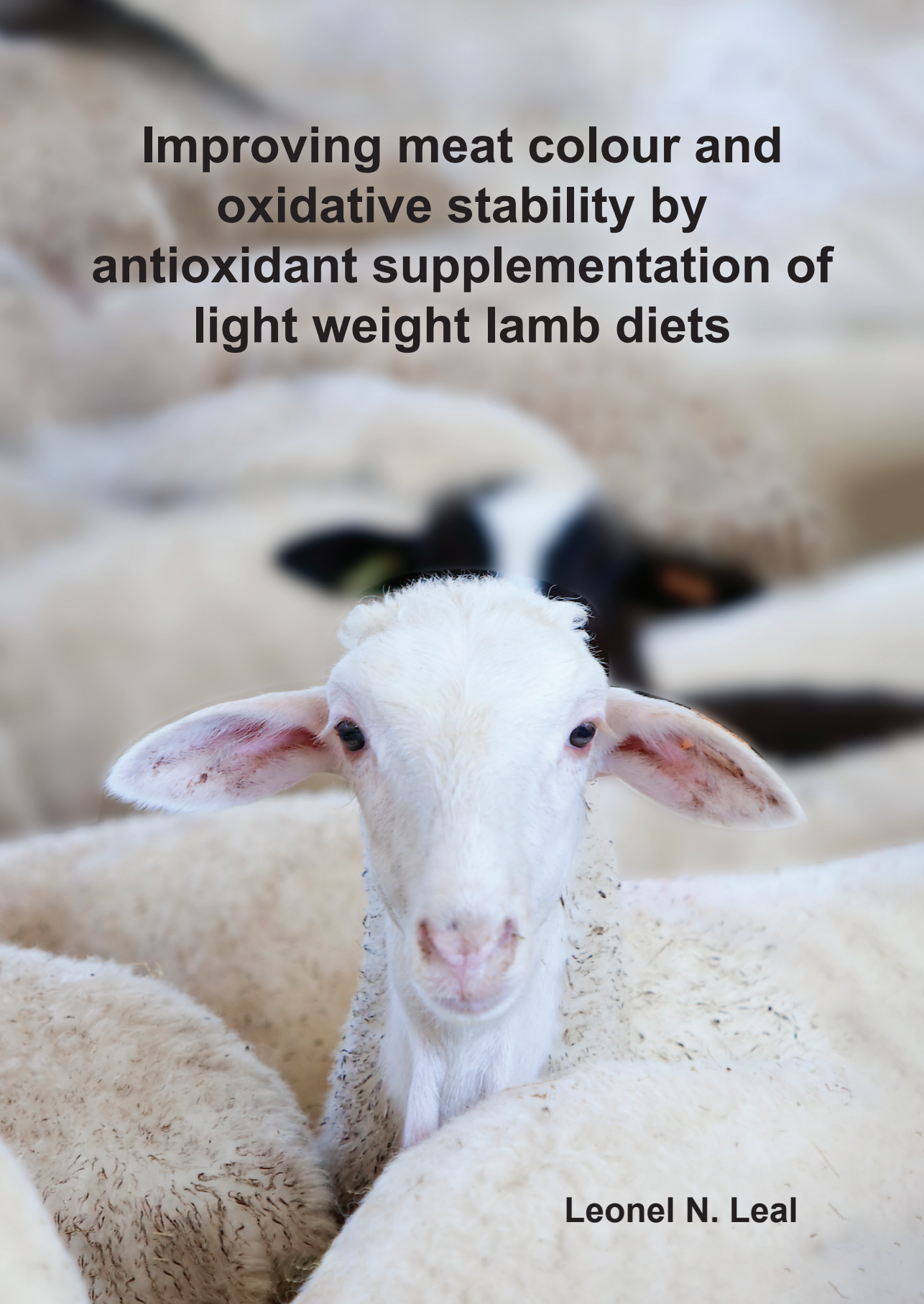
Improving meat colour and oxidative stability by antioxidant supplementation of light weight lamb diets



Leonel N. Leal

Propositions

1. The difference in the biological activity between natural and synthetic vitamin E cannot be represented by the use of a single ratio.
(this thesis)

2. Dietary supplementation strategies with plant extracts are far from becoming a “gold standard” at preventing oxidation and colour deterioration of lamb meat.
(this thesis)

3. In experiments, the absence of well-chosen “controls” produces meaningless results and incorrect conclusions unwittingly leading to poorly formulated subsequent hypotheses and ill-designed studies.

4. With the dawn of the information era, there is an urgent need for content curation.

5. The best approach to solve a problem is to start anew instead of using someone else’s draft.

6. Like with many other aspects of life, in research, knowing where we come from and why we do what we do, unveils the direction that should be followed.

7. Writing a PhD thesis confronts oneself with their past naivety and brilliance.

Propositions belonging to the thesis, entitled
Improving meat colour and oxidative stability by antioxidant supplementation of light weight lamb diets
Leonel N. Leal
Wageningen, 18 October 2019

Improving meat colour and oxidative stability by antioxidant supplementation of light weight lamb diets

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Improving meat colour and oxidative stability by antioxidant supplementation of light weight lamb diets

Leonel N. Leal

Thesis

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Chapter 1

General Introduction

Each year, one third of all global food products for human consumption are "lost" or "wasted" along the food supply chain (Food and Agriculture Organization, 2011). Apart from its economic impact, food loss and wastage represents a misuse of resources including land, water and energy. In Europe, food waste per capita at the consumer level is approximately 95-115 kg year^{-1}, which represents more than 35% of the total food wasted along the entire food chain (production, post-harvest handling, processing, distribution and consumption). For the 61 million tons of meat and meat products produced in Europe, approximately 25% is lost or wasted (**Figure 1.1**). These losses occur mainly at the end of the food chain (processing, distribution and consumer), with losses occurring by the consumer representing half of the total meat and meat products wasted (Food and Agriculture Organization, 2011).

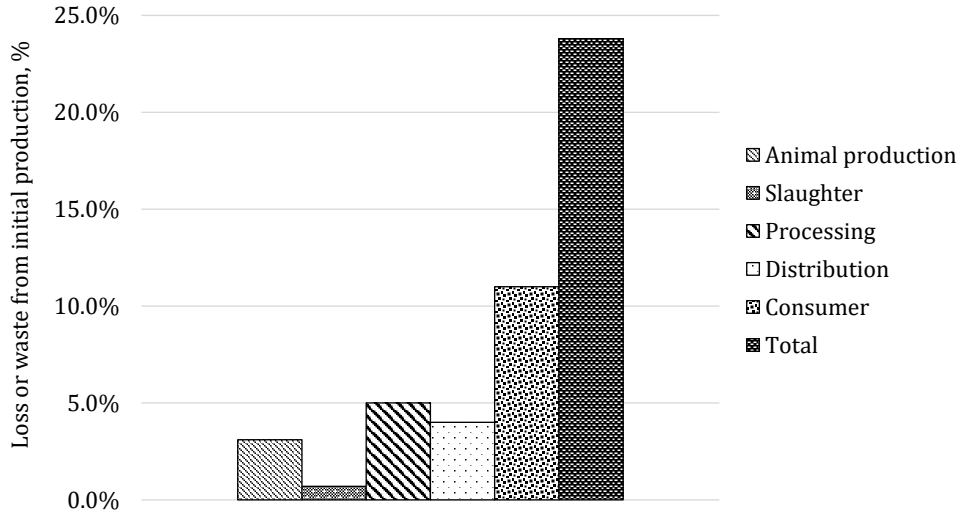
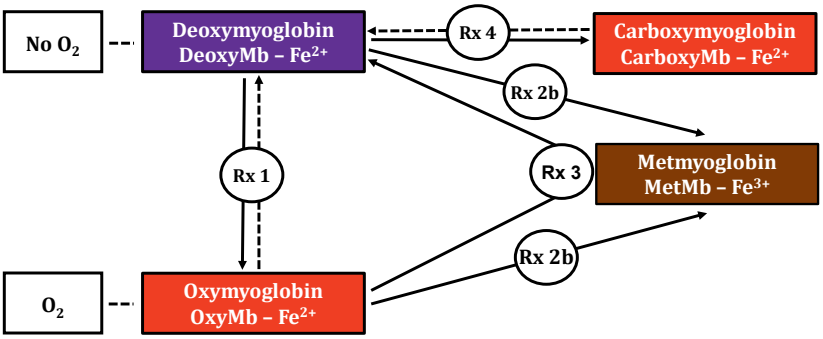


Figure 1.1 Part of the initial production of meat and meat products wasted at different stages of the food chain in Europe. Source: Food and Agriculture Organization, 2011.

It is widely recognized that the willingness of consumer to eat or purchase fresh meat or meat products is strongly associated by product appearance including its colour (Faustman and Cassens, 1990). In lamb and other ruminant derived meats, consumers perceive a "bright", "cherry-red" colour as an indicator of freshness and wholesomeness of the product, while they discriminate against meat that has turned brown (O'Grady et al., 2000; Djenane et al., 2003).

Meat Colour and Oxidative Stability

Meat colour is highly affected by the heme protein myoglobin (Faustman et al., 2010). The concentration of this protein and its oxidation state determines meat colour via the occurrence of four chemical forms of myoglobin (**Figure 1.2**). Deoxymyoglobin (DeoxyMb) is the purple pigment observed in freshly cut meat. Following its exposure to air, DeoxyMb becomes oxygenated to oxymyoglobin (OxyMb), which presents the desirable bright, cherry red colour appreciated by the consumer. After further exposure to air (hours or days), OxyMb is further oxidized to metmyoglobin (MetMb) in which a molecule of water is substituted for a molecule of oxygen resulting in brown pigmentation. Discoloration results from conversion of the ferrous iron (Fe^{2+}) form present in DeoxyMb and OxyMb to the ferric form (Fe^{3+}) present in MetMb. Carboxymyoglobin (CarboxyMb), is formed when carbon monoxide attaches to DeoxyMb, producing a stable bright-red colour in the absence of environmental oxygen. Atmospheres containing oxygen will result in the conversion of CarboxyMb to either OxyMb or MetMb (Mancini and Hunt, 2005).



Rx 1 (oxygenation) : DeoxyMb + O_2 ⟶ OxyMb
Rx 2a (oxidation): OxyMb + [oxygen consumption or low O_2 partial pressure] – e^- ⟶ MetMb
Rx 2b (oxidation): [DeoxyMb – hydroxyl ion – hydrogen ion complex] + O_2 ⟶ MetMb + O_2^-
Rx 3 (reduction): MetMb + oxygen consumption + MetMb reducing activity ⟶ DeoxyMb
Rx 4 (carboxymyoglobin): DeoxyMb + carbon monoxide ⟶ CarboxyMb

Figure 1.2 Visible myoglobin redox interconversions on the surface of meat. Adapted from: Mancini and Hunt, 2005.

Further enhancements of the red colouring of meat can be achieved with modified atmosphere packaging (MAP) technologies, which can be a highly oxidizing packaging method (70%-80% of oxygen and 30%-20% carbon dioxide) (Álvarez et al., 2008). As previously described, the high concentrations of oxygen environment increase the oxygenation of myoglobin and lead to the development of the bright red OxyMb (Gatellier et al., 2005). However, over time these high concentrations of oxygen in the MAP also

accelerate lipid oxidation and MetMb formation which, in turn, contribute to the deterioration of flavour, texture and colour of the meat or meat products (Jakobsen and Bertelsen, 2000).

Lipid oxidation is commonly described as an oxygen dependent chain reaction that is comprised of three major steps: initiation, propagation and termination (Eitenmiller and Lee, 2004).

Initiation is the first step in lipid oxidation and occurs when a hydrogen is abstracted from a methylene carbon in a fatty acid (RH) by exposure to a catalyst such as light, heat, metal ions or through the action of lipoxygenase to form a lipid (alkyl) radical ($R^{\cdot}$) (Eq. 1) (Halliwell and Chirico, 1993).

(Eq. 1) $RH \longrightarrow R^{\cdot} + H^{\cdot}$

Propagation is used to describe the high reactivity of lipid radicals with triplet oxygen which leads to the rapid formation of a peroxy radical ($ROO^{\cdot}$) (Eq. 2).

(Eq. 2) $R^{\cdot} + O2 \longrightarrow ROO^{\cdot} + H^{\cdot}$

Peroxy radicals will preferentially oxidize other unsaturated fatty acids to form a hydroperoxide and a new unstable lipid (Eq. 3). This lipid radical then reacts with oxygen to produce another peroxy radical, which represents a significant self-propagating reactant for the autocatalytic chain reaction (Eq. 4).

(Eq. 3) $ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$
(Eq. 4) $R^{\cdot} + O_2 \longrightarrow ROO^{\cdot} + H^{\cdot}$

Hydroperoxides (ROOH) are unstable in most chemical and environmental conditions and their radicals can further accelerate propagation reactions. These branching steps or secondary reactions (Eqs. 5 and 6) are often homolytic in their progression and occur rapidly at elevated temperatures.

(Eq. 5) $ROOH \longrightarrow RO^{\cdot} + OH$
(Eq. 6) $RO^{\cdot} + RH \longrightarrow ROH + R^{\cdot}$

Secondary decomposition products such as pentanal, hexanal, 4-hydroxynoneal and malondialdehyde (MDA) which are responsible for causing off-flavours and reduced shelf-life (Pearson et al., 1983; Raharjo and Sofos, 1993) may then be formed.

Termination, in the last step of the lipid peroxidation reaction and occurs when two radicals react and produce a non-radical product (Eqs. 7, 8 and 9).

(Eq. 7) $ROO^{\cdot} + R^{\cdot} \longrightarrow ROOR$

(Eq. 8) $R^{\cdot} + R^{\cdot} \longrightarrow R\text{-}R$

(Eq. 9) $ROO^{\cdot} + ROO^{\cdot} \longrightarrow ROOR + O_2$

Lipid oxidation is, therefore, another major determinant of quality and acceptability of meats and meat products. The chemistry associated with oxidation of lipids and myoglobin seems to indicate that these reactions may exacerbate one another and that they cannot be easily dissociated. As meat ages, lipid peroxidation increases the rate of MetMb formation and conversely MetMb can act as a catalyst for lipid peroxidation (Faustman et al., 2010).

Antioxidant Use in Lamb Diets

In order to delay lipid oxidation and colour deterioration, both retailers and meat producers adopted the incorporation of antioxidants into animal diets or directly added to the meat during processing (Nerín et al., 2006). Nutritional interventions are generally preferred over direct addition of the antioxidant to the muscle, since it is a convenient strategy to uniformly incorporate the antioxidant into the subcellular membranes (Govaris et al., 2004; Descalzo and Sancho, 2008). More importantly, these cell-integrated antioxidants are more effective at preventing oxidative damage than those added directly to the meat (Kerry et al., 1998).

In vivo, antioxidant molecules (such as vitamin E) and enzymes found in skeletal muscle (catalase, superoxide dismutase and glutathione peroxidase) constitute the main line of defence against free radicals (Descalzo and Sancho, 2008). Following exsanguination, cellular antioxidant enzymes maintain only a remnant activity until the onset of cell death (Renerre et al., 1996; Descalzo and Sancho, 2008). Since the enzymatic antioxidant mechanism is compromised after slaughter, a considerable amount of antioxidant molecules must be incorporated into the tissues before slaughter to effectively reduce meat oxidation and colour deterioration.

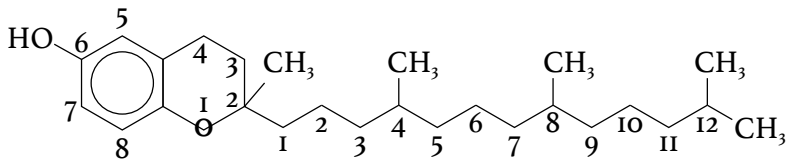
Among potential antioxidant molecules, vitamin E is the most commonly used because of its known effects on raising α-tocopherol concentrations in tissues (Ochoa et al., 1992), delaying lipid oxidation and improving the colour stability of lamb meat (López-Bote et al., 2001; Lauzurica et al., 2005).

Vitamin E

After its discovery by Evans and Bishop nearly 100 years ago, the role of vitamin E as a major chain-breaking antioxidant in biological systems has become largely undisputed. Naturally occurring vitamin E, encompasses a group of eight compounds, including α-, β-, γ-, and δ-tocopherols and four corresponding unsaturated analogues (α-, β-, γ-, and δ-tocotrienol). As presented in **Table 1.1**, all feature a chromanic nucleus (6-chromanol) with a hydroxyl group that can donate a hydrogen atom to reduce free radicals and an

aliphatic 16-carbon side chain (unsaturated side chain in case of tocotrienols) that allows its incorporation into biological membranes (Schneider, 2005).

Table 1.1 Structural terminologies of tocopherol and tocotrienol isoforms. Adapted from Ghosh et al. (2008).



Common Name	Structural Terminology
α-tocopherol	2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol
β-tocopherol	2,5,8-trimethyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol
γ-tocopherol	2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol
δ-tocopherol	2,8-dimethyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol
α-tocotrienol	2,5,7,8-tetramethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)chroman-6-ol
β-tocotrienol	2,5,8-trimethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)chroman-6-ol
γ-tocotrienol	2,7,8-trimethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)chroman-6-ol
δ-tocotrienol	2,8-dimethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)chroman-6-ol

Alpha and γ-tocopherols are the predominant forms of vitamin E found in nature, of which α-tocopherol has the greatest biological activity and is the main form of vitamin E found in blood and animal tissues (Wolf, 2006). Following intake, dietary and supplemental vitamin E is absorbed and delivered to the liver, where mainly α-tocopherol is recognized by a specific 32-kDa, α-tocopherol transfer protein (α-TTP). This selective binding of α-tocopherol by α-TTP, regulates body's vitamin E levels, metabolism and excretion (Burton and Traber, 1990;Traber and Atkinson, 2007). As demonstrated *in vitro* by Hosomi et al. (1997), when considering the affinity of α-TTP for α-tocopherol as 100%, the affinity for the remaining tocopherol analogues were the following: β-tocopherol 38%, γ-tocopherol 9% and δ-tocopherol 2%.

Alpha-tocopherol supplements used in animal nutrition are different from the natural occurring forms which consists of a single stereoisomer, 2'R, 4'R, 8R'-α-tocopherol (see **Figure 1.3**). Due to the 3 asymmetric centres in the side chain, synthetic α-tocopherol (*all-rac*-α-tocopherol) is a racemic mixture (12.5% of each) of eight stereoconfigurations of the α-tocopherol molecule (RRR-, RRS-, RSR-, RSS-, SRR-, SSR-, SRS-, and SSS-) (Weiser and Vecchi, 1981, 1982).

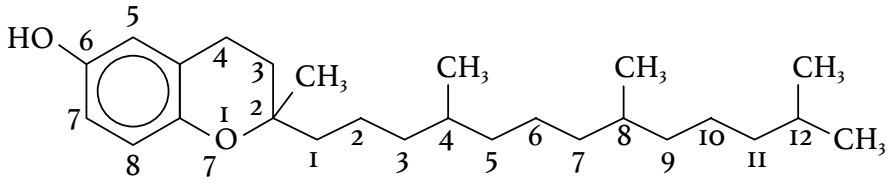


Figure 1.3 Struture of RRR-α-tocopherol, with R configuration at postion 2', 4', and 8' of the phytil tails. Source: Dersjant-Li and Peisker (2010).

Discrimination in the liver among these α-tocopherol stereoisomers also occurs through the action of α-tocopherol transfer protein (α-TTP), which preferentially binds to 2R stereoisomers, resulting in degradation of the majority of 2S stereoisomers in the *all-rac*-α-tocopherol (synthetic α-tocopherol) (Kuchan et al., 2016). Although different α-tocopherol stereoisomers may present similar antioxidant activity *in vitro* (Food and Nutrition Board, 2000), the stereoselectivity of α-TTP towards the RRR-α-tocopherol stereoisomer seems to be an important factor for the difference in bioactivity between RRR- and *all-rac*-α-tocopherol. In an attempt to standardize vitamin E applications, the United States Pharmacopeia (USP, 1979) defined 1 mg of *all-rac*-α-tocopheryl acetate to equal 1 international unit (IU) of vitamin E, 1 mg of *all-rac*-α-tocopherol to 1.1 IU, 1 mg of RRR-α-tocopheryl acetate to 1.36 IU and 1 mg of RRR-α-tocopherol to 1.49 IU. These values were mainly derived from a series of rat resorption-gestation tests first published by Harris and Ludwig (1949) and later supported by data using a similar model on the relative biopotency (i.e. capacity of a chemical substance to function in a biological system) of each α-tocopherol stereoisomer (Weiser and Vecchi, 1981; 1982). However, some concerns have been identified regarding the use of these conversion factors to discriminate between different forms of vitamin E. When feeding animals diets that are similar in vitamin E on an IU basis, concentrations of α-tocopherol in biological fluids and tissues are almost always higher in RRR-α-tocopherol fed animals when compared with *all-rac*-α-tocopherol (Lauridsen et al., 2002; Jensen et al., 2006; Weiss et al., 2009). Moreover, Blatt et al. (2004) added to the overall discussion when proposing that *all-rac*- and RRR-α-tocopherol are not equivalent in any dosage ratio. Based on previous studies, they concluded that a fixed dosage ratio of *all-rac*- and RRR-α-tocopherol cannot produce a fixed ratio of effects on all processes in all tissues at all times after all dosages. Therefore, studies focused on quantitative determination of total α-tocopherol and its stereoisomer composition in different tissues will allow a better understanding of their relative bioavailability.

Plant Extracts

Besides α-tocopherol, alternative sources of natural antioxidants have been investigated in several types of plant such as vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs (Kähkönen et al., 1999). Like in animals, plants are under constant oxidative stress caused by free radicals, reactive oxygen species, and prooxidants generated either exogenously (heat, light and water shortage) or endogenously (hydrogen peroxide). In response, plant cells have developed antioxidant systems to control free radicals, lipid oxidation catalysts, oxidation intermediates and secondary breakdown products (Lacopini et al., 2008). Interestingly, crude extracts from some plant materials rich in phenolics have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of plant extracts has been linked to their phenolic compound content, as demonstrated in several in vitro studies (Zheng and Wang, 2001; Shan et al., 2005).

All plant phenolic compounds arise from common precursors (phenylalanine or shikimic acid), so far more than 8000 polyphenolic compounds have been identified in different plants or plant materials (Pandey and Rizvi, 2009). However, based on the number of phenol rings and the structural elements binding those rings to one another, plant phenolics can be broadly classified into 4 groups (Spencer et al., 2008), as shown in **Figure 1.4**.

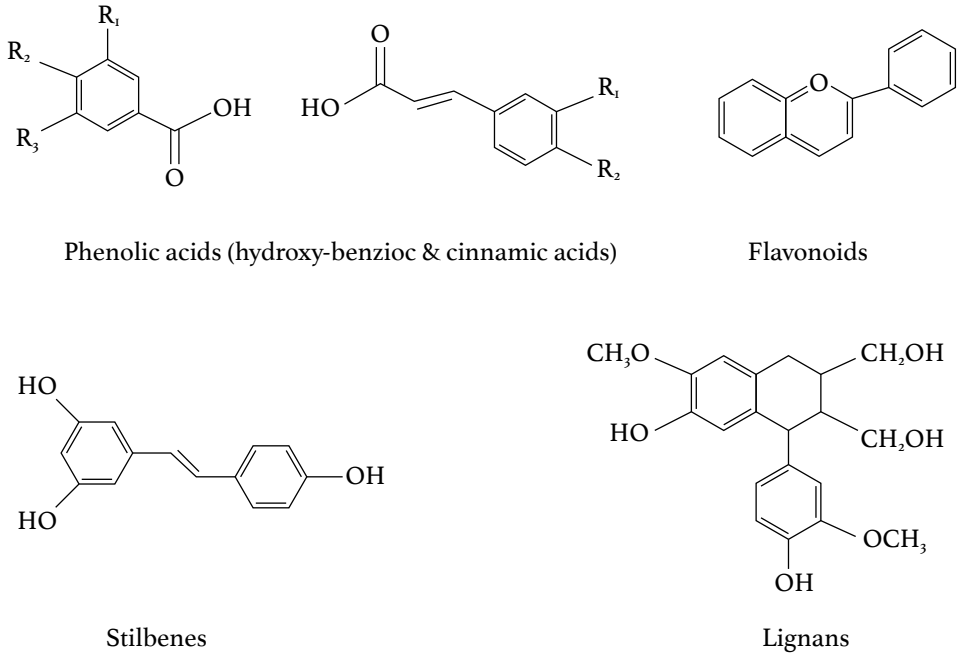


Figure 1.4 Chemical structures of the different classes of polyphenols. Source: Pandey and Rizvi (2009).

The chemical structure of phenolic compounds can greatly vary, ranging from simple molecules such as phenolic acids and flavonoids to the highly polymerized tannins (Brewer, 2011). Therefore, molecular complexity of phenolics seems to be a major factor that can limit their bioavailability (Manach et al., 2005). Like for α-tocopherol, for these phenolic compounds to exhibit any antioxidant activity post-mortem, they would need to be absorbed along the gastrointestinal tract, transported in the blood and accumulated in the target tissue (such as the skeletal muscle) (Vasta and Luciano, 2011).

The mechanism of action of these phenolic compounds on meat oxidative stability are only partly understood and several hypotheses have been proposed. Some authors suggest an indirect antioxidant effect, where phenolic compounds act in the gut by chelating pro-oxidant metals and reducing the production of lipid peroxides and toxic compounds leading to an overall improvement of the animal's antioxidant status (Halliwell et al., 2005; Kerem et al., 2006). Additionally, the direct antioxidant activity of phenolic compounds in the gut may spare other antioxidants like vitamin E from oxidation (Yamamoto et al., 2006). Based on the reduction of lipid oxidation in plasma from rats and sheep receiving diets supplemented with a polyphenol-rich plant extracts, Gladine et al. (2007a,b) hypothesized that polyphenol rich plant extracts can have a direct effect against oxidation of fatty acids. Moreover, the dietary transfer of polyphenol compounds into skeletal muscle has been demonstrated by Moñino et al. (2008), where lambs receiving milk from sheep fed a distillate of rosemary (*Rosmarinus officinalis*) leaves had increased muscular contents of rosmarinic acid, carnosol and carnosic acid. This ultimately led to an improved antioxidant capacity of the fresh meat samples when compared with lambs from non-supplemented sheep. In a later study, Nieto et al. (2010) included the distillate of rosemary leaves in sheep diets and improved redness and reduced lipid oxidation in fresh lamb meat packed in MAP and displayed for 21 d.

The mechanism of action of phenolic compounds in meat and meat products whether direct, indirect, or synergistic, remains unclear. Moreover, there is also limited information on the effect of dietary administration of polyphenols compared with vitamin E supplementation on lipid oxidation and colour stability in lamb meat. Thus, contrasting both feeding strategies and quantifying their relative value for meat colour and stability, under the same experimental conditions, is a topic of interest for animal nutritionists, as well as for meat scientists.

Lamb Production System in Mediterranean Areas

Historically, sheep farming have a peculiar importance in Mediterranean areas. The different production systems are adapted to the geographical characteristics of the region, defined by overpopulated coastlines, limited irrigated plain lands, mountainous hinterlands, and dry, warm summers. Moreover, it utilises resources that other farming sectors could not utilize.

In Spain, unlike northern European countries, consumers value lightweight lamb carcasses (<13 kg) because of a perceived loss in quality attributes if lambs are slaughter at higher carcass weights (loss of tenderness, flavour and darker meat) (Beriain et al., 2000; Boyazoglu and Morand-Fehr, 2001). Therefore, acknowledging these differences in consumer's perception, the European Union (EU) has established two different scales for carcass classification in sheep based on their carcass weight: heavy (>13 kg), and light (<13 kg) carcasses (De la Fuente et al., 2007). To ensure carcass homogeneity and to align the product with consumer preference, feedlots and fattening units were developed to act as intermediaries between the reproductive flocks, in which lambs are kept with the sheep, or dairy farms, and the abattoirs where lambs are slaughtered (Miranda-de la Lama et al., 2009). After weaning at 12-14 kg body weight (BW), lambs enter the feedlot facilities, are grouped based on body weight and fed a concentrate diet for a period between 21 to 45 d, until a slaughter weight of 22-24 kg is reached (Lobón et al., 2017).

Unlike fresh forages, that are rich in natural antioxidants (such as RRR-α-tocopherol, carotenoids, flavonoids and other phenolic compounds), most concentrate diets, that have not been supplemented with antioxidants (like vitamin E), present low concentrations of antioxidant components (Gatellier et al., 2004). Acknowledging the importance of the nutritional background to prevent fatty acid oxidation and meat discoloration, a large body of literature exits that describes the benefits of vitamin E supplementation of concentrates above the requirements of lambs. From these studies, supplementation levels between 287 mg of *all-rac*-α-tocopheryl acetate per kg diet (Álvarez et al., 2008) and 550-626 mg kg^{-1} diet (López-Bote et al., 2001) have been proposed. However, when planning a supplementation strategy in lambs, other important aspects that need attention are the antioxidant status of the animal at the beginning of the supplementation period (Descalzo et al., 2005), the length of the supplementation period (Lauzurica et al., 2005), the different slaughter weights (De la Fuente et al., 2007) and the animal breed (López-Bote et al., 2001). Therefore, the value of the available recommendations for antioxidant supplementation found in literature, require adaptation to individual meat production systems and should consider the conditions in which those recommendations were generated.

Research Objectives and Outline

Based on the knowledge gaps identified above, the objective of this thesis was to investigate the effect of supplementation level with natural (RRR-) and synthetic vitamin E (*all-rac*-) on stereoisomer deposition, meat colour and oxidative stability in light weight lambs. In **Chapter 2**, an experiment comparing RRR- and *all-rac*-α-tocopheryl acetate supplementation on α-tocopherol stereoisomer distribution in lamb tissues is described. In **Chapter 3**, the effect of α-tocopherol source and dosage on quality attributes of meat stored under retail conditions for 14 d are evaluated. Then, in **Chapter 4**, the results from *all-rac*-α-tocopheryl acetate supplementation on physicochemical and fatty acid stability of thawed lamb meat frozen for 9 months are presented. Secondly, different polyphenol

rich plant extracts were screened for their potential to alter the antioxidant potential in selected lamb tissues. In **Chapter 5**, the antioxidant potential of 11 different plant extracts in selected lamb tissues were evaluated using four different methodologies. Finally, in **Chapter 6** a standardized plant extract was compared with *all-rac*-α-tocopheryl acetate in its ability to reduce meat oxidation and improve colour stability in lamb meat stored for 14 d under retail conditions. **Chapter 7** includes an integrative discussion of the results presented in this thesis and general conclusions are drawn.

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Chapter 2

Bioavailability of α-tocopherol stereoisomers in lambs depends on dietary doses of *all-rac*- or RRR-α-tocopheryl acetate

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ABSTRACT

When supplementing lamb diets with vitamin E, an equivalence factor of 1.36 is used to discriminate between RRR-α-tocopheryl acetate and *all-rac*-α-tocopheryl acetate. However, more recent studies suggest a need for new equivalence factors for livestock animals. The current study aimed to determine the effect of RRR- and *all-rac*-α-tocopheryl acetate supplementation on α-tocopherol deposition in lamb tissues. One hundred and eight *Rasa Aragonesa* breed lambs were fed increasing amounts of *all-rac*-α-tocopheryl acetate (0.25, 0.5, 1.0 and 2.0 g kg^{-1} compound feed) or RRR-α-tocopheryl acetate (0.125, 0.25, 0.5 and 1.0 g kg^{-1} compound feed) added to a basal diet which contained 0.025 g kg^{-1} feed of *all-rac*-α-tocopheryl acetate as part of the standard vitamin and mineral mixture. The diets were fed for the last 14 d prior to slaughtering at 25.8 ± 1.67 kg BW. Within 20 min after slaughter samples of muscle, heart, liver, brain and spleen were frozen at -20°C until α-tocopherol analysis. Increased supplementation of either vitamin E sources led to a significant increase ($P<0.001$) in α-tocopherol concentration in all tissues studied. The tissue with the highest α-tocopherol concentration was the liver followed by spleen, heart and muscle. At similar supplementation levels (0.25, 0.50 and 1.0 g kg^{-1} compound feed), α-tocopherol content in the selected tissues was not affected by α-tocopherol source. However, the ratios between RRR- and *all-rac*-α-tocopheryl acetate increased with the increasing α-tocopherol supplementation (at 0.25 and 1.0 g kg^{-1} compound feed), from 1.06 to 1.16 in muscle, 1.07 to 1.15 in heart, 0.91 to 0.94 in liver and 0.98 to 1.10 in spleen. The highest relative proportion of 2S (sum of SSS-, SSR-, SRS- and SRR-α-tocopherol) configured stereoisomers was found in the liver of lambs supplemented with *all-rac*-α-tocopheryl acetate accounting with up to 35-39% of the total α-tocopherol retained, whereas the proportion of 2S configured stereoisomers in the other tissues accounted for less than 14%. Increasing *all-rac*-α-tocopheryl acetate supplementation was also found to affect the 2R configured stereoisomer profile in muscle, heart and spleen increasing the proportions of RRS-, RSR- and RSS- at the cost of RRR-α-tocopherol. In all tissues, the relative proportion of all non-RRR-stereoisomers in lambs receiving RRR-α-tocopheryl acetate was lower than RRR-α-tocopherol. These results confirm that the relative bioavailability of RRR- and *all-rac*-α-tocopheryl acetate is dose and tissue dependent and that a single ratio to discriminate the two sources cannot be used.

INTRODUCTION

After its discovery nearly 100 years ago, the role of vitamin E as a major chain-breaking antioxidant present in membranes and lipoproteins has become largely undisputed. However, vitamin E is not a single molecule but instead consists of a family of compounds including α-, β-, γ-, and δ-tocopherol and four corresponding unsaturated analogs (α-, β-, γ-, and δ-tocotrienol) (Brigelius-Flohé and Traber, 1999). Alpha- and γ-tocopherol are the predominant forms of vitamin E found in nature, of which α-tocopherol has the greatest biological activity and is the main form of vitamin E found in blood and tissues of animals (Wolf, 2006). Unlike the other vitamins, chemically synthesized α-tocopherol (*all-rac*-α-tocopherol) is not biochemically equivalent to the natural form (RRR-α-tocopherol) due to the presence of eight different stereoisomers (RRR-, RRS-, RSR-, RSS-, SRR-, SRS-, SSR-SSS-) with RRR- accounting for 12.5% of the total mixture (Weiser and Vecchi, 1981).

Interestingly, no consensus has been reached on determining an equivalence ratio between the two vitamin E sources (RRR- vs *all-rac*-α-tocopherol). The United States Pharmacopeia (USP, 1979) adopted the official biopotency factors of 1.00 for *all-rac*-α-tocopheryl acetate and 1.36 for RRR-α-tocopheryl acetate. These values were based on results from a rat resorption-gestation test which were first published by Harris and Ludwig (1949) and were later supported by data using a similar model on the relative biopotency (i.e. capacity of a chemical substance to function in a biological system) of each α-tocopherol stereoisomer (Weiser and Vecchi, 1982). However, controversy arose after human and animal studies in which deuterium-labeled forms of RRR- and *all-rac*-α-tocopheryl acetate were used, found that a ratio 2.00:1.00 (RRR-:*all-rac*-α-tocopheryl acetate) would better explain the biopotency differences between the two sources (Jensen and Lauridsen, 2007).

In many ruminant production systems, animals are kept indoors and fed ensiled forages and concentrates as an alternative to grazing. However, there is compelling evidence that animals allowed to graze present higher levels of α-tocopherol in the tissues (more specifically in muscle) than those fed preserved forages and concentrates (Lanari et al., 2002; Realini et al., 2004; Ripoll et al., 2013; Turner et al., 2002). One could argue that the lower vitamin E status found in animals kept indoors can be tackled with vitamin E supplementation. However, the most commonly used source of α-tocopherol in animal diets is *all-rac*-α-tocopheryl acetate, which is different from the RRR-α-tocopherol found in pasture and may explain to some extent the lower α-tocopherol levels in tissues of animals kept indoors.

MATERIALS AND METHODS

Animals and Diets

The animal management, care, feeding and performance have been previously reported in a study that discussed the effect of vitamin E dosage and source on meat quality parameters in lambs (Leal et al., 2018). Briefly, three hundred and sixty weaned *Rasa Aragonesa* breed lambs (males and females) with an average body weight of 22.3 ± 1.18 kg purchased from

local dealers were housed at a commercial farm (Franco and Navarro, Zaragoza, Spain). Two batches of 180 animals were used for the experiment. At arrival, lambs were weighed and brought into 20 m2 straw pens, blocked according to sex and batch of arrival following a randomized complete block design, with four blocks of nine pens each (with 10 lambs per pen). Within *each* block, pens were randomly assigned to one of nine experimental diets. All experimental diets were formulated to be adequate in protein, energy, vitamins, and minerals for this type of animal. Despite the native α-tocopherol present in the basal diet, an extra 0.025 g kg^{-1} feed of *all-rac*-α-tocopheryl acetate was included in all experimental diets as part of the mixture of vitamins and minerals. In addition, increasing amounts of α-tocopherol were added to the diets; 0.25, 0.5, 1.0 and 2.0 g kg^{-1} feed added as *all-rac*-α-tocopheryl acetate or 0.125, 0.25, 0.5 and 1.0 g kg^{-1} feed added as RRR-α-tocopheryl acetate. Diets were pelleted at 2.5 mm diameter. Supplemental Vitamin E (*all-rac*- and RRR-α-tocopheryl acetate) sourced from Trouw Nutrition Netherlands (Putten, The Netherlands). Ingredients and chemical composition of the experimental diets are shown in **Table 2.1**. For a period of 14 d after arrival, the lambs had free access to the experimental diets, wheat straw and water via separated troughs available in each pen. Lambs were weighted individually at the beginning of the experiment and before slaughter (14 d after) to determine body weight and average daily gain. Intakes of the experimental diets were recorded weekly per

Table 2.1 Composition of the basal diet and analysed α-tocopherol content in the experimental lamb diets.

Ingredients, g kg^{-1}		**α-Tocopherol content, g kg^{-1}**	
Wheat	300	Basal diet	0.040
Barley	260	*All-rac*-α-tocopheryl acetate	
Soya bean meal (480 g kg^{-1} of crude protein)	220	0.25	0.287
Maize	150	0.50	0.549
Soya oil	20	1.0	1.083
Limestone	27	2.0	2.343
Sodium bicarbonate	6	RRR-α-tocopheryl acetate	
Sodium chloride	4	0.125	0.148
Standard mineral and vitamin premix*	3	0.25	0.353
Vitamin E premix	10	0.50	0.584
		1.0	1.176

* Mineral and vitamins provided: Ca 0.24 g, Na 0.47 g, S 0.34 g, Mn 62 mg, Zn 110 mg, Cu 5 mg, I 0.6 mg, Co 0.3 mg, Se 0.1 mg, Fe 20 mg, vitamin A 8000 IU, vitamin D3 1600 IU, *all-rac*- α-tocopheryl acetate 25 mg. *all-rac*- α-tocopheryl acetate = synthetic vitamin E. RRR-α-tocopheryl acetate = natural vitamin E.

pen and orts measured at the end of the experiment. Intake of wheat straw and water were not registered.

Sample Collection

Animals were slaughtered at a local slaughterhouse (Mercazaragoza S.A., Zaragoza, Spain) within 2 hours after leaving the farm. Dissection took place within 30 min after slaughter and liver, heart, spleen and muscle (*longissimus throracis et lumborum*) samples were collected from 3 animals randomly selected from each pen, vacuum packed and frozen at -20°C until analysed for α-tocopherol concentration and stereoisomer distribution.

Tocopherol Analysis

Tocopherols were determined by high-pressure liquid chromatography (HPLC) after saponification and extraction into heptane according to Jensen et al. (1999) as follows. In the clean-up procedure, 2.0 g dry feed sample was mixed with 70 ml of 96% v/v ethanol, 30 ml methanol (Peter Mark, C 2517), 30 ml ascorbic acid (Merck, 5.00074.1000) and 20 ml KOH-water 1:1 (w/v) (B&B, 5268120). The mixture was saponified for 30 min at 80°C in the dark and cooled in cold water. Exactly 2 ml of the saponified mixture were diluted with 1 ml distilled water, after which tocopherols were quantitatively extracted with 2 × 5 ml heptane (Peter Mark, C 2514) and centrifuged at 1500 g for 10 min between each extraction (Leal et al., 2018). From the combined heptane extract, 100 µl was injected into the HPLC. Tissues were homogenized in twice the amount of ethanol by an Ultra-Turrax homogenizer (IKA-Werke GmbH, Staufen, Germany), while being kept on ice. Aliquots of the homogenates were saponified in a mixture of ethanol, methanol, ascorbic acid (20% w/v) and KOH-water 1:1 (w/v) (B&B, 5268120) at 80°C for 20 min, after cooling in the dark, the samples were extracted with two portions of 5 ml heptane after which 100 µl was injected into the HPLC. The HPLC column for determination of total tocopherols consisted of a 100 × 4.6 mm Brownlee Spheri-5 Silica 5 µm column (Perkin-Elmer GmbH, D-7770 Überlingen, Germany). The mobile phase was heptane containing 2-propanol (3.0 ml l^{-1}) and degassed with helium. The flow rate was 3.0 ml min^{-1}. A comparison of retention time and peak areas with Merck (D-6100 Damstadt, Germany) external standards of RRR-α-tocopherol and *all-rac*-α-tocopherol were used to obtain the identification and quantification of the tocopherols. Fluorescence detection was performed with an excitation wavelength of 290 nm and an emission wavelength of 327 nm (Leal et al., 2018). The HPLC system was a Perkin Elmer, Series 200.

Stereoisomers of α-tocopherol were analysed by HPLC as follows. The remaining heptane extract was evaporated to exact dryness under a stream of nitrogen at 45°C. Then the α-tocopherol was derivatized to its methyl ether based on the method described by Drotleff and Ternes (2001) and described in detail by Jensen and Lauridsen (2007). The methyl ether derivative was extracted with 1.5 ml heptane, of which 100 µL was injected into the HPLC. Chromatographic separation was achieved on a Chiralcel OD-H column (250 × 4.6

mm, 5 µm particle size, cellulose tris (3,5-dimethylphenylcarbamate) from Daicel Chemical industries, Ltd. (Tokyo, 100-6077, Japan) with heptane as eluent.
The method allowed the quantification of total tocopherol content and composition by normal phase HPLC after saponification and extraction into heptane with subsequent separation of the stereoisomers of α-tocopherol as methyl ethers by chiral HPLC. By this method, the α-tocopherol stereoisomers were separated into five peaks. The first peak consists of the sum of four Σ2S configured isomers (SSS-, SSR-, SRR-, SRS-), the second peak consisted of RSS-, the third peak consisted of RRS-, the fourth peak consisted of RRR- and the fifth peak consisted of RSR-α-tocopherol.

Statistical and Mathematical Calculations

All statistical analyses were performed using SAS, version 9.3 (SAS Institute, Inc., Cary, NC, USA). Pen was the experimental unit, and individual measurements were averaged per pen.
Total tocopherol content in the tissues and stereoisomer distribution were subjected to ANOVA analysis (PROC MIXED procedure, SAS Inst. Inc., Cary, NC, USA). The model included the fixed effects of the block, vitamin E source, dose and interaction of vitamin E source and dose. Orthogonal-polynomial contrasts were used to determine linear and quadratic effects of increasing vitamin E dosages on α-tocopherol deposition in tissues. The statistical significance level was claimed at $P<0.05$ and all the values are reported as least square means.
In addition, a non-linear regression procedure (PROC NLIN procedure, SAS Inst. Inc., Cary, NC, USA) was also used to describe the effect of the dietary vitamin E supplementation on α-tocopherol concentrations in the selected tissues. An exponential response was calculated using the equation proposed by López-Bote et al. (2001): $y = a + b (1 - e^{-cx})$, where y = dependent variable; a = intercept; a + b = asymptote; c = curvature steepness and x = independent variable.
For the non-linear regression procedure, the supplemental α-tocopherol was calculated based on the analytical values (**Table 2.1**) to represent the level of α-tocopherol supplemented above the content in the basal diet. This was achieved by subtracting the basal dietary levels found in the different treatments from the total analysed α-tocopherol. Therefore, the α-tocopherol contents used for the statistical analysis were 0.108, 0.313, 0.544 and 1.136 g kg^{-1} of compound feed for the RRR-α- tocopheryl acetate groups and 0.247, 0.509, 1.043 and 2.303 g kg^{-1} compound feed for the *all-rac*-α-tocopheryl acetate (Leal et al., 2018).

RESULTS

Animal Performance

Overall least square means and standard error of the means for body weight, average daily weight gain, feed intake and feed efficiency are presented by Leal et al. (2018). Neither

dietary α-tocopherol supplementation level, nor source had an effect (P>0.05) on the aforementioned parameters.

α-Tocopherol Concentration in Tissues

The highest α-tocopherol concentration per unit wet tissue, after the 14 d supplementation period, was found in liver followed by spleen, heart and muscle (**Figure 2.1**). Alpha-tocopherol concentration in all tissues increased with the increasing dietary level of both α-tocopherol sources (P<0.001).

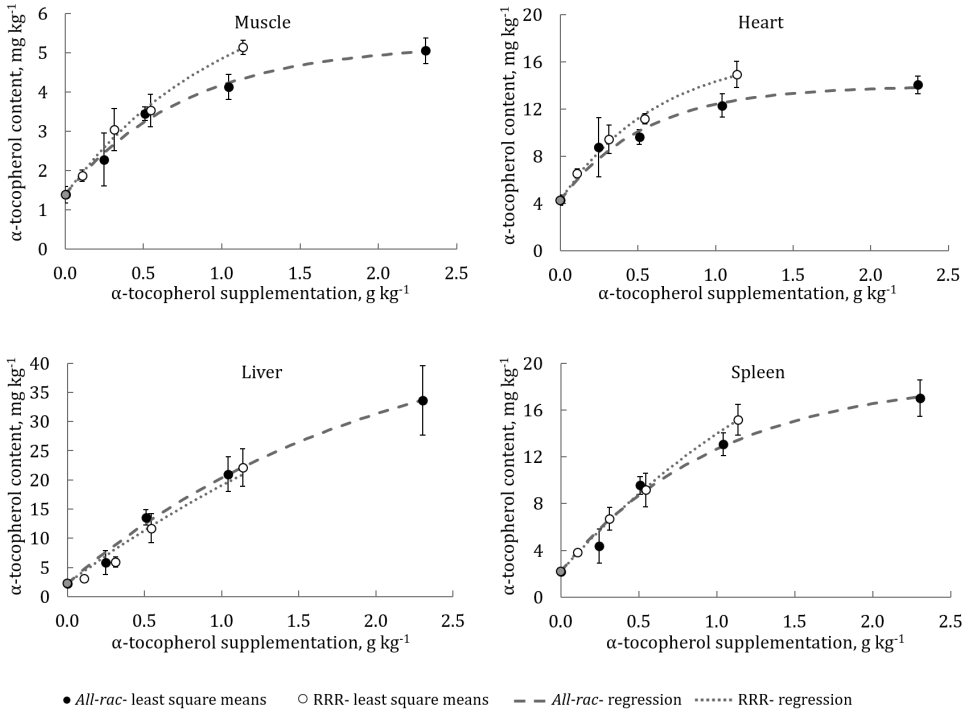

Figure 2.1 Effect of dietary *all-rac*-α-tocopheryl acetate (synthetic vitamin E) or RRR-α-tocopheryl acetate (natural vitamin E) supplementation on α-tocopherol concentrations in lamb tissues. The data, per tissue and vitamin E source, were fitted to the following exponential equation: tissue α-tocopherol concentration (mg kg^{-1} of tissue) = a + b (1 – e^{cX}), where a = common intercept for both vitamin E sources; b and c = regression coefficients; x = dietary vitamin E concentration (g kg^{-1} of feed). The regression coefficients for the non-linear model are given in Supplementary materials (Table S2.1).

In muscle, RRR-α-tocopheryl acetate supplementation led to a linear increase in α-tocopherol (linear effect, P<0.001; quadratic effect, P=0.183), whereas the effect of *all-rac*-α-tocopheryl acetate supplementation on α-tocopherol content in muscle was found to be quadratic (linear effect, P<0.001; quadratic effect, P=0.011). In the heart, however, increasing dietary inclusion of both α-tocopherol sources led to a quadratic increase in α-tocopherol content in the tissue (linear effect, P<0.001; quadratic effect, P<0.05). At similar supplementation levels (0.25, 0.50 and 1.0 g kg^{-1}), α-tocopherol content in muscle and heart was not affected by α-tocopherol source (P>0.05). However, the ratio between RRR- and *all-rac*-α-tocopherol increased in muscle from 1.06 at 0.25 g kg^{-1} feed to 1.10 and 1.16 at 0.50 and 1.0 g kg^{-1} feed. Similarly, in the heart, the ratio between the two sources increased from 1.07 at 0.25 g kg^{-1} feed to 1.11 at 0.50 g kg^{-1} feed and to 1.15 at 1.0 g kg^{-1} feed. Supplementation of 1.0 g kg^{-1} of RRR-α-tocopheryl acetate or 2.0 g kg^{-1} of *all-rac*-α-tocopheryl acetate was found to yield the highest α-tocopherol concentrations in both tissues (P<0.05) (**Figure 2.1**).
In liver, supplementation of both sources (RRR- or *all-rac*-α-tocopheryl acetate) induced a linear increase in α-tocopherol content (linear effect, P<0.001; quadratic effect, P>0.05). Similar to muscle and heart, at similar dosages (0.25, 0.50 and 1.0 g kg^{-1}), no differences were found between the two α-tocopherol sources (P>0.05). Despite that, the ratio between the two forms of α-tocopherol increased from 0.91 at 0.25 g kg^{-1} feed to 0.94 at 1.0 g kg^{-1} feed. Moreover, supplementation of 2.0 g kg^{-1} of *all-rac*-α-tocopheryl acetate led to a 1.52 fold increase in total α-tocopherol concentration in liver (P<0.05), when compared with 1.0 g kg^{-1} of RRR- α-tocopheryl acetate (33.6 mg kg^{-1} and 22.1 mg kg^{-1}, respectively) (**Figure 2.1**).
Spleen tissue showed a similar pattern to that observed for muscle, in that the lambs fed RRR-α-tocopheryl acetate showed a linear increase in α-tocopherol content (linear effect, P<0.001; quadratic effect, P=0.40), whereas, increasing supplementation levels with *all-rac*-α-tocopheryl acetate led to a quadratic increase of total α-tocopherol content in this organ (linear effect, P<0.001; quadratic effect, P=0.003). At similar levels of supplementation (0.25, 0.50 and 1.0 g kg^{-1}), no differences were found between the two α-tocopherol sources. Notwithstanding, the ratio between RRR- and *all-rac*-α-tocopherol increased in spleen from 0.98 at 0.25 g kg^{-1} feed to 1.02 at 0.5 g kg^{-1} feed and to 1.10 at 1.0 g kg^{-1} feed.

Distributions of α-Tocopherol Stereoisomers in Tissues

The relative and absolute distributions of α-tocopherol stereoisomers in muscle, heart, liver and spleen from lambs fed either RRR- or *all-rac*-α-tocopheryl acetate are described in **Figure 2.2** to **2.5**.

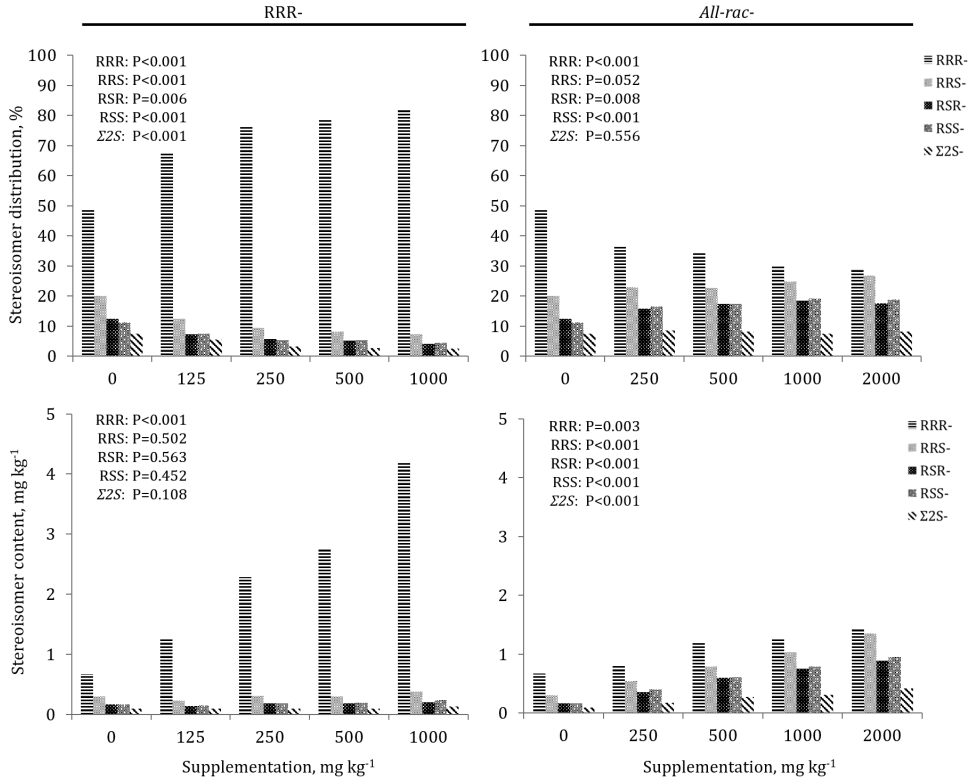

Figure 2.2 Relative and absolute proportion of α-tocopherol stereoisomers (RRR, RRS, RSR, RSS and ∑2S = SSS + SSR + SRS + SRR) in muscle of lambs supplemented with RRR-α-tocopheryl acetate (natural vitamin E) or *all-rac*-α-tocopheryl acetate (synthetic vitamin E).

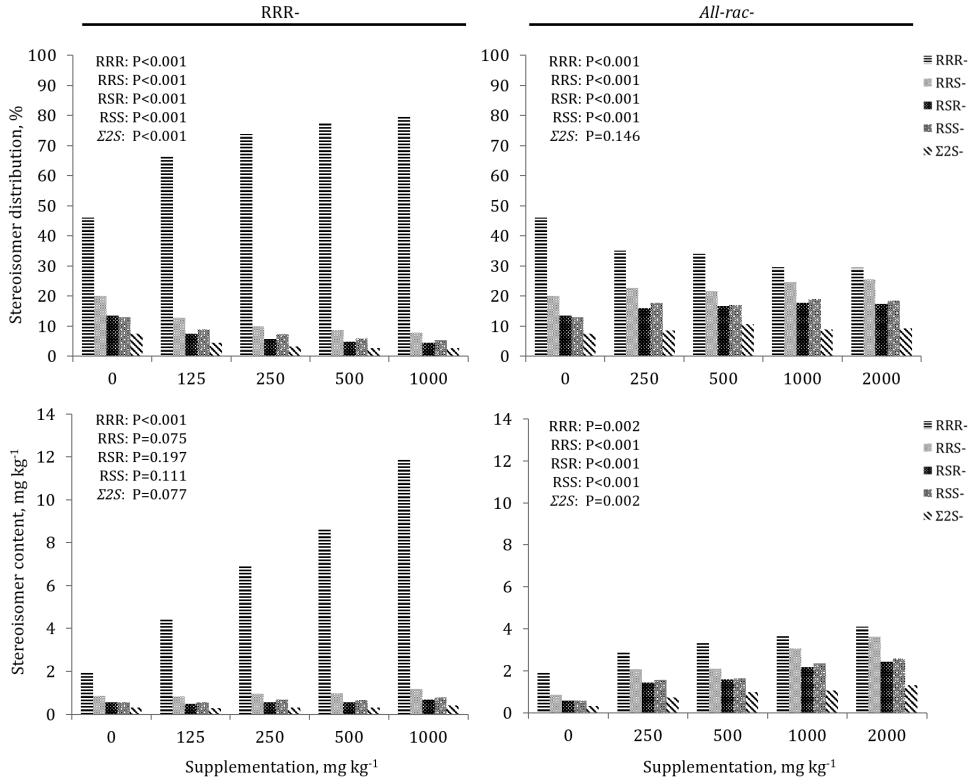

Figure 2.3 Relative and absolute proportion of α-tocopherol stereoisomers (RRR, RRS, RSR, RSS and ∑2S = SSS + SSR + SRS + SRR) in heart of lambs supplemented with RRR-α-tocopheryl acetate (natural vitamin E) or *all-rac*-α-tocopheryl acetate (synthetic vitamin E).

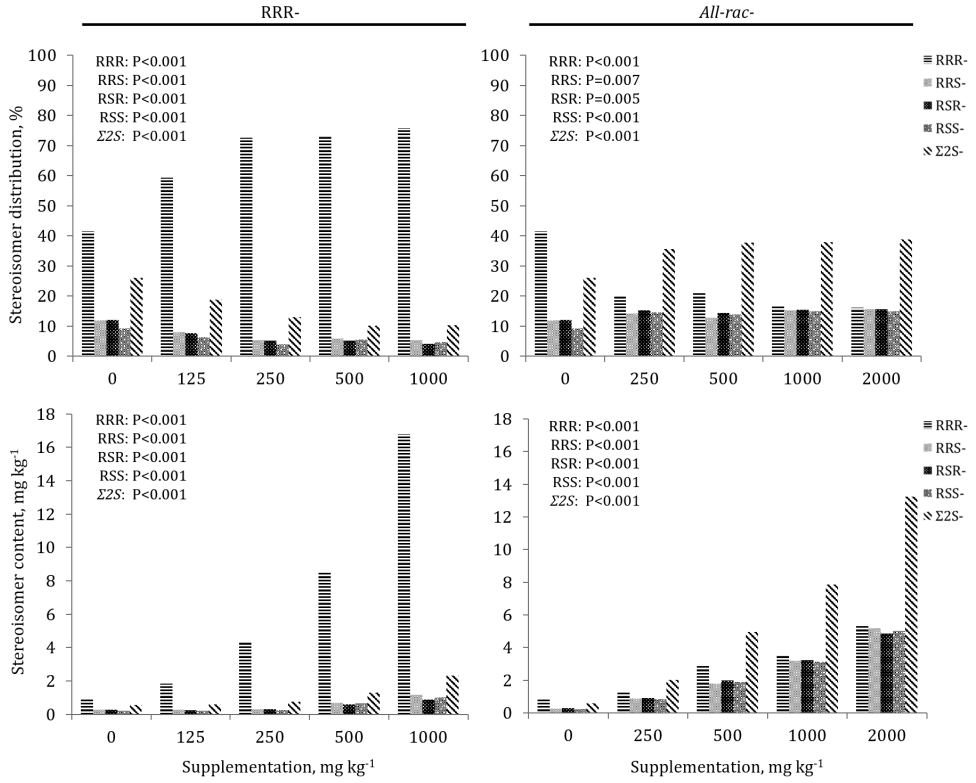

Figure 2.4 Relative and absolute proportion of α-tocopherol stereoisomers (RRR, RRS, RSR, RSS and ∑2S = SSS + SSR + SRS + SRR) in liver of lambs supplemented with RRR-α-tocopheryl acetate (natural vitamin E) or *all-rac*-α-tocopheryl acetate (synthetic vitamin E).

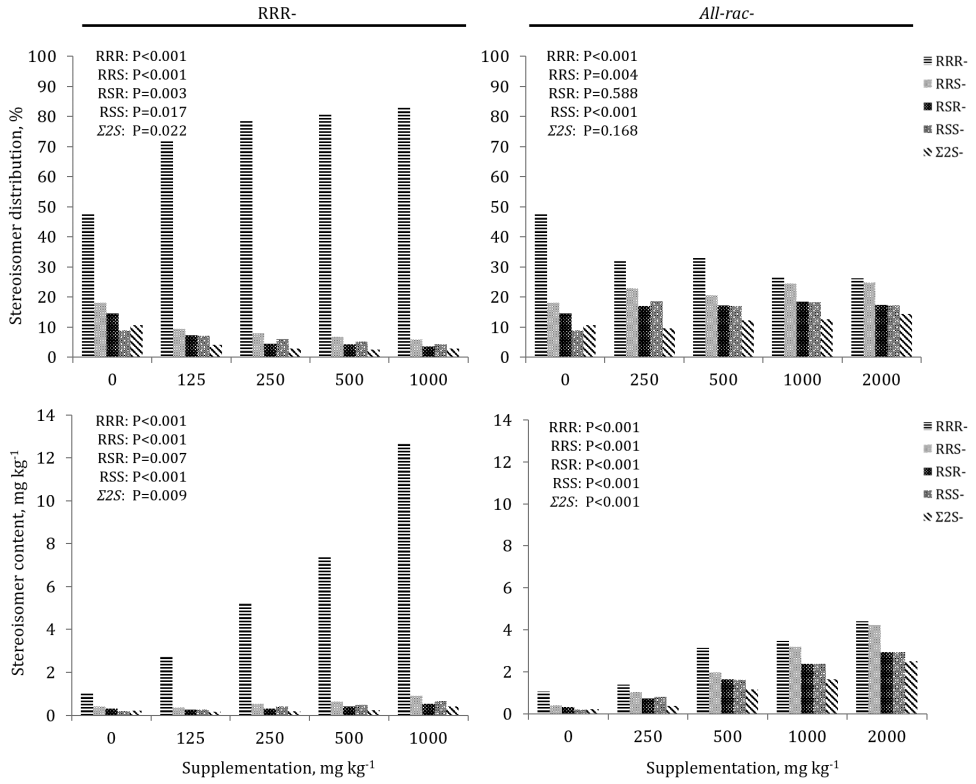

Figure 2.5 Relative and absolute proportion of α-tocopherol stereoisomers (RRR, RRS, RSR, RSS and ∑2S = SSS + SSR + SRS + SRR) in spleen of lambs supplemented with RRR-α-tocopheryl acetate (natural vitamin E) or *all-rac*-α-tocopheryl acetate (synthetic vitamin E).

With increasing dietary content of dietay RRR-α-tocopheryl acetate, the relative proportion of RRR-α-tocopherol increased in all tissues (P<0.001). The increase in the relative proportion of RRR-α-tocopherol stereoisomer led to a reduction in the proportion of the remaining stereoisomers (P<0.01). However, the relative reduction in the RRS-, RSR-, RSS-, and ∑2S- (sum of SSS-, SSR-, SRS- and SRR-α-tocopherol) stereoisomers had no effect on their absolute concentration in muscle (**Figure 2.2**) and heart (**Figure 2.3**) (P>0.05). In liver (**Figure 2.4**) and spleen (**Figure 2.5**), however, it was found when RRR-α-tocopheryl acetate concentration increased in the diets, the absolute concentration of all stereoisomers also increased (P<0.01).

When lambs were fed increasing levels of *all-rac*-α-tocopheryl acetate, the relative distribution of RRR-α-tocopherol was reduced in all tissues (P<0.01). In muscle and heart, the reduction in the relative proportion of RRR-α-tocopherol was followed by a significant increase in the relative proportions of RRS-, RSR-, and RSS-stereoisomers (P<0.01).

Whereas, the relative proportion of ∑2S stereoisomers was unaffected (P>0.05) (**Figure 2.2-2.3**). Despite all the changes in the relative stereoisomer distribution in muscle and heart, RRR-α-tocopherol was the most abundant stereoisomer (mg kg^{-1} tissue) followed by RRS-, RSS-, RSR- and ∑2S-α-tocopherol (P<0.01).

In liver, with the increase in *all-rac*-α-tocopheryl acetate supplementation, the relative proportion of ∑2S-stereoisomers increased from 26.0% in the non-supplemented lambs to 38.8% in the group supplemented with 2.0 g kg^{-1} feed (P<0.001) (**Figure 2.4**). The increase in the relative proportion of ∑2S-stereoisomers was associated with a concomitant reduction in the relative proportion of the RRR-stereoisomer (P<0.001), from 41.5% in the non-supplemented lambs to 16.1% in lambs supplemented with 2.0 g kg^{-1} feed. Although the absolute concentration (mg kg^{-1} tissue) of all stereoisomers in liver increased with *all-rac*-α-tocopheryl acetate supplementation (P<0.001), the ∑2S-stereoisomers were the most abundant followed by RRR-, RRS-, RSS- and RSR- α-tocopherol.

The relative and absolute contents of each stereoisomer in spleen are presented in **Figure 2.5**. Similar to the other tissues, feeding *all-rac*-α-tocopheryl acetate to the lambs caused a decrease in the relative proportion of RRR-α-tocopherol in spleen tissue (P<0.001). The decrease in the proportion of RRR-α-tocopherol was accompanied by an increase in the proportion of RRS- (P=0.004) and RSS- (P<0.001) stereoisomers, while no change in relative distribution was found for RSR- (P=0.588) and ∑2S- (P=0.168) stereoisomers. Despite all the changes in the relative stereoisomer distribution in spleen, RRR-α-tocopherol was the most abundant stereoisomer (mg kg^{-1} tissue) followed by RRS-, RSS-, RSR- and ∑2S-α-tocopherol (P<0.01).

DISCUSSION

As previously reported by Leal et al. (2018), neither dietary vitamin E supplementation level, nor source had an effect on average daily weight gain, feed intake and feed efficiency in light weight lambs. This is in agreement with early findings in lambs (De la Fuente et al., 2007; López-Bote et al., 2001; Turner et al., 2002) and beef (Arnold et al., 1992; Lee et al., 2008; Nassu et al., 2011), in which vitamin E supplementation had no effect on growth, feed intake nor efficiency.

Four different doses of RRR-α-tocopheryl acetate and *all-rac*-α-tocopheryl acetate were compared in this study, and as seen from **Figure 2.1**, tissue α-tocopherol concentration responded in a curvilinear increase with the increasing dietary level of either α-tocopherol source. Moreover, present data demonstrates that various tissues respond differently to increases of dietary supplementation of RRR- and *all-rac*-α-tocopheryl acetate. The tissue with the highest α-tocopherol concentration was the liver followed by spleen, heart and muscle. In line with our findings, Hidiroglou (1987) reported in sheep that after a single oral dose of 0.1 g kg^{-1} of body weight with RRR-α-tocopherol acetate, the highest α-tocopherol concentration in tissue followed the order liver, spleen, heart and muscle. Moreover, when feeding 1000 IU d^{-1} of different α-tocopherol preparations to sheep for 56 d, Ochoa et al.

(1992) reported consistently higher α-tocopherol concentrations in liver when compared with heart and muscle tissues. Conversely, Jensen et al. (2006) when studying the effect of different dietary doses of RRR- and *all-rac*-α-tocopheryl acetate in rats found higher α-tocopherol content in the spleen than in the liver. As indicated by the lack of response to incremental doses of RRR-α-tocopheryl acetate in that study, the authors argued that α-tocopherol content in spleen at the start of the study could be already close to a "plateau" or tissue saturation.

As initially postulated by Blatt et al. (2004) and later supported by the findings of Jensen et al. (2006), the relative bioavailability of RRR- and *all-rac*-α tocopherols varies between tissues, time after dosing, duration of dosing and the amount of each dose. All these factors combined led both authors to conclude that RRR- and *all-rac*-α-tocopherols are not equivalent in any dose ratio. Accordingly, at similar levels of supplementation (0.25, 0.50 and 1.0 g kg^{-1}), the ratio between α-tocopherol measured in the selected tissues of lambs fed RRR- and *all-rac*-α-tocopheryl acetate increased with increasing dietary vitamin E supplementation. Moreover, in the current study dietary RRR-α-tocopheryl acetate led to a linear increase in α-tocopherol concentration in liver, spleen and muscle. Whereas, increasing supplementation levels with *all-rac*-α-tocopheryl acetate led to a linear increase of α-tocopherol content only in liver. A linear ($P<0.05$) and quadratic ($P<0.05$) effect of dietary α-tocopherol supplementation was observed in heart from RRR- fed lambs and in muscle, heart and spleen from *all-rac*-α-tocopheryl acetate supplemented lambs. The linear plus quadratic response pattern suggested an exponential response, which was fitted as indicated in **Figure 2.1**. In an exponential response curve, the asymptote (a + b) indicates the upper limit or "plateau" in tissue α-tocopherol concentration that can be reached. Therefore, lambs fed increasing amounts of *all-rac*-α-tocopheryl acetate reached a "plateau" for α-tocopherol deposition in muscle and spleen. Whereas, no "plateau" was reached in muscle and spleen from lambs fed increasing amounts of RRR-α-tocopheryl acetate (linear effect). The non-parallel dose-concentration curves in the aforementioned tissues indicates that the relative bioavailability of RRR- and *all-rac*-α-tcopheryl acetate is not constant (dose and tissue dependent) (Blatt et al., 2004). As tissue α-tocopherol concentrations approach the "plateau", only a marginal increase in α-tocopherol deposition will be attained at higher supplementation levels. Thus, explaining the different ratios found in literature to discriminate between these two α-tocopherol sources.

The biological value of different α-tocopherol sources is influenced by a multitude of factors, such as absorption, transport, distribution, and metabolism (Bramley et al., 2000). All forms of α-tocopherol are taken up to a similar extent, in an unspecific process that includes emulsification by biliary and pancreatic secretions together with other lipids in the diet (Clifford et al., 2006; Traber, 1996). From the intestine, α-tocopherol stereoisomers are transported in chylomicrons and subsequently in remnants to the liver (Brigelius-Flohé et al., 2002). Once in the liver, the cytosolic α-tocopherol transfer protein (α-TTP)

selectively sorts out the α-tocopherol stereoisomers before their incorporation into very low-density lipoproteins, which are then released into the circulation (Traber et al., 1990). From the eight stereoisomers (RRR-, RRS-, RSR-, RSS-, SRR-, SRS-, SSR-, and SSS-) found in *all-rac*-α-tocopherol, the 2R configured stereoisomers (RRR-, RRS-, RSR-, and RSS-) when compared with the 2S configured stereoisomers (SRR-, SRS-, SSR-, and SSS-) are preferentially retained in all tissues except in the liver (Burton et al., 1998; Ingold et al., 1987). Accordingly, the highest relative proportion and tissue concentration of 2S configured stereoisomers was found in the liver of lambs supplemented with *all-rac*-α-tocopheryl acetate; accounting with up to 35-39% of the total α-tocopherol retained; whereas the proportion of 2S configured stereoisomers in the other tissues accounted for less than 14% of the total (**Figure 2.2-2.5**). The fate of the 2S configured stereoisomers retained in the liver is still unclear. However, it seems that 2S configured stereoisomers are recognized as xenobiotic molecules and are, therefore, predominantly catabolized (Lauridsen et al., 2002; Traber et al., 2017). On the other hand, the 2R configured isomers, and to a greater extent the RRR- stereoisomer, are recognized by α-TTP and re-secreted from the liver back into the plasma (Brigelius-Flohé et al., 2002). Increasing *all-rac*-α-tocopheryl acetate supplementation level in lamb diets, was also found to affect the 2R configured stereoisomer profile in muscle, heart and spleen; increasing the proportions of RRS-, RSR- and RSS- at the cost of RRR-α-tocopherol. These findings indicates that at higher supplementation levels with *all-rac*-α-tocopheryl acetate, the stereospecificity of α-TTP favours 2R configurations other than only RRR-α-tocopherol.

As expected, when lambs were supplemented with RRR-α-tocopheryl acetate, the relative proportion of all stereoisomers other than RRR-α-tocopherol, were reduced in all tissues analysed. It is noteworthy that, although the proportion of 2S configured stereoisomers was reduced with the increase of dietary dose of RRR-α-tocopheryl acetate in all tissues, the absolute content of 2S configured stereoisomers were maintained in muscle and heart; and increased in liver and spleen. According to Jensen et al. (2006), this could mean that 2S configured stereoisomers remained in the tissues for a longer period than the 2R configured stereoisomers, and only a small exchange in stereoisomers took place during the 14 d of supplementation.

CONCLUSIONS

We found that the different tissues responded differently to the increased supplementations of dietary RRR- and *all-rac*-α-tocopheryl acetate. Bioavailability of the α-tocopherol stereoisomers in all tissues was affected by vitamin E source and dose. The non-parallel dose-concentration curves found in all tissues indicates that the relative bioavailability of RRR- and *all-rac*-α-tocopheryl acetate is not constant and that a single ratio cannot accurately describe the nutritional difference between the two sources. Therefore, this study illustrates that the generally accepted equivalence value of 1.36 is inadequate to describe α-tocopherol deposition in ruminant tissues, at least specifically in intensively

fed growing lambs. The biological implications of the very distinct tissue distribution of these vitamin E sources deserves further research.

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SUPPLEMENTARY MATERIALS

Table S2.1 Regression coefficients (means ± standard error) of the effect vitamin E supplementation (RRR-α-tocopheryl acetate and *all-rac*-α-tocopheryl acetate) on α-tocopherol content in lamb tissues.

	RRR-		*all-rac*	
Muscle	Mean ± SE	*P value*	Mean ± SE	*P value*
a	1.39 ± 0.178	<0.001	1.39 ± 0.178	<0.001
b	6.20 ± 2.585	0.032	3.78 ± 0.477	<0.001
c	-0.83 ± 0.510	0.127	-1.32 ± 0.393	0.006
Liver				
a	2.29 ± 0.258	<0.001	2.29 ± 0.258	<0.001
b	60.3 ± 32.94	0.090	46.4 ± 14.97	0.009
c	-0.33 ± 0.208	0.140	-0.48 ± 0.252	0.080
Heart				
a	4.29 ± 0.369	<0.001	4.29 ± 0.369	<0.001
b	13.0 ± 2.46	<0.001	9.0 ± 0.98	<0.001
c	-1.50 ± 0.539	0.016	-2.24 ± 0.712	0.009
Spleen				
a	2.21 ± 0.164	<0.001	2.21 ± 0.164	<0.001
b	36.9 ± 32.21	0.273	16.4 ± 1.66	<0.001
c	-0.39 ± 0.406	0.360	-0.98 ± 0.223	<0.001

a = intercept. a + b = asymptote. c = curvature steepness. RRR-α-tocopheryl acetate = natural vitamin E. *all-rac*-α-tocopheryl acetate = synthetic vitamin E.

Chapter 3

Dietary vitamin E dosage and source affects meat quality parameters in light weight lambs

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ABSTRACT

Supra-nutritional vitamin E supplementation is a commonly used approach to delay lipid oxidation and colour deterioration in lamb and beef meat marketed under modified atmosphere packaging. However, these applications lack a precise calibration of dose for the desired effect and limited information is available regarding the use of natural vitamin E for this purpose. Three hundred and sixty *Rasa Aragonesa* lambs were fed diets supplemented with *all-rac*-α-tocopheryl acetate (250, 500, 1000 and 2000 mg kg^{-1} compound feed), RRR-α-tocopheryl acetate (125, 250, 500 and 1000 mg kg^{-1} compound feed) and a basal diet without vitamin E supplementation, for 14 d before slaughter at 25.8 ± 1.67 kg BW. Vitamin E supplementation had no effect ($P>0.05$) on average daily weight gain, feed intake and feed efficiency. Display time had larger effects on lipid oxidation, colour stability, myoglobin forms and meat discolouration parameters than did vitamin E supplementation. However, vitamin E source and dosage significantly extended meat shelf-life as indicated by lipid oxidation, redness, hue angle, metmyoglobin formation, deoxymyoglobin formation, $A_{580-630}$ and I_{SO_2}. Quantification of these effects demonstrated that the biological activity value of 1.36 used to distinguish both vitamin E sources is not appropriate for meat quality enhancing properties.

INTRODUCTION

Meat quality in lambs, as defined by its colour and stability properties (i.e. shelf-life) is an important factor driving consumer preference and purchase decision (Lanari et al., 1994). The "bright red" colour in meat, perceived by consumers as an indicator of freshness and wholesomeness of the meat product (Faustman et al., 1989; Djenane et al., 2003), can be further enhanced by modified atmosphere packaging (MAP) technology. This technology utilises oxidizing gas compositions (70%-80% of oxygen and 30%-20% carbon dioxide) (Álvarez et al., 2008) which, over time, leads to an increase in lipid oxidation and colour deterioration of the meat (Jakobsen et al., 2000; Baron et al., 2002). To decelerate lipid oxidation and colour deterioration, producers and retailers have adopted the use of antioxidants in meat products during processing (Nerín et al., 2006) or into lamb diets (López-Bote et al., 2001). Dietary application of antioxidants is preferred, due to uniform incorporation of the antioxidants into the subcellular membranes and local inhibition of the oxidative reactions (Govaris et al., 2004).

It is well accepted that vitamin E supplementation to lamb diets can be used as a mean to delay lipid oxidation and prolong colour stability of the meat (López-Bote et al., 2001; Lauzurica et al., 2005). Of the four tocopherol isomers found in nature (α, β, δ and γ), α-tocopherol is recognized to be the most effective antioxidant in delaying meat discolouration and lipid oxidation (Kamal-Eldin et al., 1996). Commonly, α-tocopherol supplements used in lamb diets to delay meat oxidation and colour deterioration differ from the naturally occurring α-tocopherol. Commercially available *all-rac*-α-tocopheryl acetate (synthetic vitamin E) is a racemic mixture of eight stereoisomers where only one isomer (RRR-), accounting for 12.5% of the total mixture, is found in nature (Weiser and Vecchi, 1982). The official biopotency factors, being the amount of a nutrient associated with some measured physiological endpoint, of 1.00 for *all-rac*-α-tocopheryl acetate and 1.36 for RRR-α-tocopheryl acetate (USP, 1979) are used to discriminate between the two sources. However, based on the relative bioactivity of the eight different stereoisomers, it has been reported that *all-rac*-α-tocopheryl acetate should only exhibit, on average, 58% of the biological activity of RRR-α-tocopheryl acetate (Brand et al., 2006). More recently, Dersjant-Li and Peisker (2010) compiled evidence that new biopotency factors for livestock animals need to be developed, different from the conversion factors used in the International Unit System for vitamin E. As highlighted by Liu et al. (1995) any supplementation strategy with vitamin E should target the optimal concentration of α-tocopherol in tissue that provides maximal suppression of lipid and protein oxidation.

The aim of this study was to determine the efficacy of two vitamin E supplementation strategies (RRR- and *all-rac*-α-tocopheryl acetate) on meat colour and lipid stability of lamb meat, stored for 14 d under retail conditions.

MATERIALS AND METHODS

Animals and Diets

Three hundred and sixty (equal number of males and females) *Rasa Aragonesa* lambs with an average body weight of 22.3 ± 1.18 kg were purchased from local dealers. Animals arrived at the commercial farm Franco and Navarro (Zaragoza, Spain) in two batches of 180 animals. Shortly after arrival, lambs were weighed and housed in straw pens according to sex and batch of arrival following a randomized complete block design, with four blocks of nine pens (20 m^2) each (with 10 lambs per pen). Within each block, pens were randomly allocated to one of nine treatments consisting of four supplementation levels of *all-rac*-α-tocopheryl acetate (250, 500, 1000 and 2000 mg kg^{-1} compound feed), four supplementation levels of RRR-α-tocopheryl acetate (125, 250, 500 and 1000 mg kg^{-1} compound feed) and a control group (basal diet without supplementation). The vitamin E supplements were included in the basal diet which contained 25 mg *all-rac*-α-tocopheryl acetate per kg compound feed. The experimental diets were presented as 2.5 mm diameter granules; the nutritional composition of the basal diet is shown in **Table 3.1.** For 14 d before slaughter, the lambs had free access to the experimental diets, wheat straw and water via separated troughs. After 14 d, the lambs were transported to and slaughtered at a local abattoir (Mercazaragoza S.A., Zaragoza, Spain). The experimental and slaughter procedures used met the guidelines of Council Directive 86/609/EEC (European Communities, 1986) on the protection of animals used for experimental and other scientific purposes.

Meat Processing and Packaging

Within 2 hours after slaughter, carcasses were hung under refrigerated conditions (2°C). After 24h, the *longissimus thoracis et lumborum* (LTL) muscle from 3 animals randomly selected from each pen were dissected, placed in bags (1 muscle per bag) and transported in sealed plastic containers in darkness at 4±1°C to the Meat Quality Laboratory of the Veterinary Faculty of Zaragoza (Zaragoza, Spain). Within 2 hours after arrival, the muscles were sectioned into approximately 2 cm thick steaks and 2 steaks per tray (alongside each other) were packed under modified atmosphere packaging with 70% O_2 + 30% CO_2 (Ulma Smart 500, Ulma Packaging, Guipúzcoa, Spain). Polystyrene trays were used and sealing was done with a polyethylene and polyamide laminate film (30 μm of thickness, water vapour transmission rate at 23 ˚C of <7 $g/m^2/24h$/85% relative humidity, an O_2 transmission rate at 23 ˚C of <15 cm^3/m^2 /0% R.H. and a CO_2 transmission rate at 23 ˚C of <75 $cm^3/m^2/24h$/0% R.H.; Linpac Packaging S.L., Spain). All trays were displayed in the same cabinet simulating retail conditions (4 ± 1°C, with a daily exposure to 14 hours of light at 1200 lx) for 14 d. All analyses (instrumental meat colour, myoglobin forms, meat discolouration and lipid oxidation) were performed on d 1, 7, 9, 12 and 14 of display.

Table 3.1 Ingredient and calculated composition of the basal diet.	
Ingredients, g kg^{-1}	
Wheat	300
Barley	260
Soya bean meal (480 g kg^{-1} of crude protein)	220
Maize	150
Soya oil	20
Limestone	27
Sodium bicarbonate	6
Sodium chloride	4
Mineral and vitamin premix*	3
Vitamin E premix	10
Composition, g kg^{-1}	
Dry matter	879
Crude protein	171
Starch	398
Ether extract	40
Ash	58

* Minerals and vitamins provided: Ca 0.24 g, Na 0.47 g, S 0.34 g, Mn 62 mg, Zn 110 mg, Cu 5 mg, I 0.6 mg, Co 0.3 mg, Se 0.1 mg, Fe 20 mg, Vitamin A 8000 IU, Vitamin D3 1600 IU, *all-rac*-α tocopheryl acetate 25 mg.

Instrumental Measurement of Colour

At each time point, a reflectance spectrophotometer (Minolta CM-2002, Osaka, Japan) with an illuminat D65 and 10° standard observer was used to determine colour of the steaks, after 2 h exposure to air. Each value was the mean of 10 determinations per sample, always on the top side of the steak and avoiding areas with excess fat. The parameters recorded according to the CIE $L^*a^*b^*$ system (CIE, 1976), were lightness (L^*), redness (a^*) and yellowness (b*). Values of chroma (C*) and hue angle (h) indexes were calculated as: $C^* = \sqrt{(a^{*2} + b^{*2})}$, and $h = \tan^{-1}(b^*/a^*)$; and expressed in degrees.

Myoglobin Forms and Meat Discolouration

The relative content of myoglobin forms (metmyoglobin (MetMb), oxymyoglobin (OxyMb) and deoxymyoglobin (DeoxyMb)) were calculated from the reflectance curve as described by Krzywicki (1979) using a wavelength of 690 nm. Since the reflectance spectrophotometer only measures the reflectance between 400 nm and 740 nm at 10

nm intervals, reflectance values not given by the instrument (473, 525 and 572 nm) were obtained by linear interpolation. The rate of meat discolouration was measured as A_{580}-A_{630} (van den Oord and Wesdorp, 1971) and to access the oxygen saturation of myoglobin on the meat surface, the parameter Iso_2 was measured following the technique described by Tsuruga et al. (1994).

Lipid Oxidation

Lipid oxidation, expressed as thiobarbituric acid reactive substances (TBARS) using the 2-thiobarbituric acid method (TBA), was determined according to the method of Pfalzgraf et al. (1995). Briefly, a 10 g meat sample was taken and homogenised with 10% trichloroacetic acid using an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany). Samples were centrifuged at 4000 rpm for 30 min at 10°C and the supernatants were filtered through a paper (Filterlab, Barcelona, Spain). Two ml of the filtrate were taken and mixed with 2 ml of TBA (20 mM), homogenized and incubated for 20 min in boiling water. Absorbance was measured at 532 nm and TBARS values were calculated from a standard curve of malonaldehyde (MDA). All samples were analysed in duplicate and the results expressed as mg of MDA per kg muscle.

α-Tocopherol Analysis in Feed

α-Tocopherol content in the experimental diets was determined by high-pressure liquid chromatography (HPLC) after saponification and extraction into heptane as described by Jensen et al. (1999). Briefly, 2 g of dry feed was mixed with 70 ml of 96% v/v ethanol, 30 ml methanol, 30 ml ascorbic acid and 20 ml KOH-water 1:1 (w/v). The mixture was saponified for 30 min at 80°C in the dark and cooled in cold water. Exactly 2 ml of the saponified mixture were diluted with 1 ml distilled water, after which tocopherols were quantitatively extracted with 2 x 5 ml heptane (Peter Mark, C 2514) and centrifuged at 1500 g for 10 min between each extraction. From the combined heptane extracts, 100 µl was injected into the HPLC (Perkin Elmer, Series 200) which contained a 100 x 4.6 mm Brownlee Spheri-5 Silica 5 µm column (Perkin-Elmer GmbH, D-7770 Überlingen, Germany). The mobile phase was heptane containing 2-propanol (3.0 ml l^{-1}) and degassed with helium. The flow rate was 3.0 ml min^{-1}. A comparison of retention time and peak areas with Merck (D-6100 Damstadt, Germany) external standards was used to obtain the identification and quantification of the tocopherol isomers. Fluorescence detection was performed with an excitation wavelength of 290 nm and an emission wavelength of 327 nm.

Mathematical and Statistical Analysis

All statistical analyses were performed using the SAS statistical software (SAS 9.3, SAS Inst., Inc., Cary, NC). Individual lamb data were summarized per pen which was considered the experimental unit for all the parameters studied. For all statistical models, dose of supplemental α-tocopherol was calculated based on the analytical values (**Table 3.2**), to

represent the level of α-tocopherol supplementation above the content in the basal diet. This was achieved by subtracting the basal dietary levels found in the different treatments from the total dietary α-tocopherol. Therefore, the α-tocopherol content used for the statistical analysis were 0.108, 0.353, 0.584 and 1.176 g kg^{-1} of compound feed for the RRR-α-tocopheryl acetate groups and 0.247, 0.509, 1.049 and 2.303 g kg^{-1} compound feed for the *all-rac*-α-tocopheryl acetate.

Feed intake, average daily gains (ADG) and feed efficiency data were analysed using the PROC MIXED procedure. The model included the fixed effects of treatment, initial body weight, and block. A Tukey test was performed to correct for multiple comparisons and all the values are reported as least square means.

A non-linear regression procedure, PROC NLIN and one of two models were used to fit the data on lipid oxidation (TBARS) (1), instrumental colour (*a**, b*, *L**, C* and h) (2), myoglobin forms (MetMb, OxyMb and DeoxyMb) (2) and meat discolouration (A580-630 and Iso_2) (2):

$$(1)\ Y = \text{Intercept} + \frac{\beta_1 \text{ x DT}}{e^{(\beta_2 \text{ x DS} + \beta_3 \text{ x DN})}} + \text{block} + \varepsilon$$

$$(2)\ Y = \text{Intercept} + \beta_1 \times DT + \beta_2 \times DT^2 + (\beta_3 \times DS + \beta_4 \times DN) + DT \times (\beta_5 \times DS + \beta_6 \times DN) + (\beta_7 \times DS^2 + \beta_8 \times DN^2) + DT^2 \times (\beta_9 \times DS + \beta_{10} \times DN) + DT^2 \times (\beta_{11} \times DS^2 + \beta_{12} \times DN^2) + \text{block} + \varepsilon$$

where Y is the predicted response variable, DT the display time in days (1, 7, 9, 12 or 14), DN the dosage of RRR-α-tocopheryl acetate in g kg^{-1}, DS the dosage of *all-rac*-tocopheryl acetate in g kg^{-1}, βs are regression coefficients, the effect of block (block) and ε is the random error. Akaike's information criterion (AIC) and Bayesian information criterion (BIC) were used to select the best model, with higher degree terms being progressively added to the model. Briefly, for a new term to be added to the model, a reduction in AIC >3 and BIC >0 was required. As demonstrated previously (Schwarz et al., 1978; Burnham et al., 2002), a model with lower AIC and BIC is significantly better than models with larger AIC and BIC. To access the validity of the models, an ANOVA was performed, enabling a check on the significance of all the effects. Response surface plots displaying the levels of a response as affected by the three continuous variables of interest (DT, DN and DS), were used for the final interpretation of the response.

In the exponential equation used to fit the data on TBARS (equation 1), regression coefficients β_2 and β_3 represent the slopes at dose zero (maximum response) for *all-rac*-α-tocopheryl acetate and RRR-α-tocopheryl acetate respectively. These coefficients reflect the effect of an infinitesimal increment in dose above the basal level of α-tocopherol on the development of TBARS. Consequently, the ratio between β_3 and β_2 was calculated to determine the relative efficacy between the two vitamin E sources on TBARS (**Table 3.3**). For the remaining parameters fitted with equation 2, there was not a single coefficient

indicating this maximum response to an incremental dose independently from DT. Therefore, relative efficacies for the remaining parameters were not calculated.

RESULTS AND DISCUSSION

Animal Performance

All animals remained healthy during the study and there were no missing values for any of the measurements. Neither dietary vitamin E supplementation level nor source had an effect (P>0.05) on the ADG, feed intake and feed efficiency (**Table 3.2**). This is in contrast to results of Wulf et al. (1995) who found differences in daily weight gain between lambs supplemented with 1000 mg to those supplemented with 500 mg α-tocopheryl acetate or non-supplemented lambs over 56 d of fattening. Later reports (Lauzurica et al., 2005; Ripoll et al., 2011), found no relationship between vitamin E supplementation and the productive performance in finishing lambs.

Table 3.2 Productive performance of the lambs and α-tocopherol content in feed for the control and vitamin E supplemented groups.

Item	Control	Natural (g kg^{-1} feed)				Synthetic (g kg^{-1} feed)				SEM	P value
	0	0.125	0.250	0.50	1.0	0.250	0.50	1.0	2.0		
Feed intake, kg d^{-1}	0.99	0.99	0.93	0.97	0.95	0.93	1.02	1.00	0.97	0.039	0.261
Average daily gain, kg d^{-1}	0.268	0.269	0.265	0.270	0.255	0.267	0.271	0.263	0.260	0.0112	0.474
Feed efficiency, kg kg^{-1}	3.80	3.91	4.29	3.89	4.25	3.86	3.95	3.82	3.90	0.244	0.337
α-Tocopherol, g kg^{-1} feed	0.040	0.148	0.353	0.584	1.176	0.287	0.549	1.083	2.343	-	-

Table 3.3 Mean ± standard error regression coefficients of a model describing the effect of *all-rac*-α-tocopheryl acetate (DS) and RRR-α-tocopheryl acetate (DN) supplementation to lambs before slaughter on subsequent TBARS values of *longissimus thoracis et lumborum* steaks during 14 d of display (DT) under retail conditions (modified atmosphere packing).

Parameter	Estimate	P value
Intercept	0.10 ± 0.026	<0.001
β1	0.09 ± 0.005	<0.001
β2	2.29 ± 0.307	<0.001
β3	4.11 ± 0.555	<0.001
β3:β2	1.80 ± 0.268	<0.001

$$\text{TBARS} = \text{Intercept} + \frac{\beta 1 \text{ x DT}}{e\text{^}(\beta 2 \text{ x DS } + \beta 3 \text{ x DN})}$$

Meat Oxidation

The effect of vitamin E dose and source on TBARS values of the LTL steaks are presented in **Table 3.3**. In lambs, when studying the effect of dietary α-tocopheryl acetate inclusion level for TBARS values at different storage times, López-Bote et al. (2001) found that an exponential model was the most appropriate to fit TBARS data.

As shown in **Table 3.3**, there was a significant effect of DT, DS and DN on TBARS values (P<0.001). In **Figure 3.1**, a response surface plot on TBARS values when varying supplementation level (DS and DN) and DT is presented.

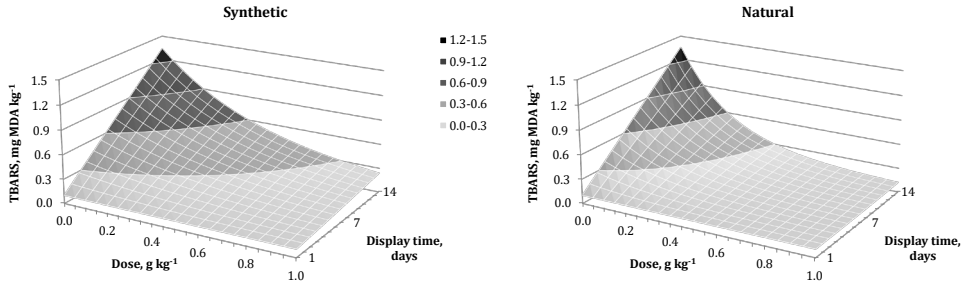

Figure 3.1 Response surface plot of predicted TBARS values of *longissimus thoracis et lumborum* steaks of lambs fed different doses of synthetic (*all-rac*-α-tocopheryl acetate) and natural (RRR-α-tocopheryl acetate) vitamin E daily for 14 d before slaughter and displayed for 14 d under retail conditions (modified atmosphere packing).

At d 0, there were no differences in TBARS values between the different vitamin E supplementation levels or sources. However, with increasing DT, the differences between treatments become apparent. Thresholds for oxidized meat acceptability have been proposed by different authors: 0.6-2.0 mg MDA kg^{-1} for beef (Greene and Cumuze, 1982; Campo et al., 2006), 0.5-1.0 mg MDA kg^{-1} for pork (Tarladgis et al., 1960; Dunshea et al., 2005), and 1.0 mg MDA kg^{-1} for lambs (Ripoll et al., 2011). In our study, in most of the supplementation levels and DT studied meat samples remained far below the threshold of 1.0 mg MDA kg^{-1} of meat. TBARS values of 1.0 mg MDA kg^{-1} in meat of non-supplemented animals were reached at 9 d of DT. Whereas, supplementation of synthetic vitamin E above 0.16 g kg^{-1} of feed or natural vitamin E above 0.09 g kg^{-1} of feed, would be sufficient to keep TBARS values below the threshold of 1.0 mg MDA kg^{-1} meat for as long as 14 d DT. When supplementing lamb diets with vitamin E, Ripoll et al. (2011) found that 0.5 g of synthetic vitamin E per kg of feed was sufficient to ensure a value of TBARS lower than 0.23 mg MDA kg^{-1} lamb meat at 14 d of DT. Such levels were achieved in our experiment at 0.56 g kg^{-1} of feed for DN and 1.01 g kg^{-1} of feed for DS. Interestingly, Ripoll et al. (2011) supplemented lambs for a period of 33 d while in the current study the supplementation period lasted for 14 d pre-slaughter. These findings are in line with previous work in lambs (Ripoll et al.,

2013), which demonstrate that for a shorter supplementation period an increased level of vitamin E supplementation is required to achieve a target value of TBARS.
The effectiveness of synthetic vitamin E to reduce lipid oxidation in lamb meat has been extensively described (López-Bote et al., 2001; Lauzurica et al., 2005; Muela et al., 2014). However, it is important to quantify the relative effectiveness of natural vitamin E compared to synthetic vitamin E to delay meat oxidation in lamb meat. According to the international unit system RRR-α-tocopheryl acetate (natural vitamin E) is 1.36 times more biopotent than *all-rac*-α-tocopheryl acetate (synthetic vitamin E) (Dersjant-Li et al., 2010). However, in the present study, natural vitamin E was 1.80 times more effective in slowing meat oxidation (TBARS) than synthetic vitamin E (**Table 3.3**). The conversion factor currently used by the international unit system to benchmark natural and synthetic vitamin E was determined using a rat foetal gestation-resorption model (Weiser et al., 1982) and its validity is applicable to the nutritional value of these vitamin E sources to prevent nutritional deficiency of vitamin E, which substantially differs from supra-nutritional applications of vitamin E to enhance meat quality.

Colour Stability

Changes in redness due to vitamin E supplementation are shown in **Table 3.4**. The main factor affecting the development of the a^* values in the current study was DT (quadratic effect) ($P<0.001$). However, significant interactions between DT and vitamin E supplementation (DS and DN) were found.

Table 3.4 Mean ± standard error regression coefficients of a model describing the effect of *all-rac*-α-tocopheryl acetate (DS) and RRR-α-tocopheryl acetate (DN) supplementation to lambs before slaughter on subsequent colour parameters (redness (*a**), yellowness (b*), luminosity (**L***), chroma (C*) and huge angle (*h*)) of *longissimus thoracis et lumborum* steaks during 14 d of display (DT) under retail conditions (modified atmosphere packing).

Coefficient	*a**	P	b*	P	L*	P	C*	P	h	P
Intercept	7.92 ± 0.324	<0.001	9.85 ± 0.196	<0.001	39.12 ± 0.434	<0.001	12.86 ± 0.237	<0.001	50.63 ± 1.003	<0.001
β1	0.96 ± 0.067	<0.001	0.54 ± 0.039	<0.001	-0.51 ± 0.085	<0.001	0.95 ± 0.048	<0.001	-1.39 ± 0.203	<0.001
β2	-0.06 ± 0.005	<0.001	-0.02 ± 0.003	<0.001	0.05 ± 0.005	<0.001	-0.05 ± 0.003	<0.001	0.13 ± 0.014	<0.001
β3	0.08 ± 0.768	0.920	-0.06 ± 0.174	0.732	0.34 ± 0.388	0.382	-0.26 ± 0.211	0.233	-0.23 ± 2.397	0.926
β4	-0.04 ± 1.477	0.980	-0.29 ± 0.349	0.412	0.34 ± 0.777	0.662	-0.59 ± 0.422	0.175	2.91 ± 4.605	0.532
β5	-0.08 ± 0.066	0.209	-0.01 ± 0.015	0.838	-0.04 ± 0.032	0.244	0.03 ± 0.018	0.112	0.19 ± 0.199	0.353
β6	-0.07 ± 0.131	0.602	0.02 ± 0.030	0.523	-0.10 ± 0.064	0.119	0.10 ± 0.036	0.009	0.61 ± 0.398	0.137
β7	-0.06 ± 0.310	0.857							0.28 ± 0.969	0.772
β8	-0.13 ± 1.215	0.916							-3.71 ± 3.794	0.335
β9	0.02 ± 0.006	<0.001							-0.08 ± 0.019	<0.001
β10	0.04 ± 0.012	0.005							-0.16 ± 0.037	<0.001
β11	-0.01 ± 0.002	0.006							0.02 ± 0.006	<0.001
β12	-0.02 ± 0.008	0.033							0.08 ± 0.037	0.002

$$\text{Parameter} = \text{Intercept} + \beta_1 \times DT + \beta_2 \times DT^2 + (\beta_3 \times DS + \beta_4 \times DN) + DT \times (\beta_5 \times DS + \beta_6 \times DN) + (\beta_7 \times DS^2 + \beta_8 \times DN^2) + DT^2 \times (\beta_9 \times DS + \beta_{10} \times DN) + DT^2 \times (\beta_{11} \times DS^2 + \beta_{12} \times DN^2)$$

In **Figure 3.2**, a response surface plot is presented to better illustrate the shape of the response and the differences between the two vitamin E sources studied. Dietary vitamin E supplementation not only increased the maximum value of *a** in the meat (11.57, 12.12 and 12.35 for non-supplemented, DS and DN, respectively), but it also increased the DT that takes to reach that maximum value of redness. The highest *a** value in the non-supplemented group was achieved after 8 d of DT, whereas, a DS supplementation of 0.45 g kg^{-1} or a DN supplementation of 0.25 g kg^{-1} delayed the peak of redness by 1 d. Moreover, if vitamin E supplementation is higher than 1.00 g kg^{-1} or 0.55 g kg^{-1} (for DS and DN, respectively), the peak of redness can be delayed to 10 d of DT. In the last day of DT (d 14), non-supplemented lambs had a redness value of 9.02, while DS supplementation above 0.85 g kg^{-1} and DN supplementation above 0.45 g kg^{-1} ensured *a** values higher than 11.0.

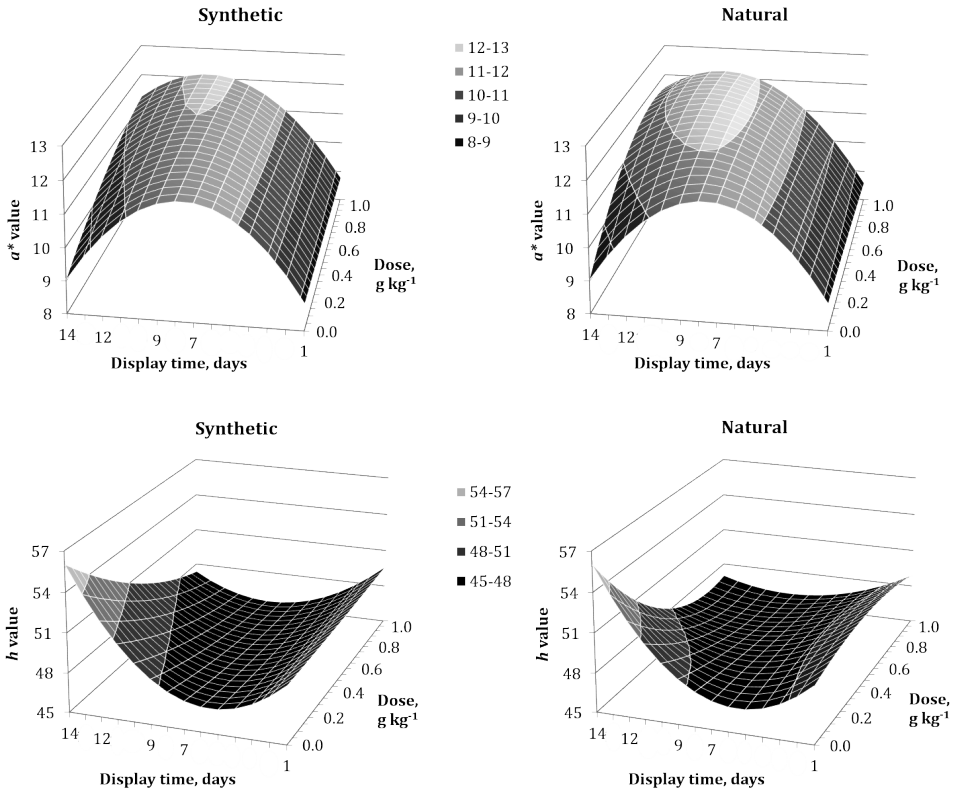

Figure 3.2 Response surface plot of predicted redness (*a**) and huge angle (*h*) values of *longissimus thoracis et lumborum* steaks of lambs fed different doses of synthetic (*all-rac*-α-tocopheryl acetate) and natural (RRR-α-tocopheryl acetate) vitamin E daily for 14 d before slaughter and displayed for 14 d under retail conditions (modified atmosphere packing).

The effect of synthetic vitamin E supplementation on meat redness values has been described in beef (Liu et al., 1995; Mitsumoto et al., 1998) and lambs (Guidera et al., 1997; Macit et al., 2003). However, to our knowledge, our study is the first to demonstrate the effect of vitamin E dose and source on *a** values in light weight lambs.

Dietary vitamin E supplementation did not affected b*, *L** and C* values in the meat (**Table 3.4**). In the current study, these parameters were only affected by DT (both linear and quadratic, P<0.001) with no effect (P>0.05) of dose or an interaction of dose with DT observed.

Assessment of the colours represented by b* (blue and yellow) are difficult to panellists (O'Sullivan et al., 2003) and are not intuitively related with meat colour (Mancini and Hunt, 2005). b* values increased consistently with display time, from values around 10 at d 1 of DT to values close to 13 at d 14 of DT. According to Wyszecki and Styles (1982) however, it is advisable to define colour in terms of lightness, chroma and hue to avoid possible errors in colour interpretation.

Lightness (*L**) values are related to muscle and protein structures which seem to play a role in water holding capacity of the meat (Macdougall et al., 1982; Huff-Lonergan and Lonergan, 2005). Lipid peroxidation is known to cause damage to membrane proteins leading to a loss of muscle cell membrane integrity and it can affect water holding capacity (Gray and Buckley, 1996). Although TBARS values were clearly affected by vitamin E supplementation, no differences were found in *L** values. Similarly to our study, Muela et al. (2014) found no effect of dietary vitamin E supplementation on lamb meat lightness during storage under MAP.

Chroma (C*) values increase with DT until they peak and progressively decrease from that point as DT increases (Bañón et al., 2012). In line with that, we found that all treatments reached their peak of C* at d 10 of DT and decreased from that point on until d 14 of DT. Synthetic vitamin E supplementation was found to have no effect on C* values (Muela et al., 2014), despite the significant differences found in *a** values (Andrés et al., 2013), which together with b* values (not significant for both sources in the current study) are the two factors that determine C* values. However, a significant interaction (P=0.009) between DT and DN was found. Which indicates different behaviour in the development of C* values between the two sources.

Just like for *a**, *h* values were affected by DT (linear and quadratic, P<0.001) but also by an interaction between DT and vitamin E supplementation (both DS and DN) (**Table 3.4**). Although the initial values for h were similar across treatment groups, meat samples from the non-supplemented lambs showed a marked increase in *h* values with DT (**Figure 3.2**). At 14 d of DT, samples from non-supplemented lambs presented an h value of 56.0. Vitamin E supplementation levels above 0.6 g kg^{-1} or 0.4 g kg^{-1} of DS and DN, respectively were able to ensure *h* values in the samples below 50. It is generally accepted that *h* is an important indicator of meat discolouration (Ripoll et al., 2008). Previous work with lambs (Ripoll et al., 2011) and beef (Albertí et al., 2005) indicated that dietary vitamin E supplementation can

delay meat discolouration, maintaining meat with a better appearance during extended periods of display. However, the present study is the first to stress the fact that different dietary vitamin E sources will yield different efficacies in delaying *h* development during DT.

Myoglobin Forms

Meat colour in lambs is primarily determined by the constant conversion between the heme-containing proteins: deoxymyoglobin (DeoxyMb), oxymyoglobin (OxyMb) and metmyoglobin (MetMb) (Minotti et al., 1992). As presented in **Table 3.5**, MetMb formation increased with DT (linearly, P>0.001) but supplementation of lamb diets with DS or DN slowed MetMb formation during DT. The surface plot for the relative percentage of MetMb during DT is shown in **Figure 3.3**. On d 1 of DT, all treatments had similar values of MetMb (between 24-25%). Similar, Guidera et al. (1997) found no effect of dietary vitamin E supplementation on MetMb formation in lamb meat at d 0 of display time. However, with an increase in DT, they found that supplementation (1.0 g kg^{-1}) resulted in lower MetMb values. This is in line with the present study, in which DS supplementation above 1.0 g kg^{-1} of feed or DN supplementation above 0.55 g kg^{-1} resulted in MetMb levels below 35% at d 14 of DT compared with the level of 43.5% of MetMb reached by the non-supplemented group. In the studies of Lauzurica et al. (2005) comparing lamb meat packed under MAP and displayed for 14 d, supplementation of 1.0 g kg^{-1} of synthetic vitamin E for a period of 34 d yielded a MetMb level of 29.2% compared with 50.6% of MetMb for the non-supplemented group. The lower MetMb levels found by these authors for the 1.0 g kg^{-1} of synthetic vitamin E compared with the current study may be explained by the differences in the vitamin E supplementation period between the two studies (34 d vs. 14 d).

High oxygen content in MAP improves meat colour due to OxyMb formation, which is associated with the bright-red colour of meat (Mancini and Hunt, 2005). In the present study, OxyMb formation was found to be mainly driven by DT (both linear and quadratic, P<0.001) (**Table 3.5**). Vitamin E source and dose had no effect on OxyMb formation, neither in its development over DT (P>0.05).

Table 3.5 Mean ± standard error regression coefficients of a model describing the effect of *all-rac*-α-tocopheryl acetate (DS) and RRR-α-tocopheryl acetate (DN) supplementation of lambs before slaughter on subsequent metmyoglobin (MetMb), oxymyoglobin (OxyMb), deoxymyoglobin (DeoxyMb), meat discolouration (A_{580}-A_{630}) and oxygen saturation (I_{SO_2}) of *longissimus thoracis et lumborum* steaks during 14 d of display (DT) under retail conditions (modified atmosphere packing.)

Coefficient	MetMb	*P*	OxyMb	*P*	DeoxyMb	*P*	A_{580}-A_{630}	*P*	I_{SO_2}	*P*
Intercept	23.05 ± 1.142	<0.001	18.11 ± 1.124	<0.001	57.13 ± 1.677	<0.001	38.57 ± 0.987	<0.001	15.82 ± 0.508	<0.001
β_1	1.08 ± 0.235	<0.001	4.04 ± 0.258	<0.001	-4.77 ± 0.382	<0.001	0.21 ± 0.208	0.313	0.97 ± 0.104	<0.001
β_2	0.03 ± 0.016	0.104	-0.21 ± 0.016	<0.001	0.15 ± 0.026	<0.001	-0.07 ± 0.013	<0.001	-0.05 ± 0.007	<0.001
β_3	1.42 ± 2.698	0.602	-1.20 ± 0.969	0.224	4.81 ± 3.806	0.215	-0.85 ± 0.869	0.338	-0.30 ± 0.451	0.507
β_4	1.52 ± 5.337	0.778	-1.05 ± 1.943	0.593	2.65 ± 7.424	0.723	-1.81 ± 1.741	0.305	-1.13 ± 0.904	0.221
β_5	0.11 ± 0.230	0.642	0.09 ± 0.097	0.349	-0.62 ± 0.373	0.107	0.21 ± 0.079	0.011	0.08 ± 0.039	0.040
β_6	0.32 ± 0.460	0.492	0.12 ± 0.195	0.535	-1.12 ± 0.747	0.143	0.54 ± 0.158	0.002	0.20 ± 0.079	0.015
β_7	-0.81 ± 1.089	0.463			-0.88 ± 1.518	0.564				
β_8	-1.53 ± 4.410	0.731			1.16 ± 6.036	0.848				
β_9	-0.07 ± 0.042	0.002			0.10 ± 0.035	0.006				
β_{10}	-0.14 ± 0.042	0.002			0.22 ± 0.069	0.003				
β_{11}	0.02 ± 0.007	0.003			-0.02 ± 0.011	0.051				
β_{12}	0.07 ± 0.026	0.015			-0.10 ± 0.043	0.020				

$$\text{Parameter} = \text{Intercept} + \beta_1 \times DT + \beta_2 \times DT^2 + (\beta_3 \times DS + \beta_4 \times DN) + DT \times (\beta_5 \times DS + \beta_6 \times DN) + (\beta_7 \times DS^2 + \beta_8 \times DN^2) + DT^2 \times (\beta_9 \times DS + \beta_{10} \times DN) + DT^2 \times (\beta_{11} \times DS^2 + \beta_{12} \times DN^2)$$

At 1 d of DT, similar OxyMb levels (21-22%) were found across all vitamin E sources and supplementation levels. Oxymyoglobin levels increased with DT until they reach a predicted maximum (at approximately 38%) on d 10, and progressively decrease from that point as DT increased to final values between 34-35% at 14 d of display. These findings are in line with previous work, which found the peak of OxyMb to be reached between 7 (Muela et al., 2014) and 14 d (Lauzurica et al., 2005) of display.

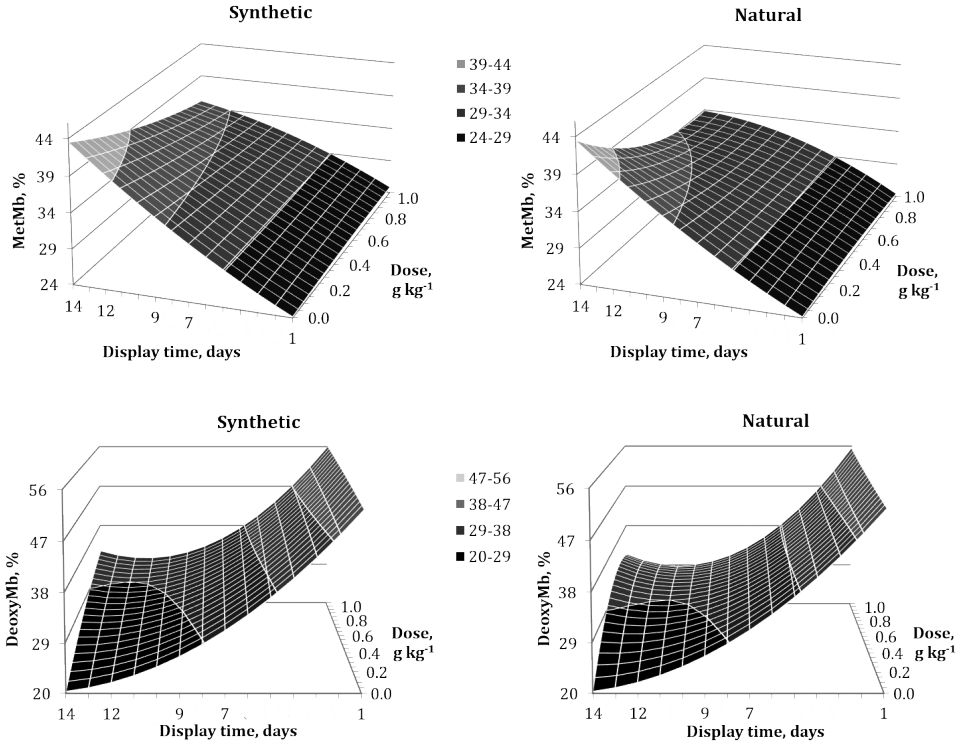

Figure 3.3 Response surface plot of predicted metmyoglobin (MetMb) and deoxymyoglobin (DeoxyMb) content of *longissimus thoracis et lumborum* steaks of lambs fed different doses of synthetic (*all-rac*-α-tocopheryl acetate) and natural (RRR-α-tocopheryl acetate) vitamin E daily for 14 d before slaughter and displayed for 14 d under retail conditions (modified atmosphere packing).

Deoxymyoglobin is characterized as the purple colour observed in freshly cut meat or vacuum packed product (Mancini and Hunt, 2005). Changes of this parameter during storage are presented also in **Table 3.5**. Display time was found to be the main factor driving DeoxyMb (Linear and Quadratic, P<0.001), however, significant (P<0.05) interactions between DT and vitamin E supplementation (DS and DN) were found. As presented in **Figure 3.3**, DeoxyMb levels were the highest for all treatments on the first day of DT (between 52.5% and 56%). Afterwards, DeoxyMb progressively decreased at a different rate depending on vitamin E source and supplementation level. On d 14 of DT, non-supplemented lambs presented 20.3% of DeoxyMb, whereas, DS supplementation above 0.75 g kg^{-1} or DN supplementation above 0.40 g kg^{-1} ensured DeoxyMb levels higher than 30%.

Ultimately, a prolonged exposure to a highly oxygenated environment (like MAP) leads OxyMb and DeoxyMb to convert into MetMb (Mancini and Hunt, 2005). In the current study, we found that vitamin E supplementation affected MetMb and DeoxyMb levels in lamb meat. However, no effect was found in OxyMb formation, which was mainly affected by DT.

Meat Discolouration

Oxygen saturation on the meat surface, assessed by I_{SO_2}, was affected by DT (linear and quadratic, P<0.001). Moreover, a linear interaction between DT and vitamin E supplementation (DS and DN) was found (P<0.05) (**Table 3.5**). As previously reported (Lauzurica et al., 2005; Minotti and Aust, 1992), the development of I_{SO_2} with DT follows the same pattern of OxyMb. Dietary vitamin E supplementation marginally affected the maximum value of I_{SO_2} in the meat (20.6% and 21.7% for non-supplemented and vitamin E supplemented groups, respectively) whilst delaying the DT necessary to reach that value. In the case of non-supplemented lambs, DS supplemented lambs up to 0.75 g kg^{-1} and DN supplemented lambs up to 0.30 g kg^{-1}, the maximum value of ISO2 is achieved after 10 d of DT. However, if a DS supplementation level above 2.0 g kg^{-1} or DN supplementations above 0.85 g kg^{-1} are considered, the time to reach the maximum value of I_{SO_2} can be delayed an additional 2 d.

Another important parameter associated with meat discolouration is A_{580}-A_{630} (Eikelenboom et al., 2000), presented in **Table 3.5**. Overall, meat discolouration increased significantly with DT in all groups (P<0.001), with the dietary supplementation level and DT interaction being statistically significant (P<0.05). After 14 d of DT, A_{580}-A_{630} values were found to be lower in the non-supplemented group when compared with the supplemented group, which suggests higher meat discolouration at lower supplementation levels. Meat from non-supplemented lambs had an A_{580}-A_{630} value of 27% at d 14 of DT, whereas, DS supplementation above 1.4 g kg^{-1} or DN supplementation above 0.5 g kg^{-1} resulted in A_{580}-A_{630} values higher than 30%. Early work from (Renerre and Mazuel, 1985) reported a value of 12.5% as the lower limit of colour acceptability based on A_{580}-A_{630}. In the current study, if

A_{580}-A_{630} values were to be considered on their own, all treatment groups showed suitable meat colour at d 14 of DT based on the minimum threshold of 12.5%.

CONCLUSIONS

Dietary vitamin E supplementation of the finishing diets of lambs for 14 d pre-slaughter increased the lipid and colour stability of meat stored under modified atmosphere packaging for 14 d of display. Vitamin E supplementation had a clear effect on lipid oxidation as measured by TBARS), instrumental colour (a^* and h), myoglobin forms (MetMb and DeoxyMb) and meat discoloration (I_{SO_2} and A_{580}-A_{630}). The effect of supplementation was clearly different between natural and synthetic vitamin E sources, being natural vitamin E substantially more efficient in affecting the different meat quality parameters during display time. The nutritionally established equivalence between the sources of 1.36 in the international system is not appropriate to be used in meat quality supra-nutritional applications of vitamin E.

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Chapter 4

Effect of dietary vitamin E on physicochemical and fatty acid stability of fresh and thawed lamb

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ABSTRACT

This study evaluated the effect of dietary vitamin E supplementation (1000 mg of DL-α-tocopheryl acetate kg^{-1} of basal diet) on physicochemical and fatty acid stability of fresh and thawed lamb leg chops frozen stored for 3, 6 and 9 months. Legs were chopped, modified atmosphere packaged (70% O_2 / 30% CO_2) and maintained under retail conditions (4 ± 0.5°C, with 14h fluorescent light) during 9 d. Muscle α-tocopherol concentration was over 3.5-fold higher in supplemented than in control lambs. The effect of dietary vitamin E was independent of frozen storage, so these effects were analysed separately. Vitamin E supplementation reduced lipid oxidation ($P<0.001$) and decreased metmyoglobin formation, leading to a more attractive colour of meat. Moreover, supplementation led to a higher percentage of polyunsaturated fatty acids. Therefore, vitamin E supplementation could be recommended for preserving either fresh or thawed lamb.

INTRODUCTION

Colour is the main sensory property of lamb used by consumers to select a product at the time of purchase (Jeremiah, 2001). A bright red colour is traditionally considered a positive aspect since it is associated with freshness and superior product quality (Berruga et al., 2005). Fresh lamb for retail sale is therefore commonly packaged in an oxygen enriched atmosphere with the aim of enhancing this colour development (Bellés et al., 2017). A disadvantage of these packaging conditions is the development of lipid oxidation, which leads to a decrease in meat quality, concerning colour, aroma, flavour, texture and even fatty acid composition (particularly polyunsaturated) (Rodríguez-Carpena et al., 2011).

The inclusion of vitamin E in the basal compound feed of lambs during the last days before slaughter has been proposed to overcome these problems, obtaining promising results (Lauzurica et al., 2005; Ripoll et al., 2011; Jose et al., 2016). Vitamin E is not degraded in the rumen (Leedle et al., 1993) and then, it accumulates in cell membranes and lipid depots, where it shows antioxidant activity (Liu et al., 1996). It has been satisfactorily applied to reduce lipid oxidation of fresh meat, showing a better colour stability and reducing off odours and flavours (Kerry et al., 2000; Ripoll et al., 2011). However, the knowledge about the effect of vitamin E on lamb quality after long-term frozen storage is scarce. Freezing is commonly used for long-term preservation as it results in a greater flexibility for distributors and retailers. Nonetheless, the possible negative effect of freezing and thawing on meat quality is still a point of concern. Primary compounds of lipid oxidation are formed during frozen storage, which lead to lipid oxidation secondary radicals after thawing. This phenomenon produces adverse changes on colour, odour, flavour and nutritional value (Owen and Lawrie, 1975). Thus, dietary vitamin E may be envisaged as a very suitable tool for inhibiting deteriorative oxidation of thawed lamb. The aim of this work was therefore to investigate the effect of dietary vitamin E on colour, lipid oxidation and fatty acid stability of fresh lamb and thawed lamb after frozen storage for three, six or nine months, maintained under retail conditions.

MATERIALS AND METHODS

The lambs used for this trial were reared in accordance with the guidelines from the Spanish Ministry of Agriculture (Boletín Oficial del Estado (BOE), 2007)

Slaughtering, Storage, Packaging and Refrigerated Storage

Sampling procedure is shown in **Figure 4.1.**

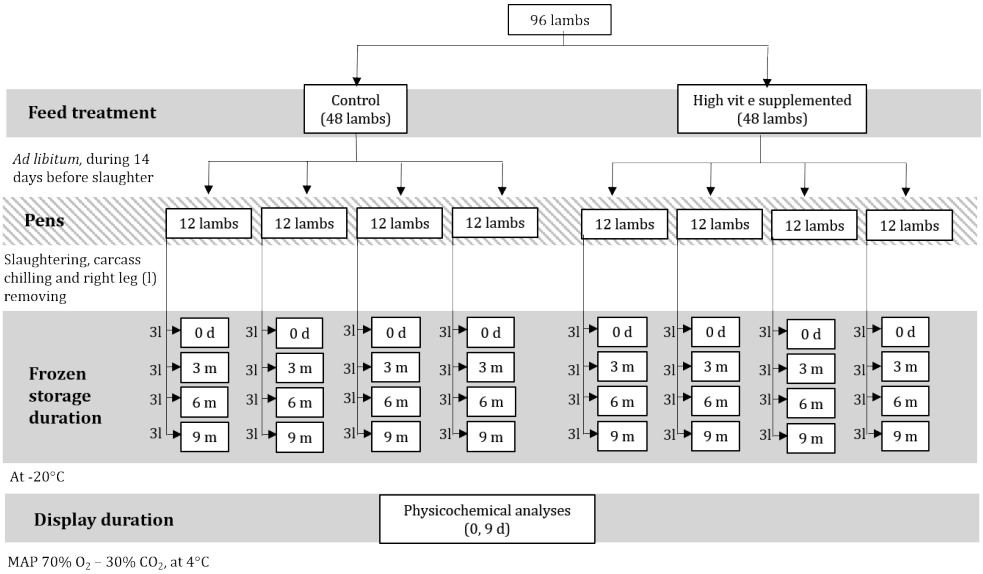

Figure 4.1 Sampling procedure.

Ninety six male *Rasa Aragonesa* lambs, a medium wool breed that is reared for meat purpose in Spain, with an average body weight of 22.3 ± 0.25 kg were randomly allocated in 8 different pens (12 lambs per pen). Four pens were fed with a basal diet containing 30 mg kg^{-1} feed of DL-α-tocopheryl acetate (control feed, C) while the other four received the basal diet plus 1000 mg kg^{-1} feed of DL-α-tocopheryl acetate (high supplemented feed, HS). Ingredients and chemical composition of the concentrates are presented in **Table 4.1**.
Lambs were offered *ad libitum* access to the experimental compound feed and wheat straw, for 14 d before slaughter. Thereafter, lambs were slaughtered at a local slaughterhouse with a body weight of 25.8 ± 0.71 kg and the carcasses were chilled during 24 h at 4°C.
The right leg of each carcass was removed and assigned (12 carcasses of each feed treatment) to one of the four frozen storage durations: 0 d (fresh meat), 3, 6 and 9 months. Prior to freezing, lamb legs were vacuum packaged (–900 mbar of pressure) in polyethylene-polyamide bags with ethyl vinyl acetate sealant layer (30 × 25 cm , 90 µm thickness, water vapour transmission rate at 23 ± 1°C of 2.8 $g/m^2/24h$ 85 ± 2% RH, an O_2 transmission rate at 23 ± 1°C of 50 $cm^3/m^2/24h/bar$ 75 ± 2% RH and a CO_2 transmission rate at 23 ± 1°C of 150 $cm^3/m^2/24h/bar$; Eurobag & Film S.L., Spain) using a Tecnotrip EV-13-L-CD-SC machine (Tecnotrip S.A., Spain) to prevent freezer burn and water losses. Frozen storage conditions (-20°C) were monitored with a data logger Testo 175-H2 (Testo S.A., Spain).

Table 4.1 Ingredients and chemical composition of the concentrate.

Raw material (%)	**Control feed**	**High vitE supplemented feed**
Barley grain	28.5	28.5
Corn grain	32.0	32.0
Wheat grain	6.0	6.0
Toasted soybean meal	26.0	26.0
Beet molasses	2.0	2.0
Powder milk serum	1.0	1.0
Animal fats	1.0	1.0
Fatty acids calcium salts	1.0	1.0
Calcium carbonate	2.0	2.0
Salt	0.5	0.5
Chemical composition (%)		
Crude protein	17.0	17.0
Crude fibre	3.9	3.9
Crude fat	4.0	4.0
Ash	6.0	6.0
Salt	0.2	0.2
Additives		
Vitamin A	13000 UI kg^{-1}	13000 UI kg^{-1}
Vitamin D_3	3000 UI kg^{-1}	3000 UI kg^{-1}
DL-α-tocopheryl acetate	30 mg kg^{-1}	1000 mg kg^{-1}
Total oligoelements	199.05 mg kg^{-1}	199.05 mg kg^{-1}
Butilhidroxitoluene (BHT)	30 mg kg^{-1}	30 mg kg^{-1}

After the corresponding frozen storage duration (0 d, 3, 6 or 9 months) legs were sliced in 20 mm chops in the facilities of Casa Ganaderos (Zaragoza, Spain) and then, chops were transported to the pilot plant of the Faculty of Veterinary (University of Zaragoza), where they were modified atmosphere packaged (70% O_2 and 30% CO_2) with a product to gas ratio of 1:3 (ULMA-SMART-500). Polystyrene trays were used and sealing was done with a polyethylene and polyamide laminate film (30 μm of thickness, water vapour transmission rate at 23°C of <7 $g/m^2/24h/bar$/ 85% relative humidity (RH), an O_2 transmission rate at 23°C of <15 cm^3/m^2 /bar 0% R.H. and a CO_2 transmission rate at 23°C of <75 $cm^3/m^2/24h/bar$ 0% R.H.; Linpac Packaging S.L., Spain). Samples were displayed under retail conditions

(4 ± 0.5ºC, with 14h fluorescent light) during 9 d of storage. Chops from frozen legs thawed within the package during the display period at 4ºC.
The content of α-tocopherol was measured in the muscle *semimembranosus* after carcass chilling. Instrumental colour, lipid oxidation and fatty acid profile analyses were performed at 0 and 9 d after packaging using only the muscle *semimembranosus*.

Content of α-Tocopherol in Semimembranosus

The content of α-tocopherol in tissue was determined following the methodology described by Liu et al. (1996) and Kasapidou et al. (2012). Samples were analysed by reverse phase HPLC (LiChrospher RP-18 column, 4.60 × 150 mm, 5 μm, 100 Å) with a mobile phase of isocratic methanol 100% at a constant flow rate of 1.2 ml min^{-1} and fluorescence detection (λexcitation = 295 nm and λemision = 330 nm; Agilent Series 1100). The injection volume was 20 μl and 9 minutes of run time. For peak identification and quantification (±)-α-tocopherol was used as external standard. Results were expressed as mg vitamin E kg^{-1} fresh meat.

Instrumental Colour

A Minolta CM-2002 (Osaka, Japan) spectrophotometer was used for measuring colour at the surface of a 20-mm-thick chop just after opening the tray. The parameters registered were L^* (lightness), a^* (redness) and b^* (yellowness) values. A D65 illuminant was used at an observation angle of 10° and with a cell opening of 30 mm. Equipment was previously calibrated using a white and black standard. Ten measurements were done in each sample. The hue angle (h) and chroma (C*) indexes were calculated as: $h = \tan^{-1}$ (b/a) expressed in degrees, and $C^* = \sqrt{(a^2 + b^2)}$. The ratio 630/580 values were calculated dividing the percentage of light reflectance at wavelength 630 nm by the percentage of light reflectance at wavelength 580 nm. The relative content of metmyoglobin was calculated from the reflectance curve according to Krzywicky (1979) using 690nm (the highest wavelength of the instrument).

Lipid Oxidation

Lipid oxidation was determined following the methodology described by Alonso et al. (2015).

Intramuscular Fat and Fatty Acid Analysis

The muscle *semimembranosus* was ground and 10 g were used to extract the fat in chloroform-methanol (1:1 v/v), with 2, 6-di-tert-butyl-4-methylphenol (BHT) (1 g/10 ml methanol) as antioxidant (Bligh and Dyer, 1959). One millilitre of chloroform phase was used to assess the percentage of intramuscular fat (IMF) by drying at 100°C for 20 min; the results were expressed as the weight percentage of wet muscle. The rest was evaporated in a sand bath under nitrogen gas at 50°C. The fatty acid methyl esters (FAMES) were formed using a KOH

solution in methanol and collected in hexane for analysis by gas chromatography following the methodology described by Carrilho et al. (2009). The FAMES were analysed in a gas chromatograph HP-6890 II (Hewlett-Packard, Waldbronn, Germany) using a capillary column SP-2380 (100 m x 0.25 mm x 0.20 μm), and oven temperature programming as follows: column temperature was set at 140ºC, then raised at a rate of 3ºC min^{-1} from 130 to 158ºC, and 1°C min-1 to 165ºC, kept for 10 min, raised at 5 to 220ºC and kept constant for 50 min. Nitrogen was used as a gas carrier at a constant flow rate of 0.8 ml min^{-1} with an injected volume of 1 μl. The methyl esters were identified using retention times of Supelco® 37 Component FAME Mix. Data regarding FAMES composition was expressed as area percentage of total identified FAMES. Fatty acid C19:0 was used as the internal standard for quantification. All chemicals were supplied by Sigma-Aldrich.

Statistical Analysis

The effects of feed treatment (FT), frozen storage duration (FSD), display duration (D), and their interactions on meat quality traits, were assessed using the GLM procedure of the SPSS statistical package, version 19.0 (IBM SPSS, 2010), with pen as random effect. The model is as follows:

$$Y_{ijkl} = \mu + FT_i + FSD_j + D_k + A_l + (FT_i \times FSD_j) + (FT_i \times D_k) + (FSD_j \times D_k) + (FT_i \times FSD_j \times D_k) + e_{ijkl}$$

Where Yijkl is the dependent variable; μ is the population average; FT_i is the fixed effect of the feeding treatment (C, HS); FSD_j is the fixed effect of frozen storage duration (0 d, 3, 6, 9 months); D_k is the fixed effect of display duration (0, 9 d); Al is the random effect of pen; ($FT_i \times FSD_j$) is the interaction effect of feed treatment and frozen storage duration; ($FT_i \times D_k$) is the interaction effect of feed treatment and display duration; ($FSD_j \times D_k$) is the interaction effect of frozen storage duration and display duration; ($FT_i \times FSD_j \times D_k$) is the interaction effect of feed treatment, frozen storage duration and display duration; and eijkl is the aleatory error. Differences were declared significant when $P<0.05$. Tukey´s post hoc test was used to assess differences between mean values when $P<0.05$.

There was not any interaction between the effects of frozen storage conditions and feed treatment on the variables measured, so both effects were analysed separately. However, significant interactions ($P<0.001$) between each one of them and display time were noted for lipid oxidation and colour, so a second model was designed for these variables where the effect of frozen storage conditions and feed treatment were analysed within the day of display and vice versa. This model determined the quality differences among treatments in each day of study and it also assessed changes of each treatment over the days of study. Fatty acid profile was not affected by the interactions among the fixed effects so the effects of frozen storage conditions, feed treatment and display duration on fatty acid profile were analysed separately.

RESULTS AND DISCUSSION

Content of α-Tocopherol in Muscle

Table 4.2 shows the content of α-tocopherol in muscle at slaughter. Following the recommendations of the Agricultural Research Council (ARC, 1980), the basal diet contained 30 mg of DL-α-tocopherol kg^{-1} in order to satisfy the minimal nutritional requirements for normal growth and health of sheep.

The content of α-tocopherol in *semimembranosus* at this vitamin E level was 1.057 mg kg^{-1} meat, which agreed with previously published data. Both Lauzurica et al. (2005) and Álvarez et al. (2009) quantified 0.95 mg of α-tocopherol per kg of muscle in lambs fed with a basal diet. Kasapidou et al. (2012) also registered a similar concentration in muscle at slaughter (0.73 mg kg^{-1}). Compared with the concentration of vitamin E obtained with the basal diet, the muscle deposition of vitamin E strongly increased with the supplementation of lambs with 1000 mg kg^{-1} of DL-α-tocopheryl acetate (P<0.001). As a result, muscle vitamin E concentration at slaughter was over 3.5-fold higher in supplemented than in control lambs.

Table 4.2 *M. semimembranosus* α-tocopherol concentration after slaughter of high (1000 mg of DL-α-tocopheryl acetate kg^{-1} of feed) and low supplemented (30 mg of DL-α-tocopheryl acetate kg^{-1} of feed) (control) lambs.

Item	C	HS	SEM	P value
α-tocopherol (mg kg^{-1})	1.057	3.908	0.201	<0.001

C = Control feed. HS = high supplemented feed. SEM = standard error of the mean.

The deposition of vitamin E in muscle depends on the length and the level of supplementation (González-Calvo et al., 2015; Jose et al., 2016). Kasapidou et al. (2012) obtained a concentration in muscle (3.73 mg α-tocopherol kg^{-1} muscle) similar to that we found using a lower level of vitamin E (500 mg kg^{-1}) but extending the period of supplementation to 63 d. In contrast, Lauzurica et al. (2005) and Álvarez et al. (2009) observed a similar deposition with a supplementation length of 37 d and a level of vitamin E of 1000 mg kg^{-1}.

Jose et al. (2016) established a concentration of 3.5-4.0 mg α-tocopherol kg^{-1} tissue as the threshold above which no added benefit of vitamin E on meat colour can be expected while Álvarez et al. (2008) did not observe any difference in inhibiting lipid oxidation when muscle α-tocopherol concentration overpasses 1.87 and 2.37mg kg^{-1} meat. Our results showed that these concentrations of vitamin E in muscle may be reached already after 14 d of high vitamin

E level supplementation, which would represent a significant economical saving. Either natural or artificial vitamin E has a high cost, so optimizing a rate of supplementation is the key to achieve the maximum improvement in product quality without an unnecessary cost (González-Calvo et al., 2015).

Instrumental Colour

Effect of dietary vitamin E

Dietary vitamin E supplementation did not modify Hue, Chroma, metmyoglobin (MetMb) content or the 630/580 index before display but its effect on colour after 9 d was significant (**Table 4.3**). Increasing the rate of supplementation resulted in lower values for Hue and higher for the ratio 630/580 than those measured in control samples at 9 d post-packaging ($P<0.001$). Similar results were obtained by Ripoll et al. (2011) when they evaluated the effect of vitamin E on lamb colour. These results could be explained by the different content of metmyoglobin noted in control and vitamin E supplemented samples after display ($P<0.001$). In fact, higher contents of metmyoglobin are related to changes in colorimetric parameters such as an increase of Hue or a decrease of the 630/580 ratio (Liu et al., 1996). According to Lawrie (1998), a brownish discolouration can be perceived onto meat surface when 60% of meat pigment is oxidised to metmyoglobin. This percentage was exceeded in control samples after 9 d of display while dietary vitamin E allowed to maintaining a better colour for a longer period of time. However, Ripoll et al. (2011) observed that consumers rejected lamb when the value of the index 630/580 fell below 2.23, which was observed either in control or supplemented lamb. Therefore, despite the great effect of vitamin E on preventing discolouration, the concentration of α-tocopherol reached in muscle was unable to maintain a desirable colour during 9 d of display.

Table 4.3 Effect of dietary vitamin E supplementation and frozen storage time (0 d, 3, 6, and 9 months) on physicochemical properties of semimembranosus muscle from lambs displayed for 0 or 9 d.

Physicochemical parameters[1]	Day of display	Frozen storage time						Dietary vitamin E			
		0d	3m	6m	9m	SEM	$P_{frozen\ storage}$	C	HS	SEM	P_{feed}
C*	0	16.53^{y}	14.81	15.47^{y}	15.88^{y}	0.273	0.061	14.84^{y}	15.51^{y}	0.47	0.332
	9	14.68^{b,x}	12.51^{a}	13.30^{ab,x}	13.35^{ab,x}	0.22	0.009	13.74^{x}	13.18^{x}	0.34	0.252
	$P_{display}$	<0.001	0.651	0.007	0.013			<0.001	<0.001		
h	0	45.27^{x}	43.73^{x}	42.69^{x}	44.16^{x}	0.483	0.301	46.86^{x}	45.56^{x}	0.83	0.282
	9	69.72^{y}	66.96^{y}	70.76^{y}	72.19^{y}	1.38	0.592	74.48^{b,y}	65.34^{a,y}	1.70	<0.001
	$P_{display}$	<0.001	<0.001	<0.001	<0.001			<0.001	<0.001		
% MetMb	0	17.27^{x}	16.99^{x}	19.23^{x}	19.86^{x}	0.475	0.121	19.71^{x}	20.97^{x}	0.81	0.282
	9	59.64^{y}	53.83^{y}	60.37^{y}	59.03^{y}	1.75	0.545	64.13^{b,y}	52.31^{a,y}	2.15	<0.001
	$P_{display}$	<0.001	<0.001	<0.001	<0.001			<0.001	<0.001		
630/580	0	2.78^{y}	2.56^{y}	2.85^{y}	2.85^{y}	0.057	0.260	2.68^{y}	2.84^{y}	0.08	0.182
	9	1.26^{x}	1.35^{x}	1.24^{x}	1.33^{x}	0.040	0.724	1.18^{a,x}	1.41^{b,x}	0.05	<0.001
	$P_{display}$	<0.001	<0.001	<0.001	<0.001			<0.001	<0.001		
mg MDA kg^{-1} of meat	0	0.07^{a,x}	0.10^{b,x}	0.11^{b,x}	0.11^{b,x}	0.11	<0.001	0.13^{x}	0.12^{x}	0.13	0.429
	9	1.75^{y}	2.25^{y}	2.26^{y}	1.80^{y}	0.30	0.442	2.83^{b,y}	1.21^{a,y}	0.11	<0.001
	$P_{display}$	<0.001	<0.001	<0.001	<0.001			<0.001	<0.001		

% MetMb = percentage of metmyoglobin. MDA = malondialdehyde. C = Control feed. HS = High supplemented feed. ^{a,b,c} Values within a row with different superscript are significantly different (within treatment). ^{x,y,z} Values within a column with different superscript are significantly different (within days of display) (P<0.05). [1]Means. SEM= standard error of the mean.

Effect of frozen storage

As it is shown in **Table 4.3**, significant differences were not found in *h* and 630/580 values between fresh and thawed lamb either before or after display. Concerning the content of metmyoglobin found in fresh lamb before display, it was similar to values previously registered in fresh meat (Ripoll et al., 2011, Muela et al., 2014). These initial values did not differ significantly from those measured in thawed meat. Moreover, frozen storage prior to thawing did not result in an increase of metmyoglobin content after display. At this time, differences were only found in Chroma, showing fresh lamb the highest values ($P<0.05$).

Lipid Oxidation

Effect of dietary vitamin E

The effect of dietary vitamin E on TBARS index is shown in **Table 4.3**. There was no difference between both treatments before display, which may be due to a residual activity of muscle enzymes post-mortem (Renerre et al., 1996) and the presence of cell antioxidant compounds. However, control samples showed a higher malondialdehyde content after 9 d ($P<0.001$). The large amount of malonaldehyde measured in controls at this time agreed with bibliography (Lauzurica et al., 2005) and it could be related to the high percentage of O2 of the atmosphere used for this trial, which has been described to enhance lipid oxidation (O´Grady et al. 2000). In contrast, oxidative reactions were significantly inhibited by the inclusion of vitamin E in the basal feed ($P<0.001$). Dietary vitamin E supplementation reduced at least half the content of MDA found in control samples after 9 d of display.

The effectiveness of vitamin E on reducing lipid oxidation in fresh lamb had been already described. Ripoll et al. (2011) reported that treatments with vitamin E kept low values of MDA (0.1-0.23 mg MDA kg^{-1}) while control treatments reached values greater than 1.5 mg kg^{-1}. Lauzurica et al. (2005) and Muela et al. (2014) also found a strong inhibition of lipid oxidation by adding vitamin E in the basal feed. According to Álvarez et al. (2008), a concentration of α-tocopherol comprised between 1.87 and 2.37 mg α-tocopherol kg^{-1} meat may be enough for optimum improvement against lipid oxidation while González-Calvo et al. (2015) suggested that it could be even lower (0.61-0.90 mg α-tocopherol kg^{-1} muscle). Nevertheless, our results showed a development of lipid oxidation reactions with a higher content of α-tocopherol in muscle (3.908 mg α-tocopherol kg^{-1} muscle). These differences may have been related to different storage conditions, which pointed out that the minimum concentration of α-tocopherol in muscle for completely inhibiting lipid oxidation depends on storage conditions and it may be higher under commonly retail conditions (high oxygen content atmospheres and 14 h of fluorescent light). In fact, oxidation affects colour, odour, flavour, texture and nutritional value, and it finally compromises lamb shelf life. Products of lipid oxidation have been associated with off flavours and off odours (Jeremiah, 2001), which decrease meat quality by overpowering its characteristic flavour and odour. TBARS has been described to be a good indicator of the development of rancid off-flavours. Several researchers have related TBARS values with human perception of rancid

compounds. Greene and Cumuze (1982) observed that untrained panellists accepted beef with TBARS values up to 2 mg kg^{-1}. Similarly, Campo et al. (2006) noted that beef flavours were overpowered when TBARS values exceeded a value of 2 mg kg^{-1}, so this point was proposed as the maximum level for positive sensory perception of beef. In our trial, control samples widely exceeded the threshold for the acceptability of oxidized beef of 2 mg MDA kg^{-1}; meanwhile a dietary dosage of 1000 mg of α-tocopherol kg^{-1} was enough to maintain oxidation levels under the limit of acceptance after display. Therefore, supplemented lamb is expected to keep better characteristics of odour and flavour, obtaining a good acceptance of consumers.

Effect of frozen storage

Table 4.3 also shows the effect of frozen storage duration before thawing on lipid oxidation of lamb. TBARS values before display were lower in fresh than in thawed lamb (P<0.001). Initial differences between fresh and thawed lamb had been already described (Muela et al., 2010) probably because of damage caused to cellular structure during freezing, frozen storage and thawing or as a result of MDA accumulation during frozen storage. Lipid oxidation has widely described to accelerate after thawing as peroxidation (primary lipid oxidation) is not stopped during frozen storage giving rise to rapid and severe secondary lipid oxidation (thiobarbituric acid forming), which results in increased TBARS values (Owen and Lawrie, 1975). In this study, however, there were no significant differences in MDA content among treatments after 9 d of display. Similar to our results, Leygonie et al. (2012) and Alonso et al. (2016) did not register differences in TBARS values among fresh and thawed meat after frozen storage during different periods of time. Muela et al. (2010) neither registered any significant difference between fresh and 3 months frozen storage meat after 10 d of display but differences became significant when thawed meat had been kept frozen up to 6 months. The lack of a higher content of MDA in thawed samples could be explained due to its consumption in other chemical pathways. According to Leygonie et al. (2012) the interaction between lipid and protein oxidation presumably leads to a lower TBARS value as malondiadehyde (MDA) acts as a substrate in one of the pathways of protein oxidation.

Intramuscular Fatty Acid Profile

Effect of dietary vitamin E

Vitamin E supplementation did not modify the content of intramuscular fat (**Table 4.3**), which was in agreement with the findings of Kasapidou et al., (2012) and Zhao et al. (2013). Nevertheless, there were some significant effects of the supplementation with vitamin E on fatty acid composition of intramuscular fat in *semimembranosus*.

Supplementation with α-tocopherol resulted in a higher percentage of PUFA (P<0.001) (**Table 4.4**), which was previously reported by Chen et al. (2008) and Álvarez et al. (2009). The higher percentage of PUFA found in vitamin E added samples may be related to its

antioxidant activity. A relationship among *in vivo* low tissue concentrations of vitamin E and lower amounts of both n-6 and n-3 PUFA has been demonstrated (Kasapidou et al., 2001), which suggests that PUFA decrease appears *in vivo* when antioxidant concentrations are low. Unsaturated fatty acids are known to be more susceptible to oxidation so the inclusion of dietary vitamin E in the basal diet may have protected PUFA from these reactions, obtaining a higher PUFA percentage in these samples than in controls. According to Álvarez et al. (2009) the supplementation with 250 mg DL-α-tocopheryl acetate kg^{-1} feed is enough to prevent PUFA from oxidation while Demirel et al. (2004) noted significant differences in intramuscular PUFA proportions when the concentration of α-tocopherol in muscle increased from 0.27 mg g^{-1} to 0.52 mg g^{-1}. Regarding to individual PUFA percentages, supplemented samples presented significant higher proportions of C18:2 n-6, C20:3 n-6 and C20:4 n-6. In a similar way, Zhao et al. (2013) registered a higher proportion of C18:2 and C20:3 in vitamin E supplemented lambs while Álvarez et al. (2009) also found a significant effect of vitamin E on preserving C18:2 n-6. This fatty acid is considered essential to humans (Beare-Rogers, 1988) and vitamin E supplementation seems to protect it against degradation.

The oxidation of PUFA in control samples may have led to a relative increase of the percentage of total SFA ($P<0.001$) as well as a significant higher percentage of some individual saturated fatty acids (C10:0, C16:0, C18:0). Regarding to the percentage of C18:1 n-9 fatty acid, as it was previously noted by Zhao et al. (2013), a lower content was found in vitamin E supplemented than in control samples ($P=0.039$). This result could be explained by the findings of Hou et al. (2013), who reported that vit E supplementation might accelerate the ruminal biohydrogenation of C18:1 unsaturated fatty acids *in vivo*. In contrast, no effect on fatty acid profile was reported by Kasapidou et al. (2012) and Berthelot et al. (2014), which suggests the need of more studies about this topic.

Table 4.4 Effect of dietary vitamin E supplementation, frozen storage time (0d, 3, 6 and 9 months) and display (0 or 9 d) on intramuscular fatty acid profile (percentage of total identified fatty acids) and intramuscular fat content of semimembranosus muscle from lamb (g fat 100g-1 of meat).

	Frozen storage time						Dietary vitamin E				Display			
Fatty acid[1]	0 d	3m	6m	9m	SEM	*P-value*	C	HS	SEM	*P-value*	0d	9d	SEM	*P-value*
C10:0	0.13^{b}	0.08^{a}	0.11^{b}	0.12^{b}	0.03	<0.001	0.12^{b}	0.10^{a}	0.03	0.019	0.11	0.11	0.04	0.340
C12:0	0.18^{ab}	0.14^{a}	0.26^{b}	0.23^{b}	0.01	0.001	0.22	0.19	0.01	0.237	0.20	0.20	0.11	0.917
C14:0	2.28^{ab}	1.96^{a}	2.64^{b}	2.60^{b}	0.06	0.001	2.47	2.26	0.06	0.135	2.35	2.39	0.07	0.798
C15:0	0.42^{a}	0.47^{ab}	0.49^{b}	0.48^{b}	0.01	0.009	0.47	0.45	0.01	0.401	0.46	0.47	0.08	0.512
C16:0	20.52^{b}	19.01^{a}	20.11^{b}	20.05^{b}	0.123	<0.001	20.34^{b}	19.51^{a}	0.12	0.002	19.65^{a}	20.20^{b}	0.13	0.036
C16:1	1.99^{b}	1.64^{a}	2.05^{b}	1.98^{b}	0.03	<0.001	1.93	1.90	0.07	0.647	1.90	1.93	0.03	0.635
C17:0	1.47^{a}	1.83^{b}	1.73^{ab}	1.69^{ab}	0.04	0.007	1.68	1.67	0.04	0.918	1.67	1.69	0.04	0.819
C18:0	11.97	12.64	12.19	12.27	0.13	0.338	12.59^{b}	11.94^{a}	0.13	0.014	12.28	12.26	0.13	0.946
tC18:1 n-9	6.27^{ab}	6.01^{a}	5.97^{a}	7.38^{b}	0.17	0.015	6.33	6.48	0.17	0.663	6.35	6.46	0.18	0.775
C18:1 n-11	0.13^{a}	0.30c	0.26^{bc}	0.21a^{b}	0.01	<0.001	0.22	0.24	0.11	0.296	0.22	0.23	0.01	0.702
C18:1 n-9	33.80	31.48	32.30	31.23	0.26	0.103	32.91^{b}	31.49^{a}	0.26	0.009	31.67	32.74	0.27	0.062
C18:2 n-6	8.52^{a}	10.57^{b}	9.25^{a}	9.43^{ab}	0.17	0.001	8.74^{a}	10.14^{b}	0.18	<0.001	9.89^{b}	9.00a	0.18	0.018
C20:1	0.16	0.18	0.17	0.16	0.01	0.432	0.16	0.17	0.00	0.072	0.17	0.16	0.01	0.653
C18:3 n-3	0.57	0.55	0.57	0.52	0.01	0.425	0.57	0.53	0.02	0.277	0.59	0.52	0.02	0.101
C20:3 n-6	0.23a	0.29^{b}	0.26^{ab}	0.25^{ab}	0.01	0.011	0.24a	0.28^{b}	0.01	0.005	0.28b	0.23a	0.07	0.001
C20:4 n-6	2.32^{a}	3.23^{b}	2.72^{b}	2.55a^{b}	0.09	0.008	2.34^{a}	3.07^{b}	0.13	<0.001	3.03^{b}	2.38^{a}	0.09	0.001
C20:5 n-3	0.28	0.29	0.34	0.26	0.00	0.617	0.30	0.28	0.03	0.600	0.34^{b}	0.24^{a}	0.02	0.027
C22:6 n-3	0.20	0.23	0.22	0.18	0.00	0.402	0.19	0.23	0.02	0.135	0.25^{b}	0.17^{a}	0.01	<0.001

C22:5 n-3	0.60	0.72	0.59	0.54	0.02	0.085	0.58	0.64	0.02	0.310	0.68[b]	0.54[a]	0.22	0.003
∑SFA	37.84	37.21	38.51	38.30	0.12	0.125	38.86[b]	37.08[a]	0.47	<0.001	37.72	38.22	0.21	0.237
∑MUFA	44.99	43.69	43.67	43.86	0.25	0.063	44.31	43.31	0.27	0.073	43.15[a]	44.65[b]	0.33	0.001
∑PUFA	13.75	16.51	14.48	14.27	0.34	0.125	13.49[a]	16.01[b]	0.37	<0.001	15.88[b]	13.62[a]	0.29	0.002
∑n-6	12.07	14.64	12.71	12.72	0.09	0.068	11.79[a]	14.28[b]	0.46	<0.001	13.96[b]	12.10[a]	0.07	0.009
∑n-3	1.68	1.88	1.77	1.55	0.08	0.495	1.70	1.74	0.40	0.815	1.92[b]	1.52[a]	0.07	0.015
IMF (%)	2.78	2.48	2.78	2.70	0.07	0.418	2.82	2.85	0.10	0.057	2.75	2.62	0.12	0.450

C = Control feed. HS = High supplemented feed. ∑SFA = saturated fatty acids summation. ∑MUFA = monounsaturated fatty acids summation. ∑PUFA = polyunsaturated fatty acids summation. ∑n-6 = polyunsaturated n-6 fatty acids summation. ∑n-3 = polyunsaturated n-3 fatty acids summation. IMF (%) = percentage of intramuscular fat. [a,b,c] Values within a row with different superscripts are significantly different ($P<0.05$). [1] Means (percentage of total fatty acids). SEM = standard error of the mean.

Effect of frozen storage

As it is widely known, PUFA are particularly susceptible to oxidative processes and enzymatic reactions are not completely inhibited during frozen storage. Therefore, it was expected a decrease in the percentage of PUFA due to lipolysis and oxidative reactions, producing a relative increase in the percentages of MUFA and SFA. In fact, this is what Alonso et al. (2016) found in pork after long-term frozen storage. Hernández et al. (1999), who reported a significant increase in free fatty acids during frozen storage, identified phospholipids hydrolysis as the major cause of this phenomenon. Alonso et al. (2016) also considered oxidative reactions a significant cause of the reduction of PUFA during frozen storage. Contrary to it was expected, frozen storage did not modify the percentage of total SFA, MUFA or PUFA (**Table 4.4**). Similar to our results Zymon et al. (2007) did not found any effect of frozen storage on meat fatty acid profile. A feasible explanation to our results could be that legs were vacuum packaged prior to be frozen, so it may have inhibited oxidative reactions (**Table 4.3**).

Effect of display

Display led to a significant decrease in the percentage of C20:5 n-3, C22:6 n-3, C22:5 n-3, C18:2 n-6, C20:3 n-6, C20:4 n-6 as well as in the summary of n-3, n-6 and total polyunsaturated fatty acids (**Table 4.4**). As it has been previously mentioned, PUFA are more susceptible to degradation, especially long chain fatty acids with a higher degree of unsaturation, so oxidative reactions throughout display may have been responsible of this decrease. In fact, malondialdehyde content was significantly higher after the storage period. Álvarez et al. (2009) studied the changes in fatty acid profile of vitamin E supplemented lamb packaged in modified atmosphere and displayed during 28 d, registering great changes in PUFA percentage. They noted a reduction in the percentage of n-3 and n-6 fatty acids of non-supplemented samples throughout display, which was attributed to lipid oxidation. In contrast, no changes were measured in the vitamin E high supplemented samples (1000 mg DL-α-tocopheryl acetate kg^{-1} feed), which demonstrated the relationship between antioxidant content, oxidative reactions and PUFA content.

Principal Components Analysis

The first two components of the principal component analysis (PCA) explained 56.98% of the variability of the results (**Figure 4.2**). The first component (PC1), which was able to explain 33.65% of the variation of the whole study, was characterised by the variables metmyoglobin, Hue, and thiobarbituric acid reactive substances (TBARS) in the positive side and ∑PUFA, ∑n-6, and the index 630/580 in the negative side. This component divided the observations in two main groups depending on the day of display. The centroids of the observations at 0 d of storage were mainly on the left quadrants of the figure, closely related to ∑PUFA, ∑n-6, ∑n-3 and the index 630/580; meanwhile, the observations at d 9 were located on the right side, characterized by the % of metmyoglobin, ∑SFA, Hue and

TBARS. Display significantly affected colour, lipid oxidation (**Table 4.3**), ∑PUFA, ∑n-6 and ∑n-3 (**Table 4.4**). A significant positive correlation was found between lipid oxidation and the percentage of metmyoglobin (0.393) and significant strong correlations were also registered among the latter and h (0.94) and the index 630/580 (-0.910).

Enriched oxygen atmospheres have been described to favour lipid oxidation, which increased throughout display in all conditions evaluated in this trial (**Table 4.3**). Primary and secondary products of lipid oxidation enhance myoglobin oxidation, leading to its conversion into metmyoglobin and resulting in meat discolouration (Faustman et al., 2010). Polyunsaturated fatty acids are known to be more susceptible to oxidative reactions which could explain its decrease during display (Ródriguez-Cárpena et al., 2011). Similarly, Álvarez et al. (2009) noted a reduction in the percentage of n-3 and n-6 fatty acids throughout display, which was attributed to lipid oxidation. In fact, a significant negative correlation was observed between TBARS and ∑PUFA (-0.326).

Moreover, a discrimination between high and low vitamin E supplemented samples within each day could be noted. The observations of the animals supplemented with 1000 mg α-tocopherol kg^{-1} were at the left of those which were supplemented with 30 mg at both days. As a result, the centroid of the animals with a high level of supplementation was located in the lower left-hand quadrant, which was related with ∑PUFA, ∑n-6, ∑n-3 and the concentration of α-tocopherol in muscle. The centroid of the animals supplemented with a dosage of 30 mg α-tocopherol kg^{-1} was on the opposed quadrant, defined by the percentage of intramuscular fat (%IMF), ∑SFA and the TBARS.

Significant negative correlations among the concentration of α-tocopherol in muscle TBARS and ∑SFA (-0.341, -0.425) were noted, while the content of tocopherol was positive correlated with ∑PUFA (0.341) and ∑n-6. Vitamin E supplementation significantly reduced lipid oxidation during display (**Table 4.3**), which could have protected PUFA from degradation. Therefore, SFA are expected to increase if the percentage of PUFA decrease. The correlation between tissue antioxidant status and fatty acid stability has been previously reported (Kasapidou et al., 2001).

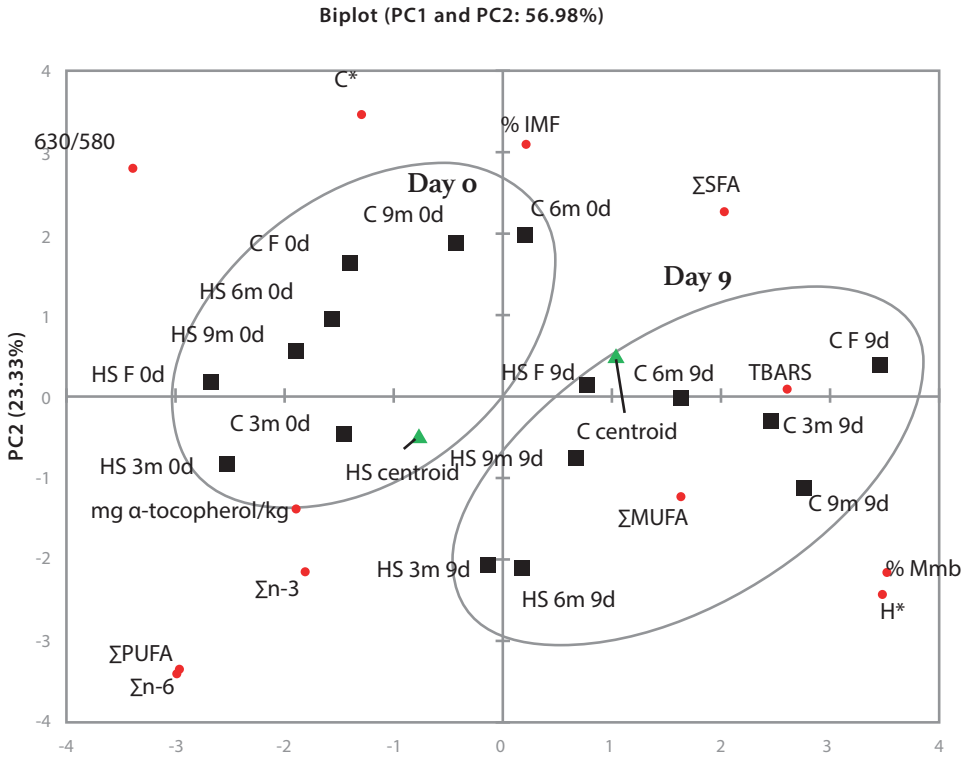

Figure 4.2 Projection in the plane of physicochemical parameters, fatty acid groups and the centroids of the observations defined by two principal components. HS: High rate of vitamin E supplementation (1000 mg α-tocopherol kg^{-1}), C: Control feed (30 mg α-tocopherol kg^{-1}), Frozen storage duration: F (fresh meat), 3m (3 months), 6m (6 months), 9m (9 months), Days of display after frozen storage: 0d or 9d. % MetMb: percentage of metmyoglobin, TBA: Thiobarbituric acid reactive substances, ∑SFA:saturated fatty acids summation; ∑MUFA: monounsaturated fatty acids summation; ∑PUFA: polyunsaturated fatty acids summation; ∑n-6:polyunsaturated n-6 fatty acids summation; ∑n-3: polyunsaturated n-3 fatty acids summation; IMF (%): percentage of intramuscular fat.

CONCLUSIONS

The effect of dietary vitamin E on lamb quality was independent from freezing and frozen storage duration. Muscle α-tocopherol concentration was over 3.5-fold higher in supplemented than in control lambs. At this vitamin E level, lipid oxidation was significantly reduced, leading to a lower metmyoglobin formation and a better colour maintenance (a lower value for Hue and a higher for the 630/580 ratio). However, a higher concentration of α-tocopherol in muscle is required to completely inhibit lipid oxidation and discolouration. Vitamin E addition also had an effect on fatty acid profile. The antioxidant effect of vitamin E protected PUFA from oxidative reactions during display, showing higher values in these samples than in controls. In conclusion, dietary vitamin E could be recommended either for fresh or frozen thawed meat, resulting in a better colour maintenance, a lower lipid oxidation and a higher nutritional value.

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Chapter 5

Dietary supplementation of 11 different plant extracts on the antioxidant capacity of blood and selected tissues in light weight lambs

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ABSTRACT

Due to the growing public concern regarding the addition of chemical antioxidants to foods, focus has shifted towards natural alternatives. Because of their antioxidant potential, culinary herbs and spices have long been used to extend the shelf-life of foods. However, a better understanding of the fate of these products following intake, is required to assess their use in lamb diets. Two hundred eighty-eight *Rasa Aragonesa* male lambs (70 d old) were supplemented (5.0 g kg^{-1} compound feed) with bay, marjoram, oregano, rosemary, thyme, turmeric, cumin, caraway, dill, cinnamon and nutmeg extracts for 14 d before slaughter. Dietary supplementation with plant extracts had no effect on intake, growth performance and antioxidant activity in blood (TEAC values). In muscle, nutmeg supplementation increased ($P<0.05$) the radical-scavenging capacity (TEAC). Whereas, a decrease in the radical-scavenging capacity was found for oregano, dill, cinnamon and nutmeg supplemented lambs (ORAC values). In liver, nutmeg supplementation increased ($P<0.05$) the antioxidant capacity (TEAC). Whereas, bay (ORAC), turmeric, cinnamon and nutmeg (DPPH• values) decreased ($P<0.05$) the radical-scavenging capacity of the tissue. In kidney, a lower ($P<0.05$) radical-scavenging capacity (TEAC values) was found in oregano, cumin and caraway supplemented lambs. Whereas, turmeric, cumin, caraway, cinnamon and nutmeg increased ($P<0.05$) the antioxidant capacity (ORAC values) in kidney. Supplementation of lamb diets with plant extracts affected the radical scavenging activity in muscle, liver and kidney. However, due to the divergent results of the different assays for the same tissue, it is not advisable to discriminate plant extracts using this approach.

INTRODUCTION

Due to the growing concern among the public opinion (consumers and meat industry) regarding the addition of chemical additives to foods, the use of synthetic antioxidants is diminishing and focus has shifted toward the use of naturally occurring antioxidants. Many natural antioxidants are recognized as promising alternatives to increase the shelf life of meat products (Moñino et al., 2008; Nieto et al., 2010; Vasta and Luciano, 2011). Supplementation of feed with α-tocopherol was found to be a simple and convenient way to introduce a natural antioxidant into cellular matrices (such in muscle) where initiation of lipid oxidation is expected to occur (Nieto et al., 2010).

In Mediterranean lamb production systems, after weaning at 12-14 kg of body weight (BW), lambs are fed *ad libitum* compound feed plus straw until they reach a slaughter weight of 22-28 kg of BW (Lobón et al., 2017; Leal et al., 2018a). Under these conditions, supplementation of lamb diets with 0.5 g vitamin E per kg diet for 7-14 d before slaughter, has been shown to elevate α-tocopherol concentrations in tissues (Leal et al., 2018b) and to delay meat oxidation.(González-Calvo et al., 2015; Leal et al., 2018a). However, other phenolics (such as flavonoids and phenolic acids) are also assumed to be able scavenge free radicals before lipid oxidation propagation.

Alternative sources of phenolics in several types of plant rich materials such as vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs have been investigated (Kähkönen et al., 1999). Apart from improving the flavour and organoleptic properties of different types of foods, culinary herbs and spices such as bay (*Laurus nobilis*), marjoram (*Origanum majorana*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*) thyme (*Thymus vulgaris*), turmeric (*Curcuma longa*), cumin (*Cuminum cyminum*), caraway (*Carum carvi*), dill (*Anethum graveolens*), cinnamon (*Cinnamomum zeylanicum*) and nutmeg (Myristica fragans), have been consistently reported to exhibit antioxidant properties (Wojdyło et al., 2007; Vallverdú-Queralt et al., 2014; Vallverdú-Queralt et al., 2015). Some authors have attributed the *in vitro* antioxidant capacity of these plants to their phenolic compound content, resulting in a positive linear correlation between them (Zheng and Wang, 2001; Shan et al., 2005).

Like α-tocopherol, the direct antioxidant capacity of a dietary polyphenol would be affected by its absorption along the gastrointestinal tract and to its deposition in the tissues (Vasta and Luciano, 2011). A better understanding of the fate of polyphenolic compounds present in culinary herbs and spices following intake is needed. Moreover, data on tissue antioxidant potential of polyphenolic compounds present in herbs and spices is scarce, even in experimental animals (Crozier et al., 2009).

The aim of the current study was to investigate whether the supplementation of lamb diets with plant extracts from 11 culinary herbs and spices affects the antioxidant capacity of different lamb tissues (plasma, muscle, liver, and kidney).

MATERIALS AND METHODS

Reagents

All the chemicals used were of AnalR grade. The 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), $FeSO_4 7H_2O$, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS), (±)-6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox), Fluorescein and potassium persulphate ($K_2S_2O_8$) were purchased from Sigma–Aldrich (Madrid, Spain). The CH_3COOH, CH_3COONa, HCl, and $FeCl_3$-6H2O were obtained from Scharlau Chemie S.A. (Barcelona, Spain). Methanol (HPLC grade) was purchased from J.T. Baker (Mallinckrodt Baker B.V., Deventer-Holland). 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, VA).

Animals and Diets

All the experimental and slaughter procedures used met the guidelines of Council Directive 86/609/EEC (European Communities, 1986) on the protection of animals used for experimental or other scientific purposes.

Two hundred eighty-eight *Rasa Aragonesa* male lambs (23.5 ± 1.38 kg) were purchased from local dealers to arrive at the commercial farm Franco and Navarro (Zaragoza, Spain) in three batches of 96 animals. After 7 d of adaptation to the basal compound feed (control), lambs were randomly allocated (per batch) based on body weight to 12 pens (8 lambs per pen in 20 m^2) containing straw bedding. For each batch, a pen was randomly assigned to one of 12 diets consisting of a basal compound feed (control) and the basal compound feed supplemented (5.0 g kg^{-1}) with 11 different plant extracts (bay, *Laurus nobilis*; marjoram, *Origanum majorana*; oregano, *Origanum vulgare*; rosemary, *Rosmarinus officinalis*; thyme,

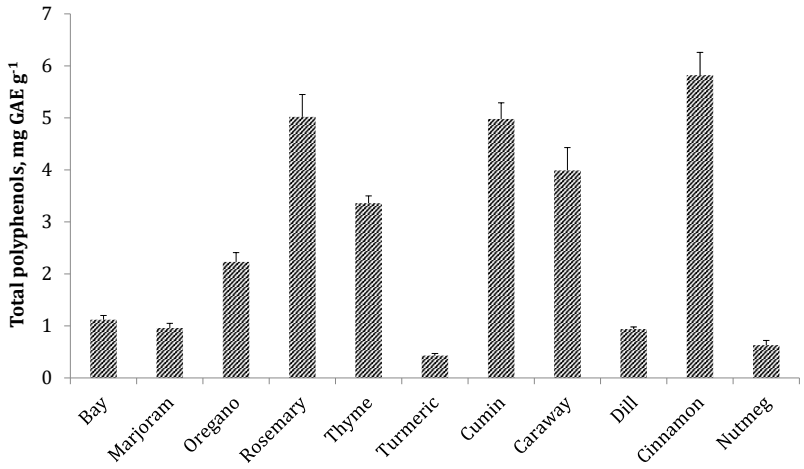

Figure 5.1 Total polyphenol content of the selected plant extracts evaluated by the Folin-Ciocalteau method and expressed per unit Gallic Acid Equivalents (GAE) of dried product. Adapted from: Vallverdú-Queralt et al. (2014) and Vallverdú-Queralt et al. (2015).

Thymus vulgaris; turmeric, *Curcuma longa*; cumin, *Cuminum cyminum*; caraway, *Carum carvi*; dill, *Anethum graveolens*; cinnamon, *Cinnamomum zeylanicum* and nutmeg, *Myristica fragans*). When lambs were supplemented with standardized rosemary extracts, dosages in the range of 0.2 to 1.2 g kg^{-1} diet have been tested (Moran et al., 2012; Ortuño et al., 2014). However, when plant extracts from different plant origins were assessed, dosages ranging from 2.0 to 40.0 g kg^{-1} diet were reported (Andrés et al., 2013; Valenti et al., 2018).

In the current study, a dosage of 5.0 g kg^{-1} compound feed was defined based on the diversity of the plant extracts investigated. Vallverdú-Queralt et al.(2014) and Vallverdú-Queralt et al. (2015) have previously described plant extract production, composition and phenolic profile. The total polyphenol content per plant extract is provided in **Figure 5.1**.
The experimental compound feed was pelleted at 62 ± 2°C to a 3.5 mm pellet. Ingredients of the experimental compound feed is shown in Table 5.1. During a period of 14 d (experimental period), lambs had free access to the experimental compound feeds, wheat straw and water via separated troughs available in each pen. Intakes of the experimental compound feed were recorded weekly and orts measured at the end of the experiment. Water and wheat straw intakes we not recorded. Lambs were weighed individually at the start and at the end of the experimental period (14 d of supplementation). After an overnight period without feed (with free access to water) of approximately 10 hours, animals were transported and slaughtered at a local abattoir (Mercazaragoza S.A., Zaragoza, Spain) within 2 hours after leaving the farm.

Table 5.1 Composition of the basal compound feed.

Ingredient	**g kg^{-1}**
Barley	285
Wheat	260
Maize	200
Soya bean meal (490 g kg^{-1} of crude protein)	190
Soya oil	17
Limestone	25
Sodium bicarbonate	6
Sodium chloride	4
Standard mineral and vitamin premix†	3
Experimental premix‡	10

† Minerals and vitamins provided: Ca 0.24 g, Na 0.47 g, S 0.23 g, Mn 30 mg, Zn 50 mg, Cu 5 mg, I 0.5 mg, Co 0.5 mg, Se 0.15 mg, Fe 50 mg, vitamin A 8000 IU, vitamin D3 1600 IU, *all-rac*-α-tocopheryl acetate 25 mg. ‡ Experimental premix: wheat bran 5g, plant extract or hydrated silica (50% water and 50% silica) 5g.

Blood and Tissue Sampling

Four lambs per pen (in total 12 lambs per treatment) were randomly selected at the start of the experimental period for blood and tissue sampling. Upon arrival and in the last day of the experimental period (d 14), blood samples (10 mL) were collected in heparinized evacuated tubes (Belliver Industrial Estate, Plymouth, U.K.). All tubes were immediately placed on ice and, within 2 h after collection were centrifuged at 2500 × g for 10 min at 4°C. The plasma was collected, acidified (1:11) with acetic acid (10 mM) and frozen at -80°C until the extraction of the polyphenolic fraction.

The lambs were slaughtered in the local abattoir according to EC Regulations. Within 30 min after slaughter, liver and kidneys (detoxification organs) were removed, cut into pieces, vacuum-packed in BB4L bags (Cryovac Packaging, Barcelona, Spain) of low gas permeability (8-12 cm^3 m^{-2} per 24h) using a discontinuous INELVI VISC 500 packer (Industrial Elétrica Vilar, Barcelona, Spain), and stored at -80°C prior to analysis. The carcasses were chilled (2°C) for 24 h before a professional butcher removed the *longissimus throracis et lumborum* muscle. The muscles were filleted (1.5 cm thick) and stored under the same conditions as described above for the detoxification organs.

Plasma Polyphenol Extraction

A modified method described by Faggian et al. (2016) was used to determine the presence of polyphenolic components in lamb plasma. In order to precipitate proteins, 2 mL of acetonitrile was added to 2 mL of acidified plasma before samples were vortexed and placed for 45 min in an ultrasound bath in ice. The sample was then centrifuged (6311 x g, 4°C for 10 min) and the clear supernatant was collected and dried at 40°C under vacuum conditions in an evaporator system (Syncore Polyvap R-96) (Buchi). The residue was redissolved in methanol and brought up to 1 mL. The extracts were kept in vials at - 80°C until their corresponding analyses.

DPPH• Radical-Scavenging Capacity

The antioxidant scavenging activities of the muscle, liver and kidney tissues were determined according to the method described by Brand-Williams et al. (1995). Methanolic extracts (500 µL) at different concentrations (400 to 25 µL mL^{-1}) were added to 1 ml of methanolic DPPH• solution (0.1 mM). The estimated time of reaction (20 min) was determined by considering the reduction of the absorbance at 517 nm (monitored every 5 min), until the reaction curve reached a plateau. The absorbance was measured at room temperature, in dark conditions, against a blank (500 µL of sample plus 1 mL of methanol). The absorbance of the control (500 µL of methanol in 1 mL of DPPH• solution) was measured daily. All the assays were conducted in triplicate. The percentage capacity for the DPPH• technique was calculated as:

$$\% \text{ Discolouration} = [1 - (\text{Abs sample}/\text{Abs control})] \times 100$$

The results were expressed as the inhibitory concentration of the fresh tissue necessary to decrease the $DPPH^{\bullet}$ absorbance by 50% (IC50). Concentrations are expressed in mg ml^{-1}.

Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC assay was used for the determination of the plasma, muscle, liver and kidney tissue antioxidant capacity according to the method described by Re et al. (1999). The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 14 mM ABTS with an equal volume of 4.9 mM potassium persulphate (final concentration: 7 mM ABTS in 2.45 mM potassium persulphate). The mixture was incubated in the dark at room temperature, 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated to 30°C. Polyphenolic extracts (15 µl) or Trolox standard (25–2000 µM in ethanol) were added to 1.5 mL of the diluted $ABTS^{\bullet+}$ solution and the absorbance was read at 30°C, 1–20 min after the initial mixing. All the absorbance readings were taken against a blank containing 15 µL of sample plus 1.5 mL of ethanol. Results are expressed as mmol of Trolox equivalent per g of fresh tissue or mL of plasma.

Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing ability of polyphenolic tissue extracts was measured according to a modified method developed by Descalzo et al. (2007). To prepare the FRAP reagent, a mixture of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride (10:1:1) was made. To 1.2 mL of reagent, 0.04 ml of methanolic extract and 0.12 mL of deionised water was added. Readings at the maximum absorption (593 nm) at 37°C were taken every 15 s, and the reaction was monitored up to 30 min. The endogenous Fe(II) content of muscle and detoxification organs (FRAP) were determined with a TPTZ/HCl solution without ferric chloride in the reaction mixture. Ferrous sulphate solutions ($FeSO_4.7H2O$) of 0.05–3 mM were used to obtain the calibration curve, and results are expressed as mmol Fe (II) equivalent per g of fresh tissue.

Oxygen Radical Absorbing Capacity (ORAC)

The automated ORAC assay was carried out with a Fluostar Optima microplate reader (BMG Labtechnologies, Durham, NC) as described by Prior et al. (2003). Methanolic extracts were diluted 1:10; 1:100 and 1:500 with phosphate buffer (75 mM, pH 7.0). The diluted extract (40 µL) was added to each well in clear 48-well Falcon plates. Phosphate buffer was used as a blank and Trolox (40 µL) was used as the standard (6.25, 12.5, 25, and 50 µM). The Fluostar Optima instrument, equipped with two automated injectors, was programmed to add 400 µL of fluorescein (0.11 µM) followed by 150 µL of AAPH (31.6 mM) to each well. Fluorescence readings (excitation 485 nm, emission 520 nm) were recorded immediately after the addition of fluorescein, immediately after the addition of AAPH, and every 192 s thereafter for 90 min to reach a 95% loss of fluorescence. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated on the

basis of differences in area under the fluorescein decay curve between the blank, samples, and Trolox standards. A standard curve was obtained by plotting the four concentrations of Trolox standards against the net area under the curve of each standard. Finally ORAC values were calculated using the regression equation between Trolox concentration and area under the curve and were expressed as mmol of Trolox equivalent per g of fresh tissue.

Statistical Analysis

All statistical analyses were performed using SAS, version 9.3 (SAS Institute, Inc., Cary, NC, USA). To assess the effects of dietary plant extract supplementation on average daily weight gain, feed intake, feed efficiency and antioxidant capacity (TEAC, FRAP, ORAC and DPPH•) in the tissues (muscle, liver and kidney), data were analysed with a MIXED procedure where the model accounted for the fixed effect of treatment, with pen and the residuals entered in the model as random effects. For initial body weight, final body weight and TEAC levels in blood, the fixed effects of treatment and time (as repeated measures), and the first-order interactions were considered. Pen and the residuals entered the model as random effects. When a treatment and time interaction was detected, a Tukey post-hoc test was used to correct for multiple comparisons. Significance was declared when $P<0.05$ and trends were declared when $P<0.10$.

RESULTS

Animal Performance

The experimental period lasted 14 d, from an initial body weight of 23.5 ± 1.38 kg (mean ± SD) to a final body weight of 27.9 ± 1.91 kg. The average daily weight gain, average daily feed intake and feed conversion ratio are presented in **Table 5.2**. Supplementation of lamb diets with the selected plant extracts had no effect on the aforementioned parameters.

Table 5.2 Productive performance of lambs fed a control diet without or with 11 plant extracts for 14 d.

Diet	Initial body weight (kg)	Final body weight (kg)	Average daily weight gain (kg d^{-1})	Feed intake (kg d^{-1})	Feed conversion ratio (kg kg^{-1})
Control	23.3 ± 0.27	27.7 ± 0.44	0.34 ± 0.023	1.06 ± 0.055	3.2 ± 0.09
Bay	23.4 ± 0.26	28.2 ± 0.41	0.37 ± 0.025	1.18 ± 0.045	3.2 ± 0.17
Marjoram	23.4 ± 0.29	27.7 ± 0.40	0.33 ± 0.022	1.10 ± 0.083	3.3 ± 0.07
Oregano	23.3 ± 0.32	27.4 ± 0.37	0.31 ± 0.020	1.05 ± 0.029	3.4 ± 0.06
Rosemary	23.5 ± 0.29	28.3 ± 0.36	0.37 ± 0.013	1.12 ± 0.017	3.1 ± 0.12
Thyme	23.4 ± 0.26	27.8 ± 0.33	0.34 ± 0.017	1.07 ± 0.039	3.2 ± 0.11
Turmeric	23.4 ± 0.31	27.8 ± 0.44	0.34 ± 0.020	1.14 ± 0.064	3.4 ± 0.37
Cumin	23.6 ± 0.28	28.0 ± 0.40	0.34 ± 0.019	1.12 ± 0.050	3.3 ± 0.08
Caraway	23.5 ± 0.30	28.2 ± 0.38	0.36 ± 0.022	1.08 ± 0.083	3.0 ± 0.16
Dill	23.6 ± 0.34	28.0 ± 0.41	0.34 ± 0.019	1.10 ± 0.061	3.3 ± 0.17
Cinnamon	23.7 ± 0.31	28.1 ± 0.44	0.34 ± 0.022	1.06 ± 0.055	3.2 ± 0.31
Nutmeg	23.5 ± 0.31	27.8 ± 0.49	0.33 ± 0.026	1.05 ± 0.042	3.3 ± 0.34

Values are means ± SEM.

Radical-Scavenging Capacity in Blood

Dietary supplementation of lamb diets with selected plant extracts had no effect (P>0.05) on TEAC values in blood (**Table 5.3**).

Table 5.3 Trolox equivalent antioxidant capacity (mM trolox g^{-1}) of blood of lambs at the start and the end of feeding a control diet without or with 11 plant extracts for 14 d.

Diet	Initial	Final
Control	41.0 ± 3.17	47.4 ± 1.74
Bay	51.5 ± 4.56	40.6 ± 3.60
Marjoram	48.1 ± 2.23	42.7 ± 3.16
Oregano	42.9 ± 3.36	39.2 ± 3.31
Rosemary	43.9 ± 3.38	44.8 ± 3.61
Thyme	46.6 ± 4.83	36.4 ± 2.54
Turmeric	50.0 ± 3.48	40.8 ± 2.97
Cumin	40.6 ± 3.87	39.5 ± 4.68
Caraway	45.6 ± 3.63	50.0 ± 1.89
Dill	44.4 ± 3.19	41.7 ± 2.67
Cinnamon	48.6 ± 4.24	42.9 ± 2.74
Nutmeg	51.3 ± 2.31	44.2 ± 3.77

Values are means ± SEM.

dical-Scavenging Capacity in Muscle

Based on the results from the TEAC assay, supplementation of lamb diets with 5.0 g kg^{-1} of nutmeg extract significantly increased the radical-scavenging capacity in muscle when compared with the Control and marjoram groups (**Table 5.4**). No differences were found among treatments when muscle radical-scavenging capacity was accessed by FRAP. When considering the results from the ORAC assay, the radical-scavenging capacity of muscle samples from the oregano, dill, cinnamon and nutmeg were significantly lower than the Control and rosemary groups (P<0.05). Moreover, higher $DPPH^{\cdot}$ values in the cinnamon group found when compared to bay, thyme and nutmeg supplemented lambs.

Table 5.4 Antioxidant capacity as measured by 4 different assays† of the longissimus throracis et lumborum muscle of lambs after feeding a control diet without or with 11 plant extracts for 14 d‡.

Diet	TEAC (mM trolox g^{-1})	FRAP (mM Fe^{+2} g^{-1})	ORAC (mM trolox g^{-1})	DPPH• (mg ml^{-1} MeOH)
Control	71.1 ± 2.09^{ab}	12.0 ± 0.84	921.1 ± 31.32^{d}	63.29 ± 2.68^{ab}
Bay	82.2 ± 3.11^{bc}	14.4 ± 1.36	846.7 ± 37.87^{bcd}	55.63 ± 3.76^{a}
Marjoram	66.6 ± 2.02^{a}	13.8 ± 1.79	798.8 ± 32.94^{bcd}	61.89 ± 3.09^{ab}
Oregano	74.0 ± 2.59^{ab}	16.7 ± 2.06	719.5 ± 48.94^{abc}	66.23 ± 4.21^{ab}
Rosemary	73.5 ± 2.98^{ab}	12.8 ± 0.74	996.0 ± 27.04^{d}	67.97 ± 6.09^{ab}
Thyme	76.9 ± 1.75^{abc}	15.4 ± 1.55	879.7 ± 50.30^{cd}	56.71 ± 2.78^{a}
Turmeric	75.2 ± 3.79^{abc}	13.2 ± 1.17	879.1 ± 59.65^{cd}	62.48 ± 4.06^{ab}
Cumin	74.2 ± 1.50^{ab}	14.6 ± 1.16	848.4 ± 35.46^{bcd}	67.66 ± 1.94^{ab}
Caraway	79.1 ± 2.31^{abc}	16.4 ± 1.03	721.7 ± 43.03^{abcd}	62.77 ± 2.91^{ab}
Dill	70.0 ± 2.62^{ab}	15.2 ± 1.34	668.2 ± 27.29^{ab}	71.94 ± 3.49^{ab}
Cinnamon	73.8 ± 4.22^{ab}	16.8 ± 1.75	592.2 ± 66.55^{a}	78.41 ± 4.39^{b}
Nutmeg	87.9 ± 2.93^{c}	15.1 ± 1.40	525.8 ± 30.73^{a}	56.26 ± 3.41^{a}

Values are means ± SEM. † TEAC = Trolox equivalent antioxidant capacity; FRAP = ferric reducing antioxidant power; ORAC = oxygen radical absorbance capacity; DPPH• = 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity. ‡ Different letters within the same column denote significant differences among treatments ($P<0.05$).

Radical-Scavenging Capacity in Liver

Data from the assessment of radical-scavenging capacity in liver samples from lambs fed the 11 different plant extracts are presented in **Table 5.5**. Nutmeg extract supplementation significant increased TEAC values compared to the Control and marjoram fed groups. Like in muscle, there was no effect of plant extract supplementation on radical-scavenging capacity in the liver, as indicated by the FRAP assay. Lambs supplemented with bay extract presented significantly lower ORAC values in liver than lambs supplemented with marjoram, oregano, rosemary and turmeric. Liver DPPH· values from nutmeg, cinnamon and turmeric supplemented lambs were significantly lower than the control, marjoram, rosemary and thyme supplemented groups.

Radical-Scavenging Capacity in Kidney

The effect of plant extract supplementation on radical-scavenging capacity of kidney tissue is presented in **Table 5.6**. According to the TEAC assay results, supplementation of lamb diets with oregano, cumin and caraway plant extracts significantly lowered the radical-scavenging capacity in kidney tissue compared to the Control, marjoram and rosemary fed lambs. Similarly to muscle and liver, no differences among treatment groups were found for FRAP in kidney tissue. When focusing on the ORAC assay values, turmeric, cumin, caraway, cinnamon and nutmeg presented significantly higher radical-scavenging capacity than the Control, bay, oregano and rosemary fed lambs. Moreover, in kidney tissue, supplementation of lamb diets with oregano extract yielded the lowest radical-scavenging capacity, as accessed by the DPPH· assay.

Table 5.5 Antioxidant capacity as measured by 4 different assays[†] of the in the liver of lambs after feeding a control diet without or with 11 plant extracts for 14 d[‡].

Diet	TEAC (mM trolox g^{-1})	FRAP (mM Fe^{+2} g^{-1})	ORAC (mM trolox g^{-1})	DPPH• (mg ml^{-1} MeOH)
Control	298.3 ± 12.40^{a}	86.8 ± 8.34	2583.2 ± 128.0^{bc}	30.6 ± 1.19^{abc}
Bay	307.8 ± 10.62^{ab}	63.8 ± 3.98	1714.0 ± 72.44^{a}	32.4 ± 2.20^{abcd}
Marjoram	282.2 ± 14.33^{a}	68.3 ± 5.63	2952.7 ± 76.70^{c}	31.3 ± 1.53^{abc}
Oregano	315.7 ± 6.73^{ab}	71.7 ± 5.05	2759.9 ± 141.75^{c}	31.9 ± 1.94^{abcd}
Rosemary	327.3 ± 14.84^{ab}	85.6 ± 9.15	2612.9 ± 182.51^{c}	29.6 ± 1.08^{ab}
Thyme	328.3 ± 10.10^{ab}	73.3 ± 3.21	2378.0 ± 87.07^{bc}	27.3 ± 1.31^{a}
Turmeric	303.8 ± 5.88^{ab}	81.4 ± 6.55	2682.4 ± 79.95^{c}	45.5 ± 2.72^{ef}
Cumin	300.8 ± 9.59^{ab}	70.7 ± 3.46	2583.1 ± 116.91^{bc}	33.1 ± 1.32^{abcd}
Caraway	310.9 ± 38.68^{ab}	68.6 ± 5.46	2543.3 ± 215.83^{bc}	37.2 ± 1.50^{bcde}
Dill	334.9 ± 7.42^{ab}	80.6 ± 6.69	2484.5 ± 113.76^{bc}	38.6 ± 2.13^{cdef}
Cinnamon	321.3 ± 8.26^{ab}	70.3 ± 4.47	2015.5 ± 106.77^{ab}	46.8 ± 2.45^{f}
Nutmeg	368.3 ± 12.73^{b}	77.3 ± 6.92	2510.2 ± 88.20^{bc}	40.3 ± 2.60^{def}

Values are means ± SEM. [†] TEAC = Trolox equivalent antioxidant capacity; FRAP = ferric reducing antioxidant power; ORAC = oxygen radical absorbance capacity; DPPH• = 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity. [‡] Different letters within the same column denote significant differences among treatments (P<0.05).

Table 5.6 Antioxidant capacity as measured by 4 different assays[†] in the kidney of lambs after feeding a control diet without or with 11 plant extracts for 14 d[‡].

Diet	TEAC (mM trolox g^{-1})	FRAP (mM Fe^{+2} g^{-1})	ORAC (mM trolox g^{-1})	DPPH• (mg ml^{-1} MeOH)
Control	196.8 ± 7.19^{c}	29.5 ± 3.20	1206.7 ± 60.73^{ab}	76.5 ± 3.09^{ab}
Bay	188.1 ± 8.30^{bc}	29.1 ± 3.27	1154.0 ± 65.05^{a}	90.1 ± 3.29^{ab}
Marjoram	192.7 ± 8.20^{c}	30.3 ± 1.58	1344.5 ± 35.72^{abcd}	76.0 ± 5.75^{ab}
Oregano	156.2 ± 6.88^{ab}	21.9 ± 1.01	1082.6 ± 52.27^{a}	117.3 ± 9.27^{c}
Rosemary	194.2 ± 4.63^{c}	28.8 ± 1.78	1218.1 ± 50.30^{abc}	81.4 ± 5.17^{ab}
Thyme	182.9 ± 6.49^{abc}	28.5 ± 2.23	1579.8 ± 164.14^{bcde}	72.5 ± 4.69^{ab}
Turmeric	168.1 ± 5.03^{abc}	28.3 ± 4.92	1762.0 ± 124.82^{ef}	91.0 ± 2.79^{ab}
Cumin	154.5 ± 6.29^{a}	26.9 ± 1.32	1628.6 ± 79.14^{cde}	87.0 ± 4.52^{ab}
Caraway	159.6 ± 6.56^{ab}	30.1 ± 2.22	1623.1 ± 45.27^{de}	89.1 ± 5.38^{ab}
Dill	178.0 ± 8.57^{abc}	31.0 ± 4.06	1608.2 ± 68.32^{bcde}	78.3 ± 7.43^{ab}
Cinnamon	187.1 ± 3.91^{bc}	32.1 ± 2.39	1761.4 ± 125.54^{ef}	68.8 ± 2.03^{a}
Nutmeg	184.4 ± 8.22^{abc}	25.5 ± 2.14	2064.5 ± 72.79^{f}	96.9 ± 6.91^{bc}

Values are means ± SEM. [†] TEAC = Trolox equivalent antioxidant capacity; FRAP = ferric reducing antioxidant power; ORAC = oxygen radical absorbance capacity; DPPH• = 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity. [‡] Different letters within the same column denote significant differences among treatments ($P<0.05$).

DISCUSSION

Supplementation of lamb diets with selected plant extracts had no effect on final body weight, average daily weight gain, feed intake and feed efficiency. This is in agreement with previous findings in lambs (Chaves et al., 2008; Smeti et al., 2018) and cattle (Devant et al., 2007; Balcells et al., 2012), in which supplementation of different plant extracts or essential oils had no effect on growth, feed intake nor efficiency.

In the current study, there was no effect of plant extract supplementation on blood radical-scavenging capacity (in mM Trolox g^{-1}), as assessed by the TEAC assay. However, when feeding plant extracts to sheep by direct ruminal infusion, Gladine et al. (2007) was able to detect the presence of epicatechins in blood. Karami et al. (2010) reported a decrease in blood lipid oxidation in goat kids as a result of dietary supplementation with *Andrographis paniculata* and turmeric, and more recently, Kafantaris et al. (2017) found that supplementation of lamb diets with grape pomace enhanced the antioxidant mechanisms in blood and tissues, as indicated by an increase in catalase and glutathione capacity. We focused on the radical scavenging ability of the plant extracts in blood by performing the TEAC assay. However, it is advisable to use more than one assay per blood/tissue sample for a better understanding of the effects and mechanisms of action of plant extracts in the target sample (Ghiselli et al., 2000).

In muscle, the radical scavenging and reducing ability of the selected plant extracts were assessed with TEAC, FRAP, ORAC and DPPH$^{\cdot}$ assays. We found that supplementation of lamb diets with nutmeg extract significantly increased TEAC values in muscle when compared with non-supplemented lambs. Phenolic compounds, or their metabolites, can be absorbed along the gastrointestinal tract and may act as direct antioxidants in the animal tissues (Moñino et al., 2008; Jordán et al., 2010). Supplementation of ewe diets during gestation and lactation with distilled rosemary leaves (up to 20% of the basal diet), increased the phenolic content in the lamb's muscle which was associated with a higher overall antioxidant activity, measured by FRAP and TEAC assays. Moreover, when supplementing lamb diets with 8.98% (dry matter basis) quebracho extract (*Schinopsis lorentzii*), Luciano et al. (2011) found an increase in total phenols in muscle (+31.3%) which resulted in higher antioxidant capacity in the tissue (TEAC and FRAP assays). Based on the ORAC assay, supplementation of lamb diets with oregano, dill, cinnamon and nutmeg significantly reduced the radical scavenging capacity (ORAC assay) in muscle compared with the non-supplemented lambs. It is recognized that phenolics present in plant extracts can act as prooxidants under certain conditions, including high concentrations of transition metal ions, alkaline pH and the presence of oxygen molecules (Blokhina et al., 2003). Earlier work from Cao et al. (1997), demonstrated that myricetin, quercetin, and kaempferol behaved as prooxidants using the ORAC assay with Cu(II) as a transition metal oxidant. Moreover, they also found that the same compounds could behave as antioxidants or prooxidants, depending on the concentration in the mixture and the free radical used in the assay.

Natural antioxidants and phytochemicals are multifunctional and differences among assays can also be attributed to their antioxidant properties (Frankel and Meyer, 2000). An antioxidant may act directly by scavenging reactive species or by inhibiting their generation. For instance, the ORAC assay is based on the inhibition of lipid peroxidation, and it is known to correlate poorly with FRAP, ABTS and DPPH techniques, which measure the capability of an antioxidant to quench free radicals either by H atom donation or by electron transfer. Therefore, an antioxidant with a high TEAC value may not perform well in preventing/delaying lipid peroxidation (Apak et al., 2016).

Determination of the radical-scavenging capacity of plant extracts in detoxification organs such as liver and kidney is of great importance to better understand the fate of plant derived polyphenols, following their intake, absorption and tissue deposition. However, data are still very scarce, even in production animals (Crozier et al., 2009). Metabolites of polyphenols are expected to follow two main routes of excretion, i.e., the urinary or biliary route. The large conjugated metabolites are more likely to follow the biliary route, whereas small conjugates are preferentially excreted in urine (Manach et al., 2004).

In liver, we found an increased radical-scavenging capacity with turmeric supplementation (TEAC assay). This could indicate that following absorption, the metabolization of turmeric in the liver could have promoted a higher concentration of this compounds in the organ, where it was potentially conjugated before being excreted in the urine and/or faeces. Interestingly, in the current study turmeric supplementation had no effect on the radical scavenging activity in muscle, but higher ORAC values were found in the kidney. Similarly, Bodas et al. (2011) when supplementing lambs with citrus flavonoids (0.15% of the diet), found elevated concentrations of naringenin in the liver but not in muscle. Interestingly, when supplementing lambs with quebracho tannins, López-Andrés et al. (2013) found a greater antioxidant capacity in liver and plasma of supplemented lambs compared with the non-supplemented animals. However, the antioxidant effects induced in the animal tissues occurred almost with null intestinal absorption of these compounds (indirect antioxidant effect). Lambs supplemented with bay extract presented significantly lower ORAC values than the non-supplemented lambs. Moreover, incorporation of turmeric, cinnamon and nutmeg plant extracts did not favour the antioxidant stability of the liver, due to the higher $DPPH^{\cdot}$ values compared with the non-supplemented lambs. As previously highlighted in muscle, phenolic compounds present in plant extracts can act as prooxidants in tissues, particularly in the presence of metals such as Cu and Fe (Eghbaliferiz and Iranshahi, 2016). Curcumin, a major phenolic compound present in turmeric extract, has shown prooxidant capacity in the presence of Cu(II), creating reactive oxygen species through the reduction of Cu(II) to Cu(I). This in turn can cause a copper-dependent DNA damage and induce apoptosis (Yoshino et al., 2004).

In kidney, supplementation of the lamb diets with oregano, cumin and caraway lowered the radical scavenging capacity. However, higher ORAC values were found for turmeric, cumin, caraway, cinnamon and nutmeg, which seems to indicate an increased radical

scavenging capacity or increased polyphenol concentration in the tissue. In a review conducted by Manach et al. (2005), urinary excretion of polyphenols in humans was found to vary from 0.3% to 43% of the polyphenol intake, which emphasizes the great variability in the bioavailability (absorption, transport, deposition and elimination) of the different polyphenols from different botanical origins.

CONCLUSIONS

The antioxidant status of muscle was increased by nutmeg supplementation (TEAC values). In liver, an increase in the radical-scavenging capacity was found in lambs supplemented with turmeric extract (TEAC values). Whereas, supplementation of lamb diets with turmeric, cumin, caraway, cinnamon and nutmeg increased the radical-scavenging capacity in kidney (ORAC values). Unfortunately, it is unclear if the divergent results on the antioxidant status registered in the tissues (as assessed by the different methods) is associated with a prooxidant effect of the plant extracts or with the specificity of the different assays.

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Chapter 6

Supplementation of lamb diets with vitamin E and rosemary extracts on meat quality parameters

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ABSTRACT

Supra-nutritional supplementation of lamb diets with α-tocopherol is an effective method to reduce lipid oxidation and colour deterioration in meat products. Alternative antioxidant sources have been proposed to replace the supra-nutritional vitamin E applications. A standardized rosemary extract (embedded or not in a fat matrix) was compared to vitamin E in ability to improve meat colour and stability selected parameters in lamb meat stored for 14 d under retail conditions. Indoor concentrate-fed light weight lambs (n = 480) were supplemented with increasing levels of *all-rac*-α-tocopheryl acetate (0.25, 0.5, 1.0 g kg^{-1} compound feed), rosemary extract (0.20, 0.40 or 0.80 g kg^{-1} compound feed) or rosemary extract embedded in a fat matrix (0.20, 0.40 or 0.80 g kg^{-1} compound feed), for 14 d before slaughter at 26.4 ± 1.29 kg BW. Supra-nutritional supplementation with antioxidants had no effect (P>0.05) on average daily weight gain, feed intake and feed efficiency. Independently from treatment, display time significantly affected lipid oxidation (TBARS), colour stability (L^*, a^* and h), myoglobin forms (DeoxyMb, OxyMb and MetMb) and meat discolouration ($A_{580-630}$ and I_{SO_2}). Rosemary extract supplementation (with or without fat embedment) had no effect on lipid oxidation, myoglobin forms or colour stability parameters, regardless of the dose. All vitamin E supplementation levels significantly affected lipid oxidation, colour stability (L^*, a^*, C^* and h), myoglobin forms and meat discolouration parameters compared to non-supplemented lambs. No effect of vitamin E dosage (0.25, 0.5, 1.0 g kg^{-1} compound feed) in the aforementioned meat oxidation and colour parameters was found. This study demonstrates that unlike vitamin E, neither dose nor protection of the rosemary extract had an effect on lipid oxidation or meat colour stability of lambs, during the 14 d of storage under retail conditions.

INTRODUCTION

Appearance, texture and flavour are important quality attributes influencing the consumer's choice to purchase meat products (Liu et al., 1995). In lamb meat, *post-mortem* biochemical changes such as lipid oxidation leads to off-odours and flavour development which have a negative impact on the shelf-life of these products (Morrissey et al., 1998). Therefore, the possibility to extend shelf-life and subsequent display time in lamb meat is a primary objective of the meat industry.

Dietary supplementation of lamb diets with vitamin E, and more specifically α-tocopherol, raises the concentrations of α-tocopherol in lamb tissues (Ochoa et al., 1992, Leal et al., 2018a), which in turn delays lipid oxidation and improves the colour stability of the meat (López-Bote et al., 2001, Lauzurica et al., 2005, Leal et al., 2018b). The protective role of α-tocopherol against lipid oxidation in lamb meat is well established. However, there is still a debate on the minimum dose of vitamin E in lamb diets that effectively protects the meat from lipid deterioration and values ranging from 287 mg kg^{-1} and 1000 mg kg^{-1} feed have been proposed (Wulf et al., 1995, López-Bote et al., 2001, Lauzurica et al., 2005, Álvarez et al., 2008, González-Calvo et al., 2015). Any recommendation on vitamin E needs to consider the wide variation of α-tocopherol concentration in feedstuffs (Yang et al., 2002), vitamin E status of the animal at the start of the supplementation period (Descalzo et al., 2005), length of the supplementation period (González-Calvo et al., 2015), differences in slaughter weight (De la Fuente et al., 2007) and animal breed (López-Bote et al., 2001). In Mediterranean farming systems, lambs are typically weaned around 12-14 kg of body weight (BW), and afterwards fed *ad libitum* concentrates plus straw until they reach 22 to 24 kg BW (Bernués et al., 2011). Under these conditions, González-Calvo et al. (2015) found that a concentrate supplemented with 500 mg of vitamin E per kg, fed for a period of 7-14 d before slaughter, was sufficient to protect lamb meat from lipid oxidation and metmyoglobin formation.

Alternative antioxidant sources to vitamin E from several types of plants and plant materials have been investigated to replace vitamin E. However, to effectively replace supra-nutritional vitamin E applications, these plant derived antioxidants need to be effectively absorbed, transported and have a considerable deposition into the lamb muscle. In recent years, special attention has been given to rosemary (*Rosmarinus officinalis* L.) products and by-products such as leaves, essential oils, distilled leaves and extracts from the second distillation of rosemary leaves with different solvents (Nieto et al., 2010, Bañón et al., 2012, Smeti et al., 2013, Ortuño et al., 2015, Smeti et al., 2018). Early work from Nieto et al. (2010), found that supplementation of pregnant ewes with 100 g of rosemary leaves kg^{-1} feed during pregnancy and lactation, improved the shelf-life of lamb meat. Interestingly, two major diterpenes present in rosemary, carnosic acid and carnosol, were later identified in lamb muscle at concentrations that could potentially lead to an antioxidant and antimicrobial effect in the meat (Jordán et al., 2014). Supplementation of lamb diets with rosemary by-products has yielded some conflicting results (Aouadi et al., 2014, Serrano et al., 2014,

Smeti et al., 2018), probably due to a lack of product standardization (Ortuño et al., 2015) or because of potential rumen degradation (Salem et al., 2006, 2010).
There are only few studies comparing supra-nutritional doses of vitamin E with rosemary by-products on their capacity to protect lamb meat from oxidative deterioration (Ortuño et al., 2015, 2017). Moreover, none of the studies performed a dose titration for both antioxidant sources in order to determine the relative value of each supplementation strategy on meat quality parameters.
The aim of the present study is to compare increasing dosages of a standardized rosemary extract with vitamin E (*all-rac*-α-tocopheryl acetate) in their ability to improve meat colour and stability parameters in lamb meat stored for 14 d under common retail conditions. Secondly, the standardized plant extract embedded in rumen inert fat will be also compared, as a mean to potentially mitigate the degradation of the rosemary extract by rumen microorganisms.

MATERIALS AND METHODS

The animal care and slaughter procedures used met the guidelines of Council Directive 86/609/EEC (European Communities, 1986) on the protection of animals used for experimental and other scientific purposes.

Animals and Diets

Four hundred and eighty *Rasa Aragonesa* male lambs were used in this study (BW = 28.8 ± 1.39 kg). Lambs were purchased from local dealers and were incorporated in the experiment in three separated batches of 160 animals each. Upon arrival at the commercial farm Franco y Navarro (Zaragoza, Spain), lambs were allocated based on body weight to 20 concrete pens (20 m^2) bedded with straw. For 14 d before slaughter, the lambs had free access to the experimental compound feed (presented as 3.5 mm diameter pellet) (**Table 6.1**), barley straw and water via separated troughs. Per batch, two pens were randomly assigned to one of 10 treatments consisting of a basal compound feed (Control), supplemented with increasing levels of *all-rac*-α-tocopheryl acetate (0.25, 0.50 or 1.0 g kg^{-1} compound feed), rosemary extract (RE) (Nutrafur S.A., Spain) (0.20, 0.40 or 0.80 g kg^{-1} compound feed) or the same rosemary extract embedded in a fat matrix (FRE) (0.20, 0.40 or 0.80 g kg^{-1} compound feed). After 14 d, animals were slaughtered at the local abattoir (Mercazaragoza S.A., Zaragoza, Spain) after an overnight period without feed but with free access to water. Three lambs per pen (in total, 18 lambs per treatment) were randomly selected at the start of the experimental period for meat analysis.
To produce the fat embedded rosemary extract (FRE treatments), hydrogenated palm fatty acids (Norel, Spain) were melted at 70 ± 2°C. Afterwards, the rosemary extract (Nutrafur S.A., Spain) was added to the melted hydrogenated palm fatty acids and stirred for 10 min at 70 ± 2°C, until an homogeneous mixture was obtained. The mixture of hydrogenated palm fatty acids and rosemary extract was then added to silica (Trouw Nutrition, the

Netherlands), stirred for 20 minutes and cooled at room temperature. The final product (free-flowing powder), was composed by 43.75% hydrogenated palm fatty acids, 31.25% silica and 25.0% rosemary extract.

Meat Processing and Packaging

The carcasses were chilled for 24 h at 2°C, before split longitudinally into two halves. The *longissimus thoracis et lumborum* (LTL) muscle from the right half was dissected, with subcutaneous fat removed, placed in bags (1 muscle per bag) and transported in sealed plastic containers in darkness at 4 ± 1°C to the Meat Quality Laboratory of the Veterinary Faculty of Zaragoza (Zaragoza, Spain).

Within 2 hours after arrival, the LTL muscles were sectioned into approximately 1.5 cm thick portions, which were placed in polystyrene trays B5-37 (Aerpack), packed under modified atmosphere packaging with 70% O_2 + 30% CO_2 (Ulma Smart 500, Ulma Packaging, Guipúzcoa, Spain) and sealed with a polyethylene and polyamine laminate film (30 μm of thickness, water vapour transmission rate at 23°C of <7 g/m²/24h/85% relative humidity, an O_2 transmission rate at 23 ° C of <15 cm³/m² /0% R.H. and a CO_2 transmission rate at 23°C of <75 cm³/m²/24h/ 0% R.H.; Linpac Packaging S.L., Spain). The trays were stored at 4 ± 1°C for 1, 7, 9, 12 or 14 d in a Zafrio display cabinet (ZAFRIO, Zaragoza, Spain) simulating retail conditions with a daily exposure to 14 h of light at 1200 lx.

Table 6.1 Composition of the basal diet and experimental premix.

Ingredients, g kg^{-1}										
Barley	295				Limestone	25				
Soya bean meal (480 g kg^{-1} of crude protein)	240				Experimental premix	10				
Wheat	200				Sodium bicarbonate	7				
Maize	200				Sodium chloride	5				
Soya oil	15				Standard mineral and vitamin premix*	3				

Experimental premix	Control	RE			FRE			SE		
		200	400	800	200	400	800	250	500	1000
Ingredients, g kg^{-1}										
Wheat bran	560	560	560	560	560	560	560	560	560	560
Hydropalm†	140	140	140	140	105	70		140	140	140
Silica‡	300	280	260	220	255	210	120	250	200	100
Rosemary§		20	40	80						
Fat embedded rosemary¥					80	160	320			
All-rac-α-tocopheryl acetate¶								50	100	200

* Mineral and vitamins provided: Ca 0.24g, Na 0.47g, S 0.23 g, Mn 30 mg, Zn 50 mg, Cu 5 mg, I 0.5 mg, Co 0.5 mg, Se 0.15 mg, Fe 50 mg, vitamin A 8000 IU, vitamin D3 1600 IU, *all-rac*-α-tocopheryl acetate 25 mg. SE = *all-rac*-α-tocopheryl acetate. RE = rosemary extract. FRE = fat embedded rosemary extract. † Hydropalm = hydrogenated palm fatty acids (Norel, Spain). ‡ Silica = silica H2O (Trouw Nutrition, the Netherlands). § Rosemary = standardized rosemary extract (Nutrafur S.A., Spain). ¥ Fat embedded rosemary = standardized rosemary extract (inclusion of 25%) mixed with melting (70 ± 2°C) Hydropalm (43.75%) and silica (31.25%). ¶ *All-rac*-α-tocopheryl acetate = contains 50 g *all-rac*-α-tocopherol per 100 g of product (Trouw Nutrition, the Netherlands).

Physical and Chemical Analysis

Colour was measured using a reflectance spectrophotometer (CM-2002; Minolta, Osaka, Japan) directly on the meat surface, after 2 h exposure to air. Each value was the mean of 10 determinations per sample. The parameters recorded according to the *CIELAB* system (CIE, 1976), were lightness (L^*), redness (a^*) and yellowness (b^*). Values of chroma (C^*) and hue angle (h) were calculated as: $C^* = \sqrt{(a^{*2} + b^{*2})}$, and $h = \tan^{-1}(b^*/a^*)$, expressed in degrees.

Lipid oxidation was expressed as thiobarbituric acid reactive substances (TBARS) and expressed as mg malondialdehyde (MDA) per kg meat, as described by Pfalzgraf et al. (1995). Briefly, 10 g of meat sample was homogenized with 10% trichloroacetic acid using an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany). After centrifugation at 1500 × g for 30 min (at 10°C) the supernatant was filtered (Filterlab, Barcelona, Spain), 2 mL of the filtrate mixed with 2 mL of TBA (20 mol L^{-1}), homogenized and incubated for 20 min (in boiling water). Absorbance was measured at 532 nm and TBARS values were calculated from a standard curve of MDA (Sigma-Aldrich, Madrid, Spain). Samples were analysed in duplicate and the results were expressed as mg of MDA kg^{-1} of meat.

Myoglobin forms [deoxymyoglobin (DeoxyMb), oxymyoglobin (OxyMb) and metmyoglobin (MetMb)] were calculated from the reflectance curve described by Krzywicki (1979) using a wavelength of 690 nm. The reflectance at 473, 525 and 572 nm was obtained by linear interpolation, since the reflectance spectrophotometer only measures the reflectance between 400-740 nm at intervals of 10 nm.

The rate of meat discoloration was accessed by the parameter A_{580}-A_{630}, according to the method proposed by van den Oord and Wesdorp (1971). Oxygen saturation of myoglobin on meat surface (I_{SO_2}) was measured following the technique described by Tsuruga et al. (1994).

Polyphenol Analysis in Feed

Feed samples were homogenized using a blender and sieved with a mesh Nº 18 (corresponding to 1 mm) to remove large particles before extraction. The phenolic compounds were extracted using methanol:water (80:20, v:v), sonicated for 5 min and centrifuged. This procedure was repeated twice with the feed residue. The supernatants were combined and the methanol evaporated. For better chromatography resolution, the extracts were cleaned using solid phase extraction (SPE) with Oasis MAX 96-well plates (30mg, 30µm) from Waters, according to Vallverdú-Queralt et al. (2014).

The identification and quantification of the major phenolic compounds was performed in a UHPLC (Waters Acquity Ultra Performace LC) coupled to an API 3000 triple quadrupole mass spectrometer (ABSciex) equipped with a Turbo ionspray source operating in negative mode. Separation was carried out using a BEH C18 (2.1 x 50 mm, 1.7 µm) (Acquity UHPLC) maintained at 30ºC. Gradient elution was performed with acetonitrile with 0.1% of formic acid (v/v) and water with 0.1% of formic acid (v/v) using an increase linear gradient flow of acetonitrile with 0.1% of formic acid (v/v) under conditions: 0 min, 20%;

0.5 min, 20%; 1.5 min, 30%; 2 min, 30%; 2.5 min, 50%; 3 min, 100%; 3.5 min, 100%; 3.7 min, 20% and 4.5 min, 20%. The flow rate was 0.4 mL min^{-1} and the injection volume was 10 µL. The identification and quantification of each compound was carried out with the mass spectrometer using multiple reaction monitoring (MRM) mode. The quantification of the phenolic compounds was performed using calibration curves with analytical standards with the internal standard method. The internal standard was ethyl gallate (400 ng g^{-1}) (Extrasynthese, Genay, France) and the results were expressed as mg kg^{-1} of feed (Martínez-Huélamo et al., 2015).

Statistical Analysis

All statistical analyses were performed using SAS Studio (SAS Institute, Inc., Cary, NC, USA). Pen was the experimental unit, and individual measurements were averaged per pen. The model included the fixed effects of antioxidant supplementation, display time and the interaction between antioxidant supplementation and display time. The effect of batch was initially included in the statistical model as a fixed effect but it was finally excluded owing as batch never reached statistical significance (P>0.05). Therefore, the model was:

$$Y_{ijk} = \mu + AS_i + D_j + (AS_i \times D_j) + e_{ijk}$$

where Y_{ijk} is the dependent variable; µ is the population average; AS_i is the fixed effect of antioxidant supplementation; D_j is the fixed effect of display time; ($AS_i \times D_j$) is the interaction between AS_i and D_j and eijk is the random error. Differences were declared significant when P<0.05. Tukey's post hoc test was used to assess differences between mean values when P<0.05.

RESULTS AND DISCUSSION

Animal Performance

Data on body weight, average daily weight gain, feed intake and feed efficiency are presented in **Table 6.2**. There were no effects of either antioxidant source (vitamin E and rosemary extract) or dosage in the assessed performance parameters. These results are in agreement with previous work in lambs, in which neither vitamin E (Lauzurica et al., 2005, Leal et al., 2018b) nor rosemary essential oil (Smeti et al., 2018) supplementation had an effect on growth, feed intake or efficiency.

Polyphenol Content in Feeds and Fat Embedment

The identification and quantification of the major polyphenols present in the experimental diets are shown in **Table 6.3**. Overall, we found high concentrations of chlorogenic acid, ferulic acid and ferulic acid-*O*-hexoside, which are commonly found in cereals (Naczk and Shahidi, 2006), which accounted for >42% of the total polyphenol content in the experimental diets. Incorporation of rosemary extracts (either protected or unprotected)

in the experimental diets, led to a substantial increase in carnosol concentration and to increases in carnosic and rosmarinic acid, recognized as major polyphenols present in rosemary extracts (Cuvelier et al., 1996).

Previous work, pointed at the potential of plant secondary metabolites or polyphenols to interact with rumen bacteria and fermentation (Salem et al., 2006, 2010). In the current study, we found no differences between the two rosemary supplementation strategies (RE and FRE) on performance (**Table 6.2**) nor quality parameters of meat stored for 14 d under retail conditions (**Table 6.4-6.7**). Due to the lack of response of both rosemary extract sources (RE and FRE) in the aforementioned parameters compared with the control group, it is not possible to draw any conclusions regarding the efficacy of protection intended by fat embedment.

Lipid Oxidation

Mean TBARS values for each dietary treatment during the 14 d of display are presented in **Table 6.4**. Lipid oxidation significantly increased with storage time in all groups ($P<0.05$). On the first day of refrigerated storage, apart from the control and SE 250 supplemented lambs, no differences in TBARS values were observed between treatments. However, from d 7 onwards, LTL muscle from vitamin E supplemented lambs (irrespective of dose) presented significantly lower TBARS values than the control, RE and FRE supplemented lambs. Vitamin E supplemented lambs presented TBARS values ranging from 0.16 to 0.31 mg MDA kg^{-1} meat on d 7, while the remaining treatments registered TBARS values between 1.63 to 1.99 mg MDA kg^{-1} meat. With increasing storage time, the differences between the vitamin E supplemented lambs and the control, RE and FRE lambs became wider. On d 14 of storage, LTL muscle from control and the rosemary supplemented lambs (RE and FRE) presented TBARS values ranging from 3.25 to 4.00 mg MDA kg^{-1} meat, while vitamin E supplemented LTL muscles showed TBARS values from 0.39 to 0.69 mg MDA kg^{-1} meat.

Table 6.2 Productive performance of the lambs fed increasing level of *all-rac*-α-tocopheryl acetate (SE), rosemary extract (RE) or fat embedded rosemary extract (FRE).

Item	Control	SE (mg kg^{-1} feed)			RE (mg kg^{-1} feed)			FRE (mg kg^{-1} feed)				
		250	500	1000	200	400	800	200	400	800	SEM	P value
IBW, kg	21.9	21.8	21.8	21.9	21.9	21.9	21.8	21.8	21.8	21.9	0.04	0.746
FBW, kg	26.4	26.2	26.5	26.4	26.2	26.7	26.5	26.4	26.3	26.5	0.29	0.848
FEED, kg d^{-1}	0.992	0.996	1.005	1.015	1.006	1.039	1.032	1.025	1.029	1.027	0.0362	0.880
ADG, kg d^{-1}	0.329	0.315	0.331	0.324	0.315	0.351	0.331	0.323	0.319	0.331	0.0205	0.832
FCR, kg kg^{-1}	3.11	3.25	3.07	3.17	3.20	3.07	3.21	3.15	3.40	3.21	0.191	0.824

SEM = standard error of the mean. IBW = initial body weight. FBW = final body weight. FEED = compound feed intake. ADG = average daily gain. FCR = feed conversion ratio.

Table 6.3 Quantification of individual bioactive compounds (mg kg^{-1}) of the different feeds.

Item	Control	SE (mg kg^{-1} feed)			RE (mg kg^{-1} feed)			FRE (mg kg^{-1} feed)		
		250	500	1000	200	400	800	200	400	800
CA	261	357	223	255	264	314	266	235	185	265
CrA	13	13	13	13	23	19	24	25	19	27
Cn	11	8	6	10	1366	1383	2149	862	553	551
ChA	1060	1084	801	837	782	634	755	777	498	742
DA	55	7	43	19	18	15	22	11	12	28
FA	926	845	810	716	753	675	872	785	594	851
FAH	1593	1423	1401	1264	1375	858	1677	1649	1035	1761
N	60	67	67	68	62	64	63	69	55	60
NG	373	392	367	355	362	338	379	379	292	363
pCA	596	491	489	429	436	444	522	480	356	528
PA	166	228	140	154	156	166	171	159	107	158
Q	55	58	57	56	57	58	62	59	58	56
RsA	31	29	30	31	40	71	128	35	50	40
R	118	73	101	73	66	92	79	76	79	83
TOTAL	5318	5075	4548	4280	5760	5131	7169	5601	3893	5513

SE = *all-rac*-α-tocopheryl acetate. RE = rosemary extract. FRE = fat embedded rosemary extract. CA = caffeic acid. CrA = carnosic acid. Cn = carnosol. ChA = chlorogenic acid. DA = dicaffeoylquinic acid. FA = ferulic acid. FAH = ferulic acid-*O*-hexoside. N = narigenin. NG = naringenin glucoside. pCA = *p*-cumaric acid. PA = protocatechuic acid. Q = quercetin. RsA = rosmarinic acid. R = rutin.

Table 6.4 Effect of dietary supplementation† with *all-rac*-α-tocopheryl acetate (SE), rosemary extract (RE) and fat embedded rosemary extract (FRE) on thiobarbituric acid reactive substances in raw lamb meat stored in modified atmospheric packaging (70% O_2:30% CO_2) kept for 1, 7, 9, 12 and 14 d under retail conditions.

Item	Days of display						P value
	1	7	9	12	14	SEM	display
Control	$0.13^{c,x}$	$1.90^{b,y}$	$2.66^{b,yz}$	$3.44^{b,z}$	$3.80^{b,z}$	0.25	<0.001
SE 250	$0.09^{ab,x}$	$0.31^{a,xy}$	$0.32^{a,xy}$	$0.41^{a,y}$	$0.69^{a,z}$	0.06	<0.001
SE 500	$0.08^{a,x}$	$0.21^{a,y}$	$0.26^{a,y}$	$0.30^{a,y}$	$0.56^{a,z}$	0.03	0.001
SE 1000	$0.09^{ab,x}$	$0.16^{a,xy}$	$0.18^{a,xy}$	$0.33^{a,yz}$	$0.39^{a,z}$	0.04	0.017
RE 200	$0.11^{abc,w}$	$1.99^{b,x}$	$2.85^{b,xy}$	$3.53^{b,yz}$	$4.00^{b,z}$	0.23	<0.001
RE 400	$0.11^{abc,w}$	$1.91^{b,x}$	$2.75^{b,xy}$	$3.61^{b,yz}$	$3.85^{b,z}$	0.21	<0.001
RE 800	$0.11^{abc,w}$	$1.86^{b,x}$	$2.55^{b,xy}$	$3.64^{b,yz}$	$3.80^{b,z}$	0.24	<0.001
FRE 200	$0.11^{abc,x}$	$1.67^{b,y}$	$2.46^{b,yz}$	$2.80^{b,yz}$	$3.25^{b,z}$	0.30	<0.001
FRE 400	$0.11^{abc,w}$	$1.63^{b,x}$	$2.29^{b,xy}$	$3.27^{b,yz}$	$3.51^{b,z}$	0.23	<0.001
FRE 800	$0.11^{abc,x}$	$1.74^{b,y}$	$2.50^{b,yz}$	$3.25^{b,z}$	$3.53^{b,z}$	0.23	<0.001
P value treatment	0.016	<0.001	<0.001	<0.001	<0.001		

† SE 250, SE 500 and SE 1000 = 250, 500 and 1000 mg *all-rac*-α-tocopheryl acetate kg^{-1} feed; RE 200, RE 400 and RE 800 = 200, 400 and 800 mg rosemary extract kg^{-1} feed; FRE 200, FRE 400 and FRE 800 = 200, 400 and 800 mg fat embedded rosemary extract kg^{-1} feed. ^{a,b,c} Values within a column with different superscript are significantly different (P<0.05). ^{w,x,y,z} Values within a row with different superscript are significantly different (P<0.05). *SEM = standard error of the mean.

Table 6.5 Effect of dietary supplementation† with *all-rac*-α-tocopheryl acetate (SE), rosemary extract (RE) and fat embedded rosemary extract (FRE) on color parameters (*L**, *a**, *b**, C*, h) in raw lamb meat stored in modified atmospheric packaging (70% O_2:30% CO_2) kept for 1, 7, 9, 12 and 14 d under retail conditions.

Item	Days of display					SEM	P value display
	1	7	9	12	14		
*L**							
Control	40.94^{x}	39.19^{x}	$40.17^{ab,x}$	$45.26^{b,y}$	$47.67^{b,z}$	0.269	<0.001
SE 250	40.22^{yz}	36.74^{w}	$37.98^{ab,wx}$	$38.78^{a,xy}$	$40.78^{a,z}$	0.173	<0.001
SE 500	40.32^{y}	37.85^{x}	$38.04^{ab,x}$	$39.21^{a,xy}$	$40.55^{a,y}$	0.256	<0.001
SE 1000	39.52^{yz}	37.31^{x}	$37.68^{a,xy}$	$38.71^{a,xyz}$	$40.67^{a,z}$	0.221	<0.001
RE 200	40.11^{x}	38.45^{x}	$40.00^{ab,x}$	$44.82^{b,y}$	$47.33^{b,y}$	0.359	<0.001
RE 400	39.41^{x}	38.38^{x}	$40.24^{ab,x}$	$45.12^{b,y}$	$48.73^{b,z}$	0.406	<0.001
RE 800	39.17^{wx}	38.15^{w}	$40.67^{b,x}$	$44.92^{b,y}$	$48.42^{b,z}$	0.252	<0.001
FRE 200	39.95^{x}	38.39^{x}	$40.09^{ab,x}$	$43.39^{b,y}$	$46.49^{b,z}$	0.324	<0.001
FRE 400	40.85^{x}	38.58^{x}	$39.93^{ab,x}$	$44.19^{b,y}$	$47.09^{b,z}$	0.389	<0.001
FRE 800	40.98^{x}	37.85^{w}	$40.66^{b,wx}$	$44.98^{b,y}$	$48.71^{b,z}$	0.356	<0.001
P value treatment	0.530	0.146	0.001	<0.001	<0.001		
*a**							
Control	8.43^{yz}	$9.95^{ab,z}$	$7.06^{a,y}$	$3.68^{a,x}$	$2.98^{a,x}$	0.295	<0.001
SE 250	9.51^{w}	$12.08^{b,z}$	$11.86^{c,yz}$	$11.10^{b,xy}$	$10.49^{b,x}$	0.103	<0.001
SE 500	8.75^{x}	11.25^{aby}	$10.93^{bc,y}$	$10.16^{b,y}$	$10.17^{b,y}$	0.150	<0.001
SE 1000	8.84^{x}	$11.51^{ab,y}$	$11.08^{bc,y}$	$10.72^{b,y}$	$10.74^{b,y}$	0.121	<0.001
RE 200	8.93^{yz}	$9.25^{a,z}$	$6.56^{a,y}$	$3.61^{a,x}$	$3.05^{a,x}$	0.307	<0.001
RE 400	8.94^{yz}	$9.26^{a,z}$	$6.65^{a,y}$	$3.01^{a,x}$	$2.52^{a,x}$	0.297	<0.001
RE 800	8.43^{yz}	$10.30^{ab,z}$	$7.11^{a,y}$	$3.58^{a,x}$	$2.51^{a,x}$	0.245	<0.001
FRE 200	9.46^{y}	$9.49^{a,y}$	$7.76a^{y}$	$5.06^{a,x}$	$3.91^{a,x}$	0.300	<0.001
FRE 400	8.45^{y}	$9.76^{a,y}$	$8.15a^{b,y}$	$4.10^{a,x}$	$3.28^{a,x}$	0.293	<0.001
FRE 800	8.97^{yz}	$9.96^{ab,z}$	$7.26^{a,y}$	$3.55^{a,x}$	$2.60^{a,x}$	0.274	<0.001
P value treatment	0.251	<0.001	<0.001	<0.001	<0.001		
*C**							
Control	13.39^{x}	$15.49^{ab,y}$	$13.54^{a,x}$	$13.15^{a,x}$	$13.55^{a,x}$	0.171	<0.001
SE 250	14.84^{x}	$16.92^{b,yz}$	$17.12^{d,z}$	$16.18^{c,yz}$	$15.94^{b,y}$	0.122	<0.001

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SE 500	13.94	16.10^{ab}	15.65^{bcd}	14.95^{b}	15.38^{b}	0.156	0.059
SE 1000	13.85^{x}	16.12^{ab,y}	16.03^{cd,y}	15.73^{bc,y}	16.08^{b,y}	0.131	0.002
RE 200	13.93	14.81^{a}	13.37^{a}	12.95^{a}	13.77^{a}	0.179	0.189
RE 400	13.97^{xy}	14.66^{a,y}	13.75^{a,xy}	12.98^{a,x}	13.74^{a,xy}	0.171	0.040
RE 800	13.20^{x}	15.42^{a,y}	13.99^{ab,x}	12.87^{a,x}	13.55^{a,x}	0.153	<0.001
FRE 200	14.84	14.89^{ab}	14.29^{abc}	13.60^{a}	13.96^{a}	0.162	0.245
FRE 400	13.62^{xy}	15.14^{a,z}	14.32^{abc,yz}	12.55^{a,x}	13.58^{a,xy}	0.154	0.006
FRE 800	14.17^{xy}	15.36^{a,y}	13.86^{ab,x}	12.91^{a,x}	13.56^{a,x}	0.156	<0.001
P value treatment	0.077	0.003	<0.001	<0.001	<0.001		
h							
Control	51.41^{x}	50.64^{abc,x}	58.85^{b,x}	74.04^{b,y}	77.15^{b,y}	1.120	<0.001
SE 250	50.15^{z}	44.36^{a,x}	46.16^{a,xy}	46.69^{a,y}	48.96^{a,z}	0.238	<0.001
SE 500	51.15^{z}	45.78^{abc,x}	45.72^{a,x}	47.37^{a,xy}	48.71^{a,yz}	0.339	<0.001
SE 1000	50.68^{z}	44.60^{ab,x}	46.25^{a,xy}	47.10^{a,xy}	48.17^{a,yz}	0.288	<0.001
RE 200	50.24^{x}	52.05^{c,xy}	61.13^{b,y}	73.90^{b,z}	76.95^{b,z}	1.185	<0.001
RE 400	50.38^{x}	51.54^{bc,x}	61.73^{b,y}	76.42^{b,z}	79.26^{b,z}	1.145	<0.001
RE 800	50.74^{x}	48.40^{abc,x}	60.00^{b,y}	73.98^{b,z}	79.29^{b,z}	0.936	<0.001
FRE 200	50.52^{x}	50.74^{abc,x}	57.74^{b,x}	68.23^{b,y}	73.35^{b,y}	1.151	<0.001
FRE 400	52.30^{x}	50.35^{abc,x}	55.32^{ab,}x	70.54^{b,y}	76.03^{b,y}	1.156	<0.001
FRE 800	51.08^{xy}	49.94^{abc,x}	58.96^{b,y}	73.93^{b,z}	78.78^{b,z}	1.040	<0.001
P value treatment	0.883	<0.001	<0.001	<0.001	<0.001		

† SE 250, SE 500 and SE 1000 = 250, 500 and 1000 mg *all-rac*-α-tocopheryl acetate kg^{-1} feed; RE 200, RE 400 and RE 800 = 200, 400 and 800 mg rosemary extract kg^{-1} feed; FRE 200, FRE 400 and FRE 800 = 200, 400 and 800 mg fat embedded rosemary extract kg^{-1} feed. ^{a,b,c} Values within a column with different superscript are significantly different (P<0.05). ^{w,x,y,z} Values within a row with different superscript are significantly different (P<0.05). * SEM = standard error of the mean.

Table 6.6 Effect of dietary supplementation† with *all-rac*-α-tocopheryl acetate (SE), rosemary extract (RE) and fat embedded rosemary extract (FRE) on myoglobin forms (DeoxyMb, OxyMb and MetMb) in raw lamb meat stored in modified atmospheric packaging (70% O_2:30% CO_2) kept for 1, 7, 9, 12 and 14 d under retail conditions.

Item	Days of display					SEM	P value display
	1	7	9	12	14		
DeoxyMb							
Control	49.04^{z}	24.37^{xy}	$16.41^{a,x}$	$22.53^{abc,xy}$	$24.59^{abc,y}$	1.80	<0.001
SE 250	50.20^{y}	28.70^{x}	$30.31^{c,x}$	$28.93^{cd,x}$	$26.99^{bc,x}$	0.90	<0.001
SE 500	52.86^{y}	28.91^{x}	$28.86^{c,x}$	$28.65^{cd,x}$	$27.76^{bc,x}$	1.07	<0.001
SE 1000	50.80^{y}	29.47^{x}	$30.48^{c,x}$	$29.71^{d,x}$	$28.36^{c,x}$	0.83	<0.001
RE 200	50.24^{y}	23.23^{x}	$18.23^{ab,x}$	$19.01^{ab,x}$	$23.49^{abc,x}$	2.22	<0.001
RE 400	49.58^{y}	23.09^{x}	$19.02^{ab,x}$	$20.00^{ab,x}$	$23.02^{ab,x}$	1.73	<0.001
RE 800	55.29^{y}	26.40^{x}	$18.35^{ab,x}$	$19.14^{ab,x}$	$23.00^{ab,x}$	1.98	<0.001
FRE 200	48.30^{y}	24.24^{x}	$20.50^{ab,x}$	$23.52^{bcd,x}$	$22.88^{ab,x}$	1.74	<0.001
FRE 400	47.42^{y}	26.76^{x}	$25.55^{bc,x}$	$20.31^{ab,x}$	$21.66^{a,x}$	1.91	<0.001
FRE 800	53.01^{z}	25.12^{y}	$19.39^{ab,xy}$	$16.40^{a,x}$	$20.59^{a,xy}$	2.02	<0.001
P value treatment	0.575	0.120	<0.001	<0.001	<0.001		
OxyMb							
Control	28.24^{y}	41.68^{z}	37.04^{yz}	$16.41^{a,x}$	$11.85^{a,x}$	2.90	<0.001
SE 250	27.02^{x}	42.07^{z}	38.97^{yz}	$38.10^{bc,y}$	$37.03^{b,y}$	0.86	<0.001
SE 500	22.34^{x}	42.98^{z}	40.59^{yz}	$38.60^{c,yz}$	$36.14^{b,y}$	1.15	<0.001
SE 1000	25.53^{x}	41.57^{z}	38.97^{yz}	$37.90^{bc,y}$	$37.29^{b,y}$	0.91	<0.001
RE 200	26.39^{xy}	40.16^{z}	33.22^{yz}	$20.03^{a,wx}$	$12.43^{a,w}$	2.73	<0.001
RE 400	26.36^{y}	40.98^{z}	31.47^{y}	$15.39^{a,x}$	$11.05^{a,x}$	2.18	<0.001
RE 800	23.14^{z}	41.73^{z}	34.46^{y}	$20.00^{a,xy}$	$12.39^{a,x}$	2.32	<0.001
FRE 200	28.83^{xy}	39.67^{z}	36.16^{yz}	$20.53^{a,wx}$	$16.23^{a,w}$	2.40	<0.001
FRE 400	28.01^{xy}	39.86^{z}	33.88^{yz}	$22.63^{a,wx}$	$17.04^{a,w}$	3.00	0.005
FRE 800	24.52^{x}	40.67^{z}	34.99^{yz}	$25.93^{a,wx}$	$13.72^{a,w}$	2.30	<0.001
P value treatment	0.928	0.738	0.593	<0.001	<0.001		
MetMb							
Control	22.73^{w}	33.95^{x}	$46.55^{b,y}$	$61.06^{b,z}$	$63.56^{b,z}$	2.50	<0.001
SE 250	22.77^{w}	29.23^{x}	$30.72^{a,xy}$	$32.97^{a,y}$	$35.98^{a,z}$	0.69	<0.001

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SE 500	24.80^{w}	28.11^{wx}	30.55^{a,xy}	32.74^{a,yz}	36.10^{a,z}	0.82	<0.001
SE 1000	23.67^{w}	28.96^{x}	30.55^{a,xy}	32.38^{a,y}	34.35^{a,z}	0.62	<0.001
RE 200	23.37^{x}	36.62^{y}	48.54^{ab,y}	60.96^{b,z}	64.08^{b,z}	2.78	<0.001
RE 400	24.07^{w}	35.93^{x}	49.51^{ab,y}	64.61^{b,z}	65.93^{b,z}	2.26	<0.001
RE 800	21.58^{w}	31.87^{x}	47.19^{b,y}	60.86^{b,z}	65.17^{b,z}	2.01	<0.001
FRE 200	22.87^{x}	36.09^{x}	43.34^{b,y}	55.94^{b,z}	60.89^{b,z}	2.70	<0.001
FRE 400	24.57^{x}	33.39^{xy}	40.57^{b,y}	57.06^{b,z}	63.53^{b,z}	2.64	<0.001
FRE 800	22.47^{w}	34.21^{x}	45.62^{b,y}	60.23^{b,z}	65.69^{b,z}	2.02	<0.001
P value treatment	0.089	0.270	<0.001	<0.001	<0.001		

† SE 250, SE 500 and SE 1000 = 250, 500 and 1000 mg *all-rac*-α-tocopheryl acetate kg^{-1} feed; RE 200, RE 400 and RE 800 = 200, 400 and 800 mg rosemary extract kg^{-1} feed; FRE 200, FRE 400 and FRE 800 = 200, 400 and 800 mg fat embedded rosemary extract kg^{-1} feed. ^{a,b,c} Values within a column with different superscript are significantly different (P<0.05). ^{w,x,y,z} Values within a row with different superscript are significantly different (P<0.05). * SEM = standard error of the mean.

Table 6.7 Effect of dietary supplementation† with *all-rac*-α-tocopheryl acetate (SE), rosemary extract (RE) and fat embedded rosemary extract (FRE) on meat discoloration ($A_{580-630}$ and I_{SO2}) in raw lamb meat stored in modified atmospheric packaging (70% O_2:30% CO_2) kept for 1, 7, 9, 12 and 14 d under retail conditions.

Item	Days of display					SEM	P value display
	1	7	9	12	14		
$A_{580-630}$							
Control	34.66^{z}	30.94^{z}	$19.29^{a,y}$	$8.88^{a,x}$	$7.60^{a,x}$	2.14	<0.001
SE 250	38.16^{z}	38.52^{z}	$36.84^{c,z}$	$33.19^{b,xy}$	$29.48^{b,x}$	0.99	<0.001
SE 500	35.98^{z}	36.97^{z}	$34.54^{bc,yz}$	$31.05^{b,xy}$	$28.93^{b,x}$	1.07	<0.001
SE 1000	37.50^{z}	36.96^{z}	$35.35^{bc,yz}$	$32.94^{b,xy}$	$31.02^{b,x}$	1.02	<0.001
RE 200	36.84^{z}	28.43^{z}	$17.77^{a,y}$	$9.00^{a,x}$	$7.55^{a,x}$	2.31	<0.001
RE 400	36.76^{z}	28.47^{z}	$17.60^{a,y}$	$6.80^{a,x}$	$6.09^{a,x}$	2.05	<0.001
RE 800	36.55^{z}	32.41^{z}	$19.25^{a,y}$	$8.41^{a,x}$	$5.93^{a,x}$	1.71	<0.001
FRE 200	38.36^{z}	29.19^{yz}	$22.47^{a,xy}$	$13.42^{a,wx}$	$9.74^{a,w}$	2.12	<0.001
FRE 400	34.42^{z}	31.18^{yz}	$24.81^{ab,y}$	$10.86^{a,x}$	$7.61^{a,x}$	2.07	<0.001
FRE 800	36.76^{z}	31.11^{z}	$20.62^{a,y}$	$8.58^{a,x}$	$5.61^{a,x}$	1.66	<0.001
P value treatment	0.138	0.124	<0.001	<0.001	<0.001		
I_{SO2}							
Control	19.28^{yz}	21.27^{z}	20.69^{yz}	$15.32^{abc,xy}$	$10.62^{a,x}$	1.38	<0.001
SE 250	18.64^{x}	21.54^{y}	21.46^{y}	$21.25^{bc,y}$	$21.08^{b,y}$	0.33	<0.001
SE 500	16.57^{x}	22.38^{y}	21.86^{y}	$21.82^{c,y}$	$20.76^{b,y}$	0.53	<0.001
SE 1000	17.44^{x}	21.80^{y}	21.85^{y}	$21.67^{c,y}$	$21.77^{b,y}$	0.34	<0.001
RE 200	17.99^{yz}	20.94^{z}	18.33^{yz}	$12.51^{a,xy}$	$8.35^{a,x}$	1.45	<0.001
RE 400	17.76^{y}	21.51^{y}	18.60^{y}	$10.91^{a,x}$	$7.20^{a,x}$	1.21	<0.001
RE 800	17.90^{yz}	21.56^{z}	18.79^{yz}	$14.31^{ab,xy}$	$9.04^{a,x}$	1.18	<0.001
FRE 200	19.07^{yz}	21.51^{z}	20.12^{yz}	$15.65^{abc,xy}$	$12.15^{a,x}$	1.28	<0.001
FRE 400	17.83^{xy}	21.03^{y}	19.76^{y}	$16.36^{abc,xy}$	$12.26^{a,x}$	1.51	<0.001
FRE 800	17.92^{yz}	21.36^{z}	20.92^{z}	$14.25^{abc,xy}$	$10.11^{a,x}$	1.06	<0.001
P value treatment	0.990	0.808	0.790	<0.001	<0.001		

† SE 250, SE 500 and SE 1000 = 250, 500 and 1000 mg *all-rac*-α-tocopheryl acetate kg^{-1} feed; RE 200, RE 400 and RE 800 = 200, 400 and 800 mg rosemary extract kg^{-1} feed; FRE 200, FRE 400 and FRE 800 = 200, 400 and 800 mg fat embedded rosemary extract kg^{-1} feed. ^{a,b,c} Values within a column with different superscript are significantly different (P<0.05). ^{w,x,y,z} Values within a row with different superscript are significantly different (P<0.05). * SEM = standard error of the mean.

Although dependent on the experience of panellist, thresholds for acceptability of oxidizing lamb meat have been proposed. In concentrate-fed lambs, slaughtered at low body weights (lower than 30 kg live weight), a threshold of 1.0 mg MDA kg^{-1} meat has been proposed to be avoided for the development of off flavours in lamb meat (Ripoll et al., 2011). After 7 d of storage, higher MDA values than 1.0 mg kg^{-1} meat were found in the LTL muscle from the control, FRE and RE lambs. Interestingly, vitamin E supplementation at 0.25, 0.50 or 1.0 g kg^{-1} feed, led to lower TBARS values (0.69, 0.56 and 0.39 mg MDA kg^{-1} meat) than the proposed threshold after 14 d of storage. In a similar study, (Leal et al., 2018b) found that a supplementation level of 0.16 g kg^{-1} feed with vitamin E (*all-rac*-α-tocopheryl acetate) for 14 d before slaughter, was sufficient to maintain TBARS values below the threshold of 1.0 mg MDA kg^{-1} meat. Dietary supplementation of lamb diets with vitamin E is a consistent strategy to increase α-tocopherol content in lamb tissues like muscle (Leal et al., 2018b) and to improve the resistance of lamb meat to oxidative deterioration (Lauzurica et al., 2005, Belles et al., 2018, Leal et al., 2018b).

Unlike vitamin E, the effects of rosemary extracts or essential oils on meat oxidative stability are not consistent. Shelf life studies in lambs have demonstrated an improvement in lipid stability of meat when rosemary by-products were included in the diet of lactating ewes (Nieto et al., 2010) or directly into lamb diets (Bañón et al., 2012, Ortuño et al., 2014). In the current study, supplementation of lamb diets with rosemary extracts (RE and FRE) failed to affect the oxidative deterioration of meat. Accordingly, Smeti et al. (2013) and Aouadi et al. (2014) found that supplementing lambs with rosemary essential oils did not exert any effect on lipid peroxidation in meat. Overall, the mechanisms by which antioxidant compounds present in plant extracts can affect the oxidative deterioration of lamb meat, are still unclear (Guerra-Rivas et al., 2016). Interestingly, several studies found a higher concentration of tocopherol in lamb muscle following dietary supplementation with a plant extracts or by-product (Ortuño et al., 2015, Luciano et al., 2017, Yagoubi et al., 2018). This is of special relevance, because even a slight increase in α-tocopherol concentration in muscle (from 0.6 to 0.9 mg kg^{-1} meat) has shown to substantially decrease lipid oxidation in 5 and 7 d of display (González-Calvo et al., 2015).

Colour Stability

Results of the colour measurements are shown in **Table 6.5**. Overall, storage time significantly affected the evolution of all meat colour parameters. The behaviour of the colour coordinates were similar for all treatments during the first 7 d of storage.

From d 7 onwards, L^* values generally increased with time of storage. Although there were some differences between treatments in L^* values at 9 d of storage, a clear reduction in L^* values was found in the meat of vitamin E supplemented lambs (regardless of dose) after 12 d of storage. When compared with the control, LTL muscle from lambs supplemented with RE or FRE presented no significant improvements in L^* values. Our results agree with Guerra-Rivas et al. (2016), who found an improvement in L^* when lamb diets were

supplemented with 0.5 g kg^{-1} vitamin E but found no effect of dietary supplementation of grape by-products (0.05 g kg^{-1} feed grape seed extract or a 5% dietary replacement with grape pomace).

Redness (*a** value) is a major determinant of consumer selection in fresh meat (De Oliveira et al., 2013). In the present study, meat of lambs fed vitamin E (0.25, 0.50 or 1.0 g kg^{-1} feed) showed higher overall *a** values than meat from the control, RE and FRE lambs from d 12 of display onwards. Rosemary extract supplementation (RE or FRE) or dosage (0.20, 0.40 or 0.8 g kg^{-1} feed) had no effect on *a** values in LTL muscle when compared with the control. The positive effect of synthetic (Guidera et al., 1997, Leal et al., 2018b) and natural (Leal et al., 2018b) vitamin E supplementation on meat redness has been already described in lambs. However, our findings conflict with earlier studies (Bañón et al., 2012, Ortuño et al., 2014) which reported a positive effect of rosemary extract supplementation in *a** values in lamb meat.

Effects on meat yellowness (*b**) were very subtle. On d 14 of storage, the meat of vitamin E supplemented lambs presented significantly lower *b** values than the RE200, RE400, RE800 and FRE800 groups. Previous work in lambs (Atay et al., 2009, Leal et al., 2018b) found no effect of vitamin E supplementation when compared with non-supplemented animals. However, lower *b** values during storage in meat from lambs fed different plant extracts have been reported previously (Luciano et al., 2009, Andrés et al., 2013).

Chroma (C*) in meat like with the *L** values, were only affected by the dietary supplementations after 12 d of storage. Independently from dosage, vitamin E supplementation led to a significant increase in C* values compared with the meat of control, RE and FRE lambs. This finding conflict with previous studies that found no effect of vitamin E supplementation in C* values in lamb meat (Muela et al., 2014, Leal et al., 2018b) and with an increase in C* values when lambs were supplemented with plant extracts (Ortuño et al., 2014).

It has been previously proposed (Ripoll et al., 2008) that human evaluators are not able to appreciate individual L*, *a**, b* coordinates. Therefore, Hue angle (*h*) provides a better estimation of meat browning than individual coordinates. Like previously reported for individual coordinates (*L**, *a**, *b**), vitamin E supplementation significantly affected *h* development in lamb meat when compared with the control, RE and FRE fed lambs. The effects on *h* value are clear after 9 d of display. No effect was found of rosemary extract supplementation in h values compared with the control. Previous work by Leal et al. (2018a) found that supplementation of lamb diets with natural or synthetic vitamin E had an effect on *h* values during the 14 d of display. However, unlike the current study, Moran et al. (2012) and Ortuño et al. (2014) found lower *h* values in meat from lambs supplemented with rosemary extracts when compared with non-supplemented animals.

Myoglobin Oxidation and Meat Discoloration

The results of meat myoglobin (metmyoglobin (MetMb), oxymyoglobin (OxyMb) and deoxymyoglobin (DeoxyMb)) oxidation are presented in **Table 6.6**. Overall, myoglobin oxidation outcomes are consistent with the colorimetric parameters previously discussed. Display time significantly affected all the myoglobin forms analysed (P<0.01). Supplementation of lamb diets with vitamin E (0.25, 0.50 or 1.0 g kg^{-1} feed) was found to consistently affect MetMb, OxyMb and DoexyMb oxidation after 12 d of storage. Whereas, rosemary extract presentation form (RE or FRE) or dosage (0.20, 0.40 or 0.8 g kg^{-1} feed) had no effect on MetMb, OxyMb and DeoxyMb percentages in the LTL muscle when compared with the control. The effects of vitamin E supplementation on MetMb development are consistent with previous work in lambs (Lauzurica et al., 2005, Leal et al., 2018b), where a clear inhibitory effect of vitamin E supplementation on MetMb formation in lamb meat, especially during longer display periods, was reported. Moreover, in line with the current study, Yagoubi et al. (2018) found no effect of supplementing lamb diets with rosemary leaf residues on MetMb and DeoxyMb percentages in meat displayed for 9 d. High levels of DeoxyMb in meat are associated with the purple colour observed in freshly cut meat (Mancini and Hunt, 2005). The highest DeoxyMb levels in all treatments were found in the first day of display. Moreover, display time was identified as the main factor affecting DeoxyMb values in meat. Although from d 9 onwards, differences among the supplemented lambs were found, there was no effect of antioxidant source nor dose on DeoxyMb levels when compared with the control lambs. The proportion of OxyMb increased significantly in all groups (P<0.01) until 7 d of storage and decreased thereafter until d 14 of storage. Apart from display time, supplementation of lamb diets with vitamin E (0.25, 0.50 or 1.0 g kg^{-1} feed) significantly reduced OxyMb oxidation when compared with the other treatments after 12 d of display. These findings contrast with previous work that found no effect of vitamin E supplementation on OxyMb oxidation in lambs (Lauzurica et al., 2005, Leal et al., 2018b). Interestingly, supplementation of lamb diets with rosemary extracts (RE and FRE) had no effect on OxyMb percentages when compared with the control group. However, Yagoubi et al. (2018) found a clear reduction in OxyMb oxidation in the LTL muscle of Barbarine lambs supplemented with rosemary distillation residues for 77 d before slaughter.

Meat discoloration, as assessed by the decrease in the A_{580}-A_{630} parameter, and oxygen saturation on the meat surface (I_{s02}) in LTL muscle are presented in **Table 6.7**. Meat discoloration and oxygen saturation were significantly affected by storage time in all the groups (P<0.001). Besides that, an interaction between antioxidant supplementation and storage time was found for the A_{580}-A_{630} and Iso2 parameters (P<0.001). After 9, 12 and 14 d of storage, the A_{580}-A_{630} values were significantly higher in the vitamin E supplemented lamb meat (0.25, 0.50 or 1.0 g kg^{-1} feed) compared with the meat of the control and rosemary supplemented lambs (RE and FRE). Similarly, after 12 and 14 d, supplementation of lamb diets with vitamin E led to significantly higher Iso2 values compared with the remaining

treatments. For meat discolouration (A_{580}-A_{630}), Renerre and Mazuel (1985) proposed a value of 12.5 as the lower limit for colour acceptability. In the current study, apart from the vitamin E supplemented lambs which had A_{580}-A_{630} values higher than 12.5 until 14 d of display, the other treatments only presented acceptable A_{580}-A_{630} values until d 9 of storage. Similarly, LTL muscle from lambs supplemented with vitamin E presented I_{502} values higher than 21.0 after 14 d of storage. Whereas, in lamb fed the control or the rosemary supplemented (RE and FRE) diets, higher Iso2 values than 21.0 were only maintained for 7 d of storage.

CONCLUSIONS

This study demonstrated that feeding lambs a concentrate diet supplemented with *all-rac*-α-tocopheryl acetate for 14 d before slaughter resulted in a general improvement in meat oxidative and colour stabilities. Moreover, supplementation of lamb diets with lowest level of α-tocopheryl acetate (0.25 g kg^{-1} compound feed) was sufficient to significantly reduce meat oxidation and colour deterioration. This study demonstrates also that unlike vitamin E, supplementation of lamb diets with a rosemary extract (dosage and fat embedment) had no effect on lipid oxidation or meat colour stability of lambs during the 14 d of storage under retail conditions.

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Chapter 7

General Discussion

INTRODUCTION

Meat quality, as defined by its colour and lipid oxidative stability (i.e. shelf-life), is the most important factor that drives consumer preference and purchase decision (Faustman and Cassens, 1990). The "bright red" colour in lamb meat that is appreciated by consumers can be further enhanced with MAP. However, overtime the high concentration of oxygen with MAP also exacerbate lipid oxidation and colour deterioration, which in turn, contributes to the deterioration of flavour, texture and general appearance of the meat or meat product (Gatellier et al., 2005). To delay this oxidative process, retailers and lamb meat producers adopted the use of antioxidants during processing or supplementation to animal diets.

Feed applications of vitamin E, and within the isomers (α-, β-, δ- and γ-), α-tocopherol is recognized as the most effective antioxidant in delaying meat discoloration and lipid oxidation. In lambs, the increase in α-tocopherol concentration in muscle can be achieved through dietary vitamin E supplementation above the nutritional recommendations for growth, without any adverse effects on feed intake, performance or carcass characteristics (López-Bote et al., 2001, Lauzurica et al., 2005). When supplementing lamb diets with vitamin E, the synthetic *all-rac*-α-tocopheryl acetate is the most commonly used α-tocopherol source. The decision to use *all-rac*-α-tocopheryl acetate instead of RRR-α-tocopheryl acetate is mainly driven by the cost difference between both sources, which overshadows the potential benefit of a 36% increase in biological activity of the RRR-source compared to *all-rac*-α-tocopheryl acetate (USP, 1979). However, there much debate regarding the need to develop new bioequivalence factors for livestock animals, different from the conversion factors used in the International Unit System for vitamins (Jensen and Lauridsen, 2007).

Besides α-tocopherol, alternative components such as polyphenols have been consistently reported as exhibiting antioxidant properties (Wojdyło et al., 2007) and have the ability to delay meat oxidation and colour deterioration in lamb meat. Interestingly, there is little information on the effect of dietary administration of polyphenols compared with vitamin E on lipid oxidation and colour stability in lamb meat (Nieto et al., 2010a, Bañón et al., 2012). Thus, contrasting both feeding strategies and quantifying their relative value for affecting meat colour and stability, under similar experimental conditions, is a relevant topic for nutritionists, as well as, meat scientists.

The aim of this thesis was to investigate the optimal supplementation level of vitamin E (natural vs. synthetic) and the use of a standardized polyphenol rich plant extract as an alternative, to extend shelf-life of lamb cuts kept under retail conditions. In the following paragraphs, the work described in this thesis, as well as conclusions and implications for the Mediterranean lamb production system, but not exclusively, are discussed.

Relative Bioavailability of RRR- and *All-rac*-α-Tocopheryl Acetate

When present in their free form, *all-rac*- or RRR-α-tocopherol can easily interact with other components in the feed during processing, manufacturing and storage. To overcome

that issue, the phenol group of α-tocopherol can be converted to an ester by using acetic acid, so commercial preparations of α-tocopherol are normally found as *all-rac*- or RRR-α-tocopheryl acetate (Bellés et al., 2018). Moreover, apart from their high stability in the feeds this acetate forms of α-tocopherol are also easily hydrolyzed and absorbed when they reach the gut (Hacquebard and Carpentier, 2005, Rigotti, 2007).

The use of a single ratio to discriminate different α-tocopherol sources

As previously described in this thesis, when formulating lamb diets, nutritionists select the α-tocopherol source based on the International Unit System (USP, 1979), which indicates that the biopotency of 1.36 mg of *all-rac*-α-tocopheryl acetate equates to the biopotency of 1.00 mg of RRR-α-tocopheryl acetate. An intense debate about the adequacy of this ratio has occured in the scientific community for the last two decades. In many publications the terms biopotency and bioavailability are frequently used interchangeably, which lead to contradictory conclusions and further fuelled the discussion (Hoppe and Krennrich, 2000, Dersjant-Li and Peisker, 2010). Bioavailability refers to the absorbed proportion of a substance after its administration, while biopotency is a measure of the biological effects exerted by the substance in a biological system. However, to date there are no biopotency studies comparing *all-rac*-α-tocopheryl acetate and RRR-α-tocopheryl acetate in humans or livestock animals, mainly due to the ethical implications associated with this type of studies (e.g. foetal reabsorption in rats) and difficulties to determine relevant clinical endpoints and targets. As an alternative, bioavailability studies have been conducted based on the principle that the magnitude of the effect of a substance (potency) is a function of its concentration at the site of action. Thus, if two different substances have the same bioavailability, it can be inferred that they may also have the same potency (Hoppe and Krennrich, 2000).

Bioavailability of RRR- and all-rac-α-tocopheryl acetate in lamb tissues

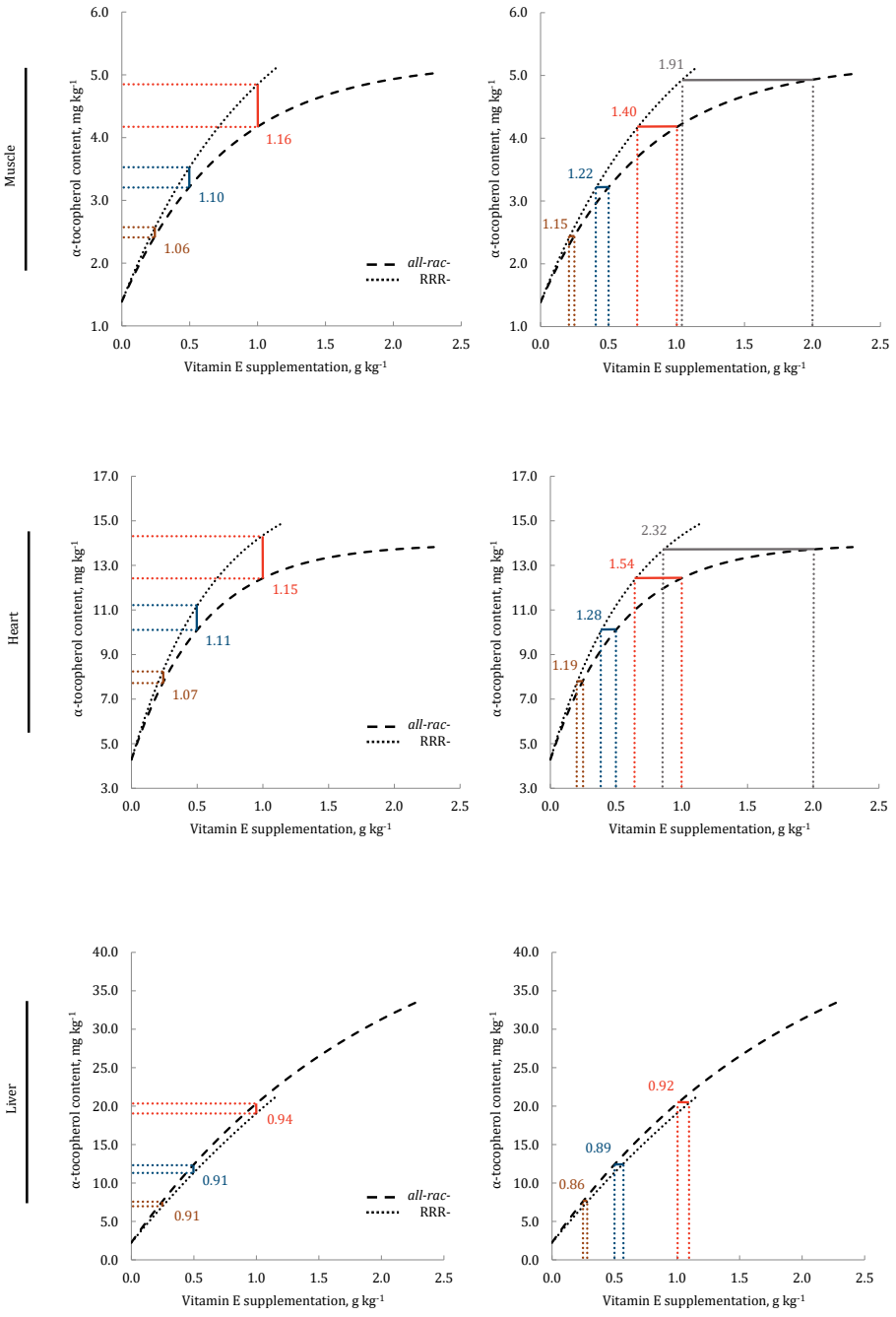
To evaluate the bioavailability of two different α-tocopherol sources (**Chapter 2**), lambs were supplemented with increasing amounts of *all-rac*-α-tocopheryl acetate (0.25, 0.5, 1.0 and 2.0 g kg^{-1} compound feed) or RRR-α-tocopheryl acetate (0.125, 0.25, 0.5 and 1.0 g kg^{-1} compound feed) for a period of 14 d before slaughter. Under those conditions, the various tissues studied (muscle, heart, liver and spleen) responded differently to increases of dietary supplementation of *all-rac*- and RRR-α-tocopheryl acetate. As summarized in **Figure 7.1** (same data as presented in **Chapter 2**), at similar supplementation levels (0.25, 0.50 or 1.0 g kg^{-1} compound feed), the bioavailability ratio between the two α-tocopherol sources is different among tissues and increases within tissue with increasing dosages. Interestingly, to attain the same α-tocopherol concentrations in muscle and heart as lambs supplemented with 2.0 g *all-rac*-α-tocopheryl acetate kg^{-1} feed, lambs supplemented with RRR-α-tocopheryl acetate required approximately half the dosage. In muscle and spleen, a 'plateau' for α-tocopherol deposition was reached with *all-rac*-α-tocopheryl acetate

supplementation. Therefore, as tissue α-tocopherol concentrations approach the 'plateau', only a marginal increase in α-tocopherol was attained at higher supplementation levels. The non-parallel dose/concentration curves between different α-tocopherol sources seems to indicate that their relative bioavailability is associated with the saturation of stereospecific pathways for distribution and elimination (Bramley et al., 2000, Blatt et al., 2004).

Regulation of α-tocopherol concentration in tissues

It is well accepted that hepatic α-TTP is the major regulatory mechanism for controlling α-tocopherol concentrations in plasma and consequently in tissues (Traber, 2007). From the eight stereoisomers found in *all-rac*-α-tocopheryl acetate, the 2R configured stereoisomers (RRR-, RRS-, RSR-, and RSS-) are preferentially retained in all tissues except in the liver (Ingold et al., 1987, Burton et al., 1998), where the 2S stereoisomers (SRR-, SRS-, SSR-, and SSS-) are retained awaiting catabolism (Lauridsen et al., 2002, Traber et al., 2017). On the other hand, the 2R configured stereoisomers, and to a greater extent the RRR- stereoisomer, are recognized by α-TTP and re-secreted from the liver back into plasma (Brigelius-Flohé et al., 2002). Although α-TTP is expressed primarily in the liver, it has also been detected in various other tissues such as brain, spleen, lung, kidney, adrenals and uterus (Hosomi et al., 1998, Copp et al., 1999, Jishage et al., 2001, Kaempf-Rotzoll et al., 2002, Kaempf-Rotzoll et al., 2003, Gohil et al., 2004). Which could explain the different bioavailability ratios found among tissues. Moreover, it leads to other questions such as tissue specific α-tocopherol requirements and other α-TTP functions beyond binding and transferring α-tocopherol into cells (Lim and Traber, 2007).

Increasing *all-rac*-α-tocopheryl acetate supplementation in lamb diets, was found to affect the 2R configured stereoisomer profiles in muscle, heart and spleen, where increasing proportions of RRS-, RSR- and RSS- were found at the cost of RRR-α-tocopherol. On the other hand, when lambs were supplemented with RRR-α-tocopheryl acetate, the relative proportion of all stereoisomers other than RRR-α-tocopherol, were reduced in all tissues analysed. Further explaining the change in the bioavailability ratios within tissue. In conclusion, the findings presented here support Blatt et al. (2004) and Jensen et al. (2006), who pointed out that bioavailability of RRR- and *all-rac*-α-tocopheryl acetate varies between tissues and with dosage. As such, the use of a single ratio to discriminate between both α-tocopherol sources is inadequate to describe α-tocopherol deposition in lamb tissues.


Muscle
Heart
Liver
α-tocopherol content, mg kg-1
Vitamin E supplementation, g kg-1
all-rac-
RRR-
1.16
1.10
1.06
1.91
1.40
1.22
1.15
1.15
1.11
1.07
2.32
1.54
1.28
1.19
0.94
0.91
0.91
0.92
0.89
0.86

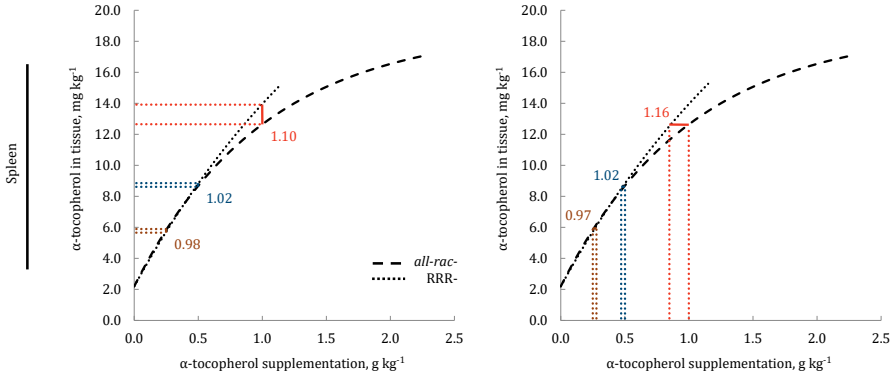


Figure 7.1 Bioavailability ratios between RRR-α-tocopheryl acetate and *all-rac*-α-tocopheryl acetate in muscle, heart, liver and spleen. Ratios presented on the left side of the figure express the difference between sources at similar dosages (0.25, 0.50 and 1.0 g kg^{-1} compound feed). Ratios presented on the right side of the figure represent the ratio between the minimum level of RRR-α-tocopheryl acetate required to produce the same α-tocopherol accumulation in muscle, heart, liver and spleen; as 0.25, 0.50 and 1.0 g kg^{-1} compound feed of *all-rac*-α-tocopheryl acetate.

Dietary Vitamin E Improves Colour and Oxidative Stability of Lamb Meat

Vitamin E plays a major role in the *in vivo* antioxidant system by protecting cells and tissues from oxidative damage. In this thesis, however, the focus was primarily on the antioxidant potential of vitamin E that is carried through to meat after slaughter. Supranutritional doses of α-tocopherol before slaughter lead to a significant increase in α-tocopherol deposition in lamb muscle (**Chapter 2**), which in turn, is related with an increased stability of meat against oxidation and colour deterioration (**Chapter 3**, **Chapter 4** and **Chapter 6**).

α-Tocopherol thresholds in meat

Similar α-tocopherol contents in muscle can be achieved with different supplementation strategies. In the work of Kasapidou et al. (2012), a level of α-tocopherol of 3.73 mg kg^{-1} muscle was achieved by supplementing lambs with 0.5 g *all-rac*-α-tocopheryl acetate kg^{-1} diet for 63 d. Higher supplementation levels with *all-rac*-α-tocopheryl acetate (1.0 g kg^{-1}) led to similar α-tocopherol concentrations in lamb muscle (3.57 mg kg^{-1}), but in a considerably shorter supplementation period (37 d) (De la Fuente et al., 2007, Álvarez et al., 2008). In **Chapter 2**, it was found that higher α-tocopherol concentrations than 3.50 mg kg^{-1} muscle were achieved when supplementing lambs with 0.5 g RRR-α-tocopheryl acetate kg^{-1} diet or with 0.65 g *all-rac*-α-tocopheryl acetate kg^{-1} diet, for 14 d. The effects of α-tocopherol on meat oxidation are dose dependent and, therefore, a reflection of its accumulation in the muscle (López-Bote et al., 2001, Lauzurica et al., 2005, Álvarez et al., 2008). Like for α-tocopherol

accumulation in muscle, above a certain threshold only marginal improvements in lipid and colour stability occur. However, a minimum amount of tocopherol in the muscle is required to effectively delay meat oxidation and colour deterioration. In line with this, Ponnampalam et al. (2014) showed that when muscle α-tocopherol concentration is lower than 2.95 mg kg^{-1} muscle, intrinsic factors such as heme iron or PUFA and extrinsic factors like illumination and packaging conditions during display also affect the lipid oxidation outcomes. Interestingly, as presented in **Figure 7.2**, which combines data on thiobarbituric acid reactive substances (TBARS) from **Chapters 3** and **6**, the levels of meat oxidation (TBARS values) from non-supplemented lambs in **Chapter 6** were approximately 3 fold higher than the TBARS of non-supplemented lambs in **Chapter 3**, after 14 d of display. This even though both experiments were conducted under similar conditions accounting for similar genetics, body weight, basal diet, housing, slaughter procedures and display conditions. It appears that the α-tocopherol background level of each individual animal plays a major role in meat oxidation and colour stability.

α-Tocopherol supplementation on meat oxidation and colour stability

The effect of vitamin E on lipid oxidation and colour stability in lamb meat has been previously demonstrated (López-Bote et al., 2001, Lauzurica et al., 2005, Álvarez et al., 2008). In an early titration study, Lauzurica et al. (2005) found that increasing dietary inclusion of vitamin E (0.25, 0.50 and 1.0 g kg^{-1} feed) for lambs, led to a significant increase in meat shelf life up to 14 d of storage.

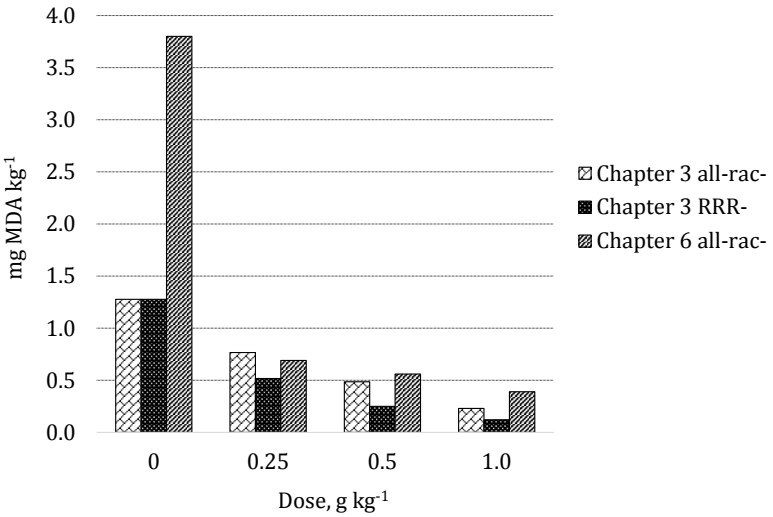


Figure 7.2 Meat oxidation (TBARS, mg malondialdehyde (MDA) kg^{-1}) values of *longissimus thoracis et lumborum* steaks of lambs fed different doses of *all-rac*-α-tocopheryl acetate (*all-rac*-) or RRR-α-tocopheryl acetate (RRR-) for 14 d before slaughter in **Chapter 3** and **6.**

Likewise, in **Chapter 3** and **6**, the evolution of TBARS values for each dietary intervention with vitamin E was assessed. Lipid oxidation significantly increased with storage time in both experiments. From d 7 of storage onwards, *longissimus thoracis et lumborum* (LTL) muscles from vitamin E supplemented lambs (irrespective of dose or source) presented already significantly lower TBARS values than the non-supplemented lambs. As shown in **Figure 7.2**, vitamin E supplemented lambs registered TBARS values ranging from 0.12 to 0.77 mg malondialdehyde (MDA) kg^{-1} meat at d 14 of storage. Whereas, non-supplemented lambs presented TBARS values ranging from 1.28 to 3.80 mg MDA kg^{-1} meat, which were above the threshold of 1.0 mg MDA kg^{-1} meat proposed by Ripoll et al. (2011) as the maximum limit for the development of off-flavours in lamb meat.

Redness (*a** value) is a major determinant of consumer preference in fresh meat (De Oliveira et al., 2013). Thus, lambs fed with increasing levels of RRR- or *all-rac*-α-tocopheryl acetate in Chapter 3 and 6, presented consistently higher *a** values than non-supplemented lambs during the 14 d of storage. These results are in line with previous work (Guidera et al., 1997), which reported a positive effect of supplementing lamb diets with *all-rac*-α-tocopheryl acetate on meat redness. However, human evaluators are not able to appreciate individual *a** values as well as other colour coordinates such as *L** and *b**. Therefore, huge angle (*h*) is often reported as a better estimate of meat browning that individual colour coordinates (Ripoll et al., 2008). Supplementation of lamb diets with α-tocopherol (RRR- or *all-rac*-) significantly affected *h* development in meat compared with non-supplemented animals. Moreover, the impact of α-tocopherol on h values are clearer as display time develops.

As previously mentioned, meat colour in lambs is primarily determined by the constant conversion between the heme-containing proteins: deoxymyoglobin (DeoxyMb), oxymyoglobin (OxyMb) and metmyoglobin (MetMb). Display time is known to be the main factor driving myoglobin oxidation in meat. However, dietary supplementation of lamb diets with α-tocopherol was found to consistently affect MetMb formation in LTL muscle when compared with non-supplemented lambs (**Chapter 3** and **6**). Thus, maintaining the 'bright red' colour of lamb meat for at least 14 d of display. These results are consistent with previous work of Lauzurica et al. (2005), who reported a clear inhibitory effect of *all-rac*-α-tocopheryl acetate supplementation on MetMb formation in lamb meat, especially at longer display periods.

Although the benefits of dietary supplementation with α-tocopherol in controlling meat oxidation and colour loss in fresh meat are well described, there is a lack of knowledge regarding the effects of α-tocopherol supplementation on lamb meat quality after long-term frozen storage. This preservation alternative is highly relevant for distributors and retailers because it provides them with great flexibility in managing meat stocks. However, there are concerns that the primary compounds of lipid oxidation formed during frozen storage cannot be controlled by the α-tocopherol present in muscle, after thawing. In **Chapter 4**, it was demonstrated that supplementation of lamb diets with 1.0 g *all-rac*-α-tocopheryl acetate kg^{-1} feed for 14 d before slaughter effectively delayed meat oxidation

(TBARS values) and colour deterioration (MetMb formation and hue values) in meat displayed for 9 d under retail conditions which was previously stored frozen (up to 9 months). Overall, these results highlight the importance of α-tocopherol supplementation not only on short-term stored meat (**Chapter 3** and **6**) but also in preserving meat from oxidation and colour deterioration in long term storage (**Chapter 4**).

α-Tocopherol source on meat oxidation and colour stability

Both α-tocopherol sources (RRR- and *all-rac*-α-tocopheryl acetate) were effective at delaying meat oxidation and colour deterioration. Although the effectiveness of *all-rac*-α-tocopheryl acetate has been extensively described before (López-Bote et al., 2001, Lauzurica et al., 2005, Muela et al., 2014). This thesis (**Chapter 3**), compares for the first time both α-tocopherol sources in their capacity to maintain the quality attributes of lamb meat preserved under retail conditions for 14 d.

Based on the surface response plots for TBARS, it was found that the effectiveness of RRR-α-tocopheryl acetate at delaying meat oxidation in lamb meat was up to 1.8 times higher than with *all-rac*-α-tocopheryl acetate. As a result, supplementation of lamb diets with 0.09 g RRR-α-tocopheryl acetate kg^{-1} compound feed was sufficient to maintain TBARS values below the threshold of 1.0 mg MDA kg^{-1} meat after 14 d of display, compared with the 0.16 g kg^{-1} compound feed required in case of *all-rac*-α-tocopheryl acetate. The difference in efficacy between sources was also noticed on colour parameters, where RRR-α-tocopheryl acetate was found to be up to 1.9, 1.5 and 1.8 times more effective than *all-rac*-α-tocopheryl acetate at controlling the development of a^* values, h values and MetMb %, respectively during the 14 d of display.

As previously discussed for α-tocopherol deposition in different tissues, a single ratio cannot accurately represent the difference between both sources for any of the meat quality parameters. Moreover, the aforementioned ratios were calculated based on the minimum dose of each α-tocopherol source required to achieve a certain threshold or target effect in meat, which can be different from the ratios obtained based on the effect created by both sources when fed at equal dosages. Instead of trying to determine the equivalence ratio between both α-tocopherol sources, future research should focus instead, on characterizing the effect of each source (with new surface responses) as two different compounds, on their ability to affect meat oxidation and colour deterioration under different production conditions (basal diet, breed, supplementation time, packaging methods and larger display periods). This information will allow, both producers and retailers to adjust their supplementation strategies based on the desired effect, and the use of either source will depend solely on their capacity to deliver the desired effect at the lowest cost.

Plant Extracts on Antioxidant Capacity of Lamb Tissues

Since the 1990's, scientists and food manufacturers have become increasingly interested in plant-derived antioxidants other than vitamin E. This interest in plant phenolics was

mainly driven by a recognition of their *in vitro* antioxidant properties (Zheng and Wang, 2001, Shan et al., 2005) and with epidemiological studies that indicated their probable role in the prevention of various diseases, such as cardiovascular disease, diabetes mellitus, and cancer (Hertog et al., 1995, Hirvonen et al., 2001, Arts and Hollman, 2005). This thesis, however, focuses solely on the antioxidant potential of plant phenolics that is carried to meat or organs after slaughter.

Screening results from the use of different polyphenols in lamb diets

Culinary herbs and spices have been mainly used to enhance or improve the flavour of food, with little attention given to their potential use in animal diets, as a mean to improve meat shelf-life. Culinary herbs and spices belonging to several families, such as *Labiatae*, *Compositae*, *Umbelliferae*, *Asteracae*, *Polygonacae* and *Myrtacae*, have been consistently reported to exhibit strong in vitro antioxidant properties (Wojdyło et al., 2007). However, to effectively act as direct antioxidants in meat systems, phenolic compounds present in these herbs and spices need to be effectively absorbed, transported and have a considerable deposition into the muscle. Therefore, it is not only important to characterize plant extracts for their content in phenolic compounds and potential *in vitro* antioxidant activity, but it is essential to know the extent to which they exhibit antioxidant activity in the target tissue.

Previous to the experimental work described in **Chapter 5**, plant extracts belonging to the main families of culinary herbs and spices described before, were fully characterized for their phenolic profiles and hydrophilic antioxidant activities (Vallverdú-Queralt et al., 2014; Vallverdú-Queralt et al., 2015). As presented in **Figure 7.3**, the lowest hydrophilic antioxidant capacities based on results from the trolox equivalent antioxidant capacity (TEAC) and 2,2-diphenyl-1-pirylhydrazyl (DPPH$^{\bullet}$) assays were determined in nutmeg and turmeric followed by dill, bay and marjoram. Whereas, cumin, cinnamon and rosemary presented the highest hydrophilic antioxidant capacity.

Although, this is information is valuable, it does not represent their accumulation and potential antioxidant activity in tissues. The transfer of the antioxidant activity of these extracts to lamb tissues was assessed *in vivo*, as described in **Chapter 5** where lambs were fed a diet consisting of a basal compound feed supplemented with 5.0 g kg^{-1} of each plant extract for a period of 14 d.

Afterwards, muscle (LTL muscle), liver and kidney samples were collected. To access the tissue antioxidant activity, four different assays were performed in parallel (TEAC, ferric reducing antioxidant power (FRAP), oxygen radical absorbing capacity (ORAC) and DPPH$^{\bullet}$), which additionally enable the comparison of these different methods to rank the plant extracts based on their radical-scavenging capacity in the different tissues.

Overall, there was no effect of plant extract supplementation of lamb diets on the tissue antioxidant capacity, as assessed by the FRAP method. Previous work in lambs (Moñino et al., 2008, Jordán et al., 2010, Luciano et al., 2011), has shown that dietary supplementation of ewes (gestation and lactation) and lambs with plant extracts led to a significant increase

in antioxidant activity of lamb muscle, which was measured by the use of the FRAP and TEAC methods. However, based on the results obtained by the FRAP method in **Chapter 5** none of the plant extract altered significantly the antioxidant potential in lamb tissues. This could indicate a poor bioavailability of these plant extracts in lambs. However, the other 3 assays have revealed significant directional changes in the antioxidant activity of the 11 plant extracts in the different tissues, as summarized in **Table 7.1**.

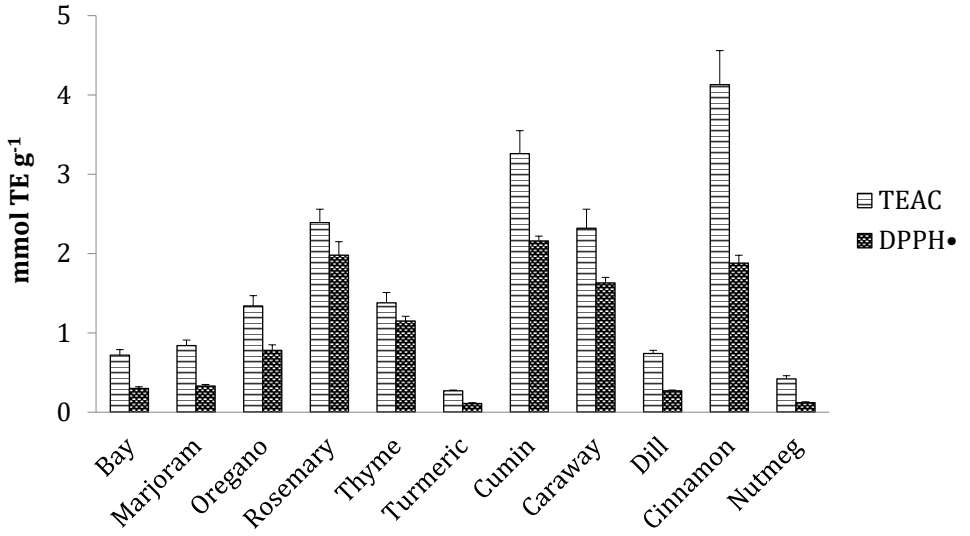


Figure 7.3 Hydrophilic antioxidant capacity of the selected plant extracts evaluated by the Trolox equivalent antioxidant capacity (TEAC) and 2,2-diphenyl-1-pirylhydrazyl (DPPH•) radical-scavenging capacity assays and expressed as mmol Trolox equivalent (TE) g^{-1}. Adapted from Vallverdú-Queralt et al. (2014) and Vallverdú-Queralt et al. (2015).

Table 7.1 Antioxidant activity of the 11 plants extracts in muscle, liver and kidney of lambs[a], assessed by TEAC (mM trolox g^{-1}), ORAC (mM Fe^{+2} g^{-1}) and DPPH• (mg ml^{-1} MeOH) assays[b].

Item	Muscle			Liver			Kidney		
	TEAC	ORAC	DPPH•	TEAC	ORAC	DPPH•	TEAC	ORAC	DPPH•
Bay	-	-	-	-	↓	-	-	-	-
Marjoram	-	-	-	-	-	-	-	-	-
Oregano	-	↓	-	-	-	-	↓	-	↑
Rosemary	-	-	-	-	-	-	-	-	-
Thyme	-	-	-	-	-	-	-	-	-
Turmeric	-	-	-	-	-	↑	-	↑	-
Cumin	-	-	-	-	-	-	↓	↑	-
Caraway	-	-	-	-	-	-	↓	↑	-
Dill	-	↓	-	-	-	-	-	-	-
Cinnamon	-	↓	-	-	-	↑	-	↑	-
Nutmeg	↑	↓	-	↑	-	↑	-	↑	-

[a] Different signs denote: no differences (-), increase (↑) or reduction (↓) of the antioxidant capacity in the different tissues compared with the non-supplemented lambs ($P<0.05$).
[b] TEAC = Trolox equivalent antioxidant capacity; FRAP = ferric reducing antioxidant power; ORAC = oxygen radical absorbance capacity; DPPH• = 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity.

As presented in **Table 7.1** and thoroughly discussed in **Chapter 5**, it is clear that no single plant extract consistently affected the tissue antioxidant activity as measured by the 3 different assays. Noteworthy, nutmeg affected the antioxidant activity in muscle (both increased and decreased), liver and kidney as assessed by different analytical methods. However, as presented in **Figure 7.3**, nutmeg was the plant extract that exhibited the lowest hydrophilic antioxidant activity of all the selected plant extracts which seems to indicate that the in vitro antioxidant activity of a phenolic compound in an animal diet does not necessarily reflect its *in vivo* antioxidant activity (D'Archivio et al., 2010). In general, supplementation of lamb diets with bay, oregano, turmeric, cumin, caraway, dill, cinnamon and nutmeg had an effect on the radical scavenging activity in lamb tissues (either antioxidant or prooxidant). Phenolic compounds are multifunctional and differences among assays can be associated with their antioxidant activity (Frankel and Meyer, 2000) but also with the reaction mechanism of each assay (Karadag et al., 2009). Therefore, it is difficult to draw clear conclusions on the antioxidant capacity of each plant extracts based on the present results, which lead to the conclusion that this approach is not advisable to discriminate or rank plant extracts from different botanical origins.

Methodological considerations of the TEAC, FRAP, ORAC and DPPH• assays

In general, the methods for determining the antioxidant capacity in food components and biological material can be divided into two categories: hydrogen atom transfer (HAT) reaction-based and electron transfer (ET) reaction-based methods (Karadag et al., 2009, Gülçin, 2012). HAT-based assays (like ORAC) typically measure the capability of an antioxidant to scavenge free radicals (generally peroxyl radicals) by hydrogen donation to form stable compounds (Prior et al., 2005). Whereas, ET-based methods such as TEAC, FRAP and DPPH• detect the ability of a potential antioxidant to transfer one electron to reduce any compound (metals, carbonyls, and radicals) (Karadag et al., 2009). Due to similarities in the mechanism of action, ET-based assays are generally assumed to correlate well among themselves, which has led some researcher to consider the application of a series of ET-based assays redundant (Apak et al., 2016). Notwithstanding, as shown in **Table 7.1**, conflicting results between TEAC and DPPH• (both ET-based assays) were obtained in the kidney of oregano supplemented lambs.

HAT- and ET-based assays are known to correlate poorly among each other, as found by (Cao and Prior, 1998) when comparing different methods for assessing total antioxidant capacity of serum in humans. Thus, a better evaluation of the overall antioxidant capacity of complex samples (like biological fluids and tissues) can be attained by the use of multiple assays. That is recommend to generate an "antioxidant profile" that contemplates both the reactivity towards lipid/organic radicals and aqueous radicals (Prior et al., 2005). However, as pointed out by Apak et al. (2016), both methods (HAR and ET-based) are known to correlate poorly with assays based on the inhibition of lipid peroxidation, which means that an antioxidant or plant extract with high antioxidant activity as measured by one of this methods may not perform well in preventing or retarding lipid peroxidation. Future work should focus on analytical methods and "endpoints" that better reflect the target antioxidant effect of plant extracts both *in vivo* as well as post-morten, which in the current thesis is to delay lipid oxidation and prevent colour deterioration in lamb meat stored under retail conditions for 14 d.

Plant Extracts as Vitamin E Alternatives at Improving the Shelf-Life Stability of Lamb Meat

Supplementation of lamb diets with phenolic rich plant by-products such as leaves, distillation residues, essential oils and extracts has been proposed as an alternative to replace the supranutritional applications of α-tocopherol to reduce meat oxidation, colour loss and increase the shelf-life of meat.

Effects of phenolic rich plant materials on meat oxidative stability

In early studies, (Nieto et al., 2010 a,b) found that the administration of thyme leaves or distilled rosemary leaves to ewes during pregnancy and lactation significantly reduced lipid oxidation (TBARS) in fresh lamb meat, stored for 14 d in modified atmosphere

packaging. These results were later corroborated by the same group (Nieto et al., 2012), who found lower lipid oxidation and increased surface redness (*a** values) in lamb meat from ewes supplemented with 5 or 10% of their diet with thyme distillation by-products. Interestingly, Serrano et al. (2014) when supplementing sheep diets (0.6 g kg^{-1} per kg feed) with a rosemary extract during pregnancy and lactation (ewe) and/or during the fattening phase (lambs), found that supplementing the ewe's diet did not offer any benefit compared to supplementing only the lambs during the fattening phase. Despite that, both supplementation strategies were found to extend the shelf-life of lamb meat by 2 d.

Nevertheless, most studies in this field focused on the supplementation of lamb diets with phenolic rich plant materials during the fattening phase. Bañón et al. (2012), reported a reduction of 73% in TBARS values after 14 d of display under retail conditions, following a supplementation of lamb diets with a rosemary extract at 0.6 g kg^{-1} feed for a period of 21 d before slaughter. Moreover, these authors reported also a significant improvement in h values during the same storage period. In line with that, supplementation of lamb diets with a rosemary extracts at 0.4 g kg^{-1} feed (for 80 d before slaughter) or 0.6 g kg^{-1} feed (for 50 d before slaughter), have been shown to reduce TBARS values after 14 d of display by 74 and 48%, respectively (Ortuño et al., 2014, Ortuño et al., 2015). Moreover, supplementation of lamb diets with carnosic acid, a major phenolic compound found in rosemary extracts at 1.2 g kg^{-1} diet, was found to reduce TBARS values after 14 d of display by 39% in *longissimus lumborum* and by 93% in *gluteus medius* muscles compared with non-supplemented lambs (Moran et al., 2012). Apart from thyme and rosemary, other phenolic rich plant materials were reported to have a positive effect on meat shelf-life stability of lambs. Andrés et al. (2014), found that quercetin supplementation (2.0 g kg^{-1} diet) was an effective tool to control meat oxidation (TBARS values) even when lambs are fed diets rich in polyunsaturated fatty acids. **Figure 7.4** summarizes the results on meat oxidation (TBARS values) results from studies that reported a positive effect of phenolic rich plant materials on meat oxidative stability in lambs, after 14 d of display under retail conditions.

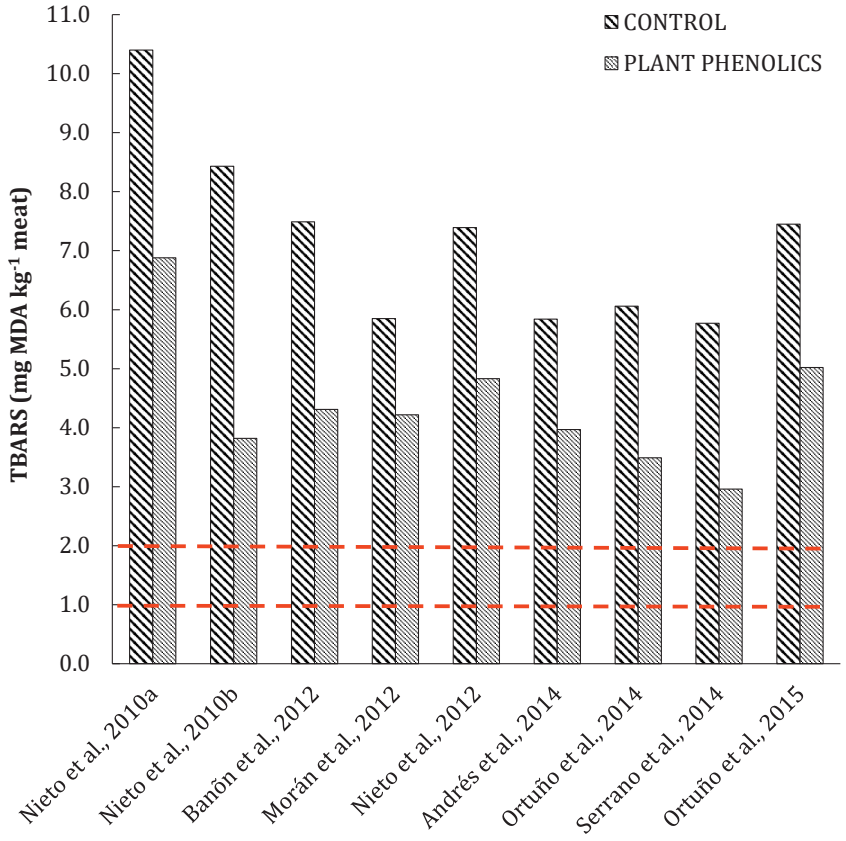


Figure 7.4 Thiobarbituric acid reactive substances (TBARS) (mg malondialdehyde (MDA) kg^{-1} meat) values of *longissimus thoracis et lumborum* muscle of lambs fed different phenolic rich plant materials (Plant Phenolics) in different studies. The dashed red lines represent the thresholds for meat oxidation (TBARS values) in lambs, proposed by (Ripoll et al., 2011) and Muíño et al. (2014).

Several studies, however, found no effect of dietary supplementation with phenolic rich plant materials of lamb diets on meat oxidation and colour stability of lambs. Aouadi et al. (2014) when feeding lambs with 0.4 g kg^{-1} diet of rosemary or with artemisia essential oils found that both supplementation strategies increased the reducing capacity of the muscle compared with non-supplemented lambs (FRAP and TEAC assay), but had no effect on oxidative stability (TBARS values) nor colour (L^*, a^*, b^*, C^* and h values) of meat over 7 d of aerobic display. In line with that, Smeti et al. (2013) found that supplementation of *Barberine* lambs with a rosemary extract (0.6 g kg^{-1} compound feed) for 60 d before slaughter, had no effect on TBARS values and meat discoloration across the storage time. Moreover, supplementation of lamb diets with red wine extract (0.9 g kg^{-1} diet), *Quillaja saponaria* bark extract (0.06 or 0.09 g kg^{-1} compound feed), sage distillation by-products

(100 g kg^{-1} compound feed), grape seed extract (0.05 g kg^{-1} diet) or dried red grape pomace (50 g kg^{-1} diet), were also found to have no effect on meat oxidative stability of lambs (Nasri et al., 2012, Muíño et al., 2014, Guerra-Rivas et al., 2016, Leticia et al., 2017).

Unlike vitamin E, the protective role of phenolic rich plant by-products and their extracts against lipid oxidation and colour deterioration in lamb meat, is not well established. When applying the thresholds of 1.0 mg MDA kg^{-1} lamb meat as the maximum limit for meat oxidation in lambs (Ripoll et al., 2011), or a less conservative threshold of 2.0 mg MDA kg^{-1} lamb meat (Muíño et al., 2014), it is clear that none of the phenolic rich plant extracts was able to maintain the TBARS values below these thresholds, after 14 d of display under retail conditions (**Figure 7.4**). Therefore, the value of supplementing lamb diets with phenolic rich plant by-products, as a mean to delay meat oxidation and colour deterioration, seems limited when compared with α-tocopherol.

Comparison between phenolic rich plant materials and all-rac-α-tocopheryl acetate

There are only few studies that compared the use of supranutritional doses of α-tocopherol with phenolic rich plant by-products on their capability to protect lamb meat from oxidative deterioration. **Chapter 6** of this thesis describes a dose-titration study where lamb diets were supplemented with increasing levels of *all-rac*-α-tocopheryl acetate (0.25, 0.5, 1.0 g kg^{-1} compound feed) or a standardized rosemary extract (0.20, 0.40 or 0.80 g kg^{-1} compound feed) with or without fat embedment.

Administration of phenolic rich plant by-products to high concentrate fed lambs has been shown to affect ruminal fermentation (Jiménez-Peralta et al., 2011). Phenolic compounds present in these plants and their by-products have been shown to possess high antimicrobial (Panizzi et al., 1993) and antiprotozoal (Calsamiglia et al., 2007) activities. The lipophilic nature of some phenolic compounds allows for their accumulation in membranes which seem associated with the disruption of membrane associated events that are required for cell metabolism such as energy (Sikkema et al., 1995) and metal ion depletion (Calsamiglia et al., 2007). Therefore, to reduce the interactions between rumen microorganisms and the phenolic compounds present in the plant extract tested in **Chapter 6**, a rosemary extract embedded in a hydrogenated fat matrix was also studied. Due the lack of differences on meat oxidation and colour stability parameters between the two rosemary supplementation strategies (with or without fat embedment) and the non-supplemented lambs, it is not possible to withdraw any conclusion regarding the efficacy of the protection intended by fat embedment.

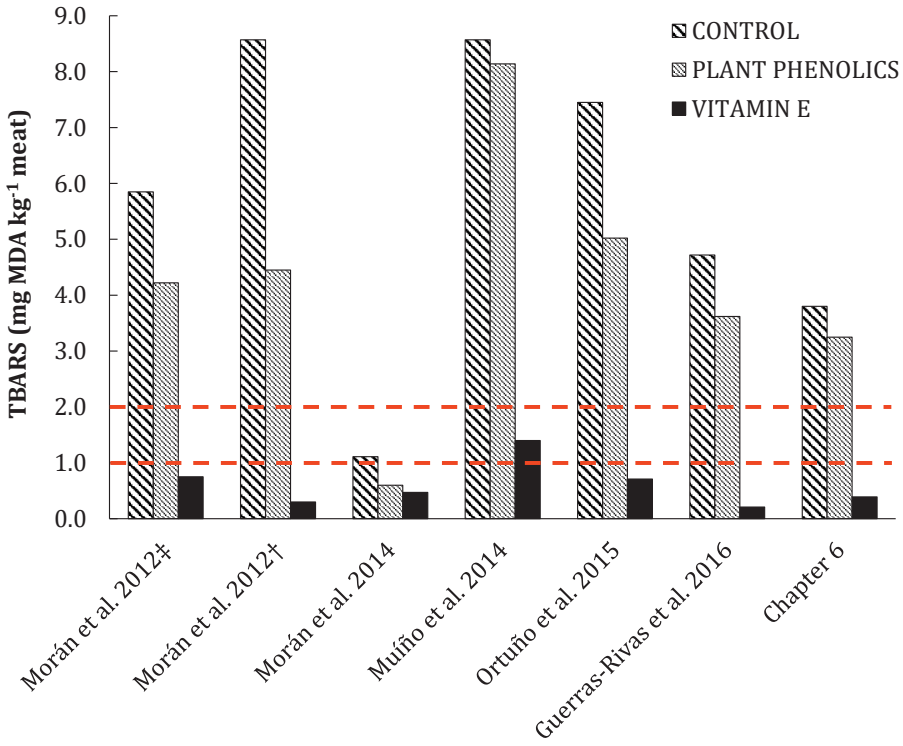


Figure 7.5 Thiobarbituric acid reactive substances (TBARS) (mg malondialdehyde (MDA) kg^{-1} meat) values obtained in lamb meat, following a dietary supplementation with different phenolic rich plant materials (Plant phenolics) or with *all-rac*-α-tocopheryl acetate (Vitamin E). The dashed red lines represent the thresholds for meat oxidation (TBARS values) in lambs, proposed by Ripoll et al. (2011) and Muíño et al.(2014). ‡ *longissimus thoracis et lumborum* muscle. † *gluteus medius* muscle.

Overall, supplementation of lamb diets with a standardized rosemary extract for 14 d before slaughter had no effect on lipid oxidation and colour stability parameters in lambs (**Chapter 6**). However, as previously highlighted, α-tocopherol supplementation significantly reduced lipid oxidation (TBARS values), improved colour stability (L^*, a^*, C^* and h values), reduced MetMb formation and meat discolouration ($A_{580-630}$ and I_{SO_2}). In **Figure 7.5**, TBARS results from studies that compared the supplementation of lamb diets with phenolic rich plant by-products with *all-rac*-α-tocopheryl acetate, on meat oxidation (TBARS values) after 14 d of display under retail conditions, are presented. Supplementation of lamb diets with *all-rac*-α-tocopheryl acetate consistently maintained the levels of TBARS within the thresholds of acceptability (1.0 to 2.0 mg MDA kg^{-1} meat). Whereas, with the exception of the study of

Morán et al. (2014), all other studies in which lambs were supplemented with phenolic rich plant by-products, reported TBARS values higher than 2.0 mg MDA kg^{-1} meat (**Figure 7.5**). Morán et al. (2014), supplemented milk fed lambs with carnosic acid (0.096 g kg^{-1} live weight) or *all-rac*-α-tocopheryl acetate (0.024 g kg^{-1}), until they were slaughtered at 11-12 kg live weight. Although MDA levels were generally low for all groups after 14 d of display (below 2.0 mg MDA kg^{-1}), significantly lower TBARS values were obtained when lambs were supplemented with both antioxidant sources. In line with that, Moran et al. (2012) found that supplementation of lamb diets with carnosic acid (1.2 g kg^{-1} dry matter) or with *all-rac*-α-tocopheryl acetate (0.6 g kg^{-1} dry matter) significantly reduced the development of TBARS in LTL and *gluteus medius* muscles when compared with non-supplemented lambs. However, supplementation of lamb diets with *all-rac*-α-tocopheryl acetate was 5.6 and 14.8 times more effective than carnosic acid at delaying meat oxidation in the LTL and *gluteus medius* muscles, respectively. Ortuño et al. (2015), also found that supplementation of lamb diets with rosemary diterpenes (0.6 g kg^{-1} compound feed) significantly reduced TBARS development in meat by 33%. However, in the same study, supplementation of lamb diets with *all-rac*-α-tocopheryl acetate (at 0.6 g kg^{-1} compound feed) was found to be 7.0 times more effective at delaying TBARS development in lamb meat than rosemary diterpenes. Likewise, supplementation of lamb diets with *all-rac*-α-tocopheryl acetate was found to be 5.8 (Muíño et al., 2014), 8.3 (**Chapter 6**) and 20.0 (Guerra-Rivas et al., 2016) times more effective than phenolic rich plant byproducts at delaying TBARS development in lamb meat stored for 14 d under retail conditions (**Figure 7.5**).

Under similar experimental conditions, the effects of phenolic rich plant by-products on oxidative stability of lamb meat are negligible, when compared with dietary applications with *all-rac*-α-tocopheryl acetate. However, the significant reductions in meat oxidation and colour deterioration parameters that have been reported following dietary supplementation of lamb diets with phenolic rich plant materials cannot be ignored.

Final considerations on the effects of phenolic rich plant materials on lamb meat

The mechanisms by which the phenolic compounds present in plant by-product affect the oxidative deterioration in lamb meat whether direct (Moñino et al., 2008), indirect/ synergistic (Halliwell et al., 2005, Kerem et al., 2006, Yamamoto et al., 2006) or through antimicrobial activity (Ortuño et al., 2014, Ortuño et al., 2015, Ortuño et al., 2017), remains unclear.

A major drawback when studying the effect of phenolic rich plant materials on the antioxidant status of lambs is the lack of product standardization. A wide range of plants and plant presentations have been tested in lambs, these include whole plants, plant parts (like leaves), essential oils, distillation residues and extracts, which vary greatly in their level of purity (**Chapter 4**). Interestingly, Yagoubi et al. (2018) when supplementing lambs with rosemary distillation residues found that supplemented lambs had a fourfold increase in α-tocopherol concentration in muscle, when compared with the non-supplemented lambs.

It is not clear, however, if the increased α-tocopherol concentrations in muscle, were due to high concentrations of α-tocopherol in the rosemary distillation residues, or due to a sparing effect of the phenolic compounds on α-tocopherol. This is an important point that has to be clarified, because even the slight increase in α-tocopherol concentration in muscle (from 0.6 to 0.9 mg kg^{-1} meat) has been shown to exponentially decrease lipid oxidation (González-Calvo et al., 2015). Therefore, future research should consider if phenolic rich plant materials might contain substantial amounts of other bioactive molecules, such as α-tocopherol, which can affect the interpretation of the results and its reproducibility.

CONCLUSIONS

The objective of this thesis was to investigate the effects of dietary supplementation of lightweight lambs with different antioxidants sources to extend the shelf life of meat under retail conditions. The studies reported in these chapters have contributed to improve our understanding on α-tocopherol physiology and tissue deposition in lambs, particularly the differences between RRR- and *all-rac*-α-tocopheryl acetate, supplemented at increasing levels, and ultimately their effect on the oxidative and colour stability of meat stored for short- or long-periods under retail conditions. Phenolic rich plant materials were also investigated as an alternative supplementation strategy for supranutritional applications of α-tocopherol, phenolic rich plant materials from different botanical origins were screened and evaluated for their tissue antioxidant capacity. In addition, a standardized phenolic rich plant extract was contrasted with *all-rac*-α-tocopheryl acetate in its effectiveness to delay meat oxidation and colour deterioration, in lamb meat stored for a short-period under retail conditions. Briefly, the studies described in this thesis have led to the following conclusions:

- Different tissues respond differently to incremental dietary supplementation of RRR- and *all-rac*-α-tocopheryl acetate indicating that a single ratio cannot accurately describe the difference between RRR- and *all-rac*-α-tocopheryl acetate.
- Supplementation of lamb diets with α-tocopherol has a clear inhibitory effect on meat oxidation and colour deterioration in lamb meat stored under retail conditions. This effect is related to the increase in α-tocopherol content in muscle, which delays the initiation and propagation of oxidative reactions in meat.
- RRR-α-tocopheryl acetate is substantially more effective than *all-rac*-α-tocopheryl acetate at improving the shelf life of lamb meat.
- Dietary *all-rac*-α-tocopheryl acetate is an effective strategy not only at extending the self-life of meat stored for short-term but also, in preserving meat from oxidation and colour deterioration in long-term storage periods.
- Supplementation of lamb diets with selected plant extracts can affect the radical scavenging activity in muscle, liver and kidney. However, the relevance of these results is debatable due to contrasting outcomes between assays in the same tissue.

- It is not advisable to discriminate or rank plant extracts in their post-morten antioxidant capacity in tissues with DPPH•, TEAC, FRAP and ORAC assays.
- Under similar experimental conditions, the effects of phenolic rich plant by-products on oxidative stability of lamb meat are negligible, when compared with dietary applications with *all-rac*-α-tocopheryl acetate.

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Appendices

SUMMARY

Meat quality, as defined by its colour stability is the main sensory attribute of lamb meat that drives consumer preference and purchase decision. The 'bright red' colour in meat, perceived by consumers as an indicator of freshness and superior product quality, can be further enhanced by highly oxidizing packaging methods. Overtime, these packaging methods also accelerate the deterioration of colour, flavour and texture. To delay that, meat processors and retailers have adopted the use of antioxidants into animal diets. As a result, the use of supra-nutritional doses of α-tocopherol (*all-rac*-α-tocopheryl acetate) has become a common practice in South European lamb production systems. Alternatively, phenolic rich plant by-products are capturing the interest of nutritionists and meat scientists, which are continuously looking for alternatives to the α-tocopherol usage. The aim of this thesis was to provide a scientific basis for the determination of the optimal supplementation level of α-tocopherol (RRR- vs. *all-rac*-α-tocopheryl acetate) and the use of phenolic rich plant by-products as an alternative to extend shelf life of lamb meat kept under typical retail conditions.

The experiment described in **Chapter 2**, aimed to investigate the effect of different dietary levels of two α-tocopherol sources (RRR- and *all-rac*-α-tocopheryl acetate) on total α-tocopherol concentration and stereoisomer distribution in muscle, heart, liver and spleen of lambs. Moreover, it was hypothesized, that the single ratio of 1.36 used to discriminate both α-tocopherol was inadequate to predict differences in α-tocopherol deposition in the different tissues. A total of 360 *Rasa Aragonesa* breed lambs, were fed increasing amounts of *all-rac*-α-tocopheryl acetate (0.25, 0.5, 1.0 and 2.0 g kg^{-1} compound feed) or RRR-α-tocopheryl acetate (0.125, 0.25, 0.5 and 1.0 g kg^{-1} compound feed) for 14 d before slaughter. Alpha-tocopherol supplementation had no effect on average daily gain, feed intake and feed efficiency. The tissue with the highest α-tocopherol concentration was the liver, followed by spleen, heart and muscle. At similar supplementation levels, the ratios between RRR- and *all-rac*-α-tocopheryl acetate increased with the increasing α-tocopherol supplementation (at 0.25 and 1.0 g kg^{-1} compound feed), from 1.06 to 1.16 in muscle, 1.07 to 1.15 in heart, 0.91 to 0.94 in liver and 0.98 to 1.10 in spleen. Increasing *all-rac*-α-tocopheryl acetate supplementation increased the proportions of RRS-, RSR-, and RSS- stereoisomers at the cost of RRR-α-tocopherol, which indicates that relative bioavailability of RRR- and *all-rac*-α-tocopheryl acetate, depends on stereospecific pathways for tissue distribution. Therefore, the relative bioavailability of RRR- and *all-rac*-α-tocopheryl acetate is dose- and tissue-dependent and a single ratio to discriminate the two sources cannot be used.

In **Chapter 3**, the effect of both α-tocopherol sources (RRR- and *all-rac*-α-tocoheryl acetate) on meat colour and lipid stability of lamb meat, stored for 14 d under retail conditions was quantified. The LTL muscle from the lambs described in **Chapter 2**, were sliced into small steaks, packed under modified atmosphere packaging (with 70% O_2 + 30% CO_2) and displayed under retail conditions (4 ± 1ºC, with a daily light exposure to 14h of light at 1200 lux) for 14 d. Display time had a larger effect on lipid oxidation, colour stability, myoglobin

forms and meat discolouration parameters than α-tocopherol supplementation. However, α-tocopherol dosage substantially extended meat shelf-life as indicated by lipid oxidation (TBARS values), redness, hue angle, metmyoglobin formation, $A_{580-630}$ and I_{s02}. Based on the surface response plots comparing the effects of RRR- and *all-rac*-α-tocopheryl acetate, was found that RRR-α-tocopheryl acetate was 1.8, 1.9, 1.5 and 1.8 times more effective than *all-rac*-α-tocopheryl acetate at controlling the development of TBARS, *a** values, *h* values and MetMb% respectively, during the 14 d of display. Thus, illustrating that the use of a single ratio to discriminate both α-tocopherol sources is not appropriate to describe their effectiveness in enhancing different meat quality properties in meat.

From literature and results presented in **Chapter 3**, the positive effect of α-tocopherol supplementation of lamb diets, on meat oxidative and colour stability during short-term refrigerated conditions is undisputed. However, there were concerns that the α-tocopherol present in muscle following a dietary supplementation with vitamin E was not effective at delaying meat oxidation and colour loss in thawed lamb meat maintained under retail conditions, after frozen stored up to 9 months. Therefore, **Chapter 4** describes an experiment that aimed to investigate the effect of single, high dose of *all-rac*-α-tocopheryl acetate (1.0 g kg^{-1} compound feed) on physicochemical and fatty acid stability of fresh and thawed lamb leg chops, frozen stored for 3, 6 and 9 months. Ninety-six male *Rasa Aragonesa* breed lambs were fed either a basal diet or the basal diet plus 1.0 g kg^{-1} compound feed of *all-rac*-α-tocopheryl acetate, for 14 d before slaughter. The right leg of each carcass was removed and assigned to one of four different frozen storage durations: 0 d, 3, 6 and 9 months. Following the frozen storage, legs were sliced and packed under modified atmosphere packaging, and displayed under retail conditions during 9 d. Supplementation of lamb diets with 1.0 g kg^{-1} compound feed of *all-rac*-α-tocopheryl acetate, effectively reduced lipid oxidation, protected polyunsaturated fatty acids from oxidation, delayed MetMb formation and improved the overall colour stability in meat, during the 9 d of display. Overall, these results highlighted the importance of α-tocopherol supplementation not only on short-term stored meat (**Chapter 3** and **6**) but also in preserving meat from oxidation and colour deterioration in long term storage (**Chapter 4**).

Because of their high *in vitro* antioxidant potential, culinary herbs and spices have been proposed as a replacement for supranutritional applications of α-tocopherol in lambs. Like α-tocopherol, the direct antioxidant capacity of a dietary phenolic rich plant material is affected by its absorption and deposition in the tissue. **Chapter 5** describes a study that aimed to investigate whether the supplementation of lamb diets with plant extracts from 11 culinary herbs and spices effects the antioxidant capacity of selected lamb tissues. Two hundred and eighty-eight *Rasa Aragonesa* male lambs (23.5 ± 1.38 kg) were assigned to one of 12 diets consisting of a basal compound feed or the basal compound feed supplemented with 5.0 g kg^{-1} with 11 different plant extracts (bay, marjoram, oregano, rosemary, thyme, turmeric, cumin, caraway, dill, cinnamon and nutmeg). To access the antioxidant capacity in muscle, liver and kidney, four antioxidant assays were performed in parallel (TEAC,

FRAP, ORAC and DPPH•). Dietary supplementation with plant extracts had no effect on feed intake, growth performance or antioxidant activity in blood. No single plant extract affected consistently the tissue antioxidant activity as assessed by the four different assays. Supplementation of lamb diets with bay, oregano, turmeric, cumin, caraway, dill, cinnamon and nutmeg revealed that although not consistent, these plant extracts led to significant directional changes in the antioxidant capacity in the different tissues. However, due to the divergent results of the different assay for the same tissue, it would be misleading to evaluate plant extracts using this approach. Instead, future work should focus on analytical methods that reflect better the target "endpoint", which in this thesis was to delay lipid oxidation and colour deterioration in lamb meat. With that in mind, **Chapter 6** of this thesis, describes a study were a standardized plant extract (rosemary extract) was compared with α-tocopherol in their ability to improve meat colour and oxidative stability parameters in fresh lamb meat stored for 14 d under retail conditions. Indoor concentrate fed lambs were supplemented with increasing levels of *all-rac*-α-tocopheryl acetate (0.25, 0.5, 1.0 g kg^{-1} compound feed) or a rosemary extract (0.2, 0.4, 0.8 g kg^{-1} compound feed), for 14 d before slaughter. Supplementation of either antioxidant source had no effects on average daily weight gain, feed intake or feed efficiency. Like in **Chapter 3** and **4**, supplementation of lamb diets with *all-rac*-α-tocopheryl acetate significantly reduced lipid oxidation, improved colour stability, affected the development of the myoglobin forms and delayed meat discoloration. However, supplementation of lamb diets with rosemary extracts had no effect, in the aforementioned meat quality parameters. Overall, based on the work described in this thesis and literature studies, under similar experimental conditions, the effect of dietary supplementation of lamb diets with phenolic rich plant materials on the oxidative stability of lamb meat is negligible, when compared with α-tocopherol.

Peer-Reviewed Scientific Publications

Vallverdú-Queralt, A., J. Regueiro, M. Martínez-Huélamo, J. F. Rinaldi Alvarenga, **L. N. Leal**, and R. M. Lamuela-Raventos. 2014. A comprehensive study on the phenolic profile of widely used culinary herbs and spices: Rosemary, thyme, oregano, cinnamon, cumin and bay. Food Chemistry 154:299-307.

Vallverdú-Queralt, A., J. Regueiro, J. F. Rinaldi Alvarenga, M. Martinez-Huelamo, **L. N. Leal**, and R. M. Lamuela-Raventos. 2015. Characterization of the phenolic and antioxidant profiles of selected culinary herbs and spices: caraway, turmeric, dill, marjoram and nutmeg. Food Science and Technology 35:189-195.

Meale, S. J., **L. N. Leal**, J. Martín-Tereso, and M. A. Steele. 2015. Delayed weaning of Holstein bull calves fed an elevated plane of nutrition impacts feed intake, growth and potential markers of gastrointestinal development. Animal Feed Science and Technology 209:268-273.

MacPherson, J. A. R., H. Berends, **L. N. Leal**, J. P. Cant, J. Martín-Tereso and M. A. Steele. 2016. Effect of plane of milk replacer intake and age on glucose and insulin kinetics and abomasal emptying in female Holstein Friesian dairy calves fed twice daily. Journal of Dairy Science 99:8007-8017.

Steele, M. A., J. H. Doelman, **L. N. Leal**, F. Soberon, M. Carson and J. A. Metcalf. 2017. Abrupt weaning reduces postweaning growth and is associated with alterations in gastrointestinal markers of development in dairy calves fed an elevated plane of nutrition during the preweaning period. Journal of Dairy Science 100:5390-5399.

Bellés, M., **L. N. Leal**, V. Díaz, V. Alonso, P. Roncalés and J. A. Beltrán. 2018. Effect of dietary vitamin E on physicochemical and fatty acid stability of fresh and thawed lamb. Food Chemistry 239:1-8.

Leal, L. N., J. A. Beltrán, V. Alonso, J. M. Bello, L. A. den Hartog, W. H. Hendriks and J. Martín-Tereso. 2018. Dietary vitamin E dosage and source affects meat quality parameters in light weight lambs. Journal of the Science of Food and Agriculture 98:1606-1614.

Leal, L. N., J. M. Romao, G. J. Hooiveld, F. Soberon, H. Berends, M. V. Boekshoten, M. E. Van Amburgh, J. Martín-Tereso and M. A. Steele. 2018. Nutrient supply alters transcriptome regulation in adipose tissue of pre-weaning Holstein calves. PLOS ONE 13:e0201929.

Berends, H., M. Vidal, M. Terré, **L. N. Leal**, J. Martín-Tereso and A. Bach. 2018. Effects of fat inclusion in starter feeds for dairy calves by mixing increasing levels of a high-fat extruded pellet with a conventional highly fermentable pellet. Journal of Dairy Science 101:10962-10972.

Leal, L. N., S. K. Jensen, J. M. Bello, L. A. Den Hartog, W. H. Hendriks and J. Martín-Tereso. 2019. Bioavailability of alpha-tocopherol stereoisomers in lambs depends on dietary doses of *all-rac*- or RRR-alpha-tocopheryl acetate. Animal 13:1874-1882.

Hare, K. S., **L. N. Leal**, J. M. Romao, G. J. Hooiveld, F. Soberon, H. Berends, M. E. Van Amburgh, J. Martín-Tereso and M. A. Steele. 2019. Preweaning nutrient supply alters mammary gland transcriptome expression relating to morphology, lipid accumulation,

DNA synthesis, and RNA expression in Holstein heifer calves. Journal of Dairy Science 102:2618-2630.

Amado, L., H. Berends, **L. N. Leal**, J. Wilms, H. Van Laar, W. J. J. Gerrits and J. Martín-Tereso. 2019. Effect of energy source in calf milk replacer on performance, digestibility, and gut permeability in rearing calves. Journal of Dairy Science 102:3994-4001.

Leal, L. N., M. J. Jordán, J. M. Bello, J. Otal, L. A. Den Hartog, W. H. Hendriks and J. Martín-Tereso. 2019. Dietary supplementation of 11 different plant extracts on the antioxidant capacity of blood and selected tissues in lightweight lambs. Journal of the Science of Food and Agriculture 99:4296-4303.

Welboren, A. C., **L. N. Leal**, M. A. Steele, M. A. Khan and J. Martín-Tereso. 2019. Performance of ad libitum fed dairy calves weaned using fixed and individual methods. Animal 21:1-8 [Epub ahead of print].

Contributions to Conferences, Symposia, and Other Scientific Output

Carvalho, I. P. C., **L. N. Leal**, H. Berends and J. Martín-Tereso. 2015. Effects of preweaning nutrient supply on growth and pre and post-weaning glucose tolerance test in male Holstein calves. In: Journal of Animal Science, Vol. 93 (Suppl. 3): 310-310. 2015 Joint Annual Meeting of ADSA-ASAS. Orlando, Florida, USA.

MacPherson, J. A. R., H. Berends, **L. N. Leal**, J. Martín-Tereso and M. A. Steele. 2015. Effects of plane of nutrition on glucose tolerance test kinetics pre- and postweaning in Holstein calves fed twice daily. In: Journal of Animal Science, Vol. 93 (Suppl. 3): 56-56. 2015 Joint Annual Meeting of ADSA-ASAS. Orlando, Florida, USA.

Steele, M. A., **L. N. Leal**, M. Carson, J. H. Doelman and J. A. Metcalf. 2015. Gradual weaning affects pre- and postweaning feed intake, growth, and gastrointestinal development in Holstein calves fed an elevated plane of nutrition during the pre-weaning stage. In: Journal of Animal Science, Vol. 93 (Suppl. 3): 242-242. 2015 Joint Annual Meeting of ADSA-ASAS. Orlando, Florida, USA.

Leal, L. N., V. Alonso, J. A. Beltrán, L. A. Den Hartog, W. H. Hendriks and J. Martín-Tereso. 2015. The effect of dietary vitamin E source and dose on lamb meat colour stability under retail conditions. In: Proceedings of the 61st International Congress of Meat Science and Technology. 2015 61st ICOMST. Clermont Ferrand, France.

Leal, L. N., G. J. Hooiveld, M. A. Steele, M. V. Boekschoten, F. Soberon, M. E. Van Amburgh and J. Martín-Tereso. 2016. Effect of nutrient supply on mammary gland development and gene expression in pre-weaned calves. In: Book of abstracts of the 67th Annual Meeting of the European Federation of Animal Science. EAAP publication No. 22: 279-279. Belfast, United Kingdom.

Leal, L. N., S. K. Jensen, J. M. Bello, L. A. Den Hartog, W. H. Hendriks and J. Martín-Tereso. 2017. Supplementation of natural and synthetic vitamin E on alpha tocopherol concentration in lamb tissues. In: Book of abstracts of the 68th Annual Meeting of

the European Federation of Animal Science. EAAP publication No. 23: 355-355. Tallin, Estonia.

Leal, L. N., G. J. Hooiveld, F. Soberon, H. Berends, M. V. Boekschoten, M. A. Steele, M. E. Van Amburgh and J. Martín-Tereso. 2017. Neonatal nutrient supply affects pancreatic development in Holstein calves. In: Book of abstracts of the 68th Annual Meeting of the European Federation of Animal Science. EAAP publication No. 23: 387-387. Tallin, Estonia.

Leal, L. N., G. J. Hooiveld, F. Soberon, H. Berends, M. V. Boekschoten, M. A. Steele, M. E. Van Amburgh and J. Martín-Tereso. 2017. Pre-weaning nutrient supply affects gene expression profiles in bone marrow and muscle in calves. In: Book of abstracts of the 68th Annual Meeting of the European Federation of Animal Science. EAAP publication No. 23: 447-447. Tallin, Estonia.

Hare, K., J. Romao, **L. Leal**, G. Hooiveld, F. Soberon, H. Berends, M. Boekschoten, M. Van Amburgh, J. Martín-Tereso and M. Steele. Increased pre-weaning metabolizable energy supply affects the expression of genes related to adipogenesis and lipid metabolism in the skeletal muscle of prepubertal Holstein heifers. In: Journal of Animal Science, Vol. 96 (Suppl. 3): 348-349. 2018 ASAS-CSAS Annual Meeting and Trade Show. Vancouver, Canada.

Romao, J., **L. Leal**, G. Hooiveld, F. Soberon, H. Berends, M. Boekschoten, M. Van Amburgh, J. Martín-Tereso and M. Steele. 2018. Nutrient supply alters adipose tissue physiology in pre-weaned calves. In: Journal of Animal Science, Vol. 96 (Suppl. 3): 417-417. 2018 ASAS-CSAS Annual Meeting and Trade Show. Vancouver, Canada.

Welboren, A., B. Hatew-Chuko, H. Berends, **L. Leal**, J. Martín-Tereso and M. Steele. 2018. Energy sources of milk replacer affects glucose homeostasis in neonatal calves. In: Journal of Animal Science, Vol. 96 (Suppl. 3): 172-172. 2018 ASAS-CSAS Annual Meeting and Trade Show. Vancouver, Canada.

Welboren, A., **L. Leal**, M. Steele, A. Khan and J. Martín-Tereso. 2018. Weaning of ad libitum fed dairy calves with automated feeders using fixed and individual methods. In: Journal of Animal Science, Vol. 96 (Suppl. 3): 449-450. 2018 ASAS-CSAS Annual Meeting and Trade Show. Vancouver, Canada.

Curriculum Vitae

Leonel Neto Leal was born on April 18, 1986 and grew up in Paços de Ferreira, Portugal. He obtained his high school diploma from Escola Secundária D. Dinis of Santo Tirso in 2005, after which he started his BSc in Zootechnical Engineering at the University of Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal. After his graduation in 2008, he started his MSc in Zootechnical Engineering at the same University. His major specialization was animal production and nutrition, and for his major thesis, he investigated the effect of high planes of milk replacer feeding on growth performance and general health status of rearing calves at the Ruminant Research Centre of Nutreco in Boxmeer, the Netherlands. In January 2011, after obtaining his MSc, Leonel joined Nutreco as a ruminant researcher. At the beginning of his professional career, his focus was devoted to small ruminant nutrition, health and productivity. In 2013, he was given the opportunity to start a PhD at the Animal Nutrition group of Wageningen University (Wageningen, the Netherlands), focusing on improving meat colour and oxidative stability of lamb meat by dietary antioxidant supplementation, resulting in this thesis. Apart from his PhD appointment, for the past 6 years, Leonel has been focusing as well on functional nutrition of calves, were he has been leading the LifeStart science platform within Trouw Nutrition R&D. After completing his PhD, Leonel aims to continue to expand his knowledge on functional nutrition of dairy and beef calves.

Training and Supervision Plan

Completed in fulfilment of the requirements for the education certificate of the Wageningen Institute of Animal Sciences (WIAS)

The Basic Package (3 ECTS1)	**Year**
WIAS Introduction Course	2014
Philosophy of Science and/or Ethics	2018
Scientific Exposure (12 ECTS)	
ADSA Discovery Conference – Immunity, nutrition, and management of calves	2013
7th ISANH World Congress on Polyphenols	2013
WIAS Science Day	2014
ICOMST – International Conference on Meat Science and Technology (1 poster presentation)	2015
EAAP – 67th Annual Meeting of the European Federation of Animal Science (1 oral presentation)	2016
EAAP – 68th Annual Meeting of the European Federation of Animal Science (3 oral presentations)	2017
XXII Congresso Internacional ANEMBE de Medicina Bovina (1 oral presentation)	2017
Disciplinary and interdisciplinary courses (5.5 ECTS)	
WIAS course: Advanced Statistics Course on Design of Experiments	2013
VLAG course: 3rd International Advanced Course on Epigenesis and Epigenetics	2014
WIAS course: Statistics for the Life Sciences	2017
VLAG course: Applied Biocatalysis	2019
VLAG course: Healthy and Sustainable Diets: synergies and trade-offs	2019
Statutory Courses (4.4 ECTS)	
Use of Laboratory Animals: basic course and species specific ruminants	2018
Professional Skills and Support Courses (3.1 ECTS)	
WIAS course: High Impact writing in Science	2017
WIAS course: Survival Guide to Peer-Review	2017
Project and Time Management	2018
Research and Didactic Skills Training (13.5 ECTS)	
Preparing Own PhD Proposal	2014
Supervising MSc Thesis (3x)	2014-2017
Lecturing "Successful Dairy Heifer Rearing" (2x)	2017-2018

Total 41.5 ECTS

[1]One ECTS credit equals a study load of 28 hours.

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