrating that dietary plant bioactive compounds can also effectively protect against protein oxidation.

Although there is no simple correlation between lipid and protein oxidation, some studies have demonstrated the two processes to be linked (Lund et al., 2011). The overall findings of the protein oxidation measurements in the current study correlate with the results of measurements of secondary lipid oxidation products through the use of 2-thiobarbituric acid assay (TBA) in the same meat samples which demonstrated that the citrus pulp groups had lower levels of lipid oxidation than the Control (Inserra et al., 2014). Statistical analysis gave positive correlations between the TBARS values and the protein radical levels (0.513, $P < 0.001$) and carbonyl levels (0.508; $P < 0.001$) measured during the storage of the meat samples. Citrus extracts used in cooked meatballs, significantly reduced lipid oxidation, and the meat colour was found to be more acceptable than the control samples (Fernández-López et al., 2005). Citrus peels and seeds have other components such as tocopherols, ascorbic acid, and limonoids (Bocco et al., 1998), which also may contribute to the antioxidative power protecting both lipid and protein from oxidation. Citrus flavonoids have been known to possess radical scavenging properties (Benavente-García et al., 1997), which may explain the lower degree of radical formation in Cp24 and Cp35 samples. Furthermore, citrus is also able to chelate metal ions (Benavente-García et al., 1997), which could contribute to lowering the level of protein carbonyl compounds in meat proteins considering that these oxidation products are primarily formed through metal catalyzed reactions (Estevez, 2011).

In conclusion, the use of dietary dried citrus pulp in feeds for lambs resulted in an improved oxidative stability of proteins in meat stored aerobically under refrigerated conditions for up to six days. The protective effects of dietary citrus pulp against oxidation of meat proteins may be caused by the bioactive compounds originating from the citrus
fruits.

# IV. EXPERIMENT 2
# Fatty acids and oxidative stability of meat from lambs fed carob-containing diets

# Fatty acids and oxidative stability of meat from lambs fed carob-containing diets[2]

## 2.1. Introduction

The increased consumption of foods with low level of saturated fatty acids (SFA) and high level of polyunsaturated fatty acids (PUFA), containing a low *n-6/n-3* fatty acid ratio correlates with favorable human health conditions (Wood et al., 2003). Consequently, ruminant meat is not considered as a part of a healthy human diet due to its high content of SFA (Scollan et al., 2006). The fatty acid composition of meat from ruminants is in part a result of the dietary intake of the corresponding fatty acids (Wood et al., 2003), thus, endeavours to modify the fatty acid composition and content of animal tissues through dietary strategies is in progress. These include dietary supplementation of PUFA-rich sources (Bessa et al., 2007; Scollan et al., 2006) or feeding animals with herbage ( Luciano et al., 2009b). On the other hand, the PUFA ingested by ruminants undergo a process in the rumen, known as biohydrogenation, leading to progressive saturation of the PUFA. Therefore feeding strategies that reduce the extent of the ruminal biohydrogenation might ultimately increase the deposition of PUFA in ruminant tissues (Buccioni et al., 2012; Wood et al., 1999).

However, elevated levels of PUFA in the phospholipid fraction of cell membranes make the meat susceptible to oxidative changes (Luciano et al., 2011a). Oxidative processes shorten the shelf life of fresh meat and negatively affect consumer acceptance as off-flavor and discoloration develop in meat. Moreover, decreased oxidative stability also influences the meat proteins. Protein oxidation has a negative impact on the nutritive and sensory properties of meat due to oxidation of indispensable amino acids or due to their reduced availability resulting in the decreased protein digestibility and tenderness of meat (Lund et al.,2011). Therefore, the oxidizable components of meat need to be protected from damages caused by the reactive oxygen species (ROS). The protection could be provided naturally through the deposition of antioxidant compounds derived from the feeds into the animal tissues. This is in accordance with the observation that the balance between the antioxidant and pro-oxidant components in meat determines the oxidative stability of lipids (Luciano et al., 2013), and correspondingly of proteins (Gravador et al., 2014).

Dietary plant secondary compounds, such as phenolics, saponins and essential oils, which are bioactive metabolites, were hypothesized to enhance the transfer of the plant-derived PUFA into the ruminant tissues by inhibiting the complete biohydrogenation of the dietary PUFA, thus improving the quality of products (Vasta & Luciano, 2011). Phenolic-rich plant materials or extracts used as feeding supplement or added to meat products exerted antioxidant properties by inhibiting lipid and protein oxidation in meat (Gravador et al., 2014; Inserra et al., 2014; Jongberg et al., 2013; Jongberg et al., 2011b; Nieto et al., 2010). Several underutilized feed resources, such as agro-industrial by-products, naturally contain high levels of these compounds, among which is

[2]Gravador, R.S., Luciano, G., Jongberg, S., Bognanno, M., Serra, M., Andersen, M.L., Lund, M., & Priolo, A. (Submitted 2014). Fatty acids and oxidative stability of meat from lambs fed carob-containing diets. *Food Chemistry*.

carob (*Ceratonia siliqua*) that is a plant native to the Mediterranean areas. It is well-adapted to the harsh climatic conditions, commercially available as a dry pulp, and commonly used in animal feeding (Vasta & Luciano, 2011).

In this study, the effects of carob pulp as an alternative feed for growing lambs on the meat fatty acid composition, and, for the first time, on the storage stability of meat colour, lipids and proteins under refrigerated and aerobic conditions, were investigated. Previous trials have demonstrated that when carob in lamb diets is equal or superior to 45% (as fed) the animal growth performances are compromised (Vasta et al., 2007a; Priolo et al., 2000). Therefore either 24% or 35% carob pulp was used in the diets in the current study in order to assure comparable lamb growth performances as the Control diet rich in barley.

## 2.2. Materials and Methods

### *2.2.1 Animal management, dietary treatments, slaughter and sampling*

The trial was conducted at an experimental farm of the University of Catania (Italy). The experimental protocol used was approved by the University of Catania in which the animals were handled by specialized personnel following the European Union Guidelines (2010/63/ EU Directive). The trial involved 26 male Comisana lambs, born in the same farm in November 2011. Lambs were weaned at age 60 days and were given free access to commercial starter concentrate and alfalfa hay until the start of the experiment. At age 90 days (average weight 20.3 kg ± 4.4 kg), the lambs were randomly assigned to three groups, individually penned and fed a total mixed diet according to three dietary treatments: Control (n=8), Carob 24 (n=9) or Carob 35 (n=9). Lambs in the Control treatment were the same used by Inserra et al. (2014) and Gravador et al. (2014). The lambs were adapted to the experimental diets over 10 days, during which the pre-experimental diet was gradually replaced with the experimental diets. Then, from 100 to 157 days of age, animals were given the experimental diets. The Control diet included 60% barley. In the Carob 24 diet, the percentage of barley was reduced to 33% and 24% carob pulp was included, while in the Carob 35 diet the 60% barley was reduced to 23% and 35% carob pulp was included. The ingredients and the chemical composition of the experimental diets are reported in Table 2.1. The diets were formulated to contain similar amounts of protein and fiber, and were ground using feed mill to pass a 5-mm screen mesh. Over the duration of the trial, the lambs had *ad libitum* access to the diets from 09.00 h to 18.00 h and fresh water was always available. Refusals were removed and weighed daily to determine the voluntary dry matter intake (DMI). The live weight of each lamb was recorded weekly at 09.00 h, before feeding lambs to calculate the average daily weight gain (ADG). Samples of the feeds offered were collected 4 times over the trial, vacuum-packed and stored at -30°C for analyses.

The lambs were slaughtered at age 158 days in a commercial abattoir, where they had access to the experimental diets and water until approximately 15 min before slaughter. The lambs were stunned by captive bolt and exsanguinated. The carcasses were weighed and halved, and the left *longissimus dorsi* muscle (LD) from the $10^{th}$-$13^{th}$ rib was excised within 20 min after the slaughter and was immediately vacuum packed and stored at -30°C until the analyses of fatty acid composition. The right half of the carcass was stored at 4°C for 24 h, after which LD was excised and pH was measured (pH meter Orion 9106). The LD was then vacuum packed and stored for 4 days at 4°C for the analyses of meat oxidative stability, described below.

*2.2.2 Chemicals*

All chemicals used were reagent grade, and water was deionized and filtered by the Milli-Q method.

*2.2.3 Analyses of feed samples*

All the analyses on feeds were performed in the pooled feed samples collected throughout the trial period. The methods of Van Soest et al. (1991) were used for the neutral detergent fiber and acid detergent fiber measurements, while the AOAC (1995) methods were used for the analyses of crude protein (CP; method 984.13) and crude fat (Ether extract; method 935.38). For the analysis of total phenols and tannins in the feeds, samples were subjected to pre-treatment following the method as described by Makkar et al. (1993) with minor modifications. Briefly, 200 mg of finely ground feeds was extracted with 5 ml of diethyl ether containing 1% acetic acid to remove the pigments and the supernate was discarded. For the extraction of total phenolic compounds, 10 ml of 70% (v/v) acetone was added and samples were subjected to ultrasonic treatment for 30 min in a cold water bath. Samples were then extracted for 2 h using a rotating device and then centrifuged at 2500 x g for 10 min at 4°C. The supernate was collected for subsequent analyses. The above extraction procedure was repeated and the supernate was collected. The residue from the acetone extraction was further extracted using a modified method described by Silanikove et al. (2006). Briefly, 9 ml of citrate-phosphate buffer containing 0.5 mg/ml of urea (pH 4.7) was added to the residue, and samples were incubated at 90°C for 2 h. The clear supernate was collected following the centrifugation at 2500 x g for 20 min. The measurements of the total phenols, using the Folin-Ciocalteu reagent, and the total tannins were measured in the same way as above after the removal of the tannins by means of polyvinylpyrrolidone (Makkar et al., 1993) were done in all the extracts. The concentration of total phenols and total tannins in feeds was calculated as the sum of the concentration measured in each extract. The assays were calibrated using standard solutions of tannic acid (TA) and results were expressed as g of TA equivalents/kg of feed (on a dry matter basis).

*2.2.4 Fatty acid analyses of feed and muscle lipids*

The fatty acids in concentrate mixtures were determined using the method of Gray et al. (1967). The total intramuscular lipids in the LD were extracted following the method of Folch et al. (1957). Briefly, a 5 g of the muscle was homogenized twice in chloroform/methanol solution (2:1, vol/vol), filtered, transferred into a separation funnel, and mixed with saline solution (0.88% KCl). The chloroform lipid fraction was washed with water/methanol solution (1:1, vol/vol), filtered and evaporated through a rotary evaporator. An aliquot of 100 mg lipid extract was methylated with 1 ml hexane and 0.05 ml 2 N methanolic KOH (IUPAC, 1987). Nonanoic acid (C9:0) was used as internal standard. The gas chromatographic analysis was carried out on a Varian model Star 3400 CX instrument equipped with a CP 88 capillary column (length: 100 m, i.d.: 0.25 mm, film thickness: 0.25 µm). Helium was used as a carrier gas at a flow rate of 0.7 ml/min. The oven temperature was programmed at 140°C and held for 4 min, then increased to 220°C at a rate of 4°C/min. The split-splitless injector temperature was 220°C and the FID detector temperature was 260°C. The injection rate was 120 ml/min with injection volume of 1 µl. Retention time and area of each peak were computed using the Varian Star 3.4.1 software. The individual fatty acid peak was identified by comparison of retention times with 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 percentage of total methylated fatty acids.

*2.2.5 Meat oxidative stability measurements*

After ageing under vacuum, three slices (2 cm thick) were prepared from each LD, using one slice for each of the three days storage: day 0 (2 h), day 3, and day 6. The slices were placed on polystyrene trays, overwrapped with oxygen permeable PVC film and kept refrigerated (4°C) in the dark. Lipid oxidation, protein oxidation and colour descriptors were measured after 2 h (day 0) and, subsequently, after 3 and 6 days of refrigerated storage. Measurements were performed as follows.

*2.2.5.1 Lipid oxidation measurement*

The 2-thiobarbituric acid reactive substances (TBARS) were quantified using the method described by Siu and Draper (1978) with some modifications. An aliquot of 2.5 g finely chopped meat trimmed of visible fat and connective tissues was homogenized in a 50 ml centrifuge tube with 12.5 ml distilled water using a tissue homogenizer (HeidolphDiax 900, HeidolphElektro GmbH & Co. KG, Kelheim, Germany) for 2 min at 9500 rpm while immersed in an ice bath. An aliquot of 12.5 ml 10% (w/v) trichloroacetic acid (TCA) was added and mixed thoroughly using a vortex mixer, and subsequently the sample was filtered through a Whatman No. 541 filter paper. To a 4.0 ml aliquot of the filtrate in screw-capped glass tube, 1.0 ml of 0.06 M thiobarbituric acid solution (TBA) was mixed prior to incubation for 90 min in a water bath at 80°C. Tubes were cooled to room temperature, and the absorbance at 532 nm (UV-Vis spectrophotometer UV-1601, Shimadzu Co., Milan, Italy) was measured. A calibration curve was plotted using 0 nmol/ml – 65.0 nmol/ml 1,1,3,3-tetraethoxypropane (TEP) in 5% TCA.

*2.2.5.2 Protein oxidation measurement*

Measurements of myofibrillar protein (MPI) oxidation were performed as described by Gravador et al. (2014). The MPI from the meat samples were isolated and were stored as freeze dried lyophilized extracts at -20°C until analysed. The MPI powder was used in the following protein oxidation measurements: (1) levels of radicals by Electron Spin Resonance (ESR), (2) oxidation of thiols determined by derivatization with Ellman's reagent, (3) formation of protein cross-links examined by SDS-PAGE and (4) levels of protein carbonyls determined through derivatization with 2,4-dinitrophenylhydrazine (DNPH). The protein concentration in the lyophilized MPI was quantified using the Pierce BCA (bicinchoninic acid) Protein Kit Assay (Thermo Specific, Pierce Biotechnology Rockford, IL, USA).

*2.2.5.3 Colour stability and myoglobin oxidation measurements*

At each day of storage, a Minolta CM-2022 spectrophotometer (*d/8°* geometry; Minolta Co., Ltd. Osaka, Japan) was used to measure meat colour descriptors lightness (L*), redness (a*), yellowness (b*), Chroma (C*), and Hue angle (H*), as well as the reflectance spectra from 400-700 nm, which were used to calculate the metmyoglobin percentage (MMb %) as described by Krzywicki (1979). Measurements were done on two different areas on meat surface and mean values were calculated. The spectrophotometer was set in the specular components excluded (SCE) mode and the illuminant A and 10° standard observer were used.

*2.2.6 Statistical analyses*

The one-way ANOVA test was used to determine the effects of dietary treatment (Diet: Control, Carob 24, or Carob 35) on animal growth performances and intramuscular fatty acid composition.

The General Linear Model with repeated measures was used to analyse the data of the meat colour stability, lipid oxidation (TBARS) and protein oxidation (radical intensity, thiol content and protein carbonyls concentration) over time of refrigerated storage. In the mixed statistical model, the individual lamb was considered as a random effect, while the dietary treatment (Diet: Control, Carob 24, or Carob 35), storage time (Time: 0, 3, or 6 days), and the Diet × Time interaction were considered as fixed effects. The Tukey's test was used for multiple comparisons of the means.

All analyses were done using the Minitab Software Version 16 (Minitab Inc., State College, PA).

## 2.3. Results

### *2.3.1 Chemical composition of the diets and lamb growth performance*

The composition of the diets fed to the animals is reported in Table 2.1. The three diets contained similar crude protein, ether extract and neutral detergent fiber, while the inclusion of carob pulp appeared to increase the acid detergent fiber content. The concentration of total phenols and tannins increased with increasing proportion of carob pulp in the diet. The Carob 24 and Carob 35 diets had higher percentage of the saturated stearic acid (C18:0), but lower levels of palmitic acid (C16:0) as compared to the Control feed. The inclusion of carob pulp in the diet increased the concentration of linolenic acid (LNA; C18:3*n-3*) compared to the Control. The dietary treatment did not affect the average daily gain, dry matter intake or feed efficiency of the lambs (Table 2.2).

### *2.3.2 Intramuscular fat content and fatty acid composition*

The use of carob pulp as a feed alternative did not affect the total intramuscular fat content of the *longissimus dorsi* muscle (LD) with a mean value of 3.26 mg/100g muscle. However, the fatty acid profiles were affected by the inclusion of carob in the diet. The concentration of saturated fatty acids (SFA) in the LD from Control-fed lambs was higher compared to Carob 24 and Carob 35 ($P < 0.01$) treatments. Similarly, the content of monounsaturated fatty acids (MUFA) was higher in the Control-fed lamb muscle than in Carob 24 or in Carob 35 groups ($P < 0.01$). Conversely, the concentration of polyunsaturated fatty acids (PUFA) was lower in the muscle of Control-fed lambs than in the muscle of both the Carob 24 and Carob 35 lambs ($P < 0.01$). Furthermore, the *n*-6/*n*-3 PUFA ratio was significantly higher in the muscle of Control lambs than in muscle of Carob 24 and Carob 35 ($P < 0.05$) lambs.

On the level of individual fatty acids, the muscle from lambs in the carob pulp fed groups had lower stearic acid content as compared to the Control treatment, despite the fact that the daily intake of this fatty acid was higher for lambs in the Carob 24 and Carob 35 groups in comparison to the Control treatment (on a dry matter basis: 0.54 and 0.37 *vs.* 0.10 g/d; $P < 0.001$; data not shown). The concentrations of LA and of LNA were affected by the dietary treatments ($P < 0.01$; Table 2.3). Specifically, higher LA concentrations were found in the muscle of animals in the Carob 24 and Carob 35 groups as compared to the muscle of animals in the Control group ($P < 0.01$). In the same manner, the LNA content in the muscle of animals in the Carob 24 and Carob 35 dietary groups was higher than in the Control ($P < 0.01$). Additionally, the content of LNA in the muscle of Carob 24 animals was lower than in the muscle of Carob 35 animals ($P < 0.05$). The higher concentration of LA and alpha-linolenic acid (ALA; C18:3 *cis-9 cis-12 cis-15*) in the muscle of lambs in either Carob 24 or Carob 35 group as compared to the muscle of lambs in the Control group ($P < 0.01$), were consistent with the daily

**Table 2.1**. Ingredients and chemical composition of the diets.

| | Diets | | |
|---|---|---|---|
| | Control | Carob 24 | Carob 35 |
| *Ingredient*[a] | | | |
| Barley | 60 | 33 | 23 |
| Carob pulp | 0 | 24 | 35 |
| Dehydrated alfalfa | 20 | 20 | 17 |
| Soybean meal | 9 | 13 | 16 |
| Wheat bran | 11 | 10 | 9 |
| *Chemical composition*[b] | | | |
| Dry matter (DM), g/kg of fresh weight | 889 | 882 | 878 |
| Crude protein, g/kg of DM | 180 | 196 | 192 |
| Ether extract, g/kg of DM | 20 | 33 | 22 |
| Neutral detergent fiber, g/kg of DM | 346 | 344 | 346 |
| Acid detergent fiber, g/kg of DM | 137 | 180 | 227 |
| Total phenols, g/kg of DM | 8.6 | 14.2 | 16.6 |
| Total tannins, g/kg of DM | 1.6 | 3.4 | 4.5 |
| *Fatty acid composition (% of total extracted fatty acids)* | | | |
| C12:0 | 0.61 | 0.1 | 0.23 |
| C14:0 | 0.31 | 0.87 | 1.03 |
| C16:0 | 22.22 | 17.82 | 17.75 |
| C16:1 | 0.6 | 0.5 | 0.32 |
| C18:0 | 1.27 | 4.17 | 3.05 |
| C18:1 *cis*-9 | 14.62 | 13.12 | 11.81 |
| C18:2 *cis*-9 *cis*-12 | 44.2 | 39.83 | 40.96 |
| C18:3 *cis*-9 *cis*-12 *cis*-15 | 13.8 | 20.75 | 22.23 |

[a]Ingredient percentages are expressed on an as-fed basis.
[b]Results are the average triplicate analysis performed on one sample pooled from sub-samples collected on a weekly basis.

**Table 2.2.** Lamb growth rate, voluntary intake and feed efficiency, fatty acid intake, and ultimate pH of lamb *longissimus dorsi* muscle as affected by dietary treatments.

| | Diets | | | | |
|---|---|---|---|---|---|
| | Control | Carob 24 | Carob 35 | SEM | *P*- value |
| No. of lambs | 8 | 9 | 9 | - | - |
| Body weight at 100 d. kg | 19.3 | 18.7 | 18.6 | 0.72 | 0.923 |
| Body weight at 157 d. kg | 29.6 | 29.9 | 28.3 | 1.08 | 0.797 |
| Average daily gain, ADG (100-157 d)/g | 181 | 198 | 170 | 8.76 | 0.418 |
| Dry matter intake, DMI, g/d[c] | 749 | 843 | 809 | 30.60 | 0.485 |
| Feed efficiency (g Body Weight, BW gain/kg DMI) | 240 | 235 | 209 | 6.86 | 0.126 |
| Ultimate pH | 5.86 | 5.77 | 5.78 | 0.02 | 0.269 |

**Table 2.3.** Effects of dietary treatments on the intramuscular fat content and fatty acid composition of lamb *longissimus dorsi* muscle.

| | Diets | | | | |
|---|---|---|---|---|---|
| | Control | Carob 24 | Carob 35 | SEM | *P*- value |
| No. of lambs | 8 | 9 | 9 | - | - |
| Total intramuscular fat, mg/100g | 2.77 | 3.12 | 3.88 | 0.236 | 0.147 |
| *Fatty acid composition, g/100g of total fatty acids* | | | | | |
| C10:0 | 0.4 | 0.38 | 0.29 | 0.028 | 0.21 |
| C12:0 | 0.79[a] | 0.65[ab] | 0.51[b] | 0.041 | 0.013 |
| C14:0 | 4.64 | 3.78 | 3.62 | 0.217 | 0.129 |
| C14:1 cis-9 | 0.12 | 0.15 | 0.17 | 0.009 | 0.126 |
| C15:0 | 0.64 | 0.52 | 0.48 | 0.037 | 0.203 |
| C15:1 | 0.19 | 0.21 | 0.26 | 0.02 | 0.317 |
| C16:0 | 16.47 | 15.72 | 15.42 | 0.234 | 0.183 |
| C16:1cis-9 | 1.089 | 0.993 | 0.963 | 0.039 | 0.407 |
| C17:0 | 1.045 | 0.96 | 0.92 | 0.031 | 0.254 |
| C17:1 cis-9 | 0.40[b] | 0.61[ab] | 0.71[a] | 0.046 | 0.014 |
| C18:0 | 14.33[a] | 13.00[b] | 13.39[b] | 0.174 | 0.003 |
| C18:1 *cis*-9 | 28.40[a] | 26.16[b] | 25.00[c] | 0.32 | <0.001 |
| C18:1 *trans*-11 | 1.29 | 1.39 | 1.47 | 0.037 | 0.141 |
| C18:2 *trans*-9 *trans* 12 | 0.59 | 0.54 | 0.51 | 0.02 | 0.27 |
| C18:2 n-6 *cis*-9 *cis*-12 | 9.20[b] | 12.51[a] | 12.96[a] | 0.418 | <0.001 |
| C18:3 n-6 *cis*-6 *cis*-9 *cis*-12 | 0.28 | 0.22 | 0.33 | 0.027 | 0.24 |
| C18:3 n-3 *cis*-9 *cis*-12 *cis*-15 | 1.00[c] | 2.11[b] | 2.60[a] | 0.15 | <0.001 |
| C18:2 *cis*-9 *trans*-11 | 0.70[b] | 1.21[a] | 1.52[a] | 0.089 | <0.001 |
| C20:2n-6 | 0.23 | 0.2 | 0.17 | 0.015 | 0.404 |
| C20:3n-6 | 0.47 | 0.35 | 0.38 | 0.024 | 0.107 |
| C20:3n-3 | 0.14[a] | 0.07[b] | 0.13[a] | 0.006 | <0.001 |
| C20:4n-6 | 8.67 | 8.21 | 8.49 | 0.301 | 0.833 |
| C20:5n-3 EPA | 0.69[b] | 1.39[a] | 1.39[a] | 0.089 | <0.001 |
| C22:5n-3 DPA | 1.94 | 1.78 | 1.76 | 0.101 | 0.737 |
| C22:6n-3 DHA | 0.71 | 1.15 | 1.35 | 0.112 | 0.058 |
| Other FA | 5.57 | 5.75 | 5.21 | 0.196 | 0.541 |
| ΣSFA[x] | 38.32[a] | 35.00[b] | 34.62[b] | 0.471 | 0.001 |
| ΣMUFA[y] | 31.49[a] | 29.51[b] | 28.57[c] | 0.28 | <0.001 |
| ΣPUFA[z] | 24.62[b] | 29.75[a] | 31.59[a] | 0.682 | <0.001 |
| *n-6* PUFA | 19.44[b] | 22.04[a] | 22.84[a] | 0.453 | 0.003 |
| *n-3* PUFA | 4.48[b] | 6.50[a] | 7.22[a] | 0.307 | <0.001 |
| *n-6/n-3* | 4.39[a] | 3.44[b] | 3.32[b] | 0.16 | 0.01 |

[x]ΣSFA = C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0. [y]ΣMUFA = C14:1 + C15:1+ C16:1 + C17:1 + C18:1 *trans-11* + C18:1 *cis-9*.
[z]ΣPUFA = C18:2 *cis-9 trans-11* + C18:2 *trans-9 cis-12* + C18:2 *cis-9 cis-12* + C18:3 *cis-6 cis-9 cis-12* + C18:3 *cis-9 cis-12 cis-15* + C20:2*n - 6* + C20:3*n - 3* + C20:4*n - 6* + C20:5*n - 3* + C22:5*n*-3 +C22:*6n -3*.
[a, b, c]Within row different superscripts indicate differences between dietary treatments ($P < 0.05$) tested using the Tukey's adjustment for multiple comparisons

intake of these fatty acids. Expressed on a dry matter basis, the intake of LA was 3.61 g/d for the Control diet, while the intake of this fatty acid by lambs in the Carob 24 and Carob 35 groups was 5.14 and 4.95 g/d, respectively ($P < 0.05$; data not shown). Likewise, the daily intake of ALA by lambs from the Control diet (1.13 g/d) was lower as compared to lambs from the Carob 24 and Carob 35 groups (2.68 and 2.69 g/d, respectively; $P < 0.01$; data not shown).

The concentration of rumenic acid (C18:2 *cis-9 trans-11*) was significantly higher in the muscle from lambs fed Carob 24 or Carob 35 diet as compared to the muscle from lambs fed Control diet ($P < 0.01$), while the level of vaccenic acid (C18:1 *trans-11*) was not affected by the dietary treatment ($P > 0.05$). On the other hand, a markedly higher concentration of stearic acid was found in the LD from lambs in the Control group than in Carob 24 ($P < 0.01$) or in Carob 35 ($P < 0.05$) group. Similarly, significantly higher oleic acid (C18:1 *cis*-9) concentration was found in muscle from Control-fed lambs in comparison with the carob pulp-fed lambs ($P < 0.01$). Additionally, there was a significantly higher lauric acid (C12:0) content in the muscle of Control lambs than in Carob 35 lambs ($P < 0.05$), similar to what was found for the concentration of heptadecenoic acid (C17:1; $P < 0.05$). The intramuscular content of eicosapentaenoic acid (EPA; C20:5*n-3*) in the muscle of Carob 24 and Carob 35 lambs was higher compared to the Control treatment ($P < 0.01$). Also, the concentration of docosahexaenoic acid (DHA; C22:6*n-3*) in the LD of lambs fed the Carob 35 diet tended to be greater as compared to the Control animals ($P = 0.051$).

*2.3.3 Oxidative stability of meat under aerobic storage conditions*

The concentration of thiobarbituric acid reactive substances (TBARS) in meat increased considerably for all the dietary treatments with time of storage ($P < 0.01$), with values from 0.16 to 2.04 mg MDA/kg meat (malondialdehyde equivalents). However, the TBARS levels in the meats were not affected by the dietary treatments ($P > 0.05$), nor by the Diet × Time interaction ($P > 0.05$) (Table 2.4).

The protein radical signal intensities increased significantly from day 0 to day 6 ($P < 0.05$) and were significantly affected by Diet × Time interaction ($P < 0.01$) (Figure 2.1). On the other hand, the animal diets only showed a tendency to affect the level of radical intensities ($P = 0.081$). The radical intensities in the MPI from the Carob 24 meat were significantly higher than Carob 35 meat ($P < 0.05$). Comparison of the radical intensities between muscle of Control and Carob 35 diets did not show significant differences, but the Control meat tended to have lower radical intensities than the Carob 24 meat ($P = 0.079$). The thiol concentrations decreased significantly from 60.10 to 51.78 nmol thiols/mg proteins from day 0 to day 6 ($P < 0.01$; Table 2.4), which corresponds to the oxidation of about 8 nmol thiols/mg proteins or 14% oxidation. The measurement of thiol concentrations was complemented with the detection of the cross-linking of the myosin heavy chains (MHC-CL) in the MPI by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). However, no MHC-CL could be detected in the gels of the MPI of meat from Control and Carob 35 stored for 0 day and 6 days (data not shown). Moreover, a significant increase in the levels of protein carbonyl compounds from day 0 to day 6 ($P < 0.01$) was observed in all the MPI. The increase in protein carbonyl concentrations accounted for 14.7%, 18.6%, 15.4% in Control, Carob 24 and Carob 35 meat samples, respectively. Overall, the dietary treatment did not exert effects on the protein oxidation parameters ($P > 0.05$; Table 2.4). Furthermore, the Diet × Time interaction (Table 2.4) demonstrated no significant effects on either thiol oxidation or carbonyls formation in the MPI.

Regarding meat colour stability, the lightness descriptor (L*) showed a tendency to change with time of storage ($P = 0.062$) and was significantly affected by the dietary treatment ($P < 0.05$). The meat from Carob 24 was lighter than meat from Carob 35 ($P < 0.01$), while the meat from Control tended to be lighter (higher L* values) than the meat from Carob 35 ($P = 0.060$). The redness descriptor (a*) in meat decreased with time of storage ($P < 0.01$) but was not affected by the diet ($P > 0.05$). The yellowness (b*) was significantly increased in meat over time of storage ($P < 0.01$) and was influenced by the dietary treatment ($P < 0.01$), but no Diet × Time interaction was found ($P > 0.05$; Table 2.4). The Carob 35 meat samples were less yellow than Control and Carob 24 ($P < 0.01$) meat samples. A less saturated (lower C* values) meat colour was measured in meat from Carob 35 fed animals compared to Carob 24 and Control-fed animals ($P < 0.01$), and values overall decreased significantly with storage duration ($P < .01$). Both the hue angle (H*) and metmyoglobin increased significantly with time of storage ($P < 0.01$), but were not affected by the dietary treatments ($P > 0.05$).

## 2.4. Discussion

The intramuscular fat content was not affected by the dietary treatment, which could be partially explained considering that the diets were formulated to be isonitrogenous and isoenergetic and that the animal growth performances were unaffected by substituting part of the barley in the diet with up to 35% carob pulp. At higher levels of carob pulp inclusion into a concentrate-based diet (45-56%) the growth performances of lambs and kids can be compromised, resulting in a reduction of the intramuscular fat content (Priolo et al., 2000; Silanikove, et al., 2006; Vasta et al., 2007a).

Inclusion of carob in the diet affected the intramuscular fatty acid composition. Higher levels of polyunsaturated fatty acids (PUFA) were present in the muscle from the carob-fed lambs as compared to the Control group, while the levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were lower. Specifically, the muscles of the Carob 24 or Carob 35 fed lambs had higher level of PUFA and a lower (more beneficial) ratio of *n-6* PUFA to *n-3* PUFA, thus the carob pulp-fed lambs are source of healthier meat as compared to the conventional concentrate-based diet-fed lambs. These could be partly explained considering that carob pulp supplementation in lamb diets led to a higher daily intake of LA and ALA in comparison to the Control diet. Consequent to the intake of more ALA was the higher level of long chain *n-3* PUFA (EPA and DHA) in the muscle of either Carob 24 or Carob 35 lamb group as compared to Control lamb group, as the synthesis of these fatty acids in animal tissues require ALA as a substrate (Wood et al., 1999).

In ruminants, the deposition of some fatty acids in the muscle is also dependent on the extent of the ruminal biohydrogenation of the ingested PUFA, which could possibly be affected by carob in the diets. Higher muscle content of stearic acid was found in Control lambs, in spite of the lower dietary intake of stearic acid provided by the Control diet in comparison to Carob 24 and Carob 35 diets. Stearic acid is the final product of biohydrogenation, thus the result suggests that biohydrogenation was inhibited to some extent in the rumen of carob pulp-fed lambs. This is in agreement with the results found by Vasta et al. (2007a), where meat from animals fed a carob-containing diet had less stearic acid compared to a control dietary treatment. Furthermore, the *Butyrivibrio fibrisolvens* in the rumen is responsible for the isomerization of LA to rumenic acid, which is an intermediate of the biohydrogenation whose final product is stearic acid (Vasta et al., 2010b). Due to the increased rumenic acid (C18:2 *cis-9 trans-11*) in carob fed lambs, it could be speculated that dietary carob to some extent inhibited the complete ruminal biohydrogenation pathway.

**Table 2.4.** Effect of the dietary treatment and time of refrigerated storage of lamb meat on lipid oxidation, protein oxidation, and colour parameters.*

| | Diets | | | Days of storage (Time) | | | SEM | *P* values | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Carob 24 | Carob 35 | 0 | 3 | 6 | | Diet | Time | Diet×Time |
| No. of lambs | 8 | 9 | 9 | | | | | | | |
| TBARS, MDA/kg meat | 1.15 | 1.32 | 1.20 | $0.16^{z}$ | $1.44^{y}$ | $2.04^{x}$ | 0.127 | 0.857 | <0.001 | 0.876 |
| Free thiol, nmol/mg proteins | 57.73 | 55.60 | 56.80 | $60.10^{x}$ | $58.12^{x}$ | $51.78^{y}$ | 0.643 | 0.290 | <0.001 | 0.433 |
| Carbonyl, nmol/mg proteins | 1.77 | 1.95 | 1.80 | $1.60^{y}$ | -- | $2.09^{x}$ | 0.089 | 0.701 | 0.007 | 0.630 |
| Lightness (L*) | $48.52^{ab}$ | $48.98^{a}$ | $46.47^{b}$ | 48.56 | 47.66 | 47.26 | 0.310 | 0.025 | 0.062 | 0.160 |
| Redness (a*) | 15.82 | 15.74 | 14.49 | $17.52^{x}$ | $14.94^{y}$ | $13.71^{y}$ | 0.284 | 0.132 | <0.001 | 0.912 |
| Yellowness (b*) | $13.16^{a}$ | $13.23^{a}$ | $11.43^{b}$ | $11.65^{y}$ | $13.00^{x}$ | $13.10^{x}$ | 0.207 | 0.003 | <0.001 | 0.100 |
| Saturation (C*) | $20.65^{a}$ | $20.65^{a}$ | $18.52^{b}$ | $21.05^{x}$ | $19.82^{xy}$ | $18.99^{y}$ | 0.297 | 0.024 | 0.001 | 0.500 |
| Hue angle (H*) | $39.98^{ab}$ | $40.13^{a}$ | $38.44^{b}$ | $33.46^{z}$ | $41.07^{y}$ | $43.70^{x}$ | 0.549 | 0.133 | <0.001 | 0.006 |
| Metmyoglobin | 44.82 | 47.05 | 44.30 | $35.33^{z}$ | $48.25^{y}$ | $52.53^{x}$ | 0.013 | 0.155 | <0.001 | 0.507 |

[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]Within row different superscripts indicate differences between days of storage ($P < 0.05$) tested using the Tukey's adjustment for multiple comparisons

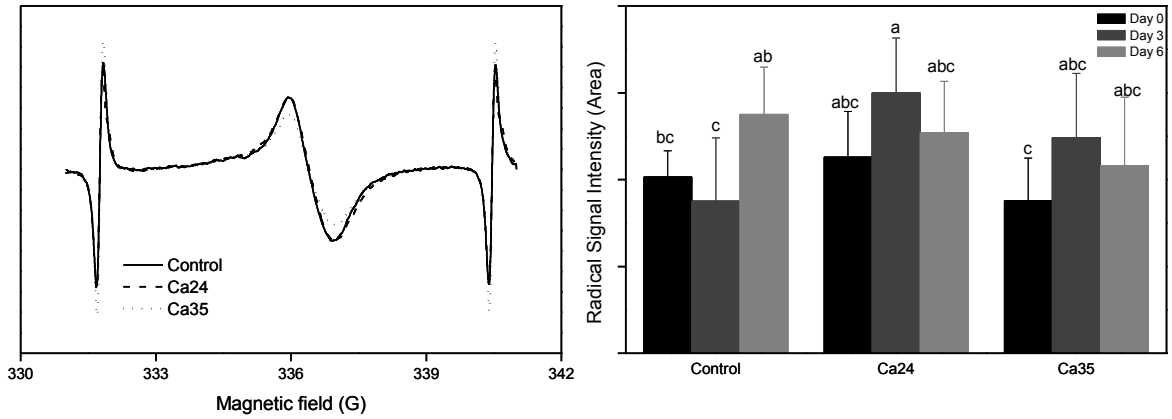


**Figure 2.1**. Electron Spin Resonance (ESR) spectra (a) and radical signal intensity (RI) (b) of myofibrillar protein isolates (MPI) extracted from lamb meat. MPI samples were stored for 0, 3 and 6 days in the dark at 4°C on polystyrene trays wrapped with oxygen-permeable PVC film. The lambs were fed with diets consisting of cereal-based concentrates (Control), or concentrates containing 24% or 35% carob pulp (Ca24 and Ca35, respectively). Values presented are means plus standard errors. Values with different superscripts are significantly different (*P<0.05*).

Another intermediate of the ruminal biohydrogenation, produced by the same microorganisms, is vaccenic acid (C18:1 *trans*-11). If the last step of ruminal biohydrogenation was inhibited by carob pulp in the diet, the muscle from Carob 24 and Carob 35-fed animals would have been expected to possess higher vaccenic acid concentration than the Control treatment, which was not the case in the present study. Vaccenic acid in the muscle originates solely from ruminal biohydrogenation, while rumenic acid can be also synthesized endogenously in the muscle from vaccenic acid, catalyzed by $\Delta^9$-desaturase enzyme (Bessa et al., 2007; Vasta et al., 2007a). Taken together, these results either indicate that: (1) the conversion of rumenic acid to vaccenic acid was slowed down, or (2) the vaccenic acid was converted back to rumenic acid in the muscle from carob-fed lambs. Considering that in the rumen, the bacteria that convert LA to rumenic acid are also responsible for the further conversion of rumenic acid to vaccenic acid, the second hypothesis appears to be more likely. A possible effect of dietary carob pulp on the ruminal biohydrogenation of PUFA could be justified taking into account that carob has been reported to contain phenolic compounds, including condensed tannins (Vasta et al., 2009a; Vasta et al., 2007a; Silanikove et al., 2006), which is in agreement with the fact that, in our study, the content of total phenols and tannins in the diets increased with increasing proportion of carob in the diet. Several results from *in vivo* and *in vitro* studies provided evidence of the inhibitory effect of purified tannins and of tannin-rich plant extracts upon the ruminal biohydrogenation, which leads to the accumulation of PUFA in animal tissues (Vasta & Luciano, 2011).

Higher concentrations of PUFA in the muscle, which in our case was found in meat from carob-fed lambs, are often associated with a reduced oxidative stability of lipids (Luciano et al., 2013; Luciano et al., 2011a). However, in this experiment the TBARS values were not affected by the dietary treatment. The increased in TBARS was lower than the values obtained in minced meat stored for seven days under HiOx-MAP in lamb fed concentrate, linseed, or linseed plus olive cake in concentrate (Luciano et al., 2013). Jerónimo et al. (2012) hypothesized that condensed tannins could have indirectly affected oxidative stability of meat through the interaction with other antioxidant compounds or with pro-oxidant compounds present in meat. Polyphenol rich plant extract incorporated in the basal diet of rats enriched with n-3 PUFA showed the ability to recycle or spare the vitamin E, and to enhance catalase activity (Gladine et al., 2007a). Moreover, the lipid and protein oxidative stability of broiler meat was also improved through dietary antioxidant supplementation containing phenolics through the enhancement of cellular antioxidant enzyme activity (Delles et al., 2014). None of these parameters were measured in the current study, however

it could be speculated that the phenolic antioxidant compounds contained in carob pulp have undergone similar mechanism of action that resulted in increased meat resistance to ROS that would otherwise attack the oxidizable PUFA. Hence oxidative damages were minimized, and were comparable to meat samples with a lower content of oxidizable PUFA throughout the six days of aerobic storage..

After six days of storage, the protein radical signal intensity increased significantly in the MPI of Control fed lamb muscle but neither the Carob 24 nor Carob 35 treatments showed considerable increase in radical signal intensity, indicating that carob feeding possibly reduced the rate of radical formation as compared to the Control feeding. Probably, a higher number of experimental units could have attenuated the variability of the data, thus allowing the observation of clearer effects of the dietary treatment on this parameter. Compared to other studies the loss of thiols observed in this study (ca. 10 nmol/mg protein) was small (Nieto et al., 2013; Zakrys-Waliwander et al., 2012). The level of thiol oxidation is linked to the formation of disulfide cross-links in the myosin heavy chains (MHC-CL) (Jongberg et al., 2013; Zakrys-Waliwander et al., 2012; Lund et al., 2007b), and in this study no MHC-CL was observed in the MPI of muscle from both Carob-fed and Control-fed lambs, which agrees with the low degree of oxidation of thiols. The MHC-CL is usually attributed to the formation of reducible disulfide bonds by oxidation of cysteine residues resulting in reduced meat tenderness (Zakrys-Waliwander et al., 2012; Lund et al., 2011; Lund et al., 2007b). The carbonyl residues, on the other hand , are formed through metal-catalyzed reactions involving the side chains of some amino acids like arginine, lysine and proline are oxidized through metal-catalyzed reactions into carbonyl residues (Lund et al., 2011). In this study the carbonyl concentration in meat was 2.09 nmol/mg protein after six days. This carbonyl concentration is lower than what was obtained by Santé-Lhoutellier et al., (2008a), in which from an initial concentration of about 2.0 nmol carbonyls/mg proteins it increased by 31.4% and 13.0% in aerobically stored meat of lambs fed concentrate and pasture, respectively.

Myoglobin oxidation to metmyoglobin, which causes browning in meat, has been found to be delayed by dietary supplementation of antioxidant compounds or by feeding on pasture (Nieto et al., 2010; Luciano et al., 2009b). Priolo et al. (2000), found reduced growth performances and a lighter coloured meat when lambs were fed 56% carob pulp. In this study, including carob pulp up to 35% did not affect growth performances and tended to reduce L* values. However, it should be stressed that fresh meat colour was measured after 2 h by Priolo et al. (2000), while in the present study colour parameters were recorded over six days of storage. In terms of meat colour stability, the oxidation of myoglobin over time of storage or display is generally associated with a corresponding decrease of the redness and saturation colour parameters (a* and C* values, respectively) and with increase of the hue angle (H*) descriptor (Luciano et al., 2011b; Khliji et al. 2010). This is in agreement with the results found in the present study, which showed that within six days of storage the a* and C* values both decreased, while the H* values increased. The meat from Control fed lambs showed a tendency to be more red (higher a*) than the meat from Carob 35, in agreement with what has been reported for meat from steers fed high tannin sorghum diets stored for seven days (Larraín et al., 2008), which may be due to the effects of tannins on muscle pigments. This effect on the a* values might have partially accounted for the lower C* values overall measured in meat from Carob 35-fed lambs, as C* descriptor results from a combination of a* and b* coordinates.

In general, the results of the meat oxidative stability parameters measured in the present study indicate that meat underwent oxidative deterioration at a rather low rate, which is evident when comparing the current results with those obtained in studies on meat subjected to higher oxidative challenges during the monitoring period. Aerobic packaging as used in the present study only contains about 20% $O_2$, while high oxygen modified atmosphere packaging, HiOx-MAP, contains at

least 70% $O_2$, thus enhancing the reactions of oxygen with the muscle components. Other treatments, such as mincing and cooking release prooxidative compounds and inactivate the antioxidant defenses of muscle by denaturation of proteins, disruption of cell membranes and depletion of antioxidant compounds (Luciano et al., 2009b). Refrigerated raw minced lamb meat packed in HiOx-MAP had levels of TBARS in the range 0.50-18.00 mg MDA/kg meat (Luciano et al., 2009) while raw whole slice of LD in HiOx-MAP had about 0.02-8.43 mg MDA/kg meat (Nieto et al., 2010) over 14 days storage. In the present study, in which fresh meat slices were subjected to aerobic refrigerated storage, the generation of TBARS (0.16-2.04 mg MDA/kg meat) indicated that lipid oxidation proceeded at a slow rate. A similar conclusion can be reached based on the results of protein oxidation found in the present study. Beef held in Hi-Ox-MAP for nine days with antioxidants had 3.3-3.7 nmol carbonyls/mg proteins, while without antioxidants the levels were 4.0-4.8 nmol carbonyls/mg proteins (Jongberg et al., 2011b), which are all higher than the values of 2.09 nmol/mg protein found in the present study. In addition, MHC-CL readily formed in meats in HiOx-MAP stored for up to 14 days such as pork patties (Nieto et al., 2013), pork and beef slices (Zakrys-Waliwander et al., 2012; Lund et al., 2007b), or beef patties (Jongberg et al., 2011b), than similar meat stored in either vacuum or aerobic package, as was the case of our study. Similarly, for the colour stability parameters, pronounced increases in H* values and metmyoglobin percentages (from 16 to 70 and from 30 % to 65%, respectively) were reported over time of storage of minced lamb meat in HiOx-MAP package (Luciano et al., 2009b). The increase in H* values and MMb% in this study were lower than the above values, which demonstrates that meat samples did not undergo severe meat browning.

Therefore, including carob pulp up to 35% into a concentrate-based diet for lambs could increase the PUFA concentration in meat without compromising the oxidative stability of colour, lipids and proteins in meat under aerobic refrigeration for six days. This could be partially explained by the action of antioxidant compounds in carob pulp.

# V. EXPERIMENT 3
# Volatiles in raw and cooked meat from lambs fed olive cake and linseed

## Volatiles in raw and cooked meat from lambs fed olive cake and linseed[3]

### 3.1. Introduction

Sheep meat is one of the dietary sources of polyunsaturated fatty acids (PUFA) such as linoleic (C18:2 *n-6*), α-linolenic (C18:3 *n-3*), and of monounsaturated fatty acids (MUFA) such as oleic acid (C18:1 *n-9*), which confer beneficial effects to human health (Enser et al., 1996). On the other hand sheep meat also contains significant amounts of saturated fatty acids (SFA) with negative impact to human health. Taking into account the beneficial effects of the healthy fatty acids and the well-established influence of diets to meat fatty acid composition, dietary manipulation for livestock has been a common practice to obtain relatively healthier products. However, an increase in unsaturation in the intramuscular fat could lead to a decrease in meat oxidative stability, as the degree of unsaturation determines the susceptibility to oxidation (Luciano et al., 2009b). During the lipid oxidation process, free radicals are produced, which can propagate leading to oxidative damage to both the lipid and protein components in the meat. These damages are correlated with the deterioration of meat quality traits including colour, flavour, and nutritional value (Santé-Lhoutellier et al., 2008b). Therefore, when animal feeding strategies are implemented to increase the levels of PUFA in the muscle, it is also important to provide adequate amounts of dietary antioxidants that could protect the meat from oxidation.

Recently, we have conducted a study to increase the concentration of PUFA *n-3* fatty acids in lamb meat by feeding diets containing linseed (Mele et al., 2014). Considering that PUFA *n-3* fatty acids are susceptible to oxidation, we have added olive cake into the diets and we found that the combination of linseed and olive cake effectively increased the content of *n-3* PUFA in the muscle (Mele et al., 2014) and preserved the meat from rapid oxidation (Luciano et al., 2013). Volatile organic compounds (VOCs) are produced through lipid oxidation, or through Maillard reaction or Strecker degradation (Mottram, 1998), which are responsible for meat flavour development. The VOCs are also strongly correlated with animal diet (Vasta and Priolo, 2006), consequently have been used as tracers of animal feeding (Priolo et al., 2004). In this study we have evaluated the effect of dietary stoned olive cake alone or in combination with rolled linseed on the meat volatile organic compounds. We have hypothesized that the differences found previously in the meat fatty acid composition and oxidative stability could lead to differences in the meat volatiles. Therefore, using the solid-phase microextraction (SPME) and subsequent GC-MS analysis, we have determined the volatile compounds in the same meat samples used by Luciano et al. (2013) and Mele et al. (2014).

[3]**Gravador, R.S.,** Serra, A., Luciano, G., Pennisi, P., Vasta, V., Pauselli, M., Mele, M., & Priolo, A. (2014). Volatiles in raw and cooked meat from lambs fed olive cake and linseed. Animal, doi:10.1017/S1751731114002730.

## 3.2. Materials and methods

*3.2.1 Animals and dietary treatments*

The detailed animal management and diet composition was published by Luciano et al. (2013) and by Mele et al. (2014). Briefly, the feeding trial was conducted at the Experimental Station of the Department of Applied Biology at the University of Perugia, Italy and involved 32 Appenninica male lambs. Animals were weaned at the age of 40 ±5 days and at the live weight of 17.8 ± 1.6 kg, and were randomly distributed into 4 groups (based on the dietary treatments) of 8 lambs each. The adaptation period to the experimental diets lasted for 20 days followed by the 40-day

experimental feeding with: barley- and oat-based concentrate with low lipid level and high non-structural carbohydrate content (diet C); concentrate containing 20% on a dry matter basis (DM) of linseed (diet L); concentrate containing 35% DM of olive cake (diet OC); or a mixture of linseed (10% DM) and olive cake (17% on DM; diet OCL). All ingredients were made into pellets (3 mm diameter) using a pelleting machine (CMS IEM-Colognola ai Colli Verona, Italy) operating at 35°C-40°C. The olive cake was prepared by the mechanical extraction of virgin olive oil using an RCM Rapanelli three phases decanter mod. 44 eco (Rapanelli Inc., Perugia, Italy). The fresh olive cake remained in the process was stored at room temperature for 36 hours, and dried through a fluid bed dryer, with an initial temperature of the drying air flow at 120°C. The maximum temperature attained by olive cake during the drying process was 45°C. The dried olive cake was stored at room temperature. The protein content and energy level of the diets were adjusted in order to obtain isonitrogenous and isoenergetic treatments. The concentrates were given to the animals, together with the grass hay (forage) following the 30:70 forage:concentrate ratio on the basis of the expected DM intake. The weights of each lamb were recorded at the beginning of the experiment and every 10 days thereafter until the day before slaughter. The amounts of feed offered and refused by animals under each group were recorded daily, and samples of these feeds were collected weekly and held at -30°C until analysis of crude protein and ether extract.

*3.2.2 Slaughter procedure*

At the end of the experimental period, the lambs were slaughtered and were weighed immediately to obtain the hot carcass weight and carcasses were kept at 4°C for 24 hours. The *longissimus dorsi* muscle (LD) between the 2$^{nd}$ and the 13$^{th}$ ribs was removed, vacuum-packed and stored at -80°C until analysis of the volatile organic compounds (VOCs).

*3.2.3 Sample preparation and volatile compounds extraction*

The analysis of VOCs was done in both raw and cooked meat. The raw meat represented the original state of animal production while the cooked meat represented the cooking condition, in which the heat employed induces the production of VOCs.

Before slicing, the visible fat of the frozen LD (n=32) was trimmed off with a scalpel to a thickness of not more than 1 mm. Six grams of the meat was placed in a 20-ml capacity glass vial and then capped with a PTFE septum. The vial was equilibrated for 20 minutes in a water bath at 45°C (±2°C). Then the solid-phase microextraction (SPME) for VOC extraction was performed by exposing a 75µm PVB/PDMS fibre (Supelco, Bellefonte, PA) to the headspace of the sample for 30 minutes, at 45°C. The same sample preparation was done on cooked meat VOC extraction except that cooking in hot water bath at an internal temperature of 70°C (±2°C) for 30 minutes was done prior to equilibration and headspace extraction.

### *3.2.4 GC-MS analysis*

Following the VOC extraction, the SPME fibre was withdrawn from the glass vial and was immediately inserted into the GC (TRACE 2000, Thermo-Finnigan, San Jose, CA). The injector temperature was set at 250°C at 4 minutes desorption time. The GC injector, with an inlet liner of 0.75 mm (Supelco, Bellefonte, PA) was operated in a splitless mode at 250°C. The carrier gas used was helium, set at a flow rate of 1.0 ml/min. The extracted volatile compounds were separated through a Supelco SPB 5 column (60m x 0.32mm x 1μm). The GC oven temperature was held at 40°C for 5 minutes and then ramped to 230°C at the rate of 3°C/min and maintained at this temperature for 5 minutes. The total acquisition time was 73 minutes. The temperature of the GC/MS interface was at 280°C. The mass spectra of the volatile compounds were obtained by using an MS equipped with an ion trap (Polaris Q, Thermo Finnigan, San Jose, CA). The electron impact acquisition mode employed was with 70 eV, and 10 microscans/s using a scanning range of 33-230 m/z. The identities of the headspace-extracted VOC were determined by comparing their mass spectra with those in the NIST 7 Mass Spectral Library (2000) and by comparing the calculated linear retention indices (LRI) with those found in literature (Kondjoyan and Berdagué, 1996; NIST Mass Spec Data Center, 2011). The LRI calculation was done by using n-alkanes standards of 5 to 17 carbon-containing atoms. The peak area of the VOCs was integrated using the specific ions for each molecule.

### *3.2.5 Statistical analysis*

The effects of the dietary treatments (C, L, OC and OCL) on the volatile organic compounds in both raw and cooked meat were analysed using one-way ANOVA of the Minitab software version 16. When ANOVA showed significant differences among samples ($P<0.05$), the means were separated by pair-wise comparison using the Tukey's test. All the data were normalized before the analysis of variance, consequently they were tabulated as $\log_{10}$ of the peak area. Pearson correlations were also performed between the VOC measured in both raw and cooked meat and data published on the tocopherol content (Luciano et al., 2013) and fatty acid composition (Mele et al., 2014) of the same muscle used in the present experiment.

## 3.3. Results and discussion

As shown by Mele et al. (2014), the inclusion of linseed in L diet increased the levels of C18:3 n-3, the inclusion of olive cake in OC diet increased the levels of C18:1 n-9, while C diet had the highest C18:2 n-6 content. Although the diets contained different quantities of SFA, MUFA and PUFA, similar growth rates among animals were observed since the diets were formulated to be isoenergetic and isonitrogenous (Mele et al., 2014; Luciano et al., 2013).

Fatty acid oxidation, and Maillard reaction or Strecker degradation (Mottram, 1998) in meat result in the production of volatile compounds. In this study, the headspace volatile organic compounds (VOCs) were extracted from raw and cooked meat of lambs fed different diets. The temperature used for the VOC extraction was 45°C (±2°C), which is lower than the 60°C used by *Vasta et al.* (2010a), to reduce the interference of volatiles formed from heat-related reactions, especially for the raw meat samples. Moreover, the cooking temperature used was 70°C (±2°C) because at low internal temperatures (72°C) minimal degradation of meat components takes place, consequently the influence of the dietary treatments and the production of volatile compounds may be easily correlated (Almela et al., 2010). Temperature is critical in VOC studies because changes in extraction temperature change the identities and total amount of volatiles derived from meat (Ahn et al. 1999). The extraction procedure used in this study was static,

which when combined with low extraction temperature, could limit the amount and number of VOCs that can be detected in the samples.

There were 20 volatile compounds identified in the raw meat samples (Table 3.1) and 30 volatile compounds were detected in the cooked meat samples (Table 3.2). The VOCs were grouped according to their chemical families: 5 aldehydes, 2 ketones, 5 hydrocarbons, 2 alcohols, 3 fatty acids, 1 ester, and 2 sulfur-containing compounds in the raw meat, and 10 aldehydes, 3 ketones, 9 hydrocarbons, 3 alcohols, 1 ester, 2 sulfur-containing, 1 furan, and 1 sulfur-and-nitrogen compound in the cooked meat. Most of the VOCs in raw meat have previously been reported in lamb meat (Vasta et al., 2010a), heifer muscle (Vasta et al., 2011), and turkey thigh (Ahn et al., 1999). The chemical families of VOCs that were found in the cooked meat were similar to those identified in cooked meat from lambs fed diets containing different fat sources (Elmore et al., 2000). Since most of the VOCs detected in meat in the current study were derived from the oxidation of fatty acids, we correlated the VOC profile of meat with the published data on the fatty acid composition (Mele et al., 2014) and tocopherol content (Luciano et al., 2013) which were measured in the same muscle samples.

The dietary treatment did not affect the concentration of aldehydes in either raw or cooked meat. Aldehydes are mainly produced from the oxidation of lipids (Vasta et al., 2011), and high amount of aldehydes can be associated with great PUFA oxidation in animal fats (Elmore et al., 2005; Vasta et al., 2012). Overall, the aldehydes concentration in the raw meat was lower than in the cooked meat, which is in agreement with the positive relation between volatile concentration and extraction temperature observed by Ahn et al. (1999). In raw meat, the total aldehyde concentration (the sum of the aldehydes detected) was not correlated with the PUFA concentration in the muscle but was positively correlated with some monounsaturated fatty acids (MUFA), such as C14:1 *trans-9* (0.353, *P=0.*047) and C16:1 *trans-10* (0.422, *P=0.016*). Conversely, in cooked meat, aldehydes concentration showed a positive correlation with the concentration of PUFA in the muscle, such as C18:3 *cis-6 cis-9 cis-12* (0.385, *P=0.030*), C22:5 *cis-4 cis-7 cis-10 cis-13 cis-16* (0.463, *P=0.008*), and in tendency with C22:4 *cis-7 cis-10 cis-13 cis-16* (0.330, *P=0.065*). This is consistent with the fact that, generally, cooking represents a stress for meat, which increases the susceptibility of PUFA to lipid peroxidation as compared to raw meat (Nieto et al., 2011). Indeed, elevated cooking temperatures increase the rate of chemical reactions (Ahn et al., 1999). Moreover, during cooking, endogenous antioxidant defences of muscle are inactivated and the iron-containing meat proteins such as myoglobin and haemoglobin are denatured, releasing free iron, a major oxidative catalyst in meat systems (Campo et al., 2003), which then enhances the interaction between the oxidants and the unsaturated fatty acid substrates (Nieto et al., 2011). Regarding the individual aldehydes, hexanal showed a tendency to be affected by the dietary treatment (P = 0.097), being lower in raw meat from lambs fed OCL diet when compared to the C diet. Hexanal and other aldehydes such as pentanal could be derived from the oxidation of linoleic acid (C18.2 n-6; Elmore et al., 2005). However, Mele et al. (2014) found no effect of the dietary treatment on the concentration of linoleic acid in the intramuscular fat from the same animals used here, and no significant correlation was found between these aldehydes and linoleic acid. Octanal and nonanal were both present in the raw and cooked meat, and originate from the oxidation of oleic acid (C18:1 *n-9;* Meynier et al., 1998), which is consistent with the fact that, in the present study, the concentration of these aldehydes in cooked meat was positively correlated with that of oleic acid (0.345, *P=0.053*). Despite the inclusion of olive cake in the diet that increased the muscle concentration of oleic acid (Mele et al., 2014), these aldehydes were not affected by the dietary treatment. The Strecker aldehydes, 2-methylbutanal and 3-methylbutanal were identified in the cooked meat and are generally associated with grilled lamb meat aroma profile (Elmore et al., 2005). Benzaldehyde was also detected in cooked meat and it has been reported to be derived from linolenic acid (C18:3*n-3*) degradation (Nuernberg et al., 2005). Linolenic acid was found at higher concentrations in meat from lamb fed linseed containing diets,

L and OCL, in comparison with the other treatments (Mele et al., 2014). However, in the current study, none of the volatile compounds that typically originate from linolenic acid degradation were found to be significantly affected by the diets. On one hand, it should be considered that individual polyunsaturated fatty acids readily form free radicals, which is the initial step in the oxidative degradation of fatty acids, but once the free radicals are formed the subsequent steps in the reaction become more independent of the nature of the fatty acids (Elmore et al., 1997; 1999). This could partly explain why, in the present study, despite the differences in the fatty acid composition of the muscle, only slight correlations were found between these lipid oxidation-derived VOCs and the individual fatty acids. Furthermore, it should be stressed that, besides the susceptibility of PUFA to oxidation (Elmore et al.,1997; Luciano et al., 2009b), the oxidative stability of meat ultimately depends on the balance between the oxidizable substrates and the antioxidants (Luciano et al., 2009b and 2013). In this trial the increased unsaturation degree of meat lipids did not probably cause extensive lipid oxidation, which could be due to the presence of antioxidants in the feeds, specifically in olive cake (Luciano et al., 2013). This is consistent with the fact that the total aldehydes in raw meat was negatively correlated with the tocopherol concentration in the muscle (-0.348, *P=0.05*), which may be an indication that the tocopherol reduced the rate of aldehyde-producing lipid oxidation process.

The 2-butanone and 3-hydroxy-2-butanone (acetoin) were the ketones detected in both the raw and cooked meat. None of the ketone was significantly affected by the dietary treatment, however in cooked meat the concentration of ketones was positively correlated with C18:3 *cis-9 trans-12 cis-15* (0.365, *P=0.040*) and C18:3 *trans-9 trans-12 cis-15* (0.359, *P=0.043*). Some studies showed a relationship between 3-hydroxy-2-butanone and fat-enriched diets (Elmore *et al.*, 2000 and 2005), while other authors reported no relationship (Vasta *et al.*, 2010a). The variations with the findings could be due to the different cooking and extraction conditions, particularly the temperature that has an impact on the volatile profile and in the samples being analyzed (Vasta et al., 2010a). The 2,3-octanedione, which has been suggested to originate from n-6 fatty acids oxidation (Meynier et al., 1998), was detected in cooked meat only. The concentration of 2,3-octanedione was observed to increase with the increase in extraction temperature, which may indicate that 2,3-octanedione could be derived from heat-induced reactions (Vasta et al., 2010a). This could explain why in the present study 2,3-octanedione was absent in the raw meat samples.

The *n-3* fatty acids are probable precursors of benzene (Elmore et al., 2005). Nute et al. (2007) found that addition of linseed oil into lamb diets increased the benzene concentration in the muscle phospholipids. On the contrary, in the current study, no effect of the dietary treatment was observed with regards to the formation of benzene. The lack of effects in the production of *n-3* PUFA-derived volatiles could be explained considering that, although the linseed-supplemented feed contained high amounts of C18:3 *n-3,* the tocopherols in the diet that were deposited in the lamb tissues (Luciano et al., 2013) were probably enough to overcome the increased unsaturation in the muscle fatty acids. Similarly, in cooked meat, none of the formed hydrocarbon was significantly influenced by the dietary treatments. The presence of hydrocarbons such as hexane, heptane and pentane in the cooked meat and their absence in the raw meat could be due to the reduced oxidative stability of the cooked samples. In fact a positive correlation was found between hydrocarbons and the fatty acids in the *longgissimus dorsi* muscle such as C18:1 *cis-9* (0.375, *P=0.034*), C18:3 *cis-6, cis-9,cis-12* (0.357, *P=0.045*), C20:3 *cis-8 cis-11 cis-14* (0.352, *P=0.048*), and C22:5 *cis-4 cis-7 cis-10 cis-13 cis-16* (0.503, *P=0.003*) and C22:4 cis-7,cis-10,cis-13,cis-16 (0.348, *P=0.05*), but not in raw meat.

Regarding the alcohols, both 2-phenoxyethanol and 1-octen-3-ol were detected in raw and cooked meat. In the raw meat, the 2-phenoxyethanol was found to be significantly affected by the dietary treatments, where OCL meat had significantly lower concentration than either C (*P=0.006*) and OC (*P=0.015*) meats. The 2-phenoxyethanol has been reported in cooked Irish

organic and conventional beef meats (Machiels et al., 2003), and 1-octen-3-ol is frequently reported among the lipid-oxidation derived VOCs in meat and meat products. The 1-pentanol was present only in cooked meat and was significantly affected by the dietary treatment, being higher in C meat in comparison with OC meat (*P=0.038*). This compound was previously reported in cooked lamb meat (Elmore et al., 2000) and cooked beef muscle (Wettasinghe et al., 2001), and could be derived from the reduction of pentanal, which is an oxidation product of linoleic acid (C18:2 *n-6*). This is consistent with the results of the compositional analysis of the feeds used in this study in which the linoleic acid content of C diet was more than double when compared with OC diet (Mele et al., 2014). Even so, no significant differences were found in the levels of volatiles, which may be due to the protective effects of the antioxidants derived from the feeds. Indeed, the concentration of volatile alcohols in cooked meat tended to be negatively correlated with the tocopherol concentration in the muscle (-0.328, *P = 0.067*).

In the raw meat, 3-methylpentanoic acid and nonanoic acid were significantly affected by the dietary treatments, while in the cooked meat, no acids were detected. The concentration of 3-methylpentanoic acid was lower in treatments L and OCL as compared to treatment C (*P=0.002* and *P=0.001*, respectively). Similarly, treatments L and OCL resulted in lower concentrations of 3-methylpentanoic acid compared to treatment OC (*P=0.001*). On the other hand, the concentration of nonanoic acid in treatment L was lower than in both treatments C (*P=0.026*) and OC (*P=0.027*) and was comparable with treatment OCL. The branched chain fatty acids are, in general, responsible for the flavour of goat and sheep meats (Vasta and Priolo, 2006) and were found to be significantly higher in intramuscular fat from lambs in the C group (Mele et al., 2014). The 3-methylpentanoic acid is one among the many volatile compounds found in sheep tissues fed either fresh grass or concentrate (Young et al., 1997). Nonanoic acid was identified in the longissimus dorsi muscle of lambs fed different diets (Vasta et al., 2010a). The dietary carbohydrates are fermented in the rumen producing propionate, which in excessive quantities is used in the synthesis of odd-chain fatty acids in the adipose tissue (Priolo et al., 2001). Branched and odd chain fatty acids may also be produced directly in the rumen by microorganisms, starting from branched amino acids and propionate, respectively (Mele et al., 2008). The lower levels of 3-methylpentanoic acid and nonanoic acid in meat from lambs fed linseed-containing diets could be a consequence of the changes in the rumen microorganism metabolism. Previous studies, indeed, reported that linseed supplementation in the diet of dairy ewes may induce a decrease in the content of odd and branched chain fatty acids in milk (Mele et al., 2011).

**Table 3.1**. Volatile organic compounds detected in the raw *longissimus dorsi* muscle from lambs fed with diets containing olive cake and linseed[A].

| Compound [m/z (relative intensity)] | Specific ion[B] | LRI[C] | Diet[D] | | | | SEM[E] | P value |
|---|---|---|---|---|---|---|---|---|
| | | | C | L | OC | OCL | | |
| *Aldehydes* | | | | | | | | |
| 2-ethyl-4-pentenal | 55 | 1029 | 3.408 | 3.334 | 3.102 | 2.945 | 0.152 | 0.712 |
| 4-ethylbenzaldehyde | 105 | 1174 | 2.552 | 2.742 | 2.117 | 1.522 | 0.215 | 0.191 |
| hexanal | 82 | 799 | 3.557 | 3.135 | 2.176 | 1.762 | 0.281 | 0.080 |
| nonanal | 67 | 1104 | 3.223 | 2.785 | 3.035 | 3.214 | 0.242 | 0.918 |
| Octanal | 81 | 1004 | 2.081 | 1.265 | 0.420 | 1.698 | 0.299 | 0.238 |
| *Ketones* | | | | | | | | |
| 2-butanone[c] | 43 | 596 | 2.458 | 1.953 | 1.926 | 1.572 | 0.276 | 0.747 |
| 3-hydroxy-2-butanone[c] | 45 | 707 | 2.118 | 2.196 | 3.273 | 3.127 | 0.289 | 0.362 |
| *Alcohols* | | | | | | | | |
| 1-octen-3-ol | 57 | 967 | 3.259 | 2.689 | 2.422 | 2.226 | 0.303 | 0.669 |
| 2-phenoxyethanol[g] | 94 | 1228 | 2.887[a] | 1.821[ab] | 2.648[a] | 0.756[b] | 0.247 | 0.004 |
| *Acids* | | | | | | | | |
| decanoic acid | 55 | 1357 | 2.786 | 1.866 | 1.935 | 1.541 | 0.265 | 0.405 |
| nonanoic acid[e] | 129 | 1259 | 2.840[a] | 0.768[b] | 2.823[a] | 2.012[ab] | 0.276 | 0.017 |
| 3-methylpentanoic acid | 60 | 947 | 2.965[a] | 0.446[b] | 3.018[a] | 0.413[b] | 0.308 | <0.001 |
| *Ester* | | | | | | | | |
| 3-butenoic acid, ethyl ester[h] | 55 | 764 | 0.436 | 0.967 | 2.212 | 2.111 | 0.315 | 0.120 |
| *Hydrocarbons* | | | | | | | | |
| 5-(1- methylethylidene)-1,3-cyclopentadiene | 91 | 849 | 3.083 | 2.608 | 2.718 | 3.080 | 0.253 | 0.883 |
| Benzene | 78 | 662 | 2.673 | 2.857 | 2.838 | 2.376 | 0.133 | 0.570 |
| trans-2,3-epoxybutane[f] | 43 | 578 | 1.877 | 1.831 | 2.859 | 2.301 | 0.330 | 0.688 |
| m-xylene[e] | 91 | 873 | 2.767 | 3.307 | 3.030 | 2.801 | 0.183 | 0.726 |
| p-xylene[d] | 106 | 855 | 3.394 | 3.608 | 3.705 | 3.074 | 0.129 | 0.331 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Sulfur-contaning* | | | | | | | | |
| carbon disulfide | 76 | 537 | 2.900 | 3.351 | 3.466 | 2.960 | 0.155 | 0.500 |
| dimethyl sulfide[c] | 62 | 519 | 3.884 | 4.039 | 3.881 | 3.722 | 0.052 | 0.206 |

[A]Values ($log_{10}$ specific ion peak area units) are means of eight lambs per dietary treatment.
[B]MS mass fragment of which area was integrated.
[C]Linear retention index
[D]The dietary treatments were: a barley- and oat-based concentrate with a low level of lipids and a high content of non-structural carbohydrate (diet C); concentrate containing 20% on a dry matter basis (DM) of linseed (diet L); a concentrate containing 35% DM olive cake (diet OC); a diet containing a mixture of linseed (10% DM) and olive cake (17% on DM; diet OCL)[E]Standard error
[a,b] Means with different letters within the same row are statistically different ($P < 0.05$)
Mass spectrum identified using Mainlib/NIST/Wiley 7 Mass Spectral Database; LRI in agreement with Kondjoyan and Berdagué (1996) and NIST Mass Spec Data Center (2011).
LRI: in agreement with linear retention index, obtained with DB5 column:
[c]Vasta *et al.*, 2007b
[d]Vasta *et al.*, 2011
LRI: in agreement with linear retention index obtained with other column:
[e]OV-101
[f]methyl silicon
[g]BPX-5
[h]SE-30

**Table 3.2.** Volatile organic compounds detected in the cooked *longissimus dorsi* muscle from lambs fed with diets containing olive cake and linseed[A].

| Compound [m/z (relative intensity)] | Specific Ion[B] | LRI[C] | Diet[D] | | | | SEM[E] | P value |
|---|---|---|---|---|---|---|---|---|
| | | | C | L | OC | OCL | | |
| *Aldehydes* | | | | | | | | |
| 2-ethyl-4-pentenal | 55 | 1029 | 3.198 | 2.877 | 2.770 | 3.206 | 0.143 | 0.629 |
| 4-ethyl-benzaldehyde | 105 | 1174 | 2.998 | 2.598 | 2.089 | 2.301 | 0.222 | 0.519 |
| hexanal | 82 | 799 | 5.290 | 5.160 | 5.133 | 5.164 | 0.033 | 0.361 |
| nonanal | 67 | 1104 | 4.670 | 4.520 | 4.486 | 4.477 | 0.037 | 0.217 |
| Octanal | 81 | 1004 | 3.736 | 1.630 | 3.009 | 3.521 | 0.337 | 0.109 |
| benzaldehyde[c] | 105 | 949 | 4.760 | 4.228 | 4.696 | 4.131 | 0.208 | 0.640 |
| 2-methyl butanal[c] | 58 | 662 | 2.924 | 2.546 | 3.346 | 2.536 | 0.220 | 0.536 |
| 3-methylbutanal[c] | 58 | 651 | 3.561 | 3.644 | 3.597 | 3.606 | 0.024 | 0.711 |
| heptanal[c] | 96 | 875 | 3.883 | 2.647 | 4.105 | 4.182 | 0.254 | 0.108 |
| Pentanal | 43 | 696 | 4.107 | 3.649 | 3.954 | 4.007 | 0.133 | 0.671 |
| *Ketones* | | | | | | | | |
| 2-butanone[c] | 43 | 596 | 4.044 | 4.053 | 4.054 | 4.037 | 0.026 | 0.996 |
| 3-hydroxy-2-butanone[c] | 45 | 707 | 1.355 | 1.824 | 2.898 | 3.095 | 0.323 | 0.166 |
| 2,3-octanedione[c] | 142 | 973 | 2.001 | 2.892 | 3.070 | 2.755 | 0.306 | 0.642 |
| *Alcohols* | | | | | | | | |
| 1-octen-3-ol | 57 | 967 | 3.693 | 3.699 | 3.941 | 3.592 | 0.220 | 0.957 |
| 2-phenoxyethanol[g] | 94 | 1228 | 2.398 | 0.993 | 1.085 | 1.026 | 0.248 | 0.124 |
| 1-pentanol | 55 | 763 | 3.899[a] | 2.041[ab] | 1.383[b] | 2.380[ab] | 0.340 | 0.052 |
| *Ester* | | | | | | | | |
| ethyl ester-3-butenoic acid[h] | 55 | 764 | 3.913 | 2.953 | 3.580 | 3.768 | 0.173 | 0.215 |
| *Hydrocarbons* | | | | | | | | |
| 5-(1- methylethylidene)-1,3-cyclopentadiene | 91 | 849 | 3.782 | 2.888 | 3.803 | 3.292 | 0.205 | 0.345 |
| trans-2,3-epoxybutane[f] | 43 | 578 | 0.802 | 0.821 | 1.753 | 1.235 | 0.287 | 0.631 |
| m-xylene[e] | 91 | 873 | 3.342 | 3.596 | 2.731 | 2.602 | 0.215 | 0.305 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| p-xylene[d] | 106 | 855 | 3.515 | 3.641 | 3.759 | 3.631 | 0.068 | 0.680 |
| 1-dodecyne[e] | 67 | 1207 | 3.159 | 1.936 | 1.600 | 1.966 | 0.263 | 0.166 |
| heptane[c] | 100 | 699 | 3.678 | 2.343 | 3.622 | 3.569 | 0.193 | 0.032 |
| Hexane | 57 | 600 | 4.010 | 3.554 | 4.108 | 3.530 | 0.179 | 0.568 |
| pentane[c] | 41 | 501 | 4.043 | 4.160 | 4.066 | 4.008 | 0.030 | 0.322 |
| toluene[c] | 92 | 768 | 3.948 | 4.027 | 3.938 | 3.915 | 0.032 | 0.639 |
| *Sulfur-containing* | | | | | | | | |
| carbon disulfide | 76 | 537 | 3.538 | 3.674 | 3.689 | 3.543 | 0.026 | 0.051 |
| dimethyl sulfide[c] | 62 | 519 | 3.868 | 3.896 | 3.769 | 3.711 | 0.032 | 0.131 |
| *Nitrogen and sulfur-contaning* | | | | | | | | |
| 2-acetyl-2-thiazoline | 101 | 1112 | 2.509 | 2.123 | 3.192 | 2.524 | 0.248 | 0.511 |
| *Furan* | | | | | | | | |
| 2-pentylfuran[c] | 81 | 991 | 3.501 | 3.516 | 3.303 | 3.883 | 0.208 | 0.813 |

[A]Values ($log_{10}$ specific ion peak area units) are means of eight lambs per dietary treatment.
[B]MS mass fragment of which area was integrated.
[C]Linear retention index
[D]The dietary treatments were: a barley- and oat-based concentrate with a low level of lipids and a high content of non-structural carbohydrate (diet C); concentrate containing 20% on a dry matter basis (DM) of linseed (diet L); a concentrate containing 35% DM olive cake (diet OC); a diet containing a mixture of linseed (10% DM) and olive cake (17% on DM; diet OCL)
[E]Standard error
[a,b] Means with different letters within the same row are statistically different ($P < 0.05$)
Mass spectrum identified using Mainlib/NIST/Wiley 7 Mass Spectral Database; LRI in agreement with Kondjoyan and Berdagué (1996) and NIST Mass Spec Data Center (2011).
LRI: in agreement with linear retention index, obtained with DB5 column:
[c]Vasta *et al.*, 2007b
[d]Vasta *et al.*, 2011
LRI: in agreement with linear retention index obtained with other column:
[e]OV-101
[f]methyl silicon
[g]BPX-5
[h]SE-30

The 3-butenoic acid ethyl ester, carbon disulfide and dimethyl sulfide were unaffected by the dietary treatment. Similarly, 2-acetyl-2-thiazoline and 2-pentylfuran, which were both detected in the cooked meat only, were not affected by the dietary treatment. According to Wettasinghe et al. (2001), the 3-butenoic acid ethyl ester, which was detected in the meat in the current study, may have little impact in the meat aroma. The carbon disulfide and dimethyl sulfide, on the other hand, are important intermediates of Maillard reaction in the formation of heterocyclic compounds (Mottram & Mottram, 2002). The 2-acetyl-2-thiazoline is considered to be a potent odorant in cooked meat patties (Kerler and Grosch, 1996), and the presence of this compound confirms the occurrence of the reaction between lipid degradation and Maillard reaction products at cooking (Elmore et al., 1997). The 2-pentylfuran could be derived from fatty acid oxidation and the fatty acid precursor is C18:2 n-6 fatty acids (Elmore et al., 2005). However, no correlation between 2-pentylfuran and any of the n-6 fatty acids was observed. The mild cooking and extraction temperature used here could explain why, unlike other studies, only one furan was detected in this study and the lack of evidence between the occurrence of these compounds and their precursors.

On the basis of the volatile compounds profile, it can be concluded the feeding lambs with diets including high proportions of olive cake and linseed in replacement of the conventional ingredients did not produce appreciable changes in the appearance of volatile organic compounds in lamb meat. Sensory evaluation of meat would be certainly of interest to explore in depth the effect of these diets upon meat sensory properties

# VI. CONCLUSIONS

The alternative feeding resources (AFR) of Mediterranean origin contain bioactive compounds able to enhance the healthy fatty acids in meat and to exert antioxidant properties. Hence, the use of AFR as replacers of the conventional ingredients of concentrate-based diets for lambs is beneficial for low-input farming system due to: the reduction of production cost, the enhancement of meat nutritional quality, and the improvement of meat oxidative stability in a natural way without sacrificing the lamb growth, health and productivity.

Specifically, the inclusion of high levels of dried citrus pulp, up to 35%, to replace part of the concentrates in lamb diet resulted in meat with high resistance to protein oxidation over time of refrigerated storage in aerobic packs. Moreover, carob pulp fed to lambs at levels of 24% or 35% in place of cereal concentrates improved the muscle content of PUFA, *n-3* PUFA, and lowered the *n-6:n-3* PUFA ratio in comparison to the meat from lambs fed a conventional cereal-based control diet. This could be explained by the higher content of PUFA in carob diet as compared to conventional diet and by the possible effect of tannins, present in carob pulp, on the reduction of the of ruminal biohydrogenation of the ingested PUFA. In spite of the susceptibility of PUFA to oxidation, meat colour, lipid and protein components in meat from the carob group were not compromised across time of aerobic and refrigerated storage. Lastly, olive cake in combination or not with linseed supplemented in to the lamb diet had minimal effect on the volatile compounds of raw or cooked meat. Cooked meat had higher concentration of volatile compounds than raw meat, which were mostly produced from fatty acid oxidation. This result is in spite of the findings published by colleagues that the studied dietary treatments affected the fatty acid composition and oxidative stability of the meat.

The protective effects of alternative feeds used in this study against oxidative damages in lamb meat is attributed to the presence and bioavailability of the antioxidant compounds, such as phenolic compounds. Higher total phenols were found in the diets containing citrus pulp or carob pulp than in the control, which could have been absorbed and deposited into the animal tissues and could have slowed down the oxidative reactions in meat by acting as antioxidants. Besides phenolics, the high amount of α-tocopherol in olive cake largely explains the high antioxidant capacity of the meat even from animals fed olive cake with linseed diets.

In conclusion, it would certainly be of interest in future studies to determine the active compounds in these feedstuffs that were able to exert antioxidant properties, and to study in depth their protective effects on meat oxidative stability. This may be done by subjecting the meat to storage that promote oxidative reactions at an extent than aerobic conditions, such as high oxygen modified atmosphere packaging, mincing or cooking. Moreover, it is also worth to see in detail the animal metabolic response to the dietary treatments as well as the influence of the proposed feedstuffs on the consumer acceptability of meat by performing sensory evaluation of the product.

## VII. REFERENCE LIST


Abbeddou, S., Rihawi, S., Hess, H. D., Iniguez, L., Mayer, A. C., & Kreuzer, M. (2011). Nutritional composition of lentil straw, vetch hay, olive leaves and saltbush leaves and their digestibility as measured in fat-tailed sheep. *Small Ruminant Research, 96,* 126-135.

Ahn D. U., Jo, C., & Olson D. G. (1999). Volatile profiles of raw and cooked turkey thigh as affected by purge temperature and holding time before purge. *Journal of Food Science 64,* 230-233.

Almela E., Jordán, M.J., Martínez, C., Sotomayor, J.A., Bedia, M., & Bañón, S. (2010). Ewe's diet (pasture vs grain-based feed) affects volatile profile of cooked meat from light lamb. *Journal of Agricultural and Food Chemistry, 58*, 9641-9646.

Ameer, B., Weintraub, R.A., Johnson, J.V., Yost, R.A., & Rouseff, R.L. (1996). Flavanone absorption after naringin, hesperidin, and citrus administration. *Clinical Pharmacology & Therapeutics, 60*(1), 34-40.

Amro, B., Aburjai, T., & Al-Khalil, S. (2002). Antioxidative and radical scavenging effects of olive cake extracts. *Fitoterapia, 73,* 456–461.

Andersen, H.J., Oksbjerg, N., Young, J.F., & Therkildsen, M. (2005). Feeding and meat quality- a future approach. A review. *Meat Science, 70(3),* 543-554.

Andrés, S., Morán, L., Aldai, N., Tejido, M. L., Prieto, N., Bodas, R., & Giráldez, F. J. (2014). Effects of linseed and quercetin added to the diet of fattening lambs on the fatty acid profile and lipid antioxidant status of meat samples. *MESC*, *97*(2), 156–163.

AOAC. (1995). Official methods of analysis. 16th ed. Assoc. Off-Anal. Chem. Arlington, WA.

Bampidis, V. A., & Robinson, P. H. (2006). Citrus by-products as ruminant feeds. A review. *Animal Feed Science and Technology*, *128*(3-4), 175–217.

Baron, C. P., & Andersen, H. J. (2002). Myoglobin-induced lipid oxidation. A review. *Journal of Agricultural and Food Chemistry*, *50*(14), 3887–97.

Batifoulier, F., Mercier, Y., Gatellier, P., & Renerre, M. (2002). Influence of vitamin E on lipid and protein oxidation induced by $H_2O_2$-activated MetMb in microsomal membranes from turkey muscle. *Meat Science, 61,* 389-395.

Benavente-García, O., Castillo, J., Marín, F. R., Ortuño, A., & Del Río, J. A. (1997). Uses and properties of citrus flavonoids. *Journal of Agricultural and Food Chemistry, 45,* 4505–4515.

Bessa, R. J. B., Alves, S. P., Jerónimo, E., Alfaia, C. M., Prates, J. A. M., & Santos-Silva, J. (2007). Effect of lipid supplements on ruminal biohydrogenation intermediates and muscle fatty acids in lambs. *European Journal of Lipid Science and Technology*, *109*(8), 868–878.

Bhattacharya, A.N., & Harb, M. (1973). Dried citrus pulp as a grain replacement for awasi lambs. *Journal of Animal Science. 36(6),* 1175-1180.

Biesalski, H.-K. (2005). Meat as a component of a healthy diet - are there any risks or benefits if meat is avoided in the diet? *Meat Science*, *70*(3), 509–24.

Bocco, A., Cuvelier, M.-E., Richard, H., & Berset, C. (1998). Antioxidant activity and phenolic composition of citrus peel and seed extracts. *Journal of Agricultural and Food Chemistry, 46,* 2123-2129.

Botsoglou, N., & Botsoglou, E. (2010). Oxidation and protection of poultry and eggs. In E. A. Decker, R. J. Elias, & J. McClements (Eds.), Oxidation in foods and beverages and antioxidant applications. Management in different industry sectors, Vol. 2. (pp.50–90) Cambridge, UK: Woodhead Publishing Limited.

Botsoglou, E., Govaris, A., Ambrosiadis, I., & Fletouris, D. (2012). Lipid and protein oxidation of α-linolenic acid-enriched pork during refrigerated storage as influenced by diet supplementation with olive leaves (*Olea europea L.*) or α-tocopheryl acetate. *Meat Science*, *92*(4), 525–32.

Botsoglou, E., Govaris, A., Ambrosiadis, I., Fletouris, D., & Botsoglou, N. (2014). Effect of olive leaf (*Olea europea L.*) extracts on protein and lipid oxidation of long-term frozen *n-3* fatty acids-enriched pork patties. *Meat Science*, *98*(2), 150-7.

Braddock, R. J. (1995). By-products of citrus fruits. *Food Technology, 49,* 74-77.

Bremner, H. A., Ford, A. L., MacFarlane, J. J., Ratcliff, D., & Russell, N. T. (1976). Meat with High Linoleic Acid Content: Oxidative Changes during Frozen Storage. *Journal of Food Science*, *41*, 757-761.

Buccioni, A., Decandia, M., Minieri, S., Molle, G., & Cabiddu, A. (2012). Lipid metabolism in the rumen: new insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. A review. *Animal Feed Science and Technology, 174,* 1-25.

Bueno, M.S., Ferrari, Jr. E., Bianchini, D., Leinz, F.F., & Rodrigues, C.F.C. (2002). Effect of replacing corn with dehydrated citrus pulp in diets of growing kids. *Small Ruminant Research. 46*, 179-185.

Campo M.M., Nute, G.R., Wood, J.D., Elmore, S.J., Mottram, D.S. & Enser, M. (2003). Modelling the effect of fatty acids in odour development of cooked meat in vitro: part I-sensory perception. *Meat Science, 63*, 367-375.

Caparra, P., Mammola, C.L., Foti, F., Sarullo, V., Verdiglione, R., & Scerra, V. (2000). Citrus pulp fresh as an ingredient in lamb diets: effects on carcass and meat quality. In: Proceedings of the Eighth International Congress of Mediterranean Federation for Health and Production of Ruminants, Giardini Naxos, Italy, 27–30 April, pp. 272–275.

Caparra, P.,Foti, F., Scerra, M., Sinatra, M.C., & Scerra, V. (2007). Solar-dried citrus pulp as an alternative energy source in lamb diets: Effects on growth and carcass and meat quality. *Small Ruminant Research, 68*, 303–311.

Contini, C., Katsikogianni, M.G., O'Neill, F.Y., O'Sullivan, M., Dowling, D.P., & Monahan, F.J. (2012). PET trays coated with citrus extract exhibit antioxidant activity with cooked turkey meat. *LWT-Food Science and Technology, 47*, 471-477.

Davies, M. J. (1990). Detection of myoglobin-derived radicals on reaction of metmyoglobin with hydrogen peroxide and other peroxidic compounds. *Free Radical Research Communications*, *10*, 361-370.

Dawson, L.E.R., Fearon, A.M., Moss, B.W., & Woods, V.B. (2010). Effects of substitution of a proportion of the concentrate in grass silage/concentrate-based diets with extruded linseed on performance and quality of dairy bulls. *Animal Feed Science and Technology, 156,* 10-18.

Deaville, E.R., Moss, A.R. & Givens, D.I., (1994). The nutritive value and chemical composition of energy-rich by-products for ruminants. *Animal Feed Science and Technology, 49,* 261-276.

Decker, E.A., Chan, W.K.M., Livisay, S.A., Butterfield, D.A., & Faustman, C. (1995). Interactions between carnosine and the different redox states of myoglobin. *Journal of Food Science*, *60*, 1201-

1204.

Decker, V., Desrumaux, C., Athias, A., Duverneuil, L., Palleau, V., Gambert, P., Masson, D., & Lagrost, L. (2002). Prevention of LDL, α-tocopherol consumption, cholesterol oxidation and vascular endothelium dysfunction by polyphenolic compounds from red wine. *Atherosclerosis, 165,* 41–50.

Delles, R.M., Xiong, Y.L., True, A.D., Ao, T.,& Dawson, K.A. (2014). Dietary antioxidant supplementation enhances lipid and protein oxidative stability of chicken broiler meat through promotion of antioxidant enzyme activity. *Poultry Science, 93,*1561-1570.

Department of Health. (1984). Diet and cardiovascular disease. Report on Health and Social Subjects No. 28. London: Her Majesty's Stationary Office HMSO, Department of Health and Social Security.

Department of Health. (1994). Report on Health and Social Subjects No. 46. Nutritional Aspects of Cardiovascular Disease. London: Her Majesty's Stationary Office (HMSO), Department of Health and Social Security.

Descalzo, A. M., & Sancho, A. M. (2008). A review of natural antioxidants and their effects on oxidative status, odor and quality of fresh beef produced in Argentina. *Meat Science*, *79*(3), 423–36.

Ellman, G.L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, *82*, 70-77.

Elmore, J.S., Mottram, D.S., Enser, M., & Wood, J.D. (1997). Novel thiazoles and 3-thiazolines in cooked beef aroma. *Journal of Agricultural and Food Chemistry, 45*, 3603-3607.

Elmore, J.S., Mottram, D.S., Enser, M., & Wood, J.D. (1999). Effect of the polyunsaturated fatty acid composition of beef muscle on the profile of aroma volatiles. *Journal of Agricultural and Food Chemistry, 47,* 1619-1625.

Elmore, J. S., Mottram, D. S., Enser, M., & Wood, J. D. (2000). The effects of diet and breed on the volatile compounds of cooked lamb, *Meat Science, 55,* 149-159.

Elmore, J.S., Cooper, S.L., Enser, M., Mottram, D.S., Sinclair, L.A., Wilkinson, R.G., & Wood, J.D. (2005). Dietary manipulation of fatty acid composition in lamb meat and its effect on the volatile aroma compounds of grilled lamb. *Meat Science 69,* 233–242.

Enser M., Hallett, K.G., Hewitt, B., Fursey, G.A.J., & Wood, J.D. (1996). Fatty acid content and composition of English beef, lamb and pork at retail. *Meat Science, 42,* 443-456.

Enser, M., Hallet, K.G., Hewett, B., Fursey, G.A.J., Wood, J.D., & Harrington, G. (1998). Fatty acid content and composition of UK beef and lamb muscle in relation to production system and implications for human nutrition. *Meat Science, 49(3),* 329-341.

Estévez, M. (2011). Protein carbonyls in meat systems: A review. *Meat Science*, *89*(3), 259-79.

Faustman, C., Sun, Q., Mancini, R., & Suman, S. P. (2010). Myoglobin and lipid oxidation interactions: mechanistic bases and control. *Meat Science*, *86*(1), 86–94.

Fernández, J., Pérez-Álvarezz, J. A., & Fernández-López, J. A. (1997). Thiobarbituric acid test for monitoring lipid oxidation in meat, *Food Chemistry, 59*(3), 345–353.

Fernández-López, J., Fernández-Ginés, J.M., Sendra, E., Sayas-Barberá, E. (2004). Application of functional citrus by-products to meat products. *Trends in Food Science & Technology, 15,* 176–185.

Fernández-López, J., Zhi, N., Aleson-Carbonell, L., Pérez-Alvarez, J. A., & Kuri, V. (2005). Antioxidant

and antibacterial activities of natural extracts: application in beef meatballs. *Meat Science*, *69*(3), 371–80.

Fernández-López, J., Sendra, E., Sayas-Barberá, E., Navarro, C., & Pérez-Alvarez, J.A. (2008). Physico-chemical and microbiological profiles of "*salchichó*n"(Spanish dry-fermented sausage) enriched with orange fiber. *Meat Science, 80,* 410–417.

Folch, J., Lees, M., & Stanley, G. S. H. (1957). A simple method for the isolation and purification of lipids from animal tissue. *Journal of Biological Chemistry, 226,* 497-509.

Frankel, E. (1980). Lipid oxidation. *Progress in Lipid Research*, *19*, 1-22.

Ganhão, R., Morcuende, D., & Estévez, M. (2010). Protein oxidation in emulsified cooked burger patties with added fruit extracts: Influence on colour and texture deterioration during chill storage. *Meat Science*, *85*(3), 402–9.

Gatellier, P., Hamelin, C., Durand, Y., & Renerre, M. (2001). Effect of a dietary vitamin E supplementation on colour stability and lipid oxidation of air- and modified atmosphere-packaged beef, *Meat Science, 59*, 133–140.

Gatellier, P., Mercier, Y., Juin, H., & Renerre, M. (2005). Effect of finishing mode (pasture- or mixed-diet) on lipid composition, colour stability and lipid oxidation in meat from Charolais cattle. *Meat Science*, *69*(1), 175–86.

Gladine, C., Morand, C., Rock, E., Gruffat, D., Bauchart, D., & Duran, D. (2007a). The antioxidative effect of plant extracts rich in polyphenols differs between liver and muscle tissues in rats fed *n-3* PUFA rich diets. *Animal Feed Science and Technology, 139,* 257–272.

Gladine, C., Rock, E., Morand, C., Bauchart, D., & Durand, D. (2007b). Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. *The British Journal of Nutrition*, *98*(4), 691–701.

Gobert, M., Gruffat, D., Habeanu, M., Parafita, E., Bauchart, D., & Durand, D. (2010). Plant extracts combined with vitamin E in PUFA-rich diets of cull cows protect processed beef against lipid oxidation. *Meat Science*, *85*(4), 676–83.

Gorinstein, S., Martín-Belloso, O., Park, Y. S., Haruenkit, R., Lojek, A., Ciz, M., Caspi, A., Libman, I., & Trakhtenberg, S. 2001. Comparison of some biochemical characteristics of different citrus fruits. *Food Chemistry, 74*, 309–315.

Govaris, A., Botsoglou, N., Papageorgiou, G., Botsoglou, E., & Ambrosiadis, I. (2004). Dietary versus post-mortem use of oregano oil and/or alpha-tocopherol in turkeys to inhibit development of lipid oxidation in meat during refrigerated storage. *International Journal of Food Sciences and Nutrition*, *55*(2), 115–23.

Grasser, L.A., Fadel, J.G., Garnett, I.,& De Peters, E.J. (1995). Quantity and economic importance of nine selected by-products used in California dairy rations. *Journal of Dairy Science, 78*, 962–971.

Gravador, R. S., Jongberg, S., Andersen, M. L., Luciano, G., Priolo, A., & Lund, M. N. (2014). Dietary citrus pulp improves protein stability in lamb meat stored under aerobic conditions. *Meat Science, 97,* 231-236.

Gray, I. K., Rumsby, M. G., & Hawke, J. C. (1967). The variations in linoleic acid and galactolipid levels in *Graminaceae* species with age of tissue and light environment. *Phytochemistry, 6,* 107-1

Huang, F.R., Zhan, Z.P., Luo, J., Liu, Z. X., & Peng, J. (2008). Duration of dietary linseed feeding affects the intramuscular fat, muscle mass and fatty acid composition in pig muscle. *Livestock Science, 118,* 132-139.

Insani, E. M., Eyherabide, A., Grigioni, G., Sancho, A. M., Pensel, N. A., & Descalzo, A. M. (2008). Oxidative stability and its relationship with natural antioxidants during refrigerated retail display of beef produced in Argentina. *Meat Science*, *79*(3), 444–52.

Inserra, L., Priolo, A., Biondi, L., Lanza, M., Bognanno, M., Gravador, R., & Luciano, G. (2014). Dietary citrus pulp reduces lipid oxidation in lamb meat. *Meat Science, 96,* 1489-1493.

IUPAC. (1987). Standard methods for the analysis of oils, fats and derivatives: Oxford: Pergamon Press.

Jerónimo, E., Alfaia, C. M. M., Alves, S. P., Dentinho, M. T. P., Prates, J. A. M., Vasta, V., Santos-Silva, J., & Bessa, R. J. B. (2012). Effect of dietary grape seed extract and *Cistus ladanifer L.* in combination with vegetable oil supplementation on lamb meat quality. *Meat Science*, *92*(4), 841–7.

Jia, N., Kong, B., Liu, Q., Diao, X., & Xia, X. (2012). Antioxidant activity of black currant (*Ribes nigrum L.*) extract and its inhibitory effect on lipid and protein oxidation of pork patties during chilled storage. *Meat Science*, *91*(4), 533–9.

Jiménez-Colmenero, F., Carballo, J., & Cofrades, S. (2001). Healthier meat and meat products: their role as functional foods. A review. *Meat Science, 59,* 5-13.

Jongberg, S., Lund, M.N., Waterhouse, A.L., & Skibsted, L.H. (2011a). Thiol-quinone adduct formation and disulfide cross-linking of myofibrillar proteins in chill-stored beef added 4-methyl catechol as antioxidant. *Journal of Agricultural and Food Chemistry, 59*, 0329-10335.

Jongberg, S., Skov, S. H., Tørngren, M. A., Skibsted, L. H., & Lund, M. N. (2011b). Effect of white grape extract and modified atmosphere packaging on lipid and protein oxidation in chill stored beef patties. *Food Chemistry*, *128*(2), 276–283.

Jongberg, S., Tørngren, M. A., Gunvig, A., Skibsted, L. H., & Lund, M. N. (2013). Effect of green tea or rosemary extract on protein oxidation in Bologna type sausages prepared from oxidatively stressed pork. *Meat Science*, *93*(3), 538–46.

Kerler, J. & Grosch, W. (1996). Odorants contributing to warmed-over flavour (WOF) of refrigerated cooked beef. *Journal of Food Science, 61,* 1271-1275.

Khliji, S., van de Ven, R., Lamb, T. A., Lanza, M., & Hopkins, D. L. (2010). Relationship between consumer ranking of lamb colour and objective measures of colour. *Meat Science*, *85*, 224–229.

Kim, Y.H.B., Huff-Lonergan, E., Sebranek, J.G., & Lonergan, S. (2010). High-oxygen modified atmosphere packaging system induces lipid and myoglobin oxidation and protein polymerization. *Meat Science*, *85*, 759-767.

Kim, Y.H.B., Bødker, S., & Rosenvold, K. (2012). Influence of lamb age and high-oxygen modified atmosphere packaging on protein polymerization of long-term aged lamb loins. *Food Chemistry*, *135,* 122–126.

Kiritsakis, A.P. (1999). Composition of olive oil and its nutritional and health effect. In Proceedings of the 10th International Rapeseed Congress, 26th–29th September 1999, Canberra, Australia, pp. 28– 33.

Koleckar, V., Kubikova, K., Rehakova, Z., Kuca, K., Jun, D., Jahodar, L., & Opletal, L. (2008). Condensed and hydrolysable tannins as antioxidants influencing the health. *Mini-Reviews in Medicinal Chemistry*, *8*(5), 436–447.

Kondjoyan, N., & Berdagué J.-L. 1996. A compilation of relative retention indices for the analysis of aromatic compounds: Laboratoire Flaveur, Clermont Ferrand, France.

Koutina, G., Jongberg, S., & Skibsted, L. (2012). Protein and lipid oxidation in Parma ham during production. *Journal of Agricultural and Food Chemistry*, *60*, 9737-9745.

Krzywicki, K. (1979). Assessment of relative content of myoglobin, oxymyoglobin and metmyoglobin at the surface of beef. *Meat Science, 3,* 1–10.

Kumazawa, S., Taniguchi, M., Suzuki, Y., Shimura, M., Kwon, M., & Nakayama, T. (2002). Antioxidant activity of polyphenols in carob pods. *Journal of Agricultural and Food Chemistry, 50,* 373−377.

Kurowska, E.M., Borradaile, N.M., Spence, J.D., & Carroll, K.K. (2000). Hypocholesterolemic effects of dietary citrus juices in rabbits. *Nutrition Research*, *20(1)*, 121-129.

Lanza, A. (1982). Dried citrus pulp in animal feeding. In Proceedings of the food industry and the environment international symposium. pp. 189–198. Budapest, Hungary.

Lanza, M., Priolo, A., Biondi, L., Bella, M., & Salem, H.B. (2001). Replacement of cereal grains by orange pulp and carob pulp on faba bean-based diets fed to lambs: effects on growth performance and meat quality. *Animal Research, 50*, 21-30.

Lanza, M., Fasone, V., Galofaro, V., Barbagallo, D., Bella, M., & Pennisi, P. (2004). Citrus pulp as an ingredient in ostrich diet: effects on meat quality. *Meat Science, 68,* 269-275.

Larraín, R. E., Schaefer, D. M., Richards, M. P., & Reed, J. D. (2008). Finishing steers with diets based on corn, high-tannin sorghum or a mix of both: color and lipid oxidation in beef. *Meat Science*, *79*(4), 656-65.

Lauridsen, C., Buckley, D. J., & Morrissey, P. A. (1997). Influence of dietary fat and vitamin E supplementation on alpha-tocopherol levels and fatty acid profiles in chicken muscle membranal fractions and on susceptibility to lipid peroxidation. *Meat Science, 46,* 9-22.

Levine, R.L., Williams, J.A., Stadtman, E.R., & Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. In Methods in enzymology, pp. 346-357. San Diego: Academic Press Inc.

Liu, H. W., Gai, F., Gasco, L., Brugiapaglia, A., Lussiana, C., Guoc, K. J., Tonga, J. M., & Zoccarato, I. (2009). Effects of chestnut tannins on carcass characteristics, meat quality, lipid oxidation and fatty acid composition of rabbits. *Meat Science*, *83*(4), 678-83.

Liu, H., Zhou, D., Tong, J., & Vaddella, V. (2012). Influence of chestnut tannins on welfare, carcass characteristics, meat quality, and lipid oxidation in rabbits under high ambient temperature. *Meat Science*, *90*(1), 164-9.

Love, J. D., & Pearson, A. M. (1970). Lipid Oxidation in Meat and Meat Products. A review. *Journal of the American Oil Chemists' Society, 48,* 28-30.

Luciano, G., Monahan, F. J., Vasta, V., Biondi, L., Lanza, M., & Priolo, A. (2009a). Dietary tannins improve lamb meat colour stability. *Meat Science*, *81*(1), 120-125.

Luciano, G., Monahan, F. J., Vasta, V., Pennisi, P., Bella, M., & Priolo, A. (2009b). Lipid and colour stability of meat from lambs fed fresh herbage or concentrate. *Meat Science*, *82*(2), 193-199.

Luciano, G., Moloney, A. P., Priolo, A., Röhrle, F. T., Vasta, V., Biondi, L., López-Andrés, P., Grasso, S., & Monahan, F. J. (2011a). Vitamin E and polyunsaturated fatty acids in bovine muscle and the oxidative stability of beef from cattle receiving grass or concentrate-based rations. *Journal of Animal Science, 89,* 3759–3768.

Luciano, G., Vasta, V., Monahan, F. J., López-Andrés, P., Biondi, L., Lanza, M., & Priolo, A. (2011b). Antioxidant status, color stability and myoglobin resistance to oxidation of *longissimus dorsi* muscle from lambs fed a tannin-containing diet. *Food Chemistry*, *124*(3), 1036–1042.

Luciano, G., Pauselli, M., Servili, M., Mourvaki, E., Serra, A., Monahan, F. J., Lanza, M., Priolo, A., Zinnai, A., & Mele, M. (2013). Dietary olive cake reduces the oxidation of lipids, including cholesterol, in lamb meat enriched in polyunsaturated fatty acids. *Meat Science*, *93*(3), 703–714.

Lund, M.N., Hviid, M.S., & Skibsted, L.H. (2007a). The combined effect of antioxidants and modified atmosphere packaging on protein and lipid oxidation in beef patties during chill storage. *Meat Science, 76*, 226-233.

Lund, M. N., Lametsch, R., Hviid, M. S., Jensen, O. N., & Skibsted, L. H. (2007b). High-oxygen packaging atmosphere influences protein oxidation and tenderness of porcine *longissimus dorsi* during chill storage. *Meat Science*, *77*(3), 295-303.

Lund, M.N., Hviid, M.S., Claudi-Magnussen, C., & Skibsted, L.H. (2008a). Effects of dietary soybean oil on lipid and protein oxidation in pork patties during chill storage. *Meat Science*, *79,* 727–733.

Lund, M.N., Luxford, C., Skibsted, L.H., & Davies, M.J. (2008b). Oxidation of myosin by haem proteins generates myosin radicals and protein cross-links. *Biochemistry Journal, 410,* 565-574.

Lund, M. N., Heinonen, M., Baron, C. P., & Estévez, M. (2011). Protein oxidation in muscle foods. A review. *Molecular Nutrition & Food Research*, *55*(1), 83–95.

Machiels, D., van Ruth, S.M., Posthumus, M.A. & Istasse, L. (2003). Gas chromatography-olfactometry analysis of the volatile compounds of two commercial Irish beef meats. *Talanta, 60,* 755-764.

Makkar, H.P.S., Blümmel, M., Borowy, N.K. & Becker, K. (1993). Gravimetric determination of tannins and their correlations with chemical and protein-precipitation methods. *Journal of the Science of Food and Agriculture, 61,* 161-165

Makkar, H. P. (2003). Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research*, *49*(3), 241–256.

Mancini, R. A., & Hunt, M. C. (2005). Current research in meat color. *Meat Science*, *71*(1), 100–21.

Marín, F. R., Martínez, M., Uribesalgo, T., Castillo, S., & Frutos, M. J. (2002). Changes in nutraceutical composition of lemon juices according to different industrial extraction systems. *Food Chemistry. 78*, 319–324.

Martín-García, A. I., Moumen, A., Yáñez-Ruiz, D. R., & Molina-Alcaide, E. (2003). Chemical composition and nutrients availability for goats and sheep of two-stages olive cake and olive leaves. *Animal Feed Science and Technology, 107,* 61–74.

McAfee, A. J., McSorley, E. M., Cuskelly, G. J., Moss, B. W., Wallace, J. M. W., Bonham, M. P., &

Fearon, A. M. (2010). Red meat consumption: an overview of the risks and benefits. *Meat Science*, *84*(1), 1–13.

Mele M., Buccioni A., Serra A., Antongiovanni, M., & Secchiari, P. (2008). Lipids of goat's milk: origin, composition and main sources of variation. In Dairy goats feeding and nutrition (eds G Pulina and A Cannas), pp. 47-70 CAB International, Wallingford, UK.

Mele, M., Contarini, G., Cercaci, L., Serra, A., Buccioni, A., Povolo, M., Conte, G., Funaro, A., Banni, S., Lercker, G., & Secchiari, P. (2011). Enrichment of Pecorino cheese with conjugated linoleic acid by feeding dairy ewes with extruded linseed: effect on fatty acid and triglycerides composition and on oxidative stability. *International Dairy Journal, 21,* 365-372.

Mele, M., Serra, A., Pauselli, M., Luciano, G., Lanza, M., Pennisi, P., Conte, G., Taticchi, A., Esposto, S., & Morbidini, L. (2014). The use of stoned olive cake and rolled linseed in the diet of intensively reared lambs: effect on the intramuscular fatty-acid composition. *Animal : An International Journal of Animal Bioscience*, *8*(1), 152-62.

Mercier, Y., Gatellier, P., Viau, M., Remignon, H., & Renerre, M. (1998). Effect of dietary fat and vitamin E on colour stability and on lipid and protein oxidation in turkey meat during storage. *Meat Science, 48*(3), 301-318.

Mercier, Y., Gatellier, P., & Renerre, M. (2004). Lipid and protein oxidation in vitro, and antioxidant potential in meat from Charolais cows finished on pasture or mixed diet. *Meat Science*, *66*(2), 467-73.

Meynier, A., Genot, C., & Gandemer, G. (1998). Volatile compounds of oxidized pork phospholipids. *Journal of the American Oil Chemists' Society, 75,* 1-7.

Molina-Alcaide, E., & Yáñez-Ruiz, D. R. (2008). Potential use of olive by-products in ruminant feeding. A review. *Animal Feed Science and Technology*, *147*(1-3), 247-264.

Monahan, F. J., Skibsted, L. H., & Andersen, M. L. (2005). Mechanism of oxymyoglobin oxidation in the presence of oxidising lipids in bovine muscle. Journal of Agricultural and Food Chemistry, 53, 5734–5738

Mottram, D.S. (1998). Flavour formation in meat and meat products. A review. *Food Chemistry, 62*, 415-424.

Mottram, D.S., & Mottram, H.R. (2002). An overview of the contribution of sulphur- containing compounds to the aroma in heated foods. In Heteroatomic aroma compounds (eds GA Reineccius and TA Reineccius), pp. 73–92. American Chemical Society, Washington, DC, USA.

Nieto, G., Díaz, P., Bañón, S., & Garrido, M. D. (2010). Effect on lamb meat quality of including thyme (*Thymus zygis ssp. gracilis*) leaves in ewes' diet. *Meat Science*, *85*(1), 82–8.

Nieto, G., Estrada, M., Jordán, M.J., Garrido, M.D., & Bañón, S. (2011). Effects in ewe diet of rosemary by-product on lipid oxidation and the eating quality of cooked lamb under retail display conditions. *Food Chemistry 124,* 1423-1429.

Nieto, G., Jongberg, S., Andersen, M. L., & Skibsted, L. H. (2013). Thiol oxidation and protein cross-link formation during chill storage of pork patties added essential oil of oregano, rosemary, or garlic. *Meat Science*, *95*(2), 177–84.

NIST Mass Spec Data Center. (2011). Retention Indices. February 12, 2012. http://webbook.nist.gov.

Nuernberg, K., Fischer, K., Nuernber, G., Kuechenmeister, U., Klosowska, D., Eliminowska-Wenda, G.,

Fiendler, I., & Ender, K. (2005). Effects of dietary olive and linseed oil on lipid composition, meat quality, sensory characteristics and muscle structure in pigs. *Meat Science, 70,* 63-74.

Nute, G. R., Richardson, R. I., Wood, J. D., Hughes, S. I., Wilkinson, R. G., Cooper, S. L., & Sinclair, L. A. (2007). Effect of dietary oil source on the flavour and the colour and lipid stability of lamb meat. *Meat Science*, *77*(4), 547-55.

Piquer, O., Ródenas, L., Casado, C., Blas, E., & Pascual, J.J. (2009). Whole citrus fruits as an alternative to wheat grain or citrus pulp in sheep diet: Effect on the evolution of ruminal parameters. *Small Ruminant Research, 83,* 14-21.

Priolo, A., Lanza, M., Biondi, L., Pappalardo, P., & Young, A. (1998). Effect of partially replacing dietary barley with 20% carob pulp on post-weaning growth, and carcass and meat characteristics of comisana lambs. *Meat Science, 50*(3), 355-363.

Priolo, A., Waghorn, G.C., Lanza, M., Biondi, L., & Pennisi, P. (2000). Polyethylene glycol as a means for reducing the impact of condensed tannins in carob pulp: Effects on lamb growth performance and meat quality. *Journal of Animal Science, 78,* 810-816.

Priolo, A., Micol, D., & Agabriel, J. (2001). Effects of grass feeding systems on ruminant meat colour and flavour. A review. *Animal Research, 50,* 185-200.

Priolo, A., Cornu, A., Prache, S., Krogmann, M., Kondjoyan, N., Micol, D., & Berdagué, J.-L. (2004). Fat volatiles tracers of grass feeding in sheep. *Meat Science 66,* 475-481.

Priolo, a., Bella, M., Lanza, M., Galofaro, V., Biondi, L., Barbagallo, D., Ben Salem, H., & Pennisi, P. (2005). Carcass and meat quality of lambs fed fresh sulla (*Hedysarum coronarium L.*) with or without polyethylene glycol or concentrate. *Small Ruminant Research*, *59*(2-3), 281–288.

Rhee, K. S., Ziprin, Y. A., Ordonez, G., & Bohac, C. E. (1988). Fatty acid profiles of the total lipids and lipid oxidation in pork muscles as affected by canola oil in the animal diet and muscle location. *Meat Science*, *23*, 201-210.

Rivas-Cañedo, A., Apeleo, E., Muiño, I., Pérez, C., Lauzurica, S., Pérez-Santaescolástica, C., & de la Fuente, J. (2013). Effect of dietary supplementation with either red wine extract or vitamin E on the volatile profile of lamb meat fed with omega-3 sources. *Meat Science*, *93*(2), 178-86.

Rodríguez-Carpena, J. G., Morcuende, D., & Estévez, M. (2011). Avocado by-products as inhibitors of color deterioration and lipid and protein oxidation in raw porcine patties subjected to chilled storage. *Meat Science*, *89*(2), 166-73.

Roginsky, V., & Lissi, E. (2005). Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry*, *92*(2), 235-254

Salminen, H., Estévez, M., Kivikari, R., & Heinonen, M. (2006). Inhibition of protein and lipid oxidation by rapeseed, camelina and soy meal in cooked pork meat patties. *European Food Research and Technology*, *223*(4), 461-468.

Salminen, H., Jaakkola, H., & Heinonen, M. (2008). Modifications of tryptophan oxidation by phenolic-rich plant materials. *Journal of Agricultural and Food Chemistry, 56,* 11178-11186

Santé-Lhoutellier, V., Engel, E., Aubry, L., & Gatellier, P. (2008a). Effect of animal (lamb) diet and meat storage on myofibrillar protein oxidation and *in vitro* digestibility. *Meat Science*, *79*(4), 777-83.

Santé-Lhoutellier, V., Engel, E., & Gatellier, P. (2008b). Assessment of the influence of diet on lamb meat oxidation. *Food Chemistry*, *109*(3), 573–579.

Scerra, V., Caparra, P., Foti, F., Lanza, M., & Priolo, A. (2001). Citrus pulp and wheat straw silage as an ingredient in lamb diets: effects on growth and carcass and meat quality. *Small Ruminant Research, 40*, 51-56.

Scerra, M., Caparra, P., Foti, F., Cilione, C., Zappia, G., Motta, C., & Scerra, V. (2011). Intramuscular fatty acid composition of lambs fed diets containing alternative protein sources. *Meat Science, 87,* 229-233.

Schieber, A., Stintzing, F. C., & Carle, R. (2001). By-products of plant food processing as a source of functional compounds—recent developments. *Trends in Food Science & Technology, 12,* 401-413.

Scollan, N., Hocquette, J.-F., Nuernberg, K., Dannenberger, D., Richardson, I., & Moloney, A. (2006). Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. *Meat Science*, *74*(1), 17-33.

Servili, M., Esposto, S., Fabiani, R., Urbani, S., Taticchi, A., Mariucci, F., Selvaggini, R., & Montedoro, G. F. (2009). Phenolic compounds in olive oil: Antioxidant, health and sensory activities according to their chemical structures. *Inflammopharmacology, 17,* 1–9.

Servili, M., Selvaggini, R., Esposto, S., Taticchi, A., Montedoro, G. F., & Morozzi, G. (2004). Health and sensory properties of virgin olive oil hydrophilic phenols: Agronomic and technological aspects of production that affect their occurrence in the oil. *Journal of Chromatography A, 1054,* 113-127.

Silanikove, N., Landau, S., Or, D., Kababya, D., Bruckental I., & Nitsan, Z. (2006). Analytical approach and effects of condensed tannins in carob pods (*Ceratonia siliqua*) on feed intake, digestive and metabolic responses of kids. *Livestock Science*, *99*, 29-38.

Simitzis, P.E., Deligeorgis, S.G., Bizelis, J.A., Dardamani, A., Theodosiou, I., & Fegeros, K. (2008). Effect of dietary oregano oil supplementation on lamb meat characteristics. *Meat Science, 79,* 217-223.

Simitzis, P.E., Ilias-Dimopoulos, V., Charismiadou, M.A., , Biniari, E. E., & Deligeorgis, S.G. (2013). The effects of dietary hesperidin supplementation on lamb performance and meat characteristics. *Animal Science Journal, 84*, 136–143.

Simopoulos, A.P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedecine & Pharmacotherapy, 56,* 365-379.

Siu, G. M., & Draper, H. H. (1978). A survey of the malonaldehyde content of retail meat and fish. *Journal of Food Science, 43,* 1147-1149.

Stadtman, E.R., Levine, R.L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino acids*, *25,* 207-218.

Uchida, K., Sakai, K., Itakura, K., Osawa, T., & Toyokuni, S. (1997). Protein modification by lipid peroxidation products: formation of malondialdehyde-derived $N^{\epsilon}$ -(2-propenal)lysine in proteins. *Archives of Biochemistry and Biophysics, 346(1),* 45-52.

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fibre, neutral detergent fibre, and no starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science, 74,* 3583-3597.

Vargas-Bello-Pérez, E., Vera, R.R., Aguilar, C., Lira, R., Peña, I., & Fernández , J. (2013). Feeding olive

cake to ewes improves fatty acid profile of milk and cheese. Short communication. *Animal Feed Science and Technology, 184,* 94-99.

Vasta, V., & Priolo, A. (2006). Ruminant fat volatiles as affected by diet. A review. *Meat Science, 73,* 218-228.

Vasta, V., Pennisi, P., Lanza, M., Barbagallo, D., Bella, M., & Priolo, A. (2007a). Intramuscular fatty acid composition of lambs given a tanniniferous diet with or without polyethylene glycol supplementation. *Meat Science*, *76*(4), 739-45.

Vasta, V., Ratel, J., & Engel, E. (2007b). Mass spectrometry analysis of volatile compounds in raw meat for the authentication of the feeding background of farm animals. *Journal of Agricultural and Food Chemistry, 55,* 4630-4639.

Vasta, V., Nudda, A., Cannas, A., Lanza, M., & Priolo, A. (2008). Alternative feed resources and their effects on the quality of meat and milk from small ruminants. *Animal Feed Science and Technology*, *147*(1-3), 223-246.

Vasta, V., Makkar, H. P. S., Mele, M., & Priolo, A. (2009a). Ruminal biohydrogenation as affected by tannins *in vitro*. *The British Journal of Nutrition*, *102*(1), 82-92.

Vasta, V., Mele, M., Serra, M., Scerra, M., Luciano, G., Lanza, M., & Priolo, A. (2009b). Metabolic fate of fatty acids involved in ruminal biohydrogenation in sheep fed concentrate or herbage with or without tannins. *Journal of Animal Science*, *87*(8), 2674-84.

Vasta, V., Priolo, A., Scerra, M., Hallett, K. G., Wood, J. D., & Doran, O. (2009c). $\Delta^9$-desaturase protein expression and fatty acid composition of *longissimus dorsi* muscle in lambs fed green herbage or concentrate with or without added tannins. *Meat Science*, *82*(3), 357-64.

Vasta, V., Jerónimo, E., Brogna, D. M. R., Dentinho, M. T. P., Biondi, L., Santos-Silva, J., & Bessa, R. J. B. (2010a). The effect of grape seed extract or *Cistus ladanifer L.* on muscle volatile compounds of lambs fed dehydrated lucerne supplemented with oil. *Food Chemistry*, *119*(4), 1339-1345.

Vasta, V., Yáñez-Ruiz, D. R., Mele, M., Serra, A., Luciano, G., Lanza, M., & Priolo, A. (2010b). Bacterial and protozoal communities and fatty acid profile in the rumen of sheep fed a diet containing added tannins. *Applied and Environmental Microbiology*, *76*(8), 2549-55.

Vasta, V., Luciano, G., Dimauro, C., Röhrle, F., Priolo, A., Monahan, F.J., & Moloney, A.P. (2011). The volatile profile of *longissimus dorsi* muscle of heifers fed pasture, pasture silage or cereal concentrate: Implication for dietary discrimination. *Meat Science, 87,* 282-289.

Vasta, V., & Luciano, G. (2011). The effects of dietary consumption of plants secondary compounds on small ruminants' products quality. *Small Ruminant Research, 101*, 150-159.

Vasta, V., Ventura, V., Luciano, G., Andronico, V., Pagano, R.I., Scerra, M., Biondi, L., Avondo, M., & Priolo, A. (2012). The volatile compounds in lamb fat are affected by the time of grazing. *Meat Science, 90,* 451-456.

Ventanas, S., Estévez, M.,Tejeda, F.J., Ruiz, J. (2006). Protein and lipid oxidation in *longissimus dorsi* and dry cured loin from Iberian pigs as affected by cross-breeding and diet. *Meat Science, 72,* 647-655.

Viljanen, K., Kylli, P., Kivikari, R., & Heinonen, M. (2004). Inhibition of protein and lipid oxidation in liposomes by berry phenolics. *Journal of Agricultural and Food Chemistry, 52,* 7419-7424.

Viuda-Martos, M., Ruiza-Navajas, Y., Fernández-López, J., & Pérez-Alvarez, J.A. (2010). Effect of added citrus fiber and spice essential oils on quality and shelf-life of mortadella. *Meat Science, 85,* 568-576.

Vuorela, S., Salminen, H., Mäkelä, M., Kivikari, R., Karonen, M., & Heinonen, M. (2005). Effect of plant phenolics on protein and lipid oxidation in cooked pork meat patties. *Journal of Agricultural and Food Chemistry*, *53*(22), 8492-7.

Warnants, N., VanOeckel, M. J., & Boucque, C. V. (1996). Incorporation of dietary polyunsaturated fatty acids in pork tissues and its implications for the quality of the end products. *Meat Science*, *44*, 125-144.

Wettasinghe, M., Vasanthan, T., Temelli, F., & Swallow, K. (2001). Volatile flavour composition of cooked by-product blends of chicken, beef and pork: a quantitative GC-MS investigation. *Food Research International, 34,* 149–158.

Williams, P. (2007). Section 2: Key nutrients delivered by red meat in the diet. Nutritional composition of red meat. *Nutrition & Dietetics, 64 (Suppl. 4),* S113-S119.

Wolf, G. (2006). How an increased intake of alpha-tocopherol can suppress the bioavailability of gamma-tocopherol. *Nutrition Reviews, 64,* 295-299.

Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Richardson, R. I., & Sheard, P. R. (1999). Manipulating meat quality and composition. *Proceedings of the Nutrition Society, 58,* 363-370.

Wood, J. D., Richardson, R. I., Nute, G. R., Fisher, A. V., Campo, M. M., Kasapidou, E., Sheard, P. R., & Enser, M. (2003). Effects of fatty acids on meat quality. A review. *Meat Science, 66,* 21-32.

Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Sheard, P. R., & Richardson, R. I. (2008). Fat deposition, fatty acid composition and meat quality. A review. *Meat Science, 78,* 343-358.

Woods, V. B., & Fearon, A. M. (2009). Dietary sources of unsaturated fatty acids for animals and their transfer into meat, milk and eggs. A review. *Livestock Science*, *126*(1-3), 1-20.

Young, O.A., Berdagué, J-L., Viallon, C., Rousset-Akrim, S. & Theriez, M. (1997). Fat-borne volatiles and sheep meat odour. *Meat Science, 45,* 183-200.

Zakrys, P. I., Hogan, S. A., O'Sullivan, M. G., Allen, P., & Kerry, J. P. (2008). Effects of oxygen concentration on the sensory evaluation and quality indicators of beef muscle packed under modified atmosphere. *Meat Science, 79,* 648-655.

Zakrys-Waliwander, P.I., O'Sullivan, M.G., O'Neill, E.E., & Kerry, J.P. (2012). The effects of high oxygen modified atmosphere packaging on protein oxidation of bovine *longissimus dorsi* muscle during chilled storage. *Food Chemistry*, *131*, 527-532.

Zhou, B., Wu, L. M., Yang, L., & Liu, Z. L. (2005). Evidence for alpha-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. *Free Radical Biology and Medicine, 38*, 78-84.