UNIVERSIDADE DE ÉVORA

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

Dietary manipulation to improve the nutritional value of lipids from lamb meat

Eliana Alexandra Sousa Jerónimo

Tese de Doutoramento em Ciências Agrárias

ORIENTADOR

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Aos meus sobrinhos, Joana e Afonso

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ABSTRACT

Dietary manipulation to improve the nutritional value of lipids from lamb meat

Lamb meat is characterized by high contents of saturated fatty acids and low levels of polyunsaturated fatty acids (PUFA), properties that are regarded as being negative to human health. To meet the nutritional recommendations is necessary improving the fatty acid (FA) composition of lamb meat. The main motivation of this thesis was explored some nutritional strategies that allows improve the nutritional value of lipid fraction from lamb meat. Data presented here show that supplementation of diets with vegetable oils rich in PUFA is an effective approach to decrease the saturation of lamb meat and increase its content in PUFA. Moreover, supplementation with blend of sunflower and linseed oils allowed increase simultaneously meat content in conjugated isomers of linoleic acid and *n*-3 long chain PUFA. Inclusion of sodium bentonite and *Cistus ladanifer* in oil supplemented diets also showed to be a good approach to improve the FA composition of lamb meat.

Keywords: lamb meat; fatty acid composition; lipid metabolism; sunflower oil; linseed oil; sodium bentonite; grape seed extract; *Cistus ladanifer*.

RESUMO

Manipulação da dieta para melhorar o valor nutricional dos lípidos da carne de borrego

A carne de borrego é caracterizada por altos teores em ácidos gordos (AG) saturados e baixos níveis de ácidos gordos polinsaturados (AGPI), propriedades que são consideradas prejudicais para a saúde humana. Para atender às recomendações nutricionais é necessário melhorar a sua composição em AG. A principal motivação desta tese foi explorar algumas estratégias nutricionais que permitam melhorar o valor nutricional da fracção lipídica da carne de borrego. Os resultados obtidos mostram que a suplementação das dietas com óleos vegetais ricos em AGPI é uma abordagem eficaz para reduzir a saturação da carne de borrego e aumentar o seu conteúdo em AGPI. Além disso, a suplementação com mistura de óleos de girassol e de linho permitiu aumentar simultaneamente o conteúdo em isómeros conjugados do ácido linoleico e em AGPI *n*-3 de cadeia longa. A inclusão de bentonite sódica e de *Cistus ladanifer* em dietas suplementadas com óleo também mostrou ser uma boa abordagem para melhorar a composição em AG da carne de borrego.

Palavras-chave: carne de borrego; composição em ácidos gordos; metabolismo lipídico; óleo de girassol; óleo de linho; bentonite sódica, extracto de grainha de uva; *Cistus ladanifer*.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS AND SYMBOLS

*a**	Redness (CIELAB colour dimension)
AOAC	Association of Official Analytical Chemists
ARA	Arachidonic acid; 20:4*n*-6; 20:4 *cis*-5, *cis*-8, *cis*-11, *cis*-14
*b**	Yellowness (CIELAB colour dimension)
BH	Biohydrogenation
BI	Biohydrogenation intermediates
°C	Degree Celsius
*C**	Chroma – colour saturation
C. ladanifer	*Cistus ladanifer* L.
CACI-MS	Covalent adduct chemical ionization mass spectrometry
CLA	Conjugated linoleic acid
cm	centimetre
CoA	Coenzyme A
CT	Condensed tannins
DHA	Docosahexaenoic acid; 22:6*n*-3; 22:6 *cis*-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19
DM	Dry matter
DMI	Dry matter intake
DPA	Docosapentaenoic acid; 22:5*n*-3; 22:5 *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19
EPA	Eicosapentaenoic acid; 20:5*n*-3; 20:5 *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17
FA	Fatty acids
FAME	Fatty acid methyl esters
g	Gram
GC-FID	Gas chromatography with a flame ionization detector
GC-MS	Gas chromatography mass spectrometry
*H**	Hue angle
HPLC	High-performance liquid chromatography
IMFA	Intramuscular fatty acids
kg	Kilogram
KKCF	Kidney knob channel fat
*L**	Lightness (CIELAB colour dimension)
LC-PUFA	Long chain polyunsaturated fatty acids - ≥ 20 carbons
LDL	Low density lipoproteins
LO	Linseed oil
m	Milimolar
MDA	Malondialdehyde
mg	Miligram
min	Minute

MJ	Mega joule
ml	Mililitre
mm	Milimetre
mM	Milimolar
mRNA	Messenger RNA
MUFA	Monounsaturated fatty acids
n-3 PUFA	Sum of *n*-3 fatty acids = 18:3*n*-3 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3
n-3 LC-PUFA	Sum of *n*-3 long chain fatty acids = 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3
n-6 PUFA	Sum of *n*-6 fatty acids = 18:2*n*-6 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6
n-6 LC-PUFA	Sum of *n*-6 long fatty acids = 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6
n-6/*n*-3	Sum of *n*-6 PUFA / Sum of *n*-3 PUFA
NDF	Neutral detergent fibre
NL	Neutral lipids
nm	Nanometre
ns	Not significant
O	Oil blend supplementation
P	Probability
pH	Potential of hydrogen
PL	Polar lipids
PUFA	Polyunsaturated fatty acids
P/S	(18:2*n*-6 + 18:3*n*-3)/(12:0 + 14:0 + 16:0 + 18:0)
%	Percentage
SAS	Statistical analysis system (Software package)
SB	Sodium bentonite
SCD	Stearoyl-CoA desaturase
SD	Standard deviation
SEM	Standard error of the mean
SFA	Saturated fatty acids
SO	Sunflower oil
TBARS	Thiobarbituric acid reactive substances
TFA	*Trans* fatty acids
rpm	Revolutions per minute
rRNA	Ribosomal RNA
µl	Microlitre
µm	Micrometre
v	volume
UV	Ultraviolet
wt	weight

INTRODUCTION

In recent years, fatty acid (FA) composition of ruminant fat has received much interest due to its implications for human health and meat quality. The edible products of ruminant have been associated to an increase of the risk of cardiovascular diseases, due to its high content in saturated fatty acids (SFA) and *trans* fatty acids (TFA) and low content in polyunsaturated fatty acids (PUFA). In rumen the dietary lipids are extensively hydrolysed and the unsaturated FA liberated (mostly C_{18} PUFA) are also extensively biohydrogenated (Jenkins *et al.*, 2008), resulting in a high level of SFA in fat, as well as in several unsaturated C_{18} FA, including *trans* FA (Bessa *et al.*, 2007). The nutritional recommendations indicate that the SFA content of most Western-type diets should be reduced and the PUFA content should be increased, particularly in *n*-3 PUFA (WHO, 2003). These recommendations have led to declining the consumption of ruminant edible products. However, ruminant edible products may also be a good dietary source of some health benefit FA, such as *n*-3 PUFA and conjugated linoleic acid isomers (CLA). The ruminant fats are naturally rich in CLA isomers, particularly of rumenic acid (18:2 *cis*-9, *trans*-11), and are the main sources of these isomers in the human diet (Chin *et al.*, 1992). The 18:2 *cis*-9, *trans*-11 is formed by ruminal biohydrogenation of linoleic acid (18:2*n*-6) (Harfoot and Hazelwood, 1997) and mainly by endogenous conversion of vaccenic acid (18:1 *trans*-11) by stearoyl-CoA desaturase (SCD) in tissues (Griinari *et al.*, 2000). The 18:1 *trans*-11 is an intermediate produced during ruminal biohydrogenation of both 18:2*n*-6 and α-linoleic acid (18:3*n*-3) (Harfoot and Hazelwood, 1997).

Manipulation of FA composition of ruminant fat, to reduce the SFA and increase the PUFA, particularly in *n*-3 long chain polyunsaturated fatty acids (≥ 20 carbons, LC-PUFA) and CLA contents, is a major target in ruminant meat and milk research. Several

studies have been show that there is an opportunity to improve the nutritional value of ruminant fat through the changes in diet. Lipid supplementation is a effective mean of improving the FA composition of ruminant fat (Chilliard and Ferlay, 2004, Scollan *et al.*, 2006, Sinclair, 2007). However, ruminal biohydrogenation (BH) of unsaturated FA constitute the main limitation to enrichment of PUFA in ruminant fat by unprotected lipid supplementation. Thus, the modulation of ruminal BH in order to increase the dietary PUFA and benefit biohydrogenation intermediates (BI) that escape from rumen may be a good approach for to improving the healthiness of ruminant meat and milk. Moreover, the intramuscular FA deposition results from balance between uptake, *de novo* synthesis and mobilization. Many these metabolic pathways are strongly regulated in order to maintain the homeostasis, but are also influenced by several factors, such as genotype, gender, age and mainly by dietary factors. The strong regulation of FA content and composition in membrane phospholipids (Raes *et al.*, 2004), the main local of PUFA deposition, suggest that there may be limitation to the incorporation of higher amount of PUFA in phospholipids. Moreover, it has been postulated that deleterious effects of some *trans* PUFA are due to their incorporation in phospholipids. However, information about deposition of most FA, as BI in intramuscular polar (PL) and neutral lipid (NL) fractions is very scarce.

Globally, this work aims to contribute for the study the lipid metabolism in ruminants, particularly contribute for current knowledge over FA metabolism in rumen and muscle. We intend explore several nutritional approach that might modulate the ruminal BH in order to increase the flow of dietary PUFA and benefit BI from rumen, increasing thus availability of these FA for deposition in muscle. We also intend to explore the intramuscular FA deposition between polar and neutral lipid fractions.

This thesis is structured in 7 chapters. In **Chapter 1**, "Scientific background and objectives", the general FA composition of ruminant meats will be present, and the nutritional value of FA, consumptions and dietary recommendations will be summarily reviewed. Subsequently, will be approached the ruminant lipid metabolism, particularly ruminal metabolism, intestinal absorption and transport, as well as deposition and endogenous synthesis in muscle. Finally, will be reviewed the dietary strategies for improve the nutritional value of lamb meat. After this brief overview, the specific objectives of this work will be described. The chapters 2 to 6 are based on scientific manuscripts, already published (4) or provisionally accepted for publication with revisions (1) in international peer reviewed journals.

Previous data obtained by our group showed that blend of sunflower and linseed oils may a good approach to obtain simultaneously lamb meat enriched in CLA and *n*-3 LC-PUFA (Bessa *et al.*, 2007). In order to explore this approach, more levels of stepwise substitution of sunflower oil with linseed oil were tested, and results are present in **Chapter 2**. Detailed C_{18} FA distribution between intramuscular PL and NL obtained in this trial is reported in **Chapter 3**.

In **Chapter 4** are presents the results obtained from one trial where we tested if incorporation of sodium bentonite in diets supplemented with vegetable oils rich in PUFA might modify the ruminal BH, increasing escape of dietary PUFA from rumen and change the BI profile.

Following the same objective, *i.e.* improve the nutritional value of lamb meat by modulation the ruminal BH, we explored the utilization of dietary condensed tannins (CT) sources in diets supplemented with vegetable oils rich in PUFA (**Chapter 5**). However, the susceptibility of PUFA to oxidation, might limit the nutritional strategies

which aim at increasing PUFA concentration in meat. Several CT sources show antioxidant activity. Thus, in this trial we also evaluated if inclusion of CT sources in diets affect the colour stability and antioxidant potential of lamb meat during storage (**Chapter 6**).

Finally, chapter 7 intends to summarise and discuss in an integrated form the results obtained in each of the five previous chapters, the main conclusions and relevant perspectives for future research are show in this topic.

CHAPTER 1

SCIENTIFIC BACKGROUND AND OBJECTIVES

1.1. FAT CONTENT AND FATTY ACID COMPOSITION OF RUMINANT MEAT

Total fat content of meat is affected by a several factors, including species, age, weight at slaughter, sex, breed, part of the carcass and diet (Valsta *et al.*, 2005, Sinclair, 2007). The intramuscular lipid content of most ruminant meat is less than 50 g/kg (Scollan *et al.*, 2006, Sinclair, 2007), a value generally considered to characterize a low-fat food.

Ruminant meats are known to have higher content of SFA and lower levels of PUFA compared to those from non-ruminant origin. In general, intramuscular fat of ruminant animals is composed of approximately 45-55% of SFA, 45-50% of monounsaturated FA (MUFA) and relatively minor amounts of PUFA (Givens *et al.*, 2006). Thus, the polyunsaturated/saturated ratio (P/S) in ruminant meats is typically low, around 0.1-0.2 (Raes *et al.*, 2004, Sinclair, 2007). Palmitic (16:0) and stearic (18:0) acids are the main SFA, while oleic (18:1 *cis*-9) is the predominant MUFA (Scollan *et al.*, 2006, Sinclair, 2007). The 18:2*n*-6, 18:3*n*-3 and arachidonic acid (20:4*n*-6; ARA) are the main PUFA (Raes *et al.*, 2004). Meat also contains important levels of *n*-3 LC-PUFA, but much lower than in fish (Howe *et al.*, 2007). Comparing meats, the highest concentrations of eicosapentaenoic (20:5*n*-3; EPA) and docosahexaenoic (22:6*n*-3; DHA) acids (EPA+DHA) were found in poultry meat (~2% of total FA), followed by sheep meat (~1%), pork (~0.6%), and beef and veal (~0.5%) (Givens *et al.*, 2006). Docosapentaenoic acid (22:5*n*-3; DPA) is the predominant *n*-3 LC-PUFA in meat, and beef and lamb meat has a higher proportion of DPA than fish (Howe *et al.*, 2006, Howe *et al.*, 2007). Ruminant meats usually have a more favourable *n*-6/*n*-3 ratio when compared with non-ruminant animals, due to the relatively higher content of 18:3*n*-3 in pastures and forages (Prates and Bessa, 2009).

Trans FA occur in higher amounts in ruminant meats than in those from non-ruminant, representing usually 2–4% of total FA in ruminant meats (Valsta *et al.*, 2005). *Trans* 18:1 isomers constitute about 80% of total TFA in meat and meat products, being the 18:1 *trans*-11 the main *trans* 18:1 isomer (about 60% of total *trans* 18:1 isomer) (Prates and Bessa, 2009). However, high concentrations of 18:1 *trans*-10 had been found in meat of ruminants fed a concentrate based diet (Bessa *et al.*, 2005).

Meat from ruminants has higher levels of CLA than meat from non-ruminant. The highest CLA concentrations were found in lamb meat (4.3–19.0 mg/g fat), followed by beef (1.2–10.0 mg/g fat), representing 0.5–2% of total FA (Schimd *et al.*, 2006, Prates and Bessa, 2009). Pork and chicken meat show small levels of CLA and usually lower than 2 mg/g fat (Parodi, 2003, Schimd *et al.*, 2006). Meat CLA content is positively related with intramuscular fat content (Raes *et al.*, 2004). Rumenic acid is the main CLA isomer in meat, constituting about 80% of total CLA (Schimd *et al.*, 2006). Usually the second most prevalent CLA isomer in meat is the 18:2 *trans*-7, *cis*-9 (Yurawecz *et al.*, 1998, Martins *et al.*, 2007). The 18:2 *trans*-10, *cis*-12 is present in residual levels in foodstuffs.

1.2. NUTRITIONAL VALUE OF FATTY ACID, CONSUMPTIONS AND DIETARY RECOMMENDATIONS

Consumers are becoming more aware of the relationship between diet and health and this has increased the consumer interest in the nutritional value of food. Particular attention has been given to FA composition of foods; especially in ruminant edible products, as milk or meat due to its implications for human health. Ruminant products have high content of SFA and low levels of PUFA, propriety that is regarded as being disadvantageous within the human diet. A high intake of SFA is positively associated with cardiovascular disease, due to increase the total cholesterol and low density

lipoprotein (LDL)-cholesterol blood levels (Givens, 2005). However, individual SFA show different cholesterolemic responses. Lauric (12:0), myristic (14:0) and palmitic (16:0) acids have a cholesterol-raising effect, whereas stearic acid (18:0) appears to have neutral effect on serum cholesterol (Williams, 2000, Givens, 2005). Within hypercholesterolemic SFA, the 14:0 is considered more potent than 12:0 and 16:0 acids in inducing the increase the total cholesterol and LDL-colesterol (Kris-Etherton and Yu, 1997). However, nutritional recommendations, not consider the specific cholesterolemic effect of the individual SFA and only indicate that intake of SFA should not exceed 10% of total energy intake (Elmadfa and Kornsteiner, 2009). Intake estimations showed that in Western Europe, SFA provides on average between 10 to 19% of total energy intake, with the lowest contribution in most Mediterranean countries (Hulshof *et al.*, 1999).

In contrast to SFA, the PUFA and MUFA are generally regarded as beneficial for human health (Scollan *et al.*, 2006). Substitution of SFA by MUFA or PUFA reduced plasma total cholesterol and LDL-colesterol, being the PUFA more potent to reduction the cholesterol than MUFA (Williams, 2000). Oleic acid (18:1 *cis*-9), the most predominant MUFA in ruminant fat, is known to have the hypocholesterolemic properties, reducing the plasma cholesterol and LDL-cholesterol levels (Kris-Etherton and Yu, 1997). The recommended intake of MUFA can amount up to 15-20% of total energy intake, and regarding the PUFA 2.5-9% of energy intake should be from *n*-6 PUFA (18:2*n*-6) and 0.5-2% from *n*-3 PUFA (Elmadfa and Kornsteiner, 2009). The minimum dietary requirement of 18:3*n*-3 for adults is 0.5% of total energy intake (Elmadfa and Kornsteiner, 2009). It is also recommended that P/S ratio of whole human diet should be higher than 0.45 (Department of Health, 1994). However, this nutritional index based only on chemical structure of FA may not be an adequate way to evaluate the nutritional value of fat, because it not considers the specific effect of individual SFA and ignore the

effects of MUFA (Santos-Silva *et al.*, 2002a). Utilization of indices based on functional effects of individual FA, as hypocholesterolaemic/hypercholesterolaemic FA ratio (Santos-Silva *et al.*, 2002a), or atherogenicity and thrombogenicity indices (Ulbricht and Southgate, 1991) seems a better approach for evaluate the potential effect of dietary fat on human health.

Western diets are generally characterized by excessive amounts of *n*-6 PUFA and deficient *n*-3 PUFA, with *n*-6/*n*-3 ratios around 15/1-17/1 (Simopoulos, 2004). Despite the higher intake of both *n*-6 PUFA and *n*-3 PUFA is associated with reduction of the cardiovascular diseases risk (Stanley *et al.*, 2007), the excessive amount of *n*-6 PUFA in diet favors the development of many diseases, such as cardiovascular diseases, cancer and inflammatory and autoimmune diseases (Simopoulos, 2004). In contrast, increased the *n*-3 PUFA levels in diets have protective effect (Simopoulos, 2004). The 18:2*n*-6 and 18:3*n*-3, essential FA for humans, are precursors of LC-PUFA via a series of stepwise dasaturations and elongations (conversion pathways of 18:2*n*-6 and 18:3*n*-3 in respective LC-PUFA are present in section 1.3.3). Linoleic acid is the precursor of ARA and the 18:3*n*-3 is converted to EPA, DPA and DHA acids. The LC-PUFA are deposited mainly in membrane phospholipids and constitute substrate for synthesis of the eicosanoids, such as prostaglandins, thromboxanes or leukotrienes (Calder, 2001). Despite the FA composition of the membrane phospholipids to be strictly controlled in order to maintain the cellular homeostasis, its composition in FA is sensitive to changes of diet FA composition (Scollan *et al.*, 2006). The membrane phospholipids FA composition of inflammatory and immune cells determines the type of eicosanoids formed (Calder, 2003), and the eicosanoid derived from *n*-6 and *n*-3 PUFA have different biological actions and potencies. The eicosanoids derived from ARA are metabolically more potent in promoting inflammation, platelet aggregation, and immune and vascular reactivity than

those derived from *n*-3 LC-PUFA (Calder, 2001, Calder, 2003). Moreover, the EPA also induced the suppression in the production of eicosanoids derived from ARA (Calder, 2001).

Nutritional recommendation indicate that the *n*-6/*n*-3 ratio in human diets should not exceed 4 (Department of Health, 1994). However, the value of dietary *n*-6/*n*-3 ratio in modifying the cardiovascular diseases has been questioned (Stanley *et al.*, 2007, Griffin, 2008). The 18:2*n*-6 and 18:3*n*-3 competes for the same elongation and desaturation enzymes, limiting the efficiency of its conversion to *n*-6 and *n*-3 LC-PUFA (Brenner, 1989) and it is generally assumed that higher dietary *n*-6/*n*-3 reduces the *n*-3 LC-PUFA synthesis. However, recently was reported that the conversion of 18:3*n*-3 in humans is influenced by the absolute amounts of 18:2*n*-6 and 18:3*n*-3 in diets and not by dietary *n*-6/*n*-3 ratio (Goyens *et al.*, 2006). Moreover, the dietary *n*-6/*n*-3 ratio does not modifies the cardiovascular diseases risk (Stanley *et al.*, 2007, Griffin, 2008). Thus, it is considered that the *n*-6/*n*-3 ratio is not a useful concept, and that distract attention from the *n*-3 LC-PUFA absolute amounts in the diet (Stanley *et al.*, 2007). The health benefits of *n*-3 PUFA are mostly associated with absolute dietary intake of *n*-3 LC-PUFA, mainly EPA and DHA, while the principal biological role of 18:3*n*-3 seems to be as precursor for to *n*-3 LC-PUFA (Burdge and Calder, 2005). The EPA and DHA shown to have anti-atherogenic, anti-trombotic and anti-inflamatory effects (Givens *et al.*, 2006) and may also have important roles in reducing the cancer, obesity and type 2 diabetes (WHO, 2003). Some studies show that intake of EPA and DHA may protect against the neurological disorders, including the Alzheime's diseases, as reviewed by Whelan and Rust (2006). The conversion of dietary 18:3*n*-3 to *n*-3 LC-PUFA is very limited in adult humans, so the 18:3*n*-3 is probably a quite limited sources of EPA and DHA (Burdge and Calder, 2005). However, several epidemiological studies shown that high intake of 18:3*n*-

3 reduces the cardiovascular disease risk (Zhao *et al.*, 2004), probably due to increase the synthesis of EPA (Givens *et al.*, 2006). The recent intake estimation of the EPA and DHA in various countries, reviewed by Givens and Gibbs (2008) showed a large variation in mean intake of these FA, ranging from 75 mg/day in Belgian children to 950 mg/day in adults in Japan. However, many of these values are below the recommended daily intake. Simopoulos (2004) reported that adequate intake of EPA + DHA is the 0.3% of diet energy (*i.e.* 650 mg/day considering diets with 2000 kcal). The minimum recommended by Scientific Advisory Committee of Nutrition and Committee on Toxicity (2004) is the 450 mg of EPA + DHA/day. Recently was reported that the adequate intake of EPA + DHA should range between 250-2000 mg/day (Elmadfa and Kornsteiner, 2009).The concentrations of *n*-3 LC-PUFA in ruminant meats are lower than those within fish or fish oil. However, the consumption of fish or fish oil in most Western countries is low (Givens and Gibbs, 2008), so may be important the contribution of meat to increase the *n*-3 LC-PUFA intake. The meat, specially the beef and lamb meat has a high proportion of DPA (Howe *et al.*, 2007), contributing substantially to the DPA intake (Astorg *et al.*, 2004, Howe *et al.*, 2006). The biological effects of DPA has been little researched, but recent evidence suggest that DPA is just as important as EPA and DHA for the health benefits associated with *n*-3 LC-PUFA (Howe *et al.*, 2007). However, the DPA is not considered in dietary recommendation for *n*-3 LC-PUFA.

Generally, TFA intake is associated to deleterious effects on human health (Hunter, 2006). However, evidence suggests that individual TFA have differential biological effects. *Trans* FA are provided in human food by ruminant products, mainly composed by 18:1 *trans*-11, and by industrial hydrogenated vegetable oils, that shown high levels of several 18:1 *trans* FA, mainly 18:1 *trans*-9 and 18:1 *trans*-10 (Pfeuffer and Schrezenmeir, 2006). Epidemiological studies showed a positive association between cardiovascular

disease risk and consumption of TFA from industrial sources, but not with consumption of TFA from ruminant sources (Chardigny *et al.*, 2008). Moreover, the 18:1 *trans*-11 has been suggest as beneficial TFA because it's the precursor of 18:2 *cis*-9, *trans*-11 in animals and man (Scollan *et al.*, 2006). However, the biological effects of TFA from ruminant origin are less known, and the some results are contradictory. Recently epidemiologic study (Motard-Bélanger *et al.*, 2008) showed that a same daily intake of TFA from ruminant and from industrial sources (3.6% of energy intake) has a same negative effect on cardiovascular disease risks factors, but moderate TFA intake from ruminant origin (1.5% of energy intake) has a neutral effect on these risk factors. The contribution of TFA from ruminant sources in ranges from 30 to 80% of total TFA intake, representing to 0.3 – 0.8% of dietary energy (Craig-Schmidt, 2006). Thus, the actual amounts of TFA from ruminant origin consumed in diets do not contribute importantly to risks of cardiovascular disease (Motard-Bélanger *et al.*, 2008). The present nutritional recommendation indicate that TFA should contribute with less than 1% of total energy intake of the human diets (Elmadfa and Kornsteiner, 2009). Intake estimations showed that in Western Europe, TFA provides on average between 0.5 to 2.1% of total energy intake, with the lowest contribution in most Mediterranean countries (Hulshof *et al.*, 1999).

In recent years, CLA have received much attention due their potential beneficial properties to human health. Conjugated linoleic acid is a collective term that refers to a mixture of positional (from carbons 6,8 to 12,14) and geometric (*trans, trans*; *trans, cis*; *cis, trans*; and *cis, cis*) isomers of 18:2*n*-6 with a conjugated double-bond system (Wahle *et al.*, 2004). Numerous beneficial physiological effects have been attributed to CLA, including anticarcinogenic, anti-adipogenic, anti-diabetogenic, anti-atherogenic and anti-inflammatory effects as reviewed by Wahle *et al.* (2004). The CLA research has focused

mainly on the effects of two isomers, 18:2 *cis*-9, *trans*-11 and 18:2 *trans*-10, *cis*-12 and specific physiology effects have been associated to each these CLA isomers. The anti-adipogenic effect of CLA has been associated to 18:2 *trans*-10, *cis*-12 isomer (Park *et al.*, 1999, Chardigny *et al.*, 2003), whereas the both 18:2 *cis*-9, *trans*-11 and 18:2 *trans*-10, *cis*-12 isomers appear to be are similarly active in anticarcinogenesis (Pariza *et al.*, 2001). Recently was reported that a mixture of *trans*, *trans* CLA isomers had a greater anticarcinogenic activity in induced rat mammary tumorigenesis as compared to 18:2 *cis*-9, *trans*-11 and 18:2 *trans*-10, *cis*-12 isomers (Islam *et al.*, 2010). Most evidence of the benefit effects of CLA isomers has been obtained by laboratory animals and by cell culture studies (Pariza *et al.*, 2001, Wahle *et al.*, 2004). In human studies inconsistent effects have been reported, and the beneficial effect of CLA for human health remains to establish (Tricon and Yaqoob, 2006). Moreover, only few studies have explored the health effects in humans of CLA naturally present in food (Prates and Bessa, 2009). The main dietary source of CLA in food is ruminant meat, milk and their products (Parodi, 2003). Martins *et al.* (2007) reviewing the daily intake estimative of 18:2 *cis*-9, *trans*-11 or CLA for several countries showed that its consumption ranges between 15 and 1000 mg. Based in consumption of food rich in CLA was estimated that daily intake of CLA for the Portuguese population is about 74 mg (Martins *et al.*, 2007). Recommendations for the intake of CLA in human yet are not established. Based on animal studies have been extrapolated the CLA intake necessary to promote health benefits in humans. Using this methodology was suggested that consumption of 720-800 mg of CLA/person/day would be necessary for anticarcinogenic protective effects in humans (Parish *et al.*, 2003, Watkins and Li, 2003).

1.3. RUMINANT LIPID METABOLISM

1.3.1. Ruminal lipid metabolism

Fatty acid metabolism in the rumen has a major influence on the FA composition of ruminant products. In rumen, the dietary lipids are extensively metabolized, resulting in marked differences between FA composition of edible products of ruminants (mostly SFA) and FA composition of its diet (mostly unsaturated FA) (Jenkins *et al.*, 2008).The main types of dietary lipids entering the rumen are triacylglycerols, galactolipids and phospholipids (Jenkins *et al.*, 2008). In ruminants fed forage the dietary lipid consists mainly in galactolpids and phospholipids, while cereals and plant oils contribute mainly with triacylglycerols (Harfoot and Hazelwood, 1997). The most abundant FA present in forages is 18:3*n*-3, whereas cereals and plant oils contain predominantly 18:2*n*-6 and 18:1 *cis*-9 (Woods and Fearon, 2009). However, in the linseed and linseed oil the most abundant FA is 18:3*n*-3 (Woods and Fearon, 2009). In rumen the dietary lipids are transformed via two major processes – lipolysis and BH.

1.3.1.1. Ruminal lypolisis

In rumen the dietary lipids are rapidly hydrolyzed, which lipases hydrolyze the ester linkages in complex lipids, causing the release the constituent FA (Garton *et al.*, 1961, Dawson *et al.*, 1977). The extent of lipolysis is generally high (>85%) (Bauchart *et al.*, 1990a). However, have been identified several factors that decrease the extent of hydrolysis, such as dietary fat lipid level (Beam *et al.*, 2000, Atkinson *et al.*, 2006) and low pH (Van Nevel and Demeyer, 1996). It is generally accepted that microbial lipases are the main responsible for hydrolysis of plant lipids in rumen (Dawson *et al.*, 1977). However, there is evidence that plant lipases may contribute for overall ruminal lipolysis.

In 1974, Faruque *et al.* (1974) showed that plant lipases remain actives for up to 5 h in rumen, and suggested that hydrolysis of triacylglycerols and galactolipids from grass was due primarily to plant enzyme activity. More recently, was reported intense lipolysis in leaves of fresh red clover and fresh ryegrass incubated in buffer (Lee *et al.*, 2002, Van Ranst *et al.*, 2009). Dierick and Decuypere (2002) showed that after 56 days of storage approximately 25 to 30% of the lipids in ground raw maize, wheat and barley was hydrolyzed, and for sorghum and oats the degree of lipolysis was 50 and 65%, respectively. Among the various types of ruminal microorganisms the bacteria are considered the most active in lipolysis. Rumen hydrolysis has been most extensively characterized in *Anaerovibrio lipolitica* which hydrolyzes triacylglycerols and *Butyrivibrio fibrosolvens* which hydrolyzes phospholipids and glycolipids (Harfoot and Hazelwood, 1997).

1.3.1.2. Ruminal biohydrogenation

After lypolisis, the unsaturated FA released are biohydrogenated. Biohydrogenation requires a free carboxyl group to proceed and thus factors that affect hydrolysis also influence the BH. The ruminal BH consist in conversion of the unsaturated FA to SFA via initial isomerization, followed by hydrogenation of double bonds mediated by rumen microorganisms (Harfoot and Hazelwood, 1997). Ruminal BH of C_{18} unsaturated FA has been extensively studied, because are the main FA present in ruminants food. These FA are extensively biohydrogenated in rumen, and the disappearance of 18:2*n*-6 and 18:3*n*-3 in the rumen averages 80 and 92%, respectively (Doreau and Ferlay, 1994). However, the biohydrogenation also occurs on 20- and 22-carbon FA, as EPA and DHA (Chilliard *et al.*, 2000). Wachira *et al.* (2000) reported the BH values between 72 and 79% for EPA

and DHA, respectively in whether sheep fed diets that contained fish oil or linseed and fish oil.

The role of BH is as yet unclear, but the most accepted theory is that ruminal biohydrogenation is a detoxification strategy to prevent the toxic effects of unsaturated FA on rumen microbe (Harfoot and Hazelwood, 1997, Jenkins *et al.*, 2008). It is reported that different unsaturated FA have differential antimicrobial effect, which the DHA, EPA and 18:3*n*-6 are more toxic for biohydrogenating bacteria than 18:3*n*-3, and this latter than 18:2*n*-6 (Maia *et al.*, 2007, Maia *et al.*, 2010). The 18:1 *trans*-11 show to have little toxic effect (Maia *et al.*, 2010), but the 18:2 *cis*-9, *trans*-11 is almost as toxic as 18:2*n*-6 (Kim *et al.*, 2000, Maia *et al.*, 2010). Moreover, the sensitivity of ruminal microorganisms to the toxic effects of PUFA is variable, being *Butyrivibrio hungatei* and *Butyrivibrio proteoclasticus* group much more sensitive than the rest of the *Butyrivibrio Pseudobutyrivibrio* cluster (Maia *et al.*, 2010). Paillard *et al.*(2007) found that the different PUFA sensitivity of ruminal species is related with their enzymatic mechanism of butyrate formation, being most sensitive the species that formed butyrate *via* butyrate kinase mechanism, as rather than acyl CoA transferase.

1.3.1.2.1. Biohydrogenation microorganisms

Bacteria play the main role in FA biohydrogenation (Jenkins *et al.*, 2008). Diverse bacterial strains with different capacities of hydrogenation were isolated, as reviewed by Harfoot and Hazelwood (1997). Thus, the bacteria involved in BH process were classified as group A and B, which group A bacteria hydrogenated 18:2*n*-6 and 18:3*n*-3 to 18:1 *trans*-11, whereas group B bacteria convert the same FA to 18:0 (Harfoot and Hazelwood, 1997). *Butyrivibrio fibrosolvens* was identified many years to undertake BH

of FA, but not form 18:0 from 18:2*n*-6 and 18:3*n*-3 (Polan *et al.*, 1964, Kepler *et al.*, 1966, Kepler and Tove, 1967). Recently, *B. proteoclasticus* was identified as a stearate producer (Wallace *et al.*, 2006). Phylogenetic analysis based on 16S ribosomal RNA (rRNA) sequence analysis, indicated that the stearate producers was clustered on branch with *B. proteoclasticus* (Jenkins *et al.*, 2008). The high capacity to metabolize 18:2*n*-6 to 18:2 *cis*-9, *trans*-11 has been associated entirely with the *Butyrivibrio* group (Kemp *et al.*, 1975, Paillard *et al.*, 2007).

Metabolism of 18:2*n*-6 by *B. fibrosolvens* or by *B. proteoclasticus* result in formation of *cis*-*trans* CLA isomers with double bonds in 9, 11 position, mainly 18:2 *cis*-9, *trans*-11, as well in 18:1 *trans*-11, but no 18:2 *trans*-10, *cis*-12 and 18:1 *trans*-10 is formed (Maia *et al.*, 2007, Wallace *et al.*, 2007). A significant production of 18:2 *trans*-10, *cis*-12 by some *Megasphaera elsdenii* strains in cultures enriched with starch was reported by Kim *et al.* (2002). Other studies indicate that *Propionibacterium acnes* may be responsible for the formation of 18:2 *trans*-10, *cis*-12 (Wallace *et al.*, 2007, McKain *et al.*, 2010). In fact, in digesta samples from cows producing high amounts of 18:2 *trans*-10, *cis*-12 was found $<10^3$/g of *M. elsdenii*, while much large number of *P. acne* were detectable (Lourenço *et al.*, 2010). However, the *P. acnes* not convert 18:2 *trans*-10, *cis*-12 to 18:1 *trans*-10 (McKain *et al.*, 2010).

Protozoa appear to have a minor role in BH, so BH in ruminal digesta was only slightly decreased following removal of protozoa from the rumen and presence of protozoa was not necessary for biohydrogenation to occur (Dawson and Kemp, 1969). Recently, was demonstrated that ruminal fungi have ability to biohydrogenate 18:2*n*-6, producing 18:2 *cis*-9, *trans*-11 and 18:1 *trans*-11 as end product (Nam and Garnsworthy, 2007), but their activity is very low in comparison of bacteria (Maia *et al.*, 2007, Nam and Garnsworthy, 2007).

1.3.1.2.2. Biohydrogenation pathways and biohydrogenation intermediates

It well known that in ruminal content occurs several BI, including 18:3, 18:2 conjugated or non-conjugated and 18:1 isomers. The origin of some BI is already established, and some BH pathway had been proposed. Specific isomers have been associated to BH of dietary 18:2*n*-6 and 18:3*n*-3, although no putative pathways for its occurrence have been proposed. Established and putative BH pathways of 18:1 *cis*-9, 18:2*n*-6 and 18:3*n*-3 are show in **Figure 1.1**. In the **Table 1.1** are present the established and putative BI of 18:1 *cis*-9, 18:2*n*-6 and 18:3*n*-3.

Oleic acid, is usually described as being hydrogenated directly to 18:0, without the formation of FA intermediates (Harfoot and Hazelwood, 1997). However, *in vitro* studies using labelled 18:1 *cis*-9, demonstrated that biohydrogenation of 18:1 *cis*-9 result in the formation of the 18:0 as well as in multiples *trans* and *cis* 18:1 isomers (Mosley *et al.*, 2002, AbuGhazaleh *et al.*, 2005, Mosley *et al.*, 2006). Harfoot and Hazelwood (1997) described the established BH pathway of 18:2*n*-6 and 18:3*n*-3. These FA are initially isomerised to their conjugated FA, followed by hydrogenation of the double bonds. The 18:2*n*-6 is isomerised to 18:2 *cis*-9, *trans*-11, which is then hydrogenated to 18:1 *trans*-11, and finally to 18:0. The 18:3*n*-3 is metabolised in a similar way, being isomerised to 18:3 *cis*-9, *trans*-11, *cis*-15, followed by reductions of double bonds at carbons 9, 15 and 11 to yield 18:2 *trans*-11, *cis*-15, 18:1 *trans*-11 and 18:0, respectively. The established pathways for biohydrogenation of 18:3*n*-3 also included the 18:1 *trans*-15 and 18:1 *cis*-15 as octadecenoic biohydrogenated intermediates.

The BI pattern is strongly dependent from the ruminal environment, and consequently dependent from the dietary regimen offered to animals. High proportion of concentrates supply induce to strong increase the flow of 18:1 *trans*-10 from rumen (Piperova *et al.*,

2002, Loor *et al.*, 2004). Griinari and Bauman (1999) proposed that in this ruminal conditions the 18:2*n*-6 and 18:3*n*-3 are isomerized by *cis*-9, *trans*-10 isomerase to octedecadienoic conjugated 18:2 *trans*-10, *cis*-12 and octadecatrienoic conjugated 18:3 *trans*-10, *cis*-12, *cis*-15, respectively. After isomerisation, the double bonds *cis*-12, and the double bond *cis*-15 in case the 18:3*n*-3 are hydrogenate, resulting in 18:1 *trans*-10.

In fact, high levels of 18:2 *trans*-10, *cis*-12 in duodenal flow (Kucuk *et al.*, 2001, Sackmann *et al.*, 2003), as well as in milk (Piperova *et al.*, 2002) and meat (Bessa *et al.*, 2005) has been found in ruminants fed diets rich in concentrate. However, as far as we know, the 18:3 *trans*-10, *cis*-12, *cis*-15 and 18:2 *trans*-10, *cis*-15 only were identified *in vitro* studies when incubated 18:3*n*-3 in rumen microorganisms cultures (Kemp *et al.*, 1975, Or-Rashid *et al.*, 2009).

Using labeled 18:2*n*-6, was found that 18:2*n*-6 may be converted in seven CLA isomers with double bonds in either the 9,11 or 10,12 positions, being all possible *cis-trans* combinations represented, except the *trans*-9, *cis*-11 (Jenkins *et al.*, 2008). Moreover, other CLA isomers have been associated to ruminal biohydrogenation of 18:2*n*-6, such as 18:2 *trans*-8, *trans*-10, 18:2 *trans*-7, *trans*-9, 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9 (Collomb *et al.*, 2004, Bessa *et al.*, 2007). Jouany *et al.* (2007) also showed that incubation of 18:2*n*-6 increase 18:2 *cis*-9, *trans*-12, as well as several octadecenoic FA compared with control incubations.

Ruminal BH of 18:3*n*-3 result in greater diversity of products. Wasowska *et al.* (2006) confirm the established 18:3*n*-3 biohydrogenation pathway when incubated 18:3*n*-3 in strained rumen fluid, and reported that 18:3 *trans*-9, *trans*-11, *cis*-15 is also a transient intermediate of 18:3*n*-3 metabolism, which is also hydrogenated to yield 18:2 *trans*-11, *cis*-15.

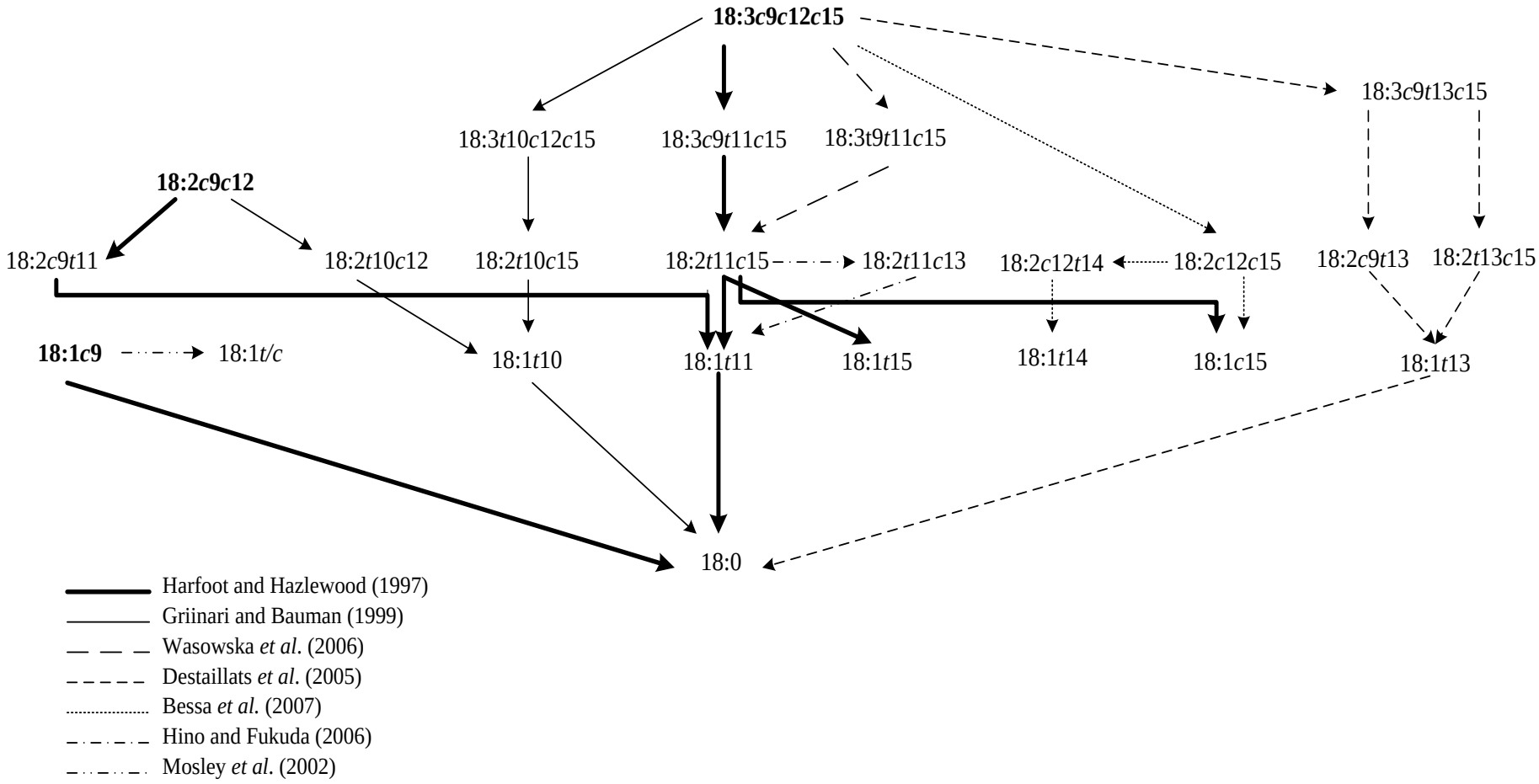

Figure 1. 1 Established and putative biohydrogenation pathways.

Table 1. 1 Established and putative biohydrogenation intermediates of oleic, linoleic and linolenic acids

18:1 *cis*-9	18:2 *cis*-9, *cis*-12	18:3 *cis*-9, *cis*-12, *cis*-15
trans-6[1]	*cis*-9, *trans*-11[3]	*cis*-9, *trans*-11, *cis*-15[3]
trans-7[1]	*trans*-10, *cis*-12[4]	*trans*-10, *cis*-12, *cis*-15[4, 5]
trans-9[1]	*trans*-9, *trans*-11[6, 8]	*trans*-9, *trans*-11, *cis*-15[10]
trans-10[1]	*trans*-9, *cis*-11[6]	*trans*-9, *cis*-11, *cis*-15[5]
trans-11[1]	*cis*-9, *cis*-11[6]	*cis*-9, *trans*-13, *cis*-15[11]
trans-12[1]	*cis*-9, *trans*-12[8]	*cis*-9, *trans*-12, *cis*-15[12]
trans-13[1]	*trans*-10, *trans*-12[6]	*cis*-9, *trans*-12, *trans*-15[12]
trans-14[1]	*cis*-10, *trans*-12[6]	*trans*-9, *trans*-12, *trans*-15[12]
trans-15[1]	*cis*-10, *cis*-12[6, 8]	*trans*-11, *cis*-15[3]
trans-16[1]	*trans*-8, *trans*-10[7]	*trans*-13, *cis*-15[11]
cis-11[2]	*trans*-8, *cis*-10[7, 8]	*cis*-9, *trans*-13[8, 9, 11]
cis-12[2]	*trans*-7, *trans*-9[7, 8]	*cis*-12, *cis*-15[8]
cis-13[2]	*trans*-7, *cis*-9[7, 8]	*cis*-9, *cis*-15[8, 13, 14]
cis-14[2]	*trans*-4[9]	*cis*-10, *cis*-15[13]
cis-15[2]	*trans*-5[9]	*cis*-11, *cis*-15[13]
cis-16[2]	*trans*-6-8[8, 9]	*trans*-10, *cis*-15[4,13]
	trans-9[8, 9]	*trans*-11, *trans*-15[15]
	trans-10[4]	*cis*-9, *cis*-11[9]
	trans-11[3]	*cis*-9, *trans*-12[9]
	trans-12[8, 9]	*trans*-9, *cis*-12[9]
	cis-12[8, 9]	*trans*-9, *trans*-12[9]
	cis-13[9]	*cis*-11, *trans*-13[7, 9]
		trans-11, *trans*-13[7, 8, 9]
		trans-11, *cis*-13[14]
		cis-/*trans*-12, 14 [7, 8]
		trans-12, *trans*-14 [7, 8]
		trans-5[9]
		trans-6-8[9]
		trans-9[9]
		trans-10[4]
		trans-11[3]
		trans-12[9]
		trans-13-14[8, 9]
		trans-15[3]
		cis-13[9]
		cis-15[3]

[1] Mosley *et al.* (2002); [2] Mosley *et al.* (2006); [3] Harfoot and Hazlewood (1997); [4] Griinari and Bauman (1999); [5] Or-Rashid *et al.* (2009), [6] Jenkins *et al.* (2008); [7] Collomb *et al.* (2004); [8] Bessa *et al.* (2007); [9] Jouany *et al* (2007); [10] Wasowska (2006); [11] Destaillats *et al.* (2005); [12] Loor *et al.* (2004); [13] Kemp *et al.* (1975); [14] Hino (2006); [15] Jerónimo *et al.* (2010).

Recently, Or-Rashid *et al*. (2009) reported that 18:3*n*-3 also may be isomerized to 18:2 *trans*-9, *cis*-11, *cis*-15. Some FA present in fat (milk and meat) or digesta of ruminats has been associated to ruminal biohydrogenation of 18:3*n*-3 and other possible pathways of 18:3*n*-3 biohydrogenation has been proposed. Destaillats *et al.* (2005) found in milk fat the 18:3 *cis*-9, *trans*-13, *cis*-15, and proposed that this conjugated octadecatrienoic FA result from the initial isomerization of 18:3*n*-3, being then reduced to 18:2 *cis*-9, *trans*-13 and 18:2 *trans*-13, *cis*-15 and subsequently to 18:1 *trans*-13. The production of 18:2 *cis*-9, *trans*-13 from 18:3*n*-3 biohydrogenation is consistent with its increase in ruminal fluid, duodenal flow, blood plasma and milk of cows fed diets supplemented with linseed oil (Loor *et al.*, 2004, Loor *et al.*, 2005). Moreover, Bessa *et al.* (2007) only detected the presence of 18:2 *cis*-9, *trans*-13 in meat from lambs fed linseed oil. However, the 18:2 *trans*-13, *cis*-15 has yet to be identified in ruminant fats.

Bessa *et al.*, (2007) reported the presence of 18:2 *cis*-12, *cis*-15 only in meat from lambs fed linseed oil, and proposed that the 18:2 *cis*-12, *cis*-15 results from the direct reduction of the 18:3*n*-3 *cis*-9 double bond. By analogy with the 18:2*n*-6 biohydrogenation pathway, which is reduced only after formation of a conjugatated intermediate, these authors proposed that 18:2 *cis*-12, *cis*-15 might be isomerized to 18:2 *cis*-12, *trans*-14 and further reduced to 18:1 *trans*-14. However, unlike 18:2*n*-6, the 18:2 *cis*-12, *cis*-15 could be reduced directly (Kemp and Lander, 1984), resulting in 18:1 *cis*-15. Kemp *et al*. (1975) described several non conjugated dienoic acids as minor intermediates of the 18:3*n*-3 biohydrogenation, including 18:2 *cis*-9, *cis*-15, 18:2 *cis*-10, *cis*-15, 18:2 *cis*-11, *cis*-15 and 18:2 *trans*-10, *cis*-15. More recently, Jouany *et al.* (2007) showed that incubation of 18:3*n*-3 also increase the 18:2 *cis*-9, *cis*-11, 18:2 *trans*-9, *cis*-12, 18:2 *cis*-9, *trans*-12, 18:2 *trans*-9, *trans*-12 and 18:2 *trans*-11, *trans*-13 compared with control

incubations. Hino and Fukuda (2006) reported that conjugated 18:2 *trans*-11, *cis*-13 is formed by isomerizarion of 18:2 *trans*-11, *cis*-15.

Loor *et al.*, (2004) reported the increase of the duodenal flow of 18:3 *cis*-9, *trans*-12, *cis*-15, 18:3 *cis*-9, *trans*-12, *trans*-15 and 18:3 *trans*-9, *trans*-12, *trans*-15 in cows with dietary linseed oil supplementation. Recently, Jerónimo *et al*. (2010a) found the 18:2 *trans*-11, *trans*-15 only in meat from lambs fed linseed oil, suggesting that this FA is probably an intermediate of 18:3*n*-3 BH. The 18:2 *trans*-12, *trans*-14 and 18:2 *cis*-/*trans*-12,14 also are closely associated with 18:3*n*-3 ingestion (Collomb *et al.*, 2004, Bessa *et al.*, 2007).

1.3.1.3. Microbial fatty acid uptake and synthesis

Ruminal microorganisms are able either to incorporate and synthesize FA of different chain lengths. Bessa (2001) estimated that the microbial lipid syntheses is about 8.8 g/kg dry matter intake (DMI). Synthesis occurs from volatile FA in most cases (Doreau and Ferlay, 1994). The saturated straight- and branched-chain FA are synthesized by a very similar mechanism and the difference between two systems is mainly related to the substrate specificity of the acyl-CoA:ACP transacylase (Kaneda, 1991). The *de novo* synthesis of straight-chain FA is achieved by repeated condensation of malonyl-coenzyme A with acetyl-CoA as primer, yielding 16:0 as the dominant end product (Fulco, 1983, Kaneda, 1991). For synthesis the linear odd-chain FA the propionyl-CoA is used as primer, instead of acetyl-CoA (Fulco, 1983, Kaneda, 1991). Odd-chain FA also can be obtained by reduction the chain length through α-oxidation (Emmanuel, 1978). Branched-chain FA are formed from branched-chain amino acids (valine, leucine, and isoleucine) and their corresponding branched short-chain carboxylic acid (isobutyric,

isovaleric, and 2-methylbutyric acid) (Vlaeminck *et al.*, 2006). The FA composition of rumen bacteria is characterized by a large proportion of odd- and branched-chain FA in their membrane lipids (Kaneda, 1991), being the odd- and branched-chain FA presents in ruminant fat largely derived from ruminal bacteria (Vlaeminck *et al.*, 2006). The odd- and branched-chain FA profile of rumen bacteria is substantial different between species, where cellulolytic bacteria present high levels of branched-chain FA, while the amylolytic bacteria are relatively enriched in linear odd-chain FA (Vlaeminck *et al.*, 2006). So, the odd- and branched-chain FA have been proposed as market for the rumen microbial ecosystem (Vlaeminck *et al.*, 2006, Bessa *et al.*, 2009). Rumen microbes also are able to synthesize unsaturated FA. Bacterial *cis*- and *trans*- monounsaturated FA may result the desaturation of saturated FA (Doreau and Chilliard, 1997). It is also reported the syntheses of dienoic FA by ruminal bacteria, but in lesser extent (Emmanuel *et al.*, 1974, Emmanuel, 1978). The *de novo* FA synthesis is influenced by amount of FA present in rumen, decreasing when ruminal FA concentration increases (Demeyer *et al.*, 1978).

The extent of dietary FA incorporation in bacterial cell lipids is higher than extent of synthesis (Doreau and Chilliard, 1997). The incorporation of FA in rumen bacteria appears to be a low selective process, reflecting the ruminal FA concentration (O´Kelly and Spiers, 1991, Jerónimo, 2004) and dietary lipid supplementation result in FA enrichment of rumen bacteria (Legay-Carmier and Bauchart, 1989, Bauchart *et al.*, 1990b, O´Kelly and Spiers, 1991, Bessa, 2001, Jerónimo, 2004). Synthesized and assimilated FA are esterified as phospholipids and sterol esters (Demeyer *et al.*, 1978). When large amounts of FA are fed, they are stored as free FA in cytosolic droplets (Bauchart *et al.*, 1990b).

1.3.2. Intestinal absorption and transport of lipids

The lipids entering the small intestine of ruminants are composed mainly by non-esterified saturated FA (Drackley, 2000). The remaining lipid components are microbial phospholipids and small amounts of triacylglycerols and glycolipids from residual feed material, which are hydrolyzed by intestinal and pancreatic lipases (Doreau and Ferlay, 1994). Fatty acids are mainly adsorbed on feed particles, microbial cells and desquamated endothelial cells. Biles salts, together with lysolecithins desorb the FA, which allow the formation of the micelles (Doreau and Chilliard, 1997). These micelles allow the lipid absorption at the jejunum. Short FA (<12 carbons) are secreted as free FA into the portal vein, while medium and long chain FA (>12 carbons) are absorbed by the epithelial cells of the small intestine and re-esterified (Hocquette and Bauchart, 1999). The lipids formed are incorporated into chylomicrons and very-low density lipoproteins (VLDL), which are mostly transported in lymph, but also via portal vein to the liver, muscle or adipose tissues (Noble, 1981). Triacylglycerols are the major lipid class in lymph with much smaller amounts of phospholipids and cholesterol esters, whereas in plasma the cholesterol esters and phospholipids are the principal components, with much smaller amounts of triacylglycerols, unesterified FA and free cholesterol (Christie, 1981).

In rumen the essential FA, 18:2*n*-6 and 18:3*n*-3 are extensively biohydrogenated, reducing significantly the availability of these FA for absorption. However, in ruminants nearly all of the 18:2*n*-6 (and presumably 18:3*n*-3) that reach the small intestine is incorporated into phospholipids and cholesterol esters (Drackley, 2000), that are lipids with a very slow turnover in body, allowing that the essential FA are retained for their critical functions (Noble, 1981).

1.3.3. Muscle fatty acids metabolism – Fatty acids syntheses and deposition in muscle

Muscle lipids are composed of PL, mainly phospholipids presented in the cell membranes, and NL consisting mainly of triacylglycerols in the adipocytes that are located along the muscle fibers and in interfascicular area (De Smet et al., 2004). Moreover, high intramyocellular triacylglycerols deposition was found in skeletal muscle with high lipid content (Malenfant et al., 2001).

Intramuscular triacylglycerols content is strongly related to the total fat content and varies from 0.2 and 5 g/100 g of muscle weight (De Smet *et al.*, 2004, Raes *et al.*, 2004). In contrast to triacylglycerols, the content of phospholipids in muscle is relatively constant and independent the total fat content (Wood *et al.*, 2008), and varies between 0.2 and 1 g/100 g of muscle weight (De Smet *et al.*, 2004, Raes *et al.*, 2004). **Figure 1.2** illustrates the increasing importance of NL fraction in total lipid with fattening proceeds and the fairly constant level of PL. However, the content of phospholipids is depends the metabolic fiber type of the muscle, which more oxidative muscle shown higher proportion of phospholipids, due to the higher content of mitochondria (Raes *et al.*, 2004).

Intramuscular FA has a dual origin, by either taken up from plasma or by *de novo* synthesis in muscle. Pre-formed FA are transported in plasma as non-esterified FA, which circulate bound to serum albumin, or as triacylglycerols incorporated into lipoproteins. Following secretion from intestinal cells or liver, chylomicrons and VLDL move through the peripheral blood, and its triacylglycerols are hydrolyzed by the lipoprotein lipase, releasing FA (Hocquette and Bauchart, 1999, Drackley, 2000). By diffusion the FA released are incorporate in cells.

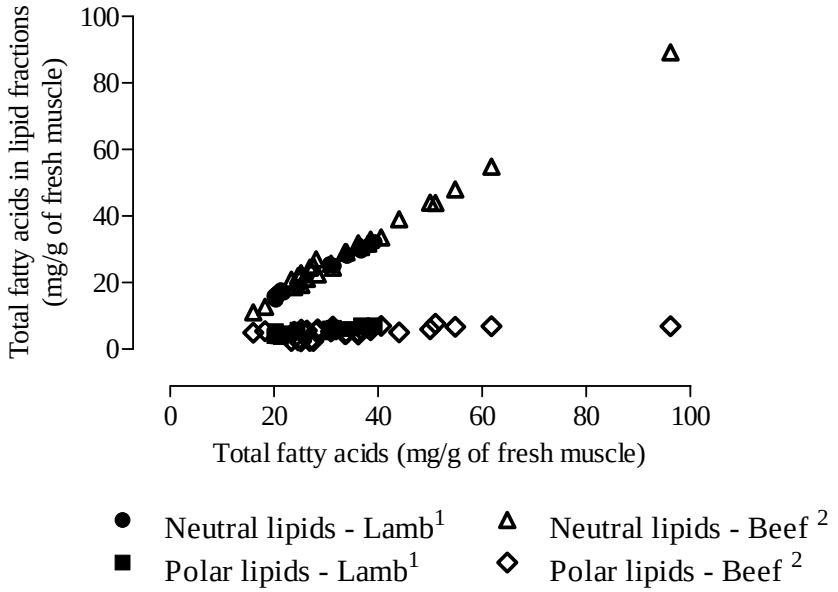

Figure 1. 2 Relationship between content of total fatty acids and total fatty acids in neutral and polar lipids in lamb meat and beef.

[1]Data of lamb meat: Demirel *et al.* (2004), Cooper *et al.* (2004), Jerónimo *et al.* (2009, 2010b); [2] Data of beef: Choi *et al.* (2000); Scollan et *al.* (2001, 2003); Noci *et al.* (2005, 2007a); Warren *et al.* (2008).

De novo FA synthesis occurs in the cytosol and is a sequential cyclical process in which acetyl (2-carbon) units are added successively to an initial starting molecule, usually acetyl-CoA (Drackley, 2000). The source of the acetyl units is acetyl-CoA, which in ruminants derived from acetate produced during fermentation of dietary carbohydrates in rumen. In the first step of FA synthesis, the acetyl-CoA carboxylase enzyme converts the acetyl-CoA to malonyl-CoA, which is the source of acetyl units in the elongation process (Drackley, 2000). Conversion of malonyl-CoA to saturated long chain FA is mediated by FA synthase complex, that consist of two multifunctional polypeptide chains, each containing seven distinct enzyme activities necessary to elongate a growing FA (Smith, 1994). In non lactating ruminants, adipose tissue is the principal site for *de novo* FA, with 16:0 being the main end product (Drackley, 2000). Palmitic acid may be desaturated

and/or elongated to form other FA. The 18:0 present in adipose tissue lipids may arise from intestinal supply or by conversion of 16:0 by the action of FA elongase.

Stearoyl-CoA desaturase (SCD) enzyme, also known as Δ9-desaturase plays a key role in the lipid metabolism, because it introduces a *cis*-double bond at the Δ9 position in SFA and octadecenoic TFA. The 18:0 and 16:0 are the main substrates for SCD, which are converted into 18:1 *cis*-9, 16:1 *cis*-9 (palmitoleic acid), respectively (Ntambi and Miyazaki, 2004). However, other SFA, and unsaturated FA, including 18:1 *trans*-11 also are substrates for the SCD. Vaccenic acid, formed during ruminal BH of both 18:2*n*-6 and 18:3*n*-3, in tissues is converted by SCD in 18:2 *cis*-9, *trans*-11 (**Figure1.3**) (Griinari *et al.*, 2000). Palmquist *et al.* (2004) and Griinari *et al.* (2000) showed that the primary source of 18:2 *cis*-9, *trans*-11 is endogenous synthesis, so content of 18:2 *cis*-9, *trans*-11 in muscle and milk depend mainly the availability of precursor (18:1 *trans*-11) and the SCD.

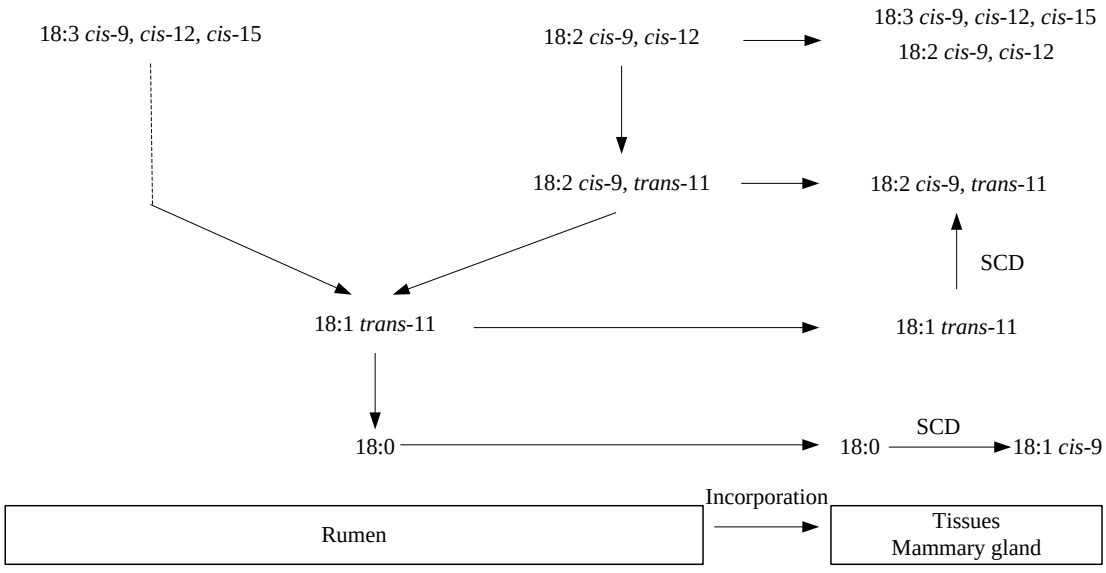

Figure 1. 3 Pathways of 18:2 *cis*-9, *trans*-11 biosynthesis

The factors that might affect the expression and activity of SCD have been extensively studied, although mainly in laboratory animals and humans. Ntambi and Miyazaki (2004) in their revision about regulation of SCD showed that developmental processes and dietary (*e.g.* PUFA, vitamin A), hormonal (*e.g.* insulin, glucagon) and environmental factors (*e.g.* temperature changes, metals) affect the SCD expression/activity in liver and adipose tissue from rodents. However, there is very limited information about regulation of expression and activity of SCD in ruminants.

Polyunsaturated FA, especially of the *n*-6 and *n*-3 series inhibits the SCD expression (Sessler *et al.*, 1996, Sessler and Ntambi, 1998). The inhibitory effect of dietary PUFA on lipogenic enzymes, including SCD are related to the degree of unsaturation and the chain length of FA (Clarke and Jump, 1993, Sessler *et al.*, 1996). The 18:3*n*-3 showed to be more effective in suppression the SCD gene expression in murine adipocytes than 18:2*n*-6 (Sessler *et al.*, 1996). In accordance with this result, Herdmann *et al*. (2010) reported recently the decrease of SCD protein expression in cattle muscle and subcutaneous adipose tissue induced by linseed oil (rich in 18:3*n*-3) supplementation of diets. Our studied also showed that dietary replacement of sunflower oil (rich in 18:2*n*-6) with linseed oil decreases the SCD messenger (mRNA) abundance in lamb muscle (Jerónimo *et al.*, 2008). Feeding forage or pasture compared to concentrate results in a depression the SCD expression in adipose tissue of lambs (Daniel *et al.*, 2004b) and steers (Duckett *et al.*, 2009), probably due to increases the 18:3*n*-3 supply. Moreover, the higher plasmatic insulin concentration in animals fed concentrate diets than fed forage diets also might explain the higher SCD expression in adipose tissue from lambs fed concentrate (Daniel *et al.*, 2004b), so insulin increases significantly the expression of ovine SCD in cultured ovine adipose tissue explants (Daniel *et al.*, 2004a). Waters *et al*. (2009) reported higher inhibitory effect of dietary fish oil (rich in *n*-3 LC-PUFA) on SCD gene expression

in cattle muscle when compared with soybean oil (rich in 18:2*n*-6). However, some studies not found effect of the changed the PUFA dietary supply on SCD expression and activity in ruminant tissues (Archibeque *et al.*, 2005, Pavan and Duckett, 2007, Bernard *et al.*, 2009). Recently was reported that SCD protein expression also may be regulated by plant secondary compounds. Vasta *et al*. (2009c) showed that supplementation of forage-based diet with CT from quebracho increases the SCD protein expression in lamb muscle.

In tissues the essentials FA, 18:2*n*-6 and 18:3*n*-3 can be desaturated and elongated to produce LC-PUFA (**Figure 1.4**). The both *n*-6 and *n*-3 PUFA are metabolized by a same desaturation/elongation pathway.

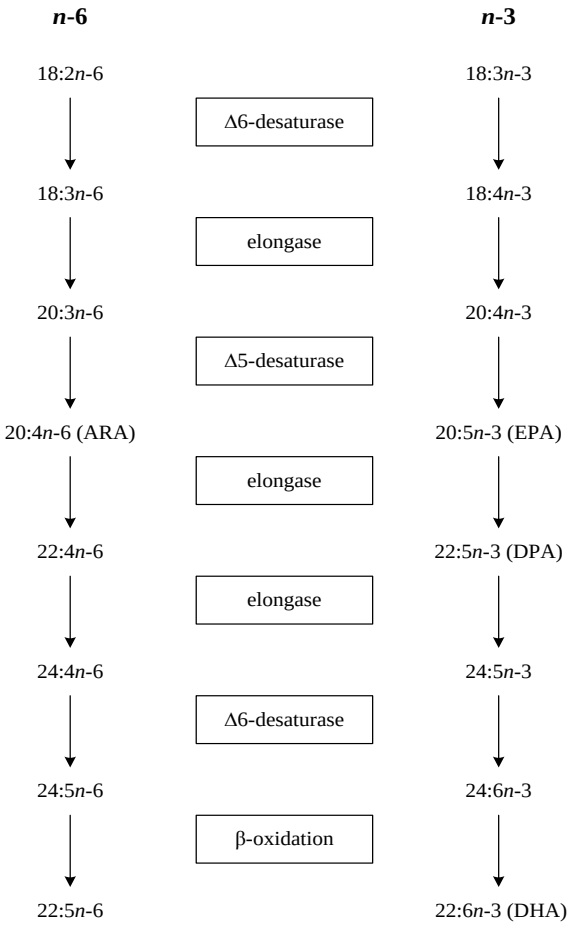

Figure 1. 4 Biosynthesis of *n*-6 and *n*-3 long chain polyunsaturated fatty acids (adapted from Brown (2005))

This pathway involves Δ5- and Δ6-desaturases and chain-elongation enzymes. The Δ6- and Δ5-desaturase introduce *cis*-double bonds at the Δ6 and Δ5 positions, respectively and elongases adds two carbons units in carbon chain. In last step of 18:2*n*-6 and 18:3*n*-3 conversion the chain is shortened in two carbons by β-oxidation. Linoleic acid is the precursor of ARA, and 18:3*n*-3 can be converted to EPA, DPA and DHA acids.

The conversion 18:3*n*-3 to *n*-3 LC-PUFA has been extensively studied, due to benefic proprieties of *n*-3 LC-PUFA. In humans, as well as in ruminants the conversion of 18:3*n*-3 to their LC-PUFA is very limited (Burdge *et al.*, 2003, Givens and Gibbs, 2008). Both *n*-3 and *n*-6 PUFA compete for same desaturase and elongase enzymes, and although these enzymes have preference for the *n*-3 PUFA (Brenner, 1989), higher dietary intake of 18:2*n*-6 limits the 18:3*n*-3 conversion to *n*-3 LC-PUFA (Burdge and Calder, 2005) and result in greater conversion of 18:2*n*-6 to *n*-6 LC-PUFA (Palmquist, 2009). Moreover, results obtained with rodents suggest that *n*-3 and *n*-6 LC-PUFA synthesis may be limited by dietary PUFA supply, so Δ6- and Δ5-desaturase mRNA levels decreases when *n*-6 PUFA and *n*-3 PUFA are supplied from diet (Cho *et al.*, 1999a, Cho *et al.*, 1999b). Recently was reported that supplementation of diet with 18:3*n*-3 (linseed oil) decreases the Δ6-desaturase protein level in cattle muscle (Herdmann *et al.*, 2010). However, this study showed that despite the lower Δ6-desaturase protein abundance, the intramuscular content of individual *n*-3 LC-PUFA increased with 18:3*n*-3 supplementation.

Saturated FA and MUFA are mainly deposited in triacylglycerols, whereas PUFA are mainly found in phospholipids. Thus, the 18:1 *cis*-9, 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 present in meat are much more predominant in NL than PL (Wood *et al.*, 2008). In triacylglycerols the PUFA, mainly of 18:2*n*-6 and 18:3*n*-3 represent 2 to 30 g/ 100 g of total triacylglycerols FA (Raes *et al.*, 2004). Phospholipids containing high levels of

PUFA (20-50 g/100 g of total FA in phospholipids), mainly 18:2*n*-6 and 18:3*n*-3, as well their longer chain derivatives, as ARA, EPA, DPA and DHA (Raes *et al.*, 2004). However, the 18:2*n*-6 and 18:3*n*-3 are deposited differentially between triacylglycerols and phospholipids, which 18:2*n*-6 is deposited preferentiality in phospholipids, while the 18:3*n*-3 is more equal partitioned between both lipids fractions (De Smet *et al.*, 2004, Wood *et al.*, 2008). Although, the distribution of these major FA is established, there is very little information about deposition of several FA between PL and NL fractions, as the most of the minor BI.

The triacylglycerols FA composition is influenced by dietary FA, but in lesser extension in ruminants than in monogastrics, due to ruminal BH of dietary FA (Raes *et al.*, 2004, Scollan *et al.*, 2006). One function of the unsaturated FA is maintaining the appropriate fluidity of cell membranes and changes in the fluidity of cell membranes can affect membrane functions and cellular activity (Wahle, 1983, Spector and Yorek, 1985). Thus, the phospholipids FA composition is strictly controlled in order to maintain membrane properties, being less influenced by diet (Raes *et al.*, 2004). However, differences in phospholipids FA composition caused by diet are observed (Scollan *et al.*, 2006), and FA composition of PL follow the same general pattern of NL (Cooper *et al.*, 2004, Demirel *et al.*, 2004, Nuernberg *et al.*, 2005). Nevertheless, some works suggest that there is adaptive mechanisms in phospholipids that allow respond of changes in FA availability, maintaining the membranes proprieties (Lands *et al.*, 1990, Scislowski *et al.*, 2004). Wood *et al.* (2008) reviewed the results of Warren *et al.* (2008) suggest that the capacity for incorporation of PUFA into phospholipids is limited. Possibly this limitation is due to strong regulation of FA composition, which may limit any strategy for meat enrichment in PUFA.

1.4. DIETARY STRATEGIES FOR IMPROVING THE FATTY ACID COMPOSITION OF LAMB MEAT

Meat FA composition is affected by several factors, such as intrinsic factors linked animal (*e.g.* species, breed, type of muscle, age/body weight) and environmental factors (*e.g.* nutrition, season, climate and accommodation). Fatness is the major animal factor influencing the FA composition of meat. The animal factors affects the fat content of meat and fat content itself have an important impact on FA composition, due differential FA deposition between triacylglycerols and phospholipids (Wood *et al.*, 2008). As referred above, triacylglycerols is characterized by a high proportion of SFA and MUFA, whereas phosoholipids show a high proportion of PUFA (De Smet *et al.*, 2004, Raes *et al.*, 2004, Wood *et al.*, 2008). With increasing the body fatness, the deposition of fat in muscle occurs mainly in the NL fraction, whereas the content of phospholipids in cell membrane remains very fairly constant (**Figure 1.1**). This variation is associated with an increased the proportion of SFA and MUFA and a decreased the proportion of PUFA in intramuscular fat, due to effect of dilution the phospholipids fraction in total lipids.

However, differences in intramuscular fat composition caused by intrinsic animal factors are smaller than those induced by dietary factors (De Smet *et al.*, 2004). In monogastrics animals the quality of meat can be relatively easy improved by dietary manipulation. However, in ruminants the BH of unsaturated FA constitute a limitation of enrichment of PUFA in ruminant fat. Modifying the ruminal BH of dietary PUFA through animal diets may be a way to improve the healthiness of ruminant fat. Thus, dietary strategies that can change the ruminal BH, increasing the dietary PUFA and benefit BI (18:2 *cis*-9, *trans*-11 and 18:1 *trans*-11) that escapes from rumen has been sought. It is identified that several dietary factors are able to modulate the ruminal BH, such as amount and type of lipid supplement, basal diet and plant secondary compounds, resulting in differences in the

amounts of PUFA that escape from rumen BH and in type and distribution of BI. Although several studies did not evaluated the ruminal BH and rumen outflow of FA, showed that dietary manipulation is the most effective means of improving the FA composition of ruminant meat (Scollan *et al.*, 2006, Sinclair, 2007).

1.4.1. Basal diet

The composition of basal diet is able to modify the ruminal BH and consequently the intramuscular FA composition. Ruminal BH of C_{18} FA reduces with increase the concentrate proportion in diet (Kalscheur *et al.*, 1997, Kucuk *et al.*, 2001, Sackmann *et al.*, 2003, Loor *et al.*, 2004), apparently because of the lower pH that is typically observed on these diets (Kalscheur *et al.*, 1997). Moreover, diets rich in concentrate change the rumen BH pathways of PUFA that favours the production of 18:2 *trans*-10, *cis*-12 and 18:1 *trans*-10 instead 18:2 *cis*-9, *trans*-11 and 18:1 *trans*-11 (Griinari and Bauman, 1999), resulting in greater flow of 18:2 *trans*-10, *cis*-12 and 18:1 *trans*-10 to duodenum (Sackmann *et al.*, 2003, Loor *et al.*, 2004). Thus, diets rich in concentrate results in lower accumulation of 18:2 *cis*-9, *trans*-11 and 18:1 *trans*-11 in lamb muscle as compared to diets rich in forage and pasture (**Table 1.2**). Generally, in muscle from lambs fed high-concentrate diets the 18:1 *trans*-10 is the predominate 18:1 *trans* isomer, however the content of 18:2 *trans*-10, *cis*-12 is residual (Daniel *et al.*, 2004b, Bessa *et al.*, 2005). Forage and pasture has shown to be one of the best strategies to increase the intramuscular content of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11, as well as *n*-3 PUFA (Raes *et al.*, 2004, Schimd *et al.*, 2006, Scollan *et al.*, 2006, Sinclair, 2007).

Table 1. 2 Effect of basal diet on 18:1 *trans*-10, 18:1 *trans*-11, 18:2 *trans*-10, *cis*-12 and 18:2 *cis*-9, *trans*-11 contents (% of total fatty acids) in intramuscular fat of lambs

	18:1*t*10	18:1*t*11	18:2*t*10*c*12	18:2*c*9*t*11	Reference
Pasture	3.34[1]		-	0.87	Santos-Silva *et al*. (2002)
Pasture supplemented with concentrate	3.16[1]		-	0.72	
Concentrate	2.87[1]		-	0.24	
Pasture – low feed rate	4.71[1]		-	0.94	Aurousseau *et al.* (2004)
Pasture – high feed rate	5.02[1]		-	1.29	
Concentrate – low feed rate	3.12[1]		-	0.60	
Concentrate – high feed rate	3.23[1]		-	0.53	
Pelleted dehydrated grass	0.38	2.25	0.01	1.29	Daniel *et al*. (2004)[2]
Concentrate	1.73	0.85	0.02	0.74	
Pasture	-	5.7	-	1.90	Nuernberg *et al*. (2005)
Concentrate	-	3.8	-	1.08	
Pelleted dehydrated lucerne	0.58	2.44	0.00	0.85	Bessa *et al.* (2005)
Concentrate	3.83	0.96	0.05	0.55	
Pasture	4.4[1]		-	1.1	Aurousseau *et al.* (2007a)
Pasture + Concentrate for a short time	4.7[1]		-	1.0	
Pasture + Concentrate for a long time	2.0[1]		-	0.9	
Concentrate	1.6[1]		-	0.7	

[1] – Sum of all 18:1 *trans* isomers; [2] – Fatty acids reported as mol/100 mol fatty acid methyl ester.

Lambs fed forage or pasture compared with concentrate have a 2-7-fold increase of 18:3*n*-3 in muscle (Aurousseau *et al.*, 2004, Daniel *et al.*, 2004b, Bessa *et al.*, 2005, Nuernberg *et al.*, 2005, Demirel *et al.*, 2006, Aurousseau *et al.*, 2007a). Although the efficiency of 18:3*n*-3 conversion to *n*-3 LC-PUFA is low, some studies showed that feeding pasture or forage increases the concentration of *n*-3 LC-PUFA in lamb muscle (Santos-Silva *et al.*, 2002a, Bessa *et al.*, 2005, Demirel *et al.*, 2006, Aurousseau *et al.*, 2007a), mainly in phospholipids (Nuernberg *et al.*, 2005, Aurousseau *et al.*, 2007b). These authors found that concentration of *n*-3 LC-PUFA was 1.2-2.5-fold more in muscle of lambs fed forage or pasture than in lambs fed diets rich in concentrate.

Within forages, it was reported that their botanical composition affects the FA metabolism in rumen (Lourenço *et al.*, 2008b) and particular forages may provide added benefits (Lourenço *et al.*, 2010). As botanically diverse pasture and silage that result in greater proportion of BI in rumen content of lambs, particularly in 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 despite the similar precursor proportion supply (Lourenço *et al.*, 2007a, Lourenço *et al.*, 2007b). These results have been associated with presence of plant secondary metabolites in botanically diverse pastures that might affect microbial BH activity in rumen (Lourenço *et al.*, 2008b).

1.4.1. Lipid supplementation

Inclusion of lipid sources rich in PUFA in ruminant diets has been extensively explored as approach to improve the nutritional value of its products. Unsaturated FA have a potent antimicrobial effects (Maia *et al.*, 2007, Maia *et al.*, 2010), changing the microbial ecology and consequently ruminal BH. Drastic changes in BH pattern due unprotected lipid supplementation, namely a strong accumulation of 18:1 *trans*-11 and low

concentrations of 18:0, have been reported *in vitro* (Fievez *et al.*, 2007). However, *in vivo* the effect of unprotected lipid supplementation on BH pattern is usually much less expressive. Dietary supplementation with vegetable oils rich in PUFA increases the rumen outflow of dietary PUFA, most individual BI and main end product of BH (18:0) (**Table 1.3**). In most dietary conditions, the supplementation of diets with vegetable oils result in a strong increases the rumen outflow of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11. However, the effect of lipid supplementation on rumen FA metabolism varies with composition of the basal diet, and in case the diet rich in concentrate, the lipid supplementation induces to strong increases the rumen outflow of 18:1 *trans*-10 (Duckett *et al.*, 2002, Loor *et al.*, 2004), being in some cases the predominant 18:1 *trans* isomer in duodenum (Duckett *et al.*, 2002).

Bessa *et al*. (2005) compared the effect of the supplementation of concentrate based diet and dehydrated lucerne with 10% of soybean oil on intramuscular FA composition of lambs (**Table 1.4**), and reported that the supplementation of concentrate diet increases the contents of 18:1 *trans*-10 and 18:2 *trans*-10, *cis*-12 but not the 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11. In contrast with concentrate, lipid supplementation of forage diet resulted in significant increase the content of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 in lamb muscle. Bas *et al*. (2007) and Manso *et al*. (2009) also not observed increase the 18:2 *cis*-9, *trans*-11 in lamb muscle when supplemented concentrate based-diets with extruded linseed or sunflower oil.

Table 1. 3 Effect of dietary supplementation with unprotected lipid sources rich in PUFA on fatty acid rumen outflow (g/day)

	18:3*n*-3	18:2*n*-6	18:2*c*9*t*11	18:1*t*11	18:1*t*10	18:0	Reference/Animals
Duodenal flow							
High-concentrate diet	1.58	30.74	0.19	5.02	3.43	180.6	Duckett *et al*. (2002) Beef steers
High-concentrate diet + 2.37% corn oil	1.61	38.21	0.25	5.22	17.9	342.3	
High-concentrate diet	0.11	1.90	0.06	1.38[1]		16.1	Kucuk *et al*. (2004) Wether lambs
High-concentrate diet + 3.2% soybean oil of DM	0.20	2.63	0.07	2.63[1]		27.5	
High-concentrate diet + 6.3% soybean oil of DM	0.23	2.91	0.08	9.07[1]		33.1	
High-concentrate diet + 9.4% soybean oil of DM	0.35	3.77	0.07	10.4[1]		51.8	
Forage and concentrate (65:35)	8.93	21.8	0.31	21.4	1.46	196.5	Loor *et al.* (2004) Dairy cows
Forage and concentrate (65:35) + 3% linseed oil of DM	12.9	20.2	0.52	61.7	6.61	454.7	
Forage and concentrate (35:65)	8.92	36.5	0.31	26.0	20.2	201.7	Loor *et al.* (2004) Dairy cows
Forage and concentrate (35:65) + 3% linseed oil of DM	29.6	42.8	0.86	139	50.6	313.7	
High-concentrate diet	0.17	1.53	0.004	2.62	-	21.7	Atkinson *et al*. (2006)[2] Wether lambs
High-concentrate diet + 3% high-linoleate safflower oil of DM	0.12	3.30	0.012	5.40	-	23.0	
High-concentrate diet + 6% high-linoleate safflower oil of DM	0.26	6.10	0.20	25.2	-	48.2	
High-concentrate diet + 9% high-linoleate safflower oil of DM	0.54	7.64	0.38	40.8	-	49.4	
Omasal flow							
Grass silage and concentrate (60:40)	1.5	5.53	1.93	14.9	1.3	237	Shingfield *et al.* (2008) Dairy cows
Grass silage and concentrate (60:40) + 250 g of sunflower oil/day	1.2	8.68	4.77	30.2	4.1	408	
Grass silage and concentrate (60:40) + 500 g of sunflower oil/day	1.0	8.72	9.23	54.7	8.6	514	
Grass silage and concentrate (60:40) + 750 g of sunflower oil/day	0.9	12.5	11.6	126	20.6	672	

[1] – Sum of all 18:1 *trans* isomers; [2] – Fatty acid flow adjusted for 50 kg of body weight.

Although some studies reported increases the 18:2 *cis*-9, *trans*-11 content in lambs muscle by supplementation of diets rich in concentrate with unsaturated vegetable lipid sources, these increases are small (Bolte *et al.*, 2002, Kott *et al.*, 2003, Boles *et al.*, 2005, Berthelot *et al.*, 2010). Intramuscular 18:2 *cis*-9, *trans*-11 content of lambs fed diets rich in concentrate supplemented with vegetable oils and oilseed rich in PUFA varied between 0.08 and 1.45% of total FA. However the most values are below to 1% of total FA and the value more frequently observed is 0.44% of total FA (**Table 1.4**). Whereas in lambs fed only forage or pasture with vegetable oils or oilseed the intramuscular content of 18:2 *cis*-9, *trans*-11 varied between 1.16 and 2.37% of total FA. Thus, supplementation of diets rich in concentrate with vegetable lipid sources rich in PUFA seems to be inadequate to obtain an expressive increases in intramuscular content of 18:2 *cis*-9, *trans*-11. Diets rich in concentrate are extensively used in ruminants fattening, and nutritional strategies that allow increased the CLA of meat from animals fed high-concentrate diet are needed.

Feeding lipid sources rich in 18:2*n*-6 and 18:3*n*-3 will increase the 18:2 *cis*-9, *trans*-11 content in ruminant meat (Schimd *et al.*, 2006). However, different responses on intramuscular CLA content are observed between vegetable lipid sources, mainly due their content in 18:2*n*-6 and 18:3*n*-3. Bessa *et al*. (2007) and Noci *et al*. (2007a) reported that linseed oil (rich in18:3*n*-3) is less effective in the increase of 18:2 *cis*-9, *trans*-11 in lamb meat and beef, respectively than sunflower oil (rich in 18:2*n*-6). This response is in accordance with higher accumulation of 18:2 *cis*-9, *trans*-11 in ruminal fluid in cows fed sunflower oil than fed linseed oil (Loor *et al.*, 2005), because during ruminal BH of 18:3*n*-3 is not produced 18:2 *cis*-9, *trans*-11.

Despite the intense BH of dietary PUFA in rumen, generally feeding dietary unprotected lipid sources rich in 18:2*n*-6 and 18:3*n*-3 increases the muscle concentration of these FA, which improves the P/S ratio in meat (**Table 1.4**). However, the lipid sources rich in 18:2*n*-6 have a negative effect on meat *n*-6:*n*-3 ratio (above 4), while feeding linseed oil or linseed improve the *n*-6:*n*-3 ratio. Previous data obtained by our group showed that blend of unprotected lipid sources rich in 18:2*n*-6 and rich in 18:3*n*-3 may a good approach to obtain simultaneously lamb meat enriched in CLA and *n*-3 PUFA (Bessa *et al.*, 2007).

Feeding a ruminally protected lipid sources allow increases the intestinal supply of unsaturated FA (Jenkins and Bridges Jr., 2007) and may be a good strategy to increase the 18:3*n*-3 content in ruminant meats (**Table 1.4**). Demirel *et al*. (2004) and Kitessa *et al*. (2009) reported that lambs fed lipid sources of 18:3*n*-3 formaldehyde-treated has 1.8-fold more 18:3*n*-3 in muscle than lambs fed control diets. Cooper *et al*. (2004) showed that protected lipid source rich of 18:3*n*-3 is more effective in increase the 18:3*n*-3 in lamb muscle than linseed oil. Despite the protected source supply only one-third of 18:3*n*-3 supplied by linseed oil, muscle from lambs fed protected lipids had 1.4-fold more 18:3*n*-3 than lambs fed linseed oil. Sinclair *et al*. (2005) tested the effect of linseed oil adsorption in clay (vermiculite) in FA duodenal flow in wethers, and reported that linseed oil adsorbed in clay result in higher flow of 18:2*n*-6 and 18:3*n*-3 and lower flow of 18:0 than linseed oil or formaldehyde-treated whole linseed oil. Although improve the P/S ratio in lamb meat this strategies results in lower deposition of CLA in muscle than unprotected vegetable lipid source (linseed oil) (Cooper *et al.*, 2004).

Table 1. 4 Effect of dietary unsaturated lipid supplementation on intramuscular fatty acid composition (% of total fatty acids) of lambs

	SFA		MUFA		PUFA				Reference
	16:0	18:0	18:1*c*9	18:1*t*11	18:2*n*-6	18:3*n*-3	*n*-3 LC-PUFA	18:2*c*9*t*11	
Dried grass + 4.4% megalac	25.4	14.5	34.2	3.83[1]	4.9	1.40	1.60	1.02	Wachira *et al*. (2002)
Dried grass + 10.5% whole linseed	21.8	14.3	30.9	6.61[1]	4.0	3.10	2.64	1.55	
Dried grass + 3.6% fish oil	25.0	11.9	25.9	7.06[1]	3.4	1.40	4.40	1.10	
Dried grass + 5.2% whole linseed + 1.8% fish oil	23.9	12.2	27.4	8.59[1]	3.5	1.99	3.26	1.66	
Concentrate	25.0	13.5	43.2	1.60	3.3	0.31	0.10	0.34	Bolte *et al*. (2002)
Concentrate + 15 % high oleate safflower	23.3	15.3	41.2	3.60	2.9	0.48	0.18	0.56	
Concentrate + 17 % high linoleate safflower	23.3	14.4	36.1	6.20	4.5	0.37	0.13	0.87	
Concentrate	23.3	13.2	42.9	-	5.5	0.82	-	0.39	Kott *et al*. (2003)
Concentrate + 15% safflower seeds	22.5	13.6	40.6	-	8.1	0.56	-	0.85	
Concentrate + 4.3% linseed oil	20.7	17.5	31.6	5.17[1]	4.80	2.70	1.60	1.09	Cooper *et al*. (2004)
Concentrate + 4.3% fish oil	24.9	13.6	28.5	4.79[1]	3.30	1.54	2.78	0.74	
Concentrate + 11.1% Protected linseed and soybean (PLS)	20.3	15.7	29.5	2.78[1]	14.5	3.68	1.34	0.68	
Concentrate + 2.1% fish oil + 15.5% Algae	24.3	13.6	27.1	4.89[1]	4.10	0.79	6.11	0.75	
Concentrate + 15.5% Algae + 11.1% PLS	23.0	13.9	28.1	5.50[1]	10.1	2.50	4.03	0.82	
Dried grass + 3.5% megalac	23.1	12.8	38.6	1.67[1]	4.1	2.01	2.22	-	Demirel *et al*. (2004)
Dried grass + 8.5% protected whole linseed	22.9	13.6	27.3	1.97[1]	3.3	3.66	2.37	-	
Dried grass + 4.2% protected whole linseed + 1.5% fish oil	23.5	13.5	33.0	3.56[1]	3.5	2.41	4.17	-	
Lucerne hay	21.4	17.7	30.4	1.45	5.91	2.50	2.80	0.55	Santos-Silva *et al*. (2004)
Lucerne hay + 8 % soybean oil	19.0	15.1	23.8	10.0	7.16	1.27	1.31	2.37	
Ground and pelleted dehydrated lucerne	24.0	15.5	33.9	2.18	6.42	1.62	1.54	0.64	
Ground and pelleted dehydrated lucerne + 8 % soybean oil	18.8	15.5	24.3	7.62	10.8	1.11	0.81	1.83	
Concentrate	22.3	10.5	34.6	-	8.44	-	-	0.62	Boles *et al*. (2005)
Concentrate + 3% safflower oil	20.6	9.51	28.3	-	14.0	-	-	0.99	
Concentrate + 6% safflower oil	19.7	9.30	25.0	-	16.1	-	-	1.45	

Concentrate	24.4	14.1	35.6	0.96	6.4	0.36	0.68	0.55	Bessa *et al.* (2005)
Concentrate + 10 % soybean oil	22.7	14.2	26.0	0.72	9.5	0.61	0.93	0.44	
Pelleted dehydrated lucerne	22.7	16.6	31.1	2.44	7.2	2.69	1.73	0.85	
Pelleted dehydrated lucerne + 10 % soybean oil	20.4	14.2	25.6	8.46	9.3	1.16	0.53	2.39	
Concentrate + 2% linseed oil + 1.5% corn oil (*n*-6/*n*-3–2.3)	20.9	12.4	28.9	3.50	16.5	2.1	2.75	0.46	Kim *et al.* (2007)
Concentrate + 3.5% soybean oil (*n*-6/*n*-3–8.8)	20.8	12.7	25.0	3.80	19.5	0.9	2.24	0.48	
Concentrate + 1.6 % corn oil + 3.5% soybean oil (*n*-6/*n*-3–12.8)	19.0	12.7	23.4	3.30	22.1	0.7	2.65	0.44	
Concentrate + 2.2 % corn oil + 1.3% soybean oil (*n*-6/*n*-3–15.6)	17.6	12.3	18.8	2.70	28.0	0.9	3.16	0.23	
Pelleted dehydrated lucerne	17.0	15.2	29.4	1.03	10.2	2.01	3.47	0.44	Bessa *et al.* (2007)
Pelleted dehydrated lucerne + 6% sunflower oil	16.0	13.7	24.2	4.75	15.7	0.51	1.63	1.50	
Pelleted dehydrated lucerne + 4% sunflower oil + 2% linseed oil	16.7	13.9	25.8	5.25	12.0	1.52	1.81	1.49	
Pelleted dehydrated lucerne + 6% linseed oil	16.5	13.3	25.3	4.09	9.5	4.08	2.73	1.16	
Concentrate	23.9	14.4	40.0	0.85	3.90	0.48	0.45	0.07	Bas *et al.* (2007)
Concentrate + 3% extruded linseed	24.4	12.6	39.7	0.65	3.65	0.78	0.42	0.08	
Concentrate + 6% extruded linseed	24.2	13.4	40.1	0.80	4.15	1.08	0.57	0.12	
Concentrate + 9% extruded linseed	23.8	14.2	37.7	0.90	4.15	1.34	0.59	0.08	
Concentrate	23.8	16.4	37.0	3.54[1]	5.99	0.44	1.73	0.35	Manso *et al.* (2009)
Concentrate + 4% sunflower oil	22.2	17.3	35.2	6.30[1]	5.75	0.30	2.03	0.39	
Concentrate	23.3	21.9	37.5	1.28	5.78	0.92	1.12	1.04	Kitessa *et al.* (2009)
Concentrate + 10% protected linseed oil and soybean seeds (3:7)	21.3	21.7	34.0	1.05	9.85	1.67	1.53	1.08	
Concentrate	22.3	11.5	35.1	0.54	9.61	0.56	1.02	0.05	Berthelot *et al.* (2010)
Concentrate + 10% extruded linseed	21.5	11.5	32.5	0.73	10.6	2.47	1.64	0.11	

[1] – Sum of all 18:1 *trans* isomers.

Supplementation of diets with lipid sources rich in 18:3*n*-3 (protected and unprotected sources) generally also increases the intramuscular concentration of *n*-3 LC-PUFA, with greater increase in EPA than DPA and DHA (Wachira *et al.*, 2002, Demirel *et al.*, 2004, Berthelot *et al.*, 2010). Although feeding lipid sources rich in 18:3*n*-3 increases significantly the individual *n*-3 LC-PUFA, in some cases these increases are little expressive, reflecting the low conversion of 18:3*n*-3 to *n*-3 LC-PUFA. Sinclair (2007) reported that there is a poor and non-significant relationship between duodenal 18:3*n*-3 flow and muscle content of EPA+DHA, being muscle content of EPA+DHA strongly related with duodenal flow of EPA+DHA. Supplementation of ruminant diets with preformed sources, as fish oil or marine algae has been shown to be more effective to increase the EPA and DHA in lamb meat than lipid sources rich in 18:3*n*-3 (Wachira *et al.*, 2002, Cooper *et al.*, 2004, Demirel *et al.*, 2004).

In contrast with vegetable lipid sources, inclusion of fish oil in ruminant diets induce to drastic changes in BH pattern *in vivo*, decreasing the flow of 18:0 to the duodenum and increasing the flow of 18:1 *trans*-11 (Kim *et al.*, 2008), suggesting that fish oil inhibit the last step of biohydrogenation. The *n*-3 LC-PUFA (EPA and DHA) are more toxic for BH bacteria than main PUFA present in vegetables oils and oilseeds, being the *B. proteoclasticus* group the most sensitive ruminal species to toxic effects of PUFA (Maia *et al.*, 2007, Maia *et al.*, 2010). Thus, it would be expected that fish oil reduces the stearate producers' bacteria in rumen. However, Kim *et al.* (2008) analysing the bacterial population in rumen by quantitative PCR (qPCR) of 16S rRNA genes did not found clear effect of fish oil on number of *B. proteoclasticus*. Nevertheless the microbial ecology of the rumen was substantially changed with addition of fish oil in diets, showing that fish oil has an inhibitory effect on the BH of FA in the rumen via its influence on microbial ecology.

Sinclair *et al*. (2005) reported that dietary supplementation with fish oil or with blend of fish oil with marine algae decrease the sheep duodenal flow of 18:0 and increase the flow of 18:1 *trans* when compared with linseed oil. In accordance with this result Wachira *et al*. (2002) and Cooper *et al.* (2004) found lower proportion of 18:0 in muscle and adipose tissue of lambs fed fish oil or blend of fish oil with algae than lambs fed whole linseed or linseed oil. However, the effect on total 18:1 *trans* in is less consistently. These two studies also showed that utilization of fish oil alone or mixture with algae is less efficient to increase the CLA content in lamb tissues when compared to lipid sources rich in PUFA. So inclusion of fish oil or blend of fish oil with algae in diets result in similar levels of 18:2 *cis*-9, *trans*-11 to control diet (without PUFA supplementation) (Cooper *et al.*, 2004), but lower than diets supplemented with lipid sources rich in 18:3*n*-3 (whole linseed or linseed oil) (Wachira *et al.*, 2002, Cooper *et al.*, 2004). Nevertheless, combining fish oil with whole linseed led to similar levels of 18:2 *cis*-9, *trans*-11 to whole linseed alone (Wachira *et al.*, 2002).

1.4.2. Plant secondary compounds

The effects of plant secondary compounds, as essential oils, saponins and tannins on ruminal BH is presently under extensive research. These classes of plant secondary metabolites are known for their ability to influence the ruminal microbes and several studies showed that some these metabolites have potential to manipulate the ruminal BH. However, evidence of the effects of plant secondary metabolites on BH has been obtained mainly by *in vitro* studies, so the effect of the most these metabolites in ruminal ecosystem is not known. Durmic *et al.* (2008) observed that some ethanolic extracts and essential oils from Australian plants to inhibit the growth and/or activity of pure cultures of some bacteria involved in ruminal BH, such *B. fibrisolvens* and *B. proteoclasticus* in

batch culture incubations. This study also showed that some plants inhibited the saturation of 18:2*n*-6 and other BI such 18:2 *cis*-9, *trans*-11 and 18:1 *trans*-11. In addition, Lourenço *et al*. (2009) reported that essential oil rich in monoterpenes limonene and carvone induces to accumulation of 18:2 *cis*-9, *trans*-11 *in vitro*. Using a continuous culture fermenter system, Lourenço *et al*. (2008a) showed that cinnamaldehyde, a main component of cinnamon bark essential oil (*Cinnamon cassia*) affected strongly the 18:2*n*-6 BH, causing a shift from the major BH pathways to a secondary pathway as evidenced by higher proportion of 18:2 *trans*-10, *cis*-12 and 18:1 *trans*-10 in fermenter effluent.

The effect of saponins on ruminal BH has been inconsistent. It was reported that range of saponins inhibited the 18:2*n*-6 metabolism in mixed digest *in vitro* (N. McCain, data published by Lourenço *et al.*, 2010). In accordance with this results Wallace *et al*. (1994) observed than a *Yucca schidigera* extract (rich in saponin) affect selectively the growth of ruminal bacteria, inhibiting the growth of *B. fibrisolvens* more than other bacteria. This result suggests that saponins might be successful used in controlling FA BH (Wallace *et al.*, 1994). However, recently was reported that extract of *Y. schidigera* and triterpene saponin from *Quillaja* bark are inefficient in modifying the BH of 18:3*n*-3 *in vitro* (Lourenço *et al.*, 2008a, Khiaosa-Ard *et al.*, 2009). Differences between results have been attributed to different types of saponins used (Lourenço *et al.*, 2008).

Two recent *in vitro* studies have shown that CT extracts from *Acacia mearnsii* and *Schinopsis lorentzii* (quebracho) inhibited the conversion of 18:1 *trans*-11 to 18:0, while no effect was detected on 18:2 *cis*-9, *trans*-11 production (Khiaosa-Ard *et al.*, 2009, Vasta *et al.*, 2009a). The inhibitory effect of CT on ruminal BH was confirmed *in vivo* studies, which the inclusion of quebracho tannins to lamb diets reduces the ruminal BH, particularly the last step of the BH, leading to accumulation of 18:1 *trans*-11 in rumen (Vasta *et al.*, 2009b, Vasta *et al.*, 2010b). The accumulation of 18:1 *trans*-11 rumen is

consistent with reported effects of CT on rumen microbial population. Durmic *et al.* (2008) showed that an extract deriving from *A. mearnsii* has a selective inhibitory effect on *B. proteoclasticus* but not on *B. fibrisolvens*. Recently, Vasta *et al.* (2010b) also showed that inclusion of quebracho tannins in lambs diets decreased the relative abundance of *B. proteoclasticus* in rumen content, however increased the abundance of *B. fibrisolvens*. These results show that inclusion of CT sources in ruminant diets may be a useful tool to increase the rumen ouflow of 18:1 *trans*-11.

To date, little information has been published on the effect of diet rich in CT on intramuscular FA composition of lambs. Moreover, the effects reported not have been consistent (**Table 1.5**). In accordance with inhibitory effect of CT on ruminal BH reported in studies *in vitro* and *in vivo*, Vasta *et al*. (2009b, 2009c) showed that lambs fed diets supplemented with quebracho powder contained lower proportion of 18:0 and higher proportions of 18:1 *trans* and PUFA in muscle than lambs fed control diet. Nevertheless, these authors not found significantly effect on content of 18:2 *cis*-9, *trans*-11.Vasta *et al*. (2007) showed that carob pulp inclusion in diets decreased the content of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 in lamb muscle. In contrast, lambs fed fresh sulla as sole diet have 1.98-fold more 18:2 *cis*-9, *trans*-11 in muscle than lambs fed concentrate. These differences observed in results may be due to differences in type and amount of CT used in experiments.

Table 1. 5 Effect of dietary condensed tannins sources on intramuscular fatty acid composition (% of total fatty acids) of lambs

	18:0	18:1*t*11	18:1*trans*	18:2*c*9*t*11	18:2*n*-6	18:3*n*-3	Reference
Control	14.5	-	2.42	0.46	17.4	1.18	Priolo *et al*. (2005)
Fresh sulla - 1.78% of CT DM	13.9	-	1.61	0.91	11.5	4.98	
Control	9.86	1.82	-	0.73	14.8	6.84	Vasta *et al*. (2007)
Control + carob pulp - 2.7% of CT DM	8.09	0.95	-	0.48	11.2	4.78	
Control	15.2	2.19	-	0.58	8.20	1.43	Vasta *et al*. (2009)
Control + quebracho powder - 4.0% of CT DM	13.7	3.71	-	0.62	11.7	2.01	
Control	14.2	0.69	2.39	0.46	9.2	1.00	Vasta *et al*. (2009)
Control + quebracho powder - 4.0% of CT DM	11.5	1.32	5.23	0.96	10.7	1.38	

1.5. RESEARCH OBJECTIVES

Facing to nutritional recommendations the alteration of the FA composition of ruminant edible products is need and has been under intense research. In this study, we aimed explore some nutritional strategies in order to improve the nutritional value of intramuscular fat of lamb meat. Thus, we conducted three *in vivo* trials with Merino Branco lambs, with following specific objectives:

- To determine the best blend of sunflower and linseed oils to obtain the simultaneously lamb meat enriched in *n*-3 PUFA and CLA (Chaper 2);

- To investigate the deposition of C_{18} BI in the intramuscular PL and NL fractions and effect of dietary replacement of sunflower oil with linseed oil on its deposition in both lipid fractions (Chaper 3);

- To evaluate the effect of sodium bentonite inclusion in diets supplemented or not with blend of vegetable oils rich in PUFA on intramuscular FA composition of lamb (Chaper 4);

- To investigate the effect of grape seed extract and leaves and stems of *Cistus ladanifer* shrub inclusion in diets supplemented or not with blend of vegetable oils rich in PUFA on the ruminal BH and intramuscular FA composition (Chaper 5), as well as on lipid oxidative and colour stability during storage (Chaper 6).

CHAPTER 2

EFFECT OF DIETARY REPLACEMENT OF SUNFLOWER OIL WITH LINSEED OIL ON INTRAMUSCULAR FATTY ACIDS OF LAMB MEAT

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ABSTRACT

The effect of stepwise replacement of dietary sunflower oil (SO) with linseed oil (LO) on carcass composition, meat colour and FA composition of intramuscular lipids of lamb meat was investigated. Thirty-six lambs were fed one of four diets consisting of pellets of lucerne with oil (60 g/kg DM): the diet varied in the composition of oil added and were: 100% SO; 66.6% SO plus 33.3% LO; 33.3% SO plus 66.6% LO; and 100% LO. The experimental period was 7 weeks. Live slaughter weight, hot carcass weight and intermuscular fat percentage of chump and shoulder increased linearly with replacement of SO by LO. Total FA content of *longissimus dorsi* muscle and PL and NL were not affected by the treatments. Replacement of SO with LO increased the content of 18:3*n*-3 and total *n*-3 LC-PUFA and decreased the 18:2*n*-6, total *n*-6 LC-PUFA and 18:2 *cis*-9, *trans*-11 in meat lipids. Maximum CLA concentration (42.9 mg/100 g fresh muscle) was observed with 100% of SO, decreasing linearly by SO with LO replacement. Maximum *n*-3 LC-PUFA was predicted to be 27 mg/100 g of fresh muscle at 78% of SO with LO replacement. Considering both CLA and *n*-3 LC-PUFA, the maximum levels were estimated to be reached at 52% of replacement of SO with LO. The utilization of blends of SO and LO is a good approach for obtaining lamb meat enriched with both CLA and *n*-3 LC-PUFA.

Keywords: lamb meat; conjugated linoleic acid; polyunsaturated fatty acids, sunflower oil; linseed oil

2.1. INTRODUCTION

Ruminant meats have been associated with an increase in the risk of cardiovascular diseases, due to their high content of SFA (Givens, 2005). However, ruminant meats may also be a good dietary source of some nutrients with health benefits including some FA such as LC-PUFA and CLA. The decrease of SFA and the increase of health-beneficial FA has been a main topic of ruminant meat research. The beneficial effects of EPA and DHA acids are well documented and include anti-atherogenic, anti-thrombotic and anti-inflammatory actions (Givens *et al.*, 2006). It is important to increase the intake of EPA and DHA in the human diet because the synthesis of these FA from dietary 18:3*n*-3 is very limited (Burdge and Calder, 2005). Although, food of marine origin are the richest sources of EPA and DHA, its contribution to human diet in most Western countries is low (Givens and Gibbs, 2008). Thus, the enrichment of foods consumed in relatively high quantities with EPA and DHA is one option to increase the intake of these *n*-3 PUFA. There are opportunities to increase the concentration of *n*-3 LC-PUFA in ruminant meats (Givens *et al.*, 2006). The inclusion of 18:3*n*-3 source in lamb diets, such as forages (Bessa *et al.*, 2005), pastures (Santos-Silva *et al.*, 2002a), linseeds (Wachira *et al.*, 2002, Demirel *et al.*, 2004) or linseed oil (Cooper *et al.*, 2004, Bessa *et al.*, 2007) increases the concentration of *n*-3 LC-PUFA in meat.

The CLA acronym refers to a group of positional and geometric isomers of 18:2*n*-6, in which the double bonds are conjugated, and many studies suggest that CLA exhibits anticarcinogenic, anti-adipogenic, anti-diabetogenic, anti-atherogenic and anti-inflammatory effects (Wahle *et al.*, 2004). Ruminant fats are among the richest natural sources of CLA isomers, particularly of the rumenic acid (18:2 *cis*-9, *trans*-11), and are the main sources of these isomers in the human diet (Chin *et al.*, 1992). The 18:2 *cis*-9, *trans*-11 is formed during the ruminal BH of 18:2*n*-6 to stearic acid (Harfoot and

Hazelwood, 1997) and by endogenous conversion of 18:1 *trans*-11 by Δ9-desaturase in tissues (Griinari *et al.*, 2000). Feeding lipid sources rich in 18:2*n*-6 and 18:3*n*-3 will increase the 18:2 *cis*-9, *trans*-11 content of ruminants meat (Santos-Silva *et al.*, 2004, Bessa *et al.*, 2005, De La Torre *et al.*, 2006, Bessa *et al.*, 2007). However, feeding linseed oil (rich in 18:3*n*-3) seems to be less effective in the increase of 18:2 *cis*-9, *trans*-11 in muscle than sunflower oil (rich in 18:2*n*-6) (Bessa *et al.*, 2007, Noci *et al.*, 2007a). Bessa *et al*. (2007) observed that a blend of sunflower and linseed oils may be a good approach to obtain simultaneously an enrichment in *n*-3 PUFA and CLA in lamb meat. Thus, in this work we intended to further explore this approach, studying more levels of stepwise substitution of sunflower oil (SO) with linseed oil (LO) in order to determine the best blend, as well as to extend the FA analysis to neutral and polar muscle lipid fractions.

2.2. MATERIALS AND METHODS

2.2.1. Animals management and sampling procedures

Animal handling followed the EU directive 86/609/EEC, concerning animal care. Thirty-six Merino Branco ram lambs were used in a trial carried out in the Centro de Experimentação do Centro Alentejo (Reguengos de Monsaraz, Portugal). The lambs were born in September 2005, and were reared on pasture with their dams until weaning, that occurred at about 90 days of age. The average initial weight of lambs was 22.9 ± 2.78 kg (mean ± SD). Animals were randomly assigned to four groups of nine lambs each. The four experimental diets were as follows: pelleted dehydrated lucerne with sunflower oil (S); pelleted dehydrated lucerne with a blend of 66.6% of sunflower oil and 33.3% of linseed oil (2SL); pelleted dehydrated lucerne with a blend of 33.3% of sunflower oil and 66.6% of linseed oil (S2L); pelleted dehydrated lucerne with of linseed oil (L). The target

for oil inclusion was 60 g/kg on a dry matter (DM) basis, resulting in pellets with ether extract range between 70 and 76 g/kg of DM. The diets were prepared in an industrial unit and oil was sprayed over the pelleted dehydrated lucerne. The chemical composition of the diets is presented in **Table 2.1**.

Table 2. 1 Chemical composition of the experimental diets.

	SO replaced with LO (%)[1]			
	0	33.3	66.6	100
g/kg dry matter				
Crude protein	153	153	153	152
Ether extract	70	71	72	76
NDF[2]	500	482	485	481
Fatty acid composition (% of total fatty acids)				
16:0	8.2	8.3	7.9	7.0
18:0	2.4	3.0	3.3	3.4
18:1 *cis*-9	20.4	19.5	17.7	15.6
18:2*n*-6	57.1	43.9	30.6	18.3
18:3*n*-3	6.3	20.4	33.6	48.0

[1] 0% of sunflower oil (SO) by linseed oil (LO) replacement – diet S; 33.3% of SO with LO replacement – diet 2SL; 66.6% of SO with LO replacement – diet S2L; 100% of SO with LO replacement – diet L; [2] Neutral detergent fibre.

After an adaptation period of 7 days to the experimental conditions, lambs stayed on trial for 7 weeks. Feed was offered daily at morning at a rate of 110% of *ad libitum* intake calculated by daily refusal weighing. The animals were weighed weekly just before feeding. At the end of trial, lambs were transported to the experimental abattoir of the Unidade de Investigação em Produção Animal – Instituto Nacional de Recursos Biológicos (UIPA-INRB; Vale de Santarém, Portugal). After weighing, to obtain the live slaughter weight, lambs were stunned and slaughtered by exsanguination. Carcasses were immediately weighed to obtain the hot carcass weight, which was used to assess the

dressing percentage. The carcasses were kept at 10 ºC for 24 h, and then chilled at 2 ºC until the third day after slaughter. The kidney knob channel fat (KKCF) and the kidneys were removed and the carcasses were split along the spine. The left sides of the carcasses were separated into eight joints (Santos-Silva *et al.*, 2002b), and the chumps and the shoulders were dissected into muscle, subcutaneous and intermuscular fats and bone. The colour of *longissimus muscle* was measured at the level of the 13th thoracic vertebra, using a Minolta CR-300 chromometer (Konica Minolta, Portugal) in the *L**, *a** and *b** system after 1 hour of exposure to air to allow blooming. After removing the epimysium, the *longissimus dorsi* muscle was minced, vacuum packed, freeze-dried and stored at -80 ºC until lipid analysis.

2.2.2. Lipid analysis

Fatty acid methyl esters (FAME) of feed lipids were prepared by a one-step extraction transesterification, using toluene and heptadecanoic acid (17:0) as internal standard, according to the procedure reported by Sukhija and Palmquist (1988).

Intramuscular lipids were extracted by the method of Folch *et al*. (1957), using dichloromethane and methanol (2:1 v/v), instead of chloroform and methanol (2:1 v/v), as described by Carlson (1985). The lipid extract was separated into neutral (NL) and polar (PL) lipids, using a solid-phase extraction procedure described by Juaneda and Rocquelin (1985) and silica gel cartridges (LiChrolut® Si, 40-63µm, 500 mg/ml, Standard, Merck KGaA, Darmstadt, Germany). The NL fraction was eluted with dichloromethane and the PL fraction with methanol. The total lipids, NL and PL of muscle were transesterified with sodium methoxide followed by hydrochloric acid in methanol (1:1 v/v) as described by Raes, *et al*. (2001). Quantification of muscle lipids FAME was done using

nonadecanoic acid (19:0) as internal standard. The FAME were analysed using a HP6890A chromatograph (Hewlett- Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GC-FID) and fused silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 µm of film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA). Helium was used as the carrier gas and the injector split ratio was 1:50. The initial column temperature of 100 ºC was held for 15 min, increased to 150 ºC at 10 ºC/min and held for 5 min. Then, was increased to 158 ºC at 1 ºC/min, held 30 min, and finally increased to 200 ºC at a rate of 1 ºC/min, and maintained for 60 min. The injector and detector temperatures were 250 and 280 ºC, respectively. Identification was accomplished by comparison of sample peak retention times with those of FAME standard mixtures (Sigma, St. Louis, MO, USA). For the resolution of 18:1 *cis*-9 from both 18:1 *trans*-13 and 18:1 *trans*-14 (that co-eluted in our GC-FID conditions) a second temperature program was used. The initial temperature column of 70 ºC was held for 4 min, increased to 110 ºC at 8 ºC/min and then increased to 170 ºC at 5 ºC/min, held 10 min, and finally increased to 220 ºC at a rate of 4 ºC/min, and maintained for 25 min. Thus, the relative amounts of 18:1 *cis*-9 and 18:1 *trans*-13/14 were calculated from the second temperature program and applied to the area of the common peak identified in initial temperature program. Also, the FA 20:3*n*-9 co-eluted with the 18:3 *cis*-9, *trans*-11, *cis*-15 in our GC-FID conditions, its quantification was conducted as describe in Bessa *et al*. (2007). CLA reported here is the GC-FID peak that included the predominant 18:2 *cis*-9, *trans*-11 isomer but also the minor 18:2 *trans*-7, *cis*-9 and the 18:2 *trans*-8, *cis*-10 isomers.

2.2.3. Statistical analysis

The effect of dietary replacement of SO with LO was analyzed using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC, USA). The model included the linear and quadratic effects of dietary oil replacement. The model used to study the growth performance and carcass traits include covariates. The average daily gain, live slaughter weight, hot carcass weight and dressing percentage were adjusted to the same initial live weight. The KKCF and muscle, bone, intermuscular fat and subcutaneous fat, obtained after chump and shoulder dissection and colour parameters were adjusted to hot carcass weight. Least square means and standard error of the mean (SEM) are presented in tables.

2.3. RESULTS

2.3.1. Growth performance, carcass composition and meat colour

Average daily weight gain, live slaughter weight, hot carcass weight, dressing percentage, carcass and meat traits are presented in **Table 2.2**. The dietary replacement of SO with LO tended to increased the average daily weight gain (P = 0.065) and increased linearly the live slaughter weight, hot carcass weight and intermuscular fat percentage on chump and shoulder. The other carcass traits and meat colour parameters were not affected by the type of dietary oil used.

Table 2. 2 Effect of dietary replacement of sunflower oil (SO) with linseed oil (LO) on growth and carcass composition of Merino Branco lambs.

	SO replaced with LO (%)[1]				SEM	*P* values	
	0	33.3	66.6	100		Linear	Quad.
Initial live weight (kg)	25.4	23.5	21.5	21.2	0.77[2]		
Average daily gain (g)	223	226	238	260	13.3	0.065	0.517
Live slaughter weight (kg)	29.6	30.4	32.7	32.1	0.49	0.003	0.546
Hot carcass weight (kg)	13.7	14.2	14.7	14.6	0.32	0.001	0.587
Dressing percentage (%)[3]	45.2	45.5	43.8	44.1	0.67	0.070	0.962
KKCF (%)[4]	2.0	2.7	2.4	2.7	0.23	0.221	0.282
Muscle (%)[5]	58.0	57.0	57.0	55.6	0.85	0.084	0.797
Intermuscular fat (%)[5]	11.2	11.9	11.9	12.8	0.40	0.011	0.816
Subcutaneous fat (%)[5]	9.62	9.90	10.0	10.6	0.680	0.321	0.825
Bone (%)[5]	19.9	19.9	19.6	19.7	0.54	0.764	0.925
Colour[6]							
*L**	38.6	39.6	39.7	38.4	0.73	0.543	0.090
*a**	12.8	13.8	13.7	14.0	0.63	0.598	0.649
*b**	4.4	5.0	5.0	5.4	0.44	0.505	0.848

[1] 0% of sunflower oil (SO) by linseed oil (LO) replacement – diet S; 33.3% of SO with LO replacement – diet 2SL; 66.6% of SO with LO replacement – diet S2L; 100% of SO with LO replacement – diet L; [2] Standard deviation; [3] Dressing percentage = (hot carcass weight x 100/ live slaughter weight); [4] kidney and knob channel fat; [5] Average of chump and shoulder; [6] *L** - lightness; *a** - redness; *b** - yellowness.

2.3.2. Meat fatty acids

The intramuscular fatty acids (IMFA) content and the pattern of muscle PL, NL fractions and total FA are presented in **Tables 2.3**, **2.4** and **2.5** respectively. The sums and ratio of FA from polar, neutral and total lipids are presented in **Table 2.6**.

The dietary replacement of SO with LO did not affect the total FA concentration in PL, NL and total muscular FA, which averaged 17.0 ± 1.70, 74.8 ± 6.15, and 91.9 ± 6.75 mg/g muscle DM (mean ± SEM), respectively. The NL:PL ratio did not changed among treatments and averaged 4.3 (19% of PL and 81% of NL).

2.3.2.1. Meat polar lipids

The major FA in PL were the 18:2*n*-6, that decreased from 24% in the lambs fed S diet to 14% in the lambs fed L diet, and the 18:1 *cis*-9, that increased from 13% in the lambs fed S diet to 19% in the lambs fed L diet, followed by 16:0, that represented 11% of total FA, 18:0, that represented 10% of total FA, and ARA, that decreased from 8% in the lambs fed S diet to 6% in the lambs fed L diet (**Table 2.3**). The dietary replacement of SO with LO did not affect the SFA, except for 17:0 which increased quadratically. The replacement of SO with LO decreased linearly all the *n*-6 PUFA and 17:1 *cis*-9. Otherwise, the 16:1 *cis*-9, 18:1 *cis*-9, 18:3*n*-3, EPA and DHA increased linearly and DPA increased quadratically with oil exchange. The 18:1 *trans*-11 and CLA were unaffected by treatments. The sum of the remaining area (others) included about 8.4% of dimethylacetals, 8.1% of BI other than 18:1 *trans*-11 and CLA, 0.25% of branched-chain FA and 4.2% of unidentified peaks.

2.3.2.2. Meat neutral lipids

The major FA in NL for all diets was the 18:1 *cis*-9 (32% of total FA), 16:0 (21% of total FA) and 18:0 (16% of total FA) (**Table 2.4**). The replacement of SO with LO in the diets decreased linearly the 18:1 *trans*-11, *n*-6 PUFA and CLA, increased linearly the 18:3*n*-3 and EPA, but did not affect the 18:1 *cis*-9, DPA and DHA. The 16:0 and 14:1 *cis*-9, 16:1 *cis*-9 and 17:1 *cis*-9 showed a quadratic response with higher concentration in treatments where both SO and LO were present (2SL and S2L) than in S and L diets. The sum of the remaining area (others) include about 10.3% of BI other than 18:1 *trans*-11 and CLA, 0.81% of branched-chain FA, a residual amount of dimethylacetals (0.06%) and 1.3% of unidentified peaks.

Table 2. 3 Effect of dietary replacement of sunflower oil (SO) with linseed oil (LO) on fatty acid concentration (mg/g fresh muscle) and composition (g/100 g total fatty acids) of polar lipids in *longissimus muscle* from Merino Branco lambs.

	SO replaced with LO (%)[1]				SEM	*P* values	
	0	33.3	66.6	100		Linear	Quad.
Total fatty acids	3.9	3.9	3.7	3.8	0.38	0.756	0.818
14:0	0.20	0.23	0.25	0.22	0.038	0.632	0.411
15:0	0.17	0.19	0.19	0.17	0.009	0.738	0.071
16:0	10.7	11.3	10.7	10.6	0.25	0.403	0.164
16:1 *cis*-9	0.28	0.38	0.39	0.43	0.026	0.001	0.298
17:0	0.49	0.54	0.54	0.53	0.012	0.027	0.019
17:1 *cis*-9	1.40	1.42	1.37	1.21	0.063	0.049	0.143
18:0	10.2	10.2	10.4	10.4	0.21	0.484	0.935
18:1 *trans*-11	2.80	2.77	2.92	2.26	0.101	0.091	0.062
18:1 *cis*-9	12.1	15.7	14.4	18.0	0.67	<0.001	0.975
18:2*n*-6	23.9	19.6	17.8	13.5	0.58	<0.001	0.983
CLA[2]	1.10	1.22	1.12	1.05	0.050	0.308	0.055
18:3*n*-3	1.29	2.55	4.73	5.93	0.225	<0.001	0.898
20:0	0.12	0.13	0.16	0.12	0.011	0.781	0.056
20:2*n*-6	0.15	0.13	0.14	0.08	0.014	0.004	0.266
20:3*n*-9	0.73	0.80	0.73	0.73	0.044	0.738	0.455
20:3*n*-6	0.64	0.57	0.50	0.41	0.015	<0.001	0.682
20:3*n*-3	0.07	0.07	0.10	0.08	0.016	0.448	0.659
20:4*n*-6	8.3	7.0	6.8	6.0	0.22	<0.001	0.242
20:5*n*-3	0.72	1.12	1.80	1.98	0.068	<0.001	0.147
22:4*n*-6	0.70	0.55	0.48	0.40	0.026	<0.001	0.153
22:5*n*-3	1.41	1.70	1.98	1.87	0.050	<0.001	<0.001
22:6*n*-3	0.30	0.33	0.52	0.43	0.029	<0.001	0.103
Others[3]	22.2	21.3	22.1	23.6	0.40	0.019	0.006

[1] 0% of sunflower oil (SO) by linseed oil (LO) replacement – diet S; 33.3% of SO with LO replacement – diet 2SL; 66.6% of SO with LO replacement – diet S2L; 100% of SO with LO replacement – diet L; [2] Include 18:2 *cis*-9, *trans*-11, 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9 isomers; [3] Include branched-chain fatty acids, biohydrogenation intermediates other than 18:1 *trans*-11 and CLA, dimethylacetls and unidentified peaks.

Table 2. 4 Effect of dietary replacement of sunflower oil (SO) with linseed oil (LO) on fatty acid concentration (mg/g fresh muscle) and composition (g/100 g total fatty acids) of neutral lipids fraction of *longissimus muscle* from Merino Branco lambs.

	SO replaced with LO (%)[1]				SEM	*P* values	
	0	33.3	66.6	100		Linear	Quad.
Total fatty acids	16.0	16.8	17.7	16.9	1.38	0.565	0.582
12:0	0.12	0.12	0.13	0.11	0.011	0.657	0.689
14:0	1.99	2.15	2.35	2.11	0.102	0.211	0.066
14:1 *cis*-9	0.05	0.06	0.06	0.05	0.005	0.723	0.039
15:0	0.30	0.29	0.29	0.27	0.015	0.173	0.858
16:0	20.5	21.6	21.5	20.6	0.43	0.931	0.030
16:1 *cis*-9	1.26	1.35	1.30	1.12	0.058	0.114	0.031
17:0	0.91	0.84	0.85	0.84	0.026	0.098	0.317
17:1 *cis*-9	0.17	0.31	0.20	0.18	0.020	0.589	0.002
18:0	16.7	16.4	16.0	16.6	0.53	0.831	0.358
18:1 *trans*-11	6.35	5.99	7.08	5.42	0.402	0.008	0.832
18:1 *cis*-9	30.6	31.4	30.5	29.4	0.84	0.262	0.286
18:2*n*-6	4.56	3.54	2.99	2.26	0.126	<0.001	0.271
CLA[2]	2.40	2.27	2.03	1.60	0.087	<0.001	0.181
18:3*n*-3	0.70	1.08	1.77	1.87	0.102	<0.001	0.204
20:0	0.09	0.08	0.09	0.13	0.008	0.002	0.014
20:2*n*-6	0.07	0.04	0.04	0.03	0.003	<0.001	0.013
20:3*n*-9	0.08	0.05	0.05	0.06	0.006	0.0280	0.003
20:3*n*-6	0.04	0.03	0.03	0.02	0.003	<0.001	0.675
20:3*n*-3	0.01	0.02	0.04	0.04	0.003	<0.001	0.004
20:4*n*-6	0.24	0.17	0.14	0.12	0.015	<0.001	0.084
20:5*n*-3	0.04	0.03	0.04	0.05	0.004	0.047	0.090
22:4*n*-6	0.05	0.03	0.02	0.02	0.003	<0.001	0.156
22:5*n*-3	0.11	0.11	0.11	0.11	0.006	0.964	0.686
22:6*n*-3	0.04	0.03	0.03	0.03	0.006	0.777	0.632
Others[3]	11.9	11.7	13.6	16.9	0.37	<0.001	<0.001

[1] 0% of sunflower oil (SO) by linseed oil (LO) replacement – diet S; 33.3% of SO with LO replacement – diet 2SL; 66.6% of SO with LO replacement – diet S2L; 100% of SO with LO replacement – diet L; [2] Include 18:2 *cis*-9, *trans*-11, 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9 isomers; [3] Include branched-chain fatty acids, biohydrogenation intermediates other than 18:1 *trans*-11 and CLA, dimethylacetls and unidentified peaks.

2.3.2.3. Meat total lipids

The total FA in muscle reflects the weighted combination of PL and NL fractions. The major FA in total lipids for all diets were the 18:1 *cis*-9 (27% of total FA), 16:0 (19% of total FA) and 18:0 (15% of total FA), which were not affected by the treatments (**Table 2.5**). Most of the minor SFA (12:0, 14:0, 15:0 and 20:0) and 17:1 *cis*-9 were also not affected by treatments. The 17:0 decreased, whereas the 14:1 *cis*-9 increased, linearly with replacement of SO with LO, although the concentration of 16:1 *cis*-9 showed a quadratic response with higher concentration in treatments where both SO and LO were present (2SL and S2L) than in S and L diets.

The replacement of SO with LO in the diets decreased linearly the 18:1 *trans*-11, *n*-6 PUFA (except the 20:2*n*-6), CLA and 20:3*n*-9 and increased linearly all *n*-3 PUFA although, both EPA and DPA (and hence the *n*-3 LC-PUFA) showed also positive quadratic response. Lambs fed S diet had higher 18:2*n*-6 (+46%) and *n*-6 PUFA (+40%) than lambs fed L diet. However, the proportion of *n*-6 LC-PUFA in total *n*-6 PUFA was lower in lambs fed S diet (24%) than in lambs fed L diet (28%).

2.3.2.4. Sums and ratios of fatty acids

The total SFA in muscle remained unaffected by dietary treatments averaging 36% of total FA and 80% in the NL fraction. The total MUFA in muscle averaged 40% of total FA and 88% were in the NL fraction. The total PUFA in muscle, tended to decreased linearly (P = 0.054) from 27% in S diet to 24% in L diet. Polar lipids contributed with 51% of total PUFA in the S diet, which decreased linearly to 41% of total PUFA in the L diet. However, the contribution of PL to total LC-PUFA is higher (86% for *n*-6 LC-PUFA and 78% for *n*-3 LC-PUFA) when compared to NL.

Table 2. 5 Effect of dietaty replacement of sunflower oil (SO) with linseed oil (LO) on fatty acid concentration (mg/g fresh muscle) and composition (g/100 g total fatty acids) of total lipids in *longissimus muscle* from Merino Branco lambs.

	SO replaced with LO (%)[1]				SEM	*P* values	
	0	33.3	66.6	100		Linear	Quad.
Total fatty acids	20.0	20.7	21.3	20.7	1.52	0.67	0.656
12:0	0.12	0.11	0.12	0.12	0.007	0.705	0.599
14:0	1.55	1.72	1.78	1.70	0.076	0.135	0.109
14:1 *cis*-9	0.06	0.07	0.07	0.09	0.007	0.043	0.726
15:0	0.27	0.26	0.25	0.25	0.012	0.278	0.794
16:0	18.1	19.2	18.6	18.2	0.43	0.896	0.075
16:1 *cis*-9	0.73	0.89	0.83	0.73	0.040	0.848	0.003
17:0	0.79	0.74	0.73	0.71	0.022	0.010	0.522
17:1 *cis*-9	0.36	0.37	0.38	0.32	0.025	0.491	0.145
18:0	14.8	14.8	14.4	14.3	0.35	0.206	0.805
18:1 *trans*-11	5.90	5.50	5.18	4.85	0.349	0.038	0.909
18:1 *cis*-9	26.3	28.0	26.5	26.9	0.71	0.936	0.336
18:2*n*-6	9.36	7.52	6.74	5.09	0.379	<0.001	0.786
CLA[2]	2.13	2.06	1.84	1.56	0.086	<0.001	0.251
20:0	0.10	0.07	0.07	0.08	0.084	0.081	0.062
18:3*n*-3	0.93	1.57	2.62	3.05	0.109	<0.001	0.404
20:3*n*-9	0.27	0.22	0.19	0.19	0.016	<0.001	0.051
20:2*n*-6	0.07	0.07	0.07	0.07	0.005	0.721	0.965
20:3*n*-6	0.22	0.16	0.15	0.13	0.011	<0.001	0.304
20:3*n*-3	0.01	0.05	0.08	0.09	0.008	<0.001	0.068
20:4*n*-6	2.45	1.91	1.87	1.52	0.148	<0.001	0.510
20:5*n*-3	0.19	0.29	0.51	0.50	0.021	<0.001	0.041
22:4*n*-6	0.21	0.16	0.16	0.15	0.016	0.010	0.195
22:5*n*-3	0.46	0.54	0.62	0.54	0.030	0.018	0.014
22:6*n*-3	0.14	0.15	0.19	0.20	0.016	0.013	0.964
Others[3]	14.5	13.4	16.0	18.8	0.55	<0.001	0.002

[1] 0% of sunflower oil (SO) by linseed oil (LO) replacement – diet S; 33.3% of SO with LO replacement – diet 2SL; 66.6% of SO with LO replacement – diet S2L; 100% of SO with LO replacement – diet L; [2] Include 18:2 *cis*-9, *trans*-11, 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9 isomers; [3] Include branched-chain fatty acids, biohydrogenation intermediates other than 18:1 *trans*-11 and CLA, dimethylacetls and unidentified peaks.

Table 2. 6 Effect of dietary replacement of sunflower oil (SO) with linseed oil (LO) on sums of fatty acids (g/100 g total fatty acids) and nutritional indices value of total, polar and neutral lipids in *longissimus muscle* from Merino Branco lambs.

	SO replaced with LO (%)[1]				SEM	*P* values	
	0	33.3	66.6	100		Linear	Quad.
Polar lipids							
SFA	22.0	22.8	22.5	22.2	0.38	0.847	0.183
MUFA	23.5	27.0	25.6	27.7	0.66	0.002	0.326
PUFA	40.8	38.0	39.7	36.4	0.74	0.006	0.777
n-6 PUFA[2]	33.7	27.9	25.7	20.5	0.61	<0.001	0.679
n-6 LC-PUFA[3]	9.81	8.28	7.89	6.93	0.242	<0.001	0.255
n-3 PUFA[4]	3.79	5.77	9.13	10.3	0.314	<0.001	0.247
n-3 LC-PUFA[5]	2.50	3.22	4.40	4.36	0.122	<0.001	0.011
Neutral lipids							
SFA	40.9	41.7	41.5	41.1	0.52	0.833	0.247
MUFA	46.9	46.1	44.2	43.5	0.52	<0.001	0.995
PUFA	9.89	10.3	12.1	13.0	0.443	<0.001	0.520
n-6 PUFA[2]	4.95	3.82	3.21	2.45	0.132	<0.001	0.185
n-6 LC-PUFA[3]	0.40	0.28	0.23	0.19	0.020	<0.001	0.180
n-3 PUFA[4]	0.89	1.28	2.00	2.11	0.105	<0.001	0.190
n-3 LC-PUFA[5]	0.19	0.20	0.23	0.23	0.012	0.011	0.924
Total lipids							
SFA	36.0	37.2	36.2	35.6	0.58	0.471	0.119
MUFA	40.6	41.6	39.2	39.8	0.57	0.106	0.743
PUFA	26.9	24.5	25.9	23.6	1.01	0.054	0.728
P/S[6]	0.30	0.25	0.27	0.24	0.016	0.029	0.569
n-6 PUFA[2]	12.3	9.82	8.98	6.96	0.51	<0.001	0.650
n-6 LC-PUFA[3]	2.94	2.30	2.25	1.87	0.169	<0.001	0.448
n-3 PUFA[4]	1.74	2.61	4.01	4.38	0.134	<0.001	0.106
n-3 LC-PUFA[5]	0.81	1.04	1.39	1.33	0.058	<0.001	0.024
n-6/*n*-3	7.04	3.78	2.26	1.60	0.143	<0.001	<0.001

SFA - sum of saturated fatty acids; MUFA - sum of monounsaturated fatty acids; PUFA - sum of polyunsaturated fatty acids; [1] 0% of SO with LO replacement – diet S; 33.3% of SO with LO replacement – diet 2SL; 66.6% of SO with LO replacement – diet S2L; 100% of SO with LO replacement – diet L; [2] *n*-6 PUFA = (18:2*n*-6 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6); [3] *n*-6 LC-PUFA = (20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6); [4] *n*-3 PUFA = (18:3*n*-3 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3); [5] *n*-3 LC-PUFA = (20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3); [6] P/S = (18:2*n*-6 + 18:3*n*-3)/(12:0 + 14:0 + 16:0 + 18:0).

In PL fraction, the percentage of *n*-6 LC-PUFA on total *n*-6 PUFA increased from 29% on the S diet to 34% on the L diet. The percentage of *n*-3 LC-PUFA on total *n*-3 PUFA was higher than the observed for *n*-6 PUFA and decreased from 66% on the S diet to 43% on the L diet. The percentage of LC-PUFA on total PUFA was much lower on NL. The *n*-6 LC-PUFA was not affected by treatments and averaged 8% of *n*-6 PUFA in the NL fraction. Otherwise, the percentage of *n*-3 LC-PUFA on *n*-3 PUFA decreased from 21% on the S diet to 11% on the L diet. The replacement of SO with LO in the diets decreased linearly the P/S ratio and quadratically the *n*-6/*n*-3 ratio in total muscle FA.

2.4. DISCUSSION

2.4.1. Growth performance and carcass composition

Generally, the type of dietary lipid supplement has no effect on growth performance and carcass traits of lambs (Wachira *et al.*, 2002, Cooper *et al.*, 2004, Demirel *et al.*, 2004, Bessa *et al.*, 2007, Kim *et al.*, 2007). However, in the present trial, stepwise replacement of SO with LO increased linearly the live slaughter weight and hot carcass weight of lambs and modified the proportions of intermuscular fat obtained after dissection of the chump and shoulder. These results might be explained by the slight differences found in ether extract concentration of experimental diets (**Table 2.1**), or by eventual differences on feed intake (not monitored).

2.4.2. Meat fatty acid content

The total IMFA content is determined mostly by the amount of FA in the NL fraction, since the level of FA in the PL fraction is fairly constant (Scollan *et al.*, 2006, Wood *et*

al., 2008). In agreement with other reports (Wachira *et al.*, 2002, Kim *et al.*, 2007) the IMFA content was independent of the lipid type included in the diet. Moreover, Bessa *et al*. (2007) did not observe differences in IMFA content between lambs supplemented with sunflower oil or linseed oil, although the animals supplemented with a blend of these two oils showed a higher IMFA content. The experiment described here did not confirm the increase in IMFA with dietary blends of sunflower and linseed oils. Consistently, the PL:NL ratio did not change with the diet, and the values are in agreement with those described in the literature (Cooper *et al.*, 2004, Demirel *et al.*, 2004).

2.4.3. Meat fatty acid composition

As expected, the FA pattern of NL fraction was characterized by a high proportion of SFA and MUFA, whereas the PL fraction showed a high proportion of PUFA (Raes *et al.*, 2004). The FA composition in PL is less influenced by dietary factors than NL, although changes induced by diet in PUFA of PL have been reported (Scollan *et al.*, 2006). The replacement of SO with LO in the diet induced changes in PL that follow the same general pattern observed in NL, as reported by others (Cooper *et al.*, 2004, Demirel *et al.*, 2004, Nuernberg *et al.*, 2005). Nevertheless, some selective deposition in lipid fractions was evident. Both 18:2*n*-6 and 18:3*n*-3 were selectively incorporated in PL although, as it was previously shown, the selectivity for PL (wt% FA in PL/wt% FA in NL) was higher for 18:2*n*-6 (5.7) than for 18:3*n*-3 (2.5) (De Smet *et al.*, 2004).

The FA composition of membrane phospholipids is the major determinant of membrane fluidity and, thus, membrane function and cellular metabolism (Wahle, 1983, Spector and Yorek, 1985). In the present experiment, the dietary replacement of SO with LO decreased the proportion of 18:2*n*-6 (-10.3%) and increased 18:3*n*-3 (+4.6%) and 18:1

cis-9 (+5.9%) in PL. This suggests that the presence of a homeoviscous adaptation mechanism, in which the degree of unsaturation of C_{18} FA in membrane PL is maintained fairly constant. Thus, 18:2*n*-6 (2 double bonds) seems to be replaced by a pondered mixture of 18:3*n*-3 (3 double bonds) and 18:1 *cis*-9 (1 double bond). A similar homeoviscous adaptation was suggested by Scislowski *et al*. (2004), that found that dietary 18:2*n*-6 supplementation did not change the fluidity of bovine lipoproteins although extensive 18:2*n*-6 incorporation can occur. Moreover, the dietary replacement of SO with LO led to a substitution of *n*-6 LC-PUFA (mainly 20:4*n*-6) by *n*-3 LC-PUFA, maintaining fairly constant the proportion of *cis* PUFA with more than 3 double bonds in PL (about 10%), as already reported by Lands *et al*. (1990) in rats. The proportion of LC-PUFA in PL is 20 times more than in NL, which is consistent with the very high selectivity of LC-PUFA for PL. Thus, this might imply that any strategy for meat enrichment in LC-PUFA will be restricted by a fixed ceiling allowed in PL by metabolic regulation mechanisms. Wood *et al*. (2008), reviewing the results of Warren *et al*. (2008) also suggested that the capacity for incorporation of PUFA into phospholipids is limited.

The capacity of conversion of 18:3*n*-3 to health promoting *n*-3 LC-PUFA is limited in humans (Burdge and Calder, 2005) which reinforces the importance of its dietary supply. Dietary replacement of SO with LO resulted in a partial substitution of *n*-6 LC-PUFA by *n*-3 LC-PUFA in membranes. The competition between 18:2*n*-6 and 18:3*n*-3 for desaturation and elongation enzymes might affect the conversion to LC derivatives (Brenner, 1989). The higher proportion of *n*-3 LC-PUFA in total *n*-3 PUFA, relative to *n*-6 LC-PUFA in total *n*-6 PUFA, is likely due to the preference of these enzymes for 18:3*n*-3 (Brenner, 1989).

The increase of *n*-3 LC-PUFA in muscle lipids was linear until 66.6% of replacement of SO with LO in the diets was reached, and then stabilize, whereas the decrease of *n*-6 LC-

PUFA remains linear. This difference between *n*-3 LC-PUFA and *n*-6 LC-PUFA suggests that 18:3*n*-3 might be more powerful in the down-regulation of the expression of desaturases and elongases involved in the conversion to LC derivatives, than 18:2*n*-6. In fact, it is well established that the Δ6- and Δ5-desaturase mRNA levels are low when *n*-6 PUFA and *n*-3 PUFA are supplied from the diet (Cho *et al.*, 1999a, Cho *et al.*, 1999b). However, to our knowledge the comparison of the effects of 18:2*n*-6 and 18:3*n*-3 on Δ6- and Δ5-desaturase expression has not yet been evaluated. Notwithstanding this, it was reported that the inhibitory potency of dietary PUFA on other enzymes of lipid metabolism, like fatty acid synthase and Δ9-desaturase increases with the degree of unsaturation and chain length (Clarke and Jump, 1993, Sessler *et al.*, 1996). An alternative explanation for the lack of linearity of the increase of *n*-3 LC-PUFA in PL may be related to homeoviscous regulation of the incorporation of these highly unsaturated FA in membranes.

The higher deposition of CLA in NL fraction (88% of total CLA), relative to PL fraction, has been previously reported (Wood *et al.*, 2008). The content of 18:2 *cis*-9, *trans*-11 in meat decreased with replacement of dietary SO with LO, confirming the previous results obtain by our group (Bessa *et al.*, 2007). In addition, Noci *et al*. (2007) observed that the 18:2 *cis*-9, *trans*-11 content in *longissimus muscle* was higher in heifers supplemented with sunflower oil than with linseed oil. The explanation might be found in ruminal BH pathways of 18:2 *n*-6 and 18:3 *n*-3. Most of the 18:2 *cis*-9, *trans*-11 present in tissues derive from endogenous desaturation of 18:1 *trans*-11 (Palmquist *et al.*, 2004), which is originated during BH of both 18:2 *n*-6 and 18:3 *n*-3. However, 18:2 *cis*-9, *trans*-11 is also synthesized by direct isomerisation of 18:2 *n*-6 in the rumen.

2.4.4. Nutritional quality of meat lipids

The P/S and *n*-6/*n*-3 ratio are indices used to evaluate the nutritional value of fat for human consumption. Lamb meat has usually a high SFA concentration and low P/S values (Sinclair, 2007). As previously reported (Szumacher-Strabel *et al.*, 2004, Bessa *et al.*, 2007), the proportion of SFA in muscle lipids was not affected by the lipid type included in the diet. The major determinant of both SFA and P/S in the meat lipids is the IMFA content and the diet (De Smet *et al.*, 2004, Scollan *et al.*, 2006). Increasing the PUFA content of the diet, by including sources rich in either *n*-6 or *n*-3 PUFA, generally improves the P/S ratio (Sinclair, 2007). This was also observed in the present trial, and in all diets, the P/S ratio was always lower than 0.45, which is the minimum value recommended for human diet by the Department of Health (1994).

The *n*-6/*n*-3 ratio is highly influenced by FA composition of the diet fed to the animals (Raes *et al.*, 2004). Consistently, the decrease in the dietary *n*-6/*n*-3 ratio, caused by replacement of SO with LO in the diets, decreased the *n*-6/*n*-3 ratio of intramuscular lipids. All the diets with LO resulted in *n*-6/*n*-3 ratios below 4, which is the maximum recommended value for human diets by public health agencies (Department of Health, 1994). Recently, the value of *n*-6/*n*-3 in modifying cardiovascular disease has been questioned (Stanley *et al.*, 2007). The health benefits of *n*-3 PUFA may be mostly associated with absolute *n*-3 LC-PUFA (mainly EPA and DHA) dietary intake. Thus, 100 g of fresh muscle of lambs fed the S diet supply 16 mg of *n*-3 LC-PUFA, whereas that from lambs fed the L diet supply 27 mg. These values correspond to 3.6 and 6% of the average recommended daily intake for human diet (450 mg/person/day - Scientific Advisory Committee on Nutrition/Committee on Toxicity (2004)), respectively.

The supplementation of ruminant diets with fish oil has been showed to be more effective to increase the EPA and DHA in meat than lipid sources rich in 18:3*n*-3 (Scollan *et al.*, 2001, Cooper *et al.*, 2004, Demirel *et al.*, 2004). However, there are concerns that the fish oil may give rise to meat of shorter shelf life and with impaired flavour (Nute *et al.*, 2007), as well as on the sustainability of increased use of fish oil in the food chain (Givens *et al.*, 2006). Despite, the synthesis of EPA and DHA from dietary 18:3*n*-3 being limited; our results showed that the vegetable oil source rich in 18:3*n*-3 may be one possible alternative to increase the *n*-3 LC-PUFA in lamb meat.

The CLA intake, mainly the 18:2 *cis*-9, *trans*-11 isomer, is expected to give protective anticarcinogenic effects on humans (Banni *et al.*, 2003). The optimal dietary intake of CLA remains to be established, although extrapolation from animal trials, suggests that the ingestion of 720-800 mg/person/day would be necessary for anticancerinogenic protective effects in humans (Parish *et al.*, 2003, Watkins and Li, 2003). In lambs fed the S diet, 100 g of fresh muscle supply 43 mg of CLA, whereas that in lambs fed L diet only supply 31 mg. Moreover, about 19% of dietary 18:1 *trans*-11may be converted to 18:2 *cis*-9, *trans*-11 by Δ9-desaturase (Turpeinen *et al.*, 2002), contributing to CLA concentration. Thus, considering the 18:1 *trans*-11 content of lamb meat the potential CLA supply would increase by 22 mg/100 g muscle for lambs fed S diet and 19 mg/100 g fresh muscle in lambs fed L diet.

The response of meat CLA and *n*-3 LC-PUFA to the dietary replacement of SO with LO is plotted in **Figure 2.1**. Assuming that both FA groups have the same health value, the better oil blend can be estimated from the sum of CLA and *n*-3 LC-PUFA. The CLA content decreases linearly with the replacement of dietary SO with LO, but the maximum concentration of *n*-3 LC-PUFA is obtained with 78% of replacement of SO with LO (27

mg/100 g fresh muscle) and the maximum of the sum of CLA and *n*-3 LC-PUFA were obtained with 52% of replacement of SO with LO.

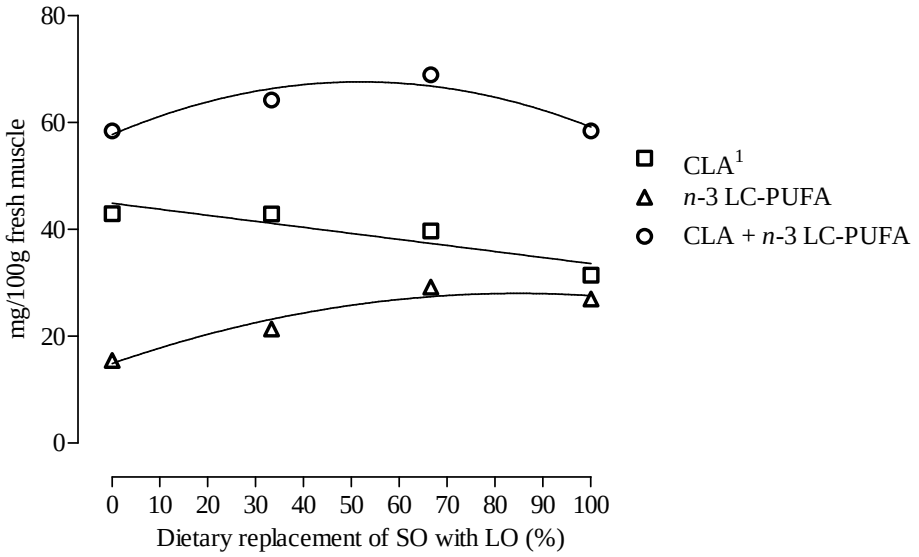

Figure 2. 1 Effect of dietary replacement of sunflower oil (SO) with linseed oil (LO) on concentration of CLA, *n*-3 LC-PUFA and sum of both group FA.

CLA = 44.87 ± 2.431 – 0.11 ± 0.039 *x*, R^2 = 0.81; *n*-3 LC-PUFA = 14.88 ± 2.631 + 0.31 ± 0.127 *x* - 0.002 ± 0.0012 x^2, R^2 = 0.94 and CLA + *n*-3 LC-PUFA = 57.70 ± 3.072 + 0.38 ± 0.148 *x* – 0.004 ± 0.0014 x^2, R^2 = 0.87; [1] Include 18:2 *cis*-9, *trans*-11, 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9 isomers.

2.5. CONCLUSIONS

The dietary replacement of SO with LO increased significantly the *n*-3 LC-PUFA in lamb meat, with the highest value of *n*-3 LC-PUFA achieved with 78% of SO with LO replacement. However, the synthesis of EPA and DHA from dietary 18:3*n*-3 seems to be limited, and thus the EPA and DHA enriched lamb meat contributes only in a small amount to the recommended daily intake for human diet. The results indicate that the maximum of 18:2 *cis*-9, *trans*-11 concentration is observed with 100% of SO, decreasing linearly by SO with LO replacement. Thus, the data indicate that the utilization of blends

of sunflower and linseed oil is a valid approach for obtaining lamb meat enriched with both CLA and *n*-3 LC-PUFA.

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CHAPTER 3

BIOHYDROGENATION INTERMEDIATES ARE DIFFERENTIALLY DEPOSITED BETWEEN POLAR AND NEUTRAL INTRAMUSCULAR LIPIDS OF LAMBS

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ABSTRACT

The deposition in intramuscular PL and NL of C_{18} FA, with emphasis on rumen BI were studied using twenty-six lambs fed oil supplemented diets where sunflower oil (SO) was progressively replaced by linseed oil (LO). Lambs were fed one of four diets consisting on dehydrated lucerne with either: 6% SO, 4% SO plus 2% LO, 2% SO plus 4% LO and 6% LO. The profile of C_{18} FA was greatly affected by replacement of SO with LO in both lipid fractions. In PL, oil replacement led to an extensive substitution of 18:2*n*-6 with 18:3*n*-3 and 18:1 *cis*-9 resulting in a fairly constant degree of unsaturation of C_{18} FA in membrane PL. C_{18} FA were differentially incorporated in NL and PL. *Cis* isomers like 18:1 *cis*-11, 18:1 *cis*-12, 18:1 *cis*-15 and 18:2 *cis*-12, *cis*-15 were preferentially incorporated in PL with the exception of 18:2 *cis*-9, *cis*-15. *Trans* C_{18} FA, including CLA isomers, were preferentially incorporated in NL with the exception of 18:2 *cis*-11, *trans*-13. The preferential deposition of biohydrogenation derived *trans* C_{18} FA, including CLA isomers in NL, suggests that their potential for competitive interactions with elongation and desaturation metabolic pathways of essential FA might be low.

***Keywords*:** neutral lipids; polar lipids; C_{18} fatty acids; selective deposition

3.1 INTRODUCTION

The FA composition of ruminant edible fats is mostly determined by complex interactions between dietary factors and rumen metabolism (Harfoot and Hazelwood, 1997). Dietary lipids are extensively hydrolysed in the rumen and the unsaturated FA liberated (mostly C_{18} PUFA) are also extensively biohydrogenated (Jenkins *et al.*, 2008). The extension of rumen BH will determine the amount of essential PUFA absorbed and, thus, available for tissue deposition. However, only a variable proportion of essential PUFA that disappears in the rumen is completely biohydrogenated to 18:0. The rumen BH of C_{18} PUFA pathways produces several unsaturated C_{18} FA derived from geometric and positional isomerisations and partial reductions of the substrates, hereafter named as BI. The extent of BH and particularly the pattern of BI are mainly affected by the type and amount of dietary lipids (Harfoot and Hazelwood, 1997) and basal diet (Bessa *et al.*, 2005).

The BI include mostly FA with *trans* double bonds, like *trans* octadecenoates and the conjugated and non-conjugated isomers of 18:2*n*-6, whose identification and quantification can be a difficult analytical task. The *cis*-9, *trans*-11 and *trans*-10, *cis*-12 conjugated isomers of linoleic acid have been extensively studied and many animal studies suggest some isomeric specific effects such as anticarcinogenic, anti-adipogenic, anti-diabetogenic, anti-atherogenic and anti-inflammatoty effects (Wahle *et al.*, 2004). However, the bioactive properties of most of the BI have not been extensively studied. The consumption of TFA has been associated with adverse effects in health (Hunter, 2006). Several studies compared the effect of TFA from industrial sources, mainly composed by 18:1 *trans*-9, with TFA from ruminant sources, mainly composed by 18:1 *trans*-11. Epidemiological evidence indicates that TFA from ruminant sources, in actual amounts consumed in diets, do not significantly contribute to the risk of coronary heart

disease (Motard-Bélanger *et al.*, 2008). However, results obtained were contradictory and biological activities of individual TFA are still to be established.

It has been postulated that deleterious effects of some *trans* PUFA are due to their incorporation in membrane lipids, thus allowing possible competition with essential FA to FA elongation and desaturation systems (Wahle and James, 1993, Chardigny *et al.*, 2007). It is expected that FA that are preferentially deposited in membrane lipids have greater potential to exert either deleterious or beneficial biological activities. Although the information about the selectivity of BI distribution to PL and NL might be highly relevant for the disclosure of potential biological effects of these isomers, it is very scarce and for most of the minor BI it is missing.

We conducted a trial where the effect of stepwise dietary substitution of sunflower oil (SO) with linseed oil (LO) of lambs fed forage on intramuscular FA was studied (Jerónimo *et al.*, 2009). Detailed information on BI distribution between intramuscular PL and NL is reported here and provides novel data on their selective deposition into muscle lipids. Moreover, the original experimental design allows us to explore if the selectivity of BI changes over a range of concentrations and different patterns induced by high dietary 18:2*n*-6 and 18:3*n*-3.

3.2 MATERIALS AND METHODS

3.2.1 Animal management and sampling procedures

Animals, diets and the experimental design were described in detail by Jerónimo *et al*. (2009, chapter 2). Briefly, four groups of nine lambs each were fed one of the four experimental diets: pelleted dehydrated lucerne with 6% of sunflower oil (diet S); pelleted dehydrated lucerne with 4% of sunflower oil and 2% of linseed oil (diet 2SL); pelleted

dehydrated lucerne with 2% of sunflower oil and 4% of linseed oil (S2L); pelleted dehydrated lucerne with 6% of linseed oil (L). After 7 weeks of trial, lambs were slaughtered in the experimental abattoir. Samples of *longissimus dorsi* muscle were collected and processed as described by Jerónimo *et al*. (2009, chapter 2).

3.2.2 Lipid analysis

Intramuscular lipid extraction, separation of NL and PL fractions, FAME preparation and gas-liquid chromatography conditions are fully described by Jerónimo *et al*. (2009, chapter 2). Briefly, lipids were extracted according to a modified Folch *et al*. (1957) procedure, lipid fractions were separated by solid-phase extraction columns according to Juaneda and Rocquelin (1985) using silica gel cartridges (LiChrolut® Si, 40-63µm, 500 mg/ml, Standard, Merck KGaA, Darmstadt, Germany). Fatty acid methyl esters were prepared by basic/acid sequential transesterification reaction as described by Raes *et al*. (2001). Quantification of FAME was done using 19:0 as internal standard. The FAME were analysed using a HP6890A chromatograph (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GC-FID) and fused silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 µm of film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA).

Identification of common FA was accomplished by comparison of sample peak retention times with those of FAME standard mixtures (Sigma, St. Louis, MO, USA) or synthesized standards and, when no standards were available, by using published chromatograms obtained with similar analytic conditions (Alves and Bessa, 2009). The synthesis of 18:2 *cis*-9, *cis*-15 and 18:2 *cis*-12, *cis*-15 was performed as described by Alves and Bessa (2007), and the synthesis of others non-conjugated 18:2 isomers, the

18:2 *trans*-9, *cis*-12 and the 18:2 *cis*-9, *trans*-12 was performed by isomerisation reaction of 18:2*n*-6 with iodine and UV light (Delmonte *et al.*, 2003). Structural analyses of some unknown peaks were conducted by gas chromatography mass spectrometry (GC-MS) using a Varian Saturn 2200 system (Varian Inc., Walnut Creek, CA, USA) equipped with a CP-Sil 88 capillary column. The characterization of the 18:2 *trans*-11, *trans*-15 was conducted by GC-MS analysis of its 4,4-dimethyloxazoline derivative. The molecular ion at *m/z* 333 confirmed the presence of the octadecadienoic FA structure. Furthermore, the strong ion at *m/z* 264 confirmed the location of the 11,15 double bond system due to fragmentation between C-13 and C-14, which is typical of octadecadienoic bis-methylene interrupted FA. Additionally, gaps of 12 amu between *m/z* 224 and 236, and between *m/z* 278 and 290 confirmed the location of the double bond position at 11 and 15, respectively. The mass spectrum of the 18:2 *trans*-11, *trans*-15 is presented in **Figure 3.1**. The region of non conjugated 18:2 isomers on the GC-FID chromatogram is displayed in **Figure 3.2**.

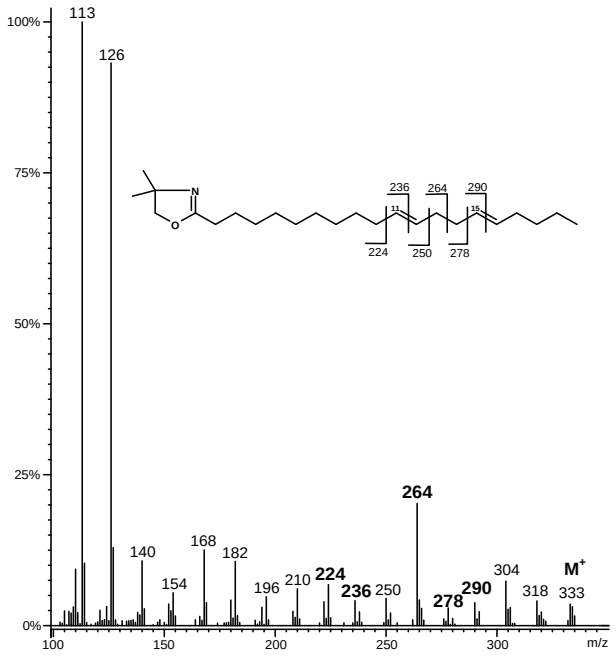

Figure 3. 1 GC-MS mass spectrum of the 4,4-dimethyloxazoline (DMOX) derivative of 18:2 *trans*-11, *trans*-15.

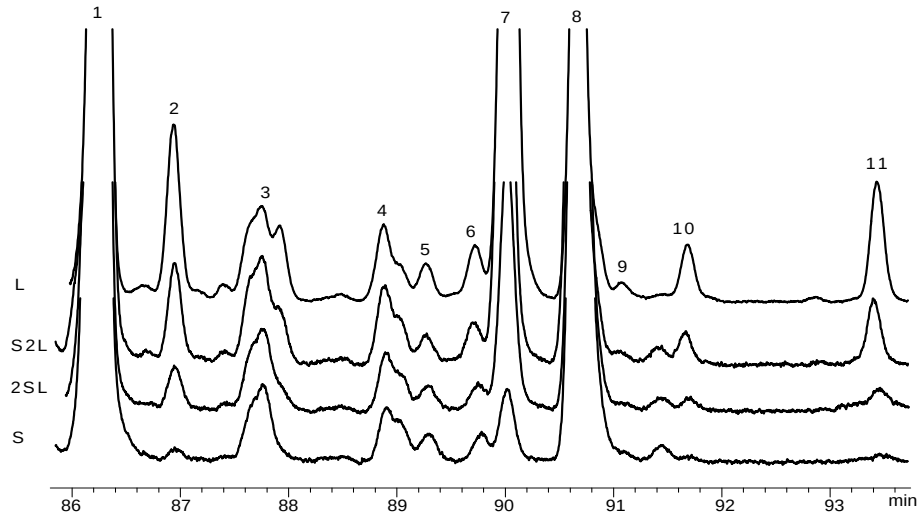

Figure 3. 2 Partial GC-FID chromatogram of neutral lipids from lambs fed: S - pellet dehydrated lucerne with 6% of sunflower oil; 2SL - pelleted dehydrated lucerne with 4% of sunflower oil and 2% of linseed oil; S2L - pelleted dehydrated lucerne with 2% of sunflower oil and 4% of linseed oil; L - pelleted dehydrated lucerne with 6% of linseed oil.

Peak identification: 1) 19:0 (standard); 2) 18:2 *trans*-11, *trans*-15; 3) 18:2 *cis*-9, *trans*-13 + 18:2 *trans*-8, *cis*-12 + 17 *cyclo;* 4) 18:2 *trans*-8, *cis*-13 + 18:2 *cis*-9, *trans*-12; 5) 18:1 *cis*-16; 6) 18:2 *trans*-9, *cis*-12; 7) 18:2 *trans*-11, *cis*-15; 8) 18:2*n*-6; 9) 19:1; 10) 18:2 *cis*-9, *cis*-15; 11) 18:2 *cis*-12, *cis*-15.

Two partially conjugated 18:3 isomers were tentatively identified by acetonitrile covalent adduct chemical ionization mass spectrometry (CACI-MS). As already published (Lawrence and Brenna, 2006), homoallylic FAME exhibit higher CACI-MS $[M+54]^+/[M+54-32]^+$ intensity ratios compared to partially conjugated FAME. Indeed, these ratios ranged from 8.1 for the 18:3*n*-3 to 1.1 and 1.2 for the other two 18:3 isomers, suggesting their partially conjugated structure. The GC-FID region of conjugated 18:2 and 18:3 isomers, showing the homoallylic 18:3*n*-3 (peak 1) and both partially conjugated 18:3 isomers (peaks 5 and 7), is displayed in **Figure 3.3**.

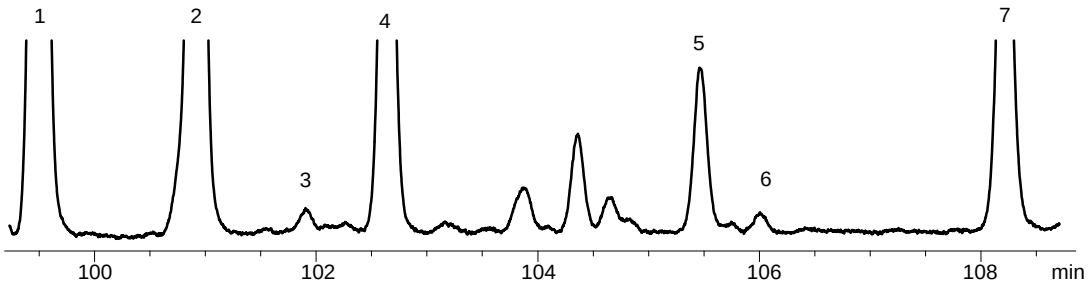

Figure 3. 3 Partial GC-FID chromatogram of neutral lipids from lambs fed diet L.

Peak identification: 1) 18:3*n*-3; 2) 18:2 *cis*-9, *trans*-11 + 18:2 *trans*-8, *cis*-10 + 18:2 *trans*-7, *cis*-9; 3) 21:0 + 18:2 *trans*-10, *cis*-12; 4) 18:2 *trans*-11, *cis*-13; 5) partially conjugated 18:3; 6) 20:2*n*-6; 7) 18:3 *cis*-9, *trans*-11, *cis*-15 + 20:3*n*-9.

For the resolution of 18:1 *cis*-9 from both 18:1 *trans*-13 and 18:1 *trans*-14 (that co-eluted in our GC-FID conditions) a second temperature program was used. The initial temperature column of 70 ºC was held for 4 min, increased to 110 ºC at 8 ºC/min and then increased to 170 ºC at 5 ºC/min, held for 10 min, and finally increased to 220 ºC at a rate of 4 ºC/min, and maintained for 25 min. Thus, the relative amounts of 18:1 *cis*-9 and 18:1 *trans*-13/*trans*-14 were calculated from the second temperature program and applied to the area of the common peak identified in the initial temperature program. The 20:3*n*-9 co-eluted with 18:3 *cis*-9, *trans*-11, *cis*-15 in our GC-FID conditions; their relative amounts were thus calculated from the GC-MS abundance relative to the area of the main peak identified by GC-FID. Methyl esters of CLA isomers were individually analysed by triple column silver-ion in series (ChromSpher 5 Lipids, 250 mm × 4.6 mm i.d. × 5 μm particle size, Chrompack, Bridgewater, NJ, USA), using a HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA), as described by Bessa *et al*. (2007).

3.2.3 Calculation of selectivity indices

To evaluate the selective deposition of C_{18} FA in PL and NL fractions, selectivity index (SI) was computed as follows*:*

SI of FA(x) = (% of FA (x) in total FA of PL) / (% of FA (x) in total FA of NL).

The interpretation of the SI is straightforward if we make the following assumptions: a) PL are phospholipids; b) NL are triacylglycerols with a small amount of cholesterol esters; c) the FA incorporation in both lipid fractions is under active metabolic control. Thus, if for a given FA value the SI = 1, no selective deposition in PL and NL is present; if SI > 1, FA is selectively deposited in PL; if SI < 1, FA is selectively deposited in NL.

3.2.4 Statistical analysis

The effect of diets on FA composition of PL, NL and SI was analyzed using the GLM procedure of SAS. Least squares means and SEM are presented in tables.

3.3 RESULTS

3.3.1 Composition of C_{18} fatty acids in polar and neutral lipid fractions

The concentration of total FA (mg/g muscle DM) and the detailed composition of C_{18} FA (mg/100 g of total FA) in PL and NL fractions are presented in **Table 3.1** and **Table 3.2**, respectively. Concentration of total C_{18} FA in PL and NL were not affected by the dietary replacement of SO with LO, and averaged 59% of total FA in PL fraction and 70% of total FA in NL fraction.

Table 3. 1 Polar fatty acid concentration (mg/g muscle dry matter) and composition (mg/100 g of total polar fatty acids) of *longissimus* muscle from lambs fed graded levels of sunflower and linseed oils.

	Diets[1]				SEM	*P* values
	S	2SL	S2L	L		
Total fatty acids	17.4	17.3	16.4	17.0	1.70	0.971
18:0	10205	10223	10391	10373	212.7	0.981
18:1 isomers						
trans-6 + *trans*-7 + *trans*-8	145	149	150	153	8.4	0.920
trans-9	187	193	182	180	9.4	0.772
trans-10	293^{c}	249^{bc}	216^{ab}	161^{a}	18.6	<0.001
trans-11	2802^{b}	2770^{b}	2915^{b}	2263^{a}	150.8	0.030
trans-12	452^{a}	483^{ab}	520^{b}	531^{b}	19.9	0.036
trans-13 + *trans*-14	585^{a}	656^{a}	749^{b}	781^{b}	29.5	<0.001
cis-9	12129^{a}	15747^{b}	14405^{b}	18038^{c}	666.4	<0.001
trans-15	142^{a}	152^{ab}	183^{b}	267^{c}	12.5	<0.001
cis-11	1525^{b}	1404^{b}	1434^{b}	1196^{a}	46.9	<0.001
cis-12	2101^{c}	2024^{c}	1663^{b}	1164^{a}	113.9	<0.001
cis-13	107^{ab}	77^{a}	118^{b}	119^{b}	10.7	0.034
cis-14 + *trans*-16	145^{a}	153^{a}	174^{a}	214^{b}	12.1	0.003
cis-15	121	132	122	133	10.4	0.793
cis-16	248^{b}	272^{b}	238^{b}	173^{a}	18.7	0.008
Total	20983^{a}	24461^{bc}	23070^{b}	25374^{c}	354	<0.001
18:2 non-conjugated isomers						
trans-11, *trans*-15	45^{a}	118^{b}	171^{c}	120^{b}	13.4	<0.001
cis-9, *trans*-13 + *trans*-8, *cis*-12[2]	233^{a}	266^{ab}	310^{b}	406^{c}	20.1	<0.001
trans-8, *cis*-13 + *cis*-9, *trans*-12[3]	155^{a}	194^{a}	165^{a}	263^{b}	17.2	<0.001
trans-9, *cis*-12	268^{c}	202^{b}	168^{b}	103^{a}	14.5	<0.001
trans-11, *cis*-15	112^{a}	266^{b}	538^{c}	714^{d}	43.0	<0.001
cis-9, *cis*-12	23859^{d}	19648^{c}	17806^{b}	13531^{a}	581.6	<0.001
cis-9, *cis*-15	0^{a}	0^{a}	57^{ab}	146^{b}	20.2	<0.001
cis-12, *cis*-15	0^{a}	153^{b}	255^{c}	448^{d}	36.5	<0.001
Total	24671^{c}	20847^{b}	19464^{b}	15731^{a}	574.5	<0.001
18:2 conjugated isomers						
trans-12, *trans*-14	3^{a}	11^{b}	20^{c}	32^{d}	1.2	<0.001
trans-11, *trans*-13	13^{a}	34^{b}	53^{c}	71^{d}	3.0	<0.001
trans-10, *trans*-12	4^{a}	4^{a}	7^{b}	7^{b}	0.8	0.008
trans-9, *trans*-11	20^{a}	27^{ab}	35^{c}	34^{bc}	2.6	0.001
trans-8, *trans*-10	3^{a}	3^{a}	5^{b}	4^{ab}	0.6	0.021
trans-7, *trans*-9	4	6	6	6	0.7	0.095
cis/trans-12, 14	5^{a}	5^{a}	9^{b}	17^{c}	1.0	<0.001
trans-11, *cis*-13	35^{a}	126^{b}	309^{c}	441^{d}	23.6	<0.001
cis-11, *trans*-13	15^{a}	14^{a}	15^{a}	21^{b}	1.5	0.011
cis-9, *trans*-11	1082	1186	1092	1000	48.5	0.094
trans-7, *cis*-9	27^{a}	35^{b}	32^{ab}	46^{c}	2.4	<0.001
Total	1224^{a}	1470^{b}	1627^{bc}	1758^{c}	72.8	<0.001
Total 18:2 isomers	25895^{c}	22317^{b}	21091^{b}	17489^{a}	562.1	<0.001
18:3 isomers						
cis-9, *cis*-12, *cis*-15	1287^{a}	2550^{b}	4730^{c}	5925^{d}	225	<0.001
partially conjugated unidentified[4]	0^{a}	52^{b}	101^{c}	125^{c}	11.9	<0.001
cis-9, *trans*-11, *cis*-15	0^{a}	66^{b}	221^{c}	405^{d}	15.8	<0.001
Total	1287^{a}	2668^{b}	5052^{c}	6456^{d}	227.7	<0.001
Total C_{18}	58478	59667	59578	59700	487.4	0.252

[1] S - pelleted dehydrated lucerne with 6% of sunflower oil; 2SL - pelleted dehydrated lucerne with 4% of sunflower oil and 2% of linseed oil; S2L - pelleted dehydrated lucerne with 2% of sunflower oil 4% of linseed oil; L - pelleted dehydrated lucerne with 6% of linseed oil; [2] peak includes 18:2 *cis*-9, *trans*-13 + 18:2 *trans*-8, *cis*-12 + 17-*cyclo* (methyl 11-cyclohexylundecanoate); [3] peak includes 18:2 *trans*-8, *cis*-13 + 18:2 *cis*-9, *trans*-12; [4] Peak 5 of Figure 3.3.

Table 3. 2 Neutral fatty acid concentration (mg/g muscle dry matter) and composition (mg/100 g of total neutral fatty acids) of *longissimus* muscle from lambs fed graded levels of sunflower and linseed oils.

	Diets[1]				SEM	*P* values
	S	2SL	S2L	L		
Total fatty acids	71.3	74.7	78.5	74.9	6.15	0.878
18:0	16650	16341	15947	16644	533.8	0.769
18:1 isomers						
trans-6 + *trans*-7 + *trans*-8	517	466	441	489	22.7	0.134
trans-9	497^{b}	451^{a}	411^{a}	442^{a}	15.0	0.003
trans-10	1228^{c}	846^{b}	677^{ab}	571^{a}	69.6	<0.001
trans-11	7084	6348	5990	5416	404.2	0.054
trans-12	804^{b}	694^{a}	634^{a}	721^{ab}	29.8	0.004
trans-13 + *trans*-14	1094^{b}	1054^{ab}	953^{a}	1798^{c}	36.3	<0.001
cis-9	30625	31389	30489	29426	843.1	0.473
trans-15	377^{a}	350^{a}	375^{a}	572^{b}	21.5	<0.001
cis-11	912^{c}	823^{b}	725^{b}	695^{a}	20.5	<0.001
cis-12	1207^{b}	1097^{b}	854^{a}	731^{a}	60.2	<0.001
cis-13	218^{c}	190^{b}	195^{b}	146^{a}	6.4	<0.001
cis-14 + *trans*-16	270^{a}	279^{a}	320^{a}	498^{b}	20.5	<0.001
cis-15	103	93	98	150	21.6	0.273
cis-16	137	134	100	111	11.7	0.094
Total	45074^{b}	44214^{b}	42302^{a}	41766^{a}	483.6	<0.001
18:2 non-conjugated isomers						
trans-11, *trans*-15	51^{a}	129^{b}	302^{c}	419^{d}	21.2	<0.001
cis-9, *trans*-13 + *trans*-8, *cis*-12 [2]	373^{a}	453^{a}	572^{b}	711^{c}	34.6	<0.001
trans-8, *cis*-13 + *cis*-9, *trans*-12 [3]	289^{a}	317^{a}	305^{a}	388^{b}	15.2	<0.001
trans-9, *cis*-12	98^{a}	100^{a}	125^{b}	166^{c}	6.8	<0.001
trans-11, *cis*-15	278^{a}	914^{b}	1881^{c}	2611^{d}	107.1	<0.001
cis-9, *cis*-12	4555^{d}	3543^{c}	2992^{b}	2262^{a}	125.8	<0.001
cis-9, *cis*-15	26^{a}	59^{b}	107^{c}	178^{d}	8.4	<0.001
cis-12, *cis*-15	0^{a}	95^{b}	176^{c}	356^{d}	20.0	<0.001
Total	5671^{a}	5611^{a}	6462^{b}	7092^{b}	242.2	<0.001
18:2 conjugated isomers						
trans-12, *trans*-14	7^{a}	17^{b}	32^{c}	51^{d}	2.3	<0.001
trans-11, *trans*-13	18^{a}	43^{b}	70^{c}	109^{d}	5.9	<0.001
trans-10, *trans*-12	22^{c}	15^{b}	11^{a}	11^{a}	0.9	<0.001
trans-9, *trans*-11	42	45	46	47	2.0	0.338
trans-8, *trans*-10	7^{c}	5^{b}	4^{b}	3^{a}	0.3	<0.001
trans-7, *trans*-9	5^{b}	5^{b}	5^{b}	4^{a}	0.2	0.023
cis/trans-12, 14	10^{a}	19^{b}	24^{b}	46^{c}	2.8	<0.001
trans-11, *cis*-13	53^{a}	204^{b}	459^{c}	734^{d}	46.7	<0.001
cis-11, *trans*-13	0^{a}	0.9^{ab}	1^{b}	3^{c}	0.38	<0.001
trans-10, *cis*-12	25^{c}	6^{b}	6^{b}	0.2^{a}	1.37	<0.001
cis-9, *trans*-11	2290^{c}	2165^{bc}	1932^{b}	1559^{a}	84	<0.001
trans-7, *cis*-9	109	104	98	101	3.4	0.113
Total	2591	2629	2688	2668	126.7	0.951
Total 18:2 isomers	8262^{a}	8240^{a}	9149^{ab}	9760^{b}	330.5	0.008
18:3 isomers						
cis-9, *cis*-12, *cis*-15	699^{a}	1083^{b}	1772^{c}	1874^{c}	101.5	<0.001
partially conjugated unidentified [4]	0^{a}	76^{b}	155^{c}	208^{d}	6.8	<0.001
cis-9, *trans*-11, *cis*-15	0^{a}	152^{b}	351^{c}	572^{d}	24.9	<0.001
Total	699^{a}	1311^{b}	2278^{c}	2654^{d}	120.1	<0.001
Total C_{18}	70701	70106	69676	70825	497.5	0.355

[1] S - pelleted dehydrated lucerne with 6% of sunflower oil; 2SL - pelleted dehydrated lucerne with 4% of sunflower oil and 2% of linseed oil; S2L - pelleted dehydrated lucerne with 2% of sunflower oil 4% of linseed oil; L - pelleted dehydrated lucerne with 6% of linseed oil; [2] peak includes 18:2 *cis*-9, *trans*-13 + 18:2 *trans*-8, *cis*-12 + 17-*cyclo* (methyl 11-cyclohexylundecanoate); [3] peak includes 18:2 *trans*-8, *cis*-13 + 18:2 *cis*-9, *trans*-12; [4] Peak 5 of Figure 3.3.

Independently of the diet, C_{18} FA were mainly present in the NL fraction that contained 87, 90 and 64% of total muscle 18:0, 18:1 *cis*-9 and 18:3*n*-3, respectively. The 18:2*n*-6 was mainly present in PL which contained 66% of total muscle 18:2*n*-6.

The proportion of 18:0 was not affected by diets in both lipid fractions, and averaged 10% and 16% of total FA in PL and NL fractions, respectively. Most of 18:1, 18:2 and 18:3 isomers were affected by the diet in both lipid fractions, although some differential effects were present. The 18:1 *cis*-9 was the predominant 18:1 isomer in both lipid fractions for all diets, averaging 70% of total 18:1 isomers in the NL fraction ($P = 0.219$) and ranging from 58 to 71% of total 18:1 isomer in the S and L diets, respectively ($P < 0.001$) in the PL fraction. Considering the other 18:1 isomers, the dietary replacement of SO with LO increased the 18:1 *trans*-15 and 18:1 *c*is-14 + *trans*-16 and decreased the 18:1 *trans*-10, 18:1 *cis*-11 and 18:1 *cis*-12 in both lipid fractions. Whereas, 18:1 *trans*-12, 18:1 *trans*-13 + *trans*-14 increased and 18:1 *trans*-11 and 18:1 *cis*-16 decreased with the replacement of SO with LO in PL but not in NL. The 18:1 *trans*-9 and 18:1 *cis*-13 decreased with replacement of SO with LO in NL but not in PL.

The dietary replacement of SO with LO increased all non-conjugated 18:2 isomers in both lipid fractions, except for 18:2 *trans*-9, *cis*-12 in PL and 18:2*n*-6 in PL and NL which decreased. In PL fraction the 18:2*n*-6 was the major non-conjugated 18:2 isomer for all diets, ranging from 97 to 86% of total non-conjugated 18:2 isomers in the S and L diets, respectively ($P < 0.001$). However, in NL fraction the 18:2*n*-6 was only the major non-conjugated 18:2 isomer in lambs fed S, 2SL and S2L whereas in lambs fed L diet 18:2 *trans*-11, *cis*-15 was the predominant non-conjugated 18:2 isomer. Linoleic acid ranged from 80% of total non-conjugated 18:2 isomer in the S diet to 32% in the L diet ($P < 0.001$) and 18:2 *trans*-11, *cis*-15 ranged from 5% of total non conjugated 18:2 isomers in the S diet to 37% in the L diet ($P < 0.001$). In both lipid fractions, 18:2 *cis*-12, *cis*-15

was only detected in lambs fed LO, and in PL fraction the 18:2 *cis*-9, *cis*-15 was only detected in lambs fed S2L and L diets.

In PL fraction, the dietary replacement of SO with LO increased all the CLA isomers except 18:2 *trans*-7, *trans*-9 and 18:2 *cis*-9, *trans*-11 that remained unchanged. In NL fraction, 5 CLA isomers increased (18:2 *trans*-12, *trans*-14, 18:2 *trans*-11, *trans*-13, 18:2 *cis/trans*-12, 14, 18:2 *trans*-11, *cis*-13 and 18:2 *cis*-11, *trans*-13) and 5 CLA isomers decreased (18:2 *trans*-10, *trans*-12, 18:2 *trans*-8, *trans*-10, 18:2 *trans*-7, *trans*-9, 18:2 *trans*-10, *trans*-12 and 18:2 *cis*-9, *trans*-11) with dietary replacement of SO with LO. The 18:2 *cis*-9, *trans*-11 was the predominant CLA isomer in both lipid fractions for all diets, ranging from 88% of total CLA in the S diet to 57% in the L diet in PL fraction ($P < 0.001$) and from 88% of total CLA in the S diet to 59% in the L diet in NL fraction ($P < 0.001$). The 18:2 *trans*-10, *cis*-12 isomer was only detected in NL fraction.

The 18:3 isomers increased with the replacement of SO with LO in both lipid fractions. The two partially conjugated 18:3 isomers were only detected in lambs fed LO.

3.3.2 Selective deposition of C_{18} fatty acids between polar and neutral lipid fractions

The selectivity indices of the C_{18} FA are presented in **Table 3.3**. In general, there was a discrimination against the deposition of most *trans* C_{18} FA in PL, with an apparent selectivity in NL (SI < 1). The 18:1 *cis*-11, 18:1 *cis*-12, 18:1 *cis*-15, 18:1 *cis*-16, 18:2*n*-6, 18:2 *cis*-12, *cis*-15, 18:2 *cis*-11, *trans*-13 and 18:3*n*-3 showed a selective deposition in PL in all diets (SI > 1). SI of some C_{18} FA were affected by the diet. Selectivity index of 18:1 *trans*-12, 18:1 *cis*-9, 18:1 *cis*-13, 18:2 *trans*-10, *trans*-12, 18:2 *trans*-9, *trans*-11, 18:2 *cis*- 9, *trans*-11, 18:2 *trans*-7, *cis*-9 and the 18:3 *cis*-9, *trans*-11, *cis*-15 increased with dietary replacement of SO with LO, although they remained below 1.

Table 3. 3 Selectivity indices of C_{18} fatty acids between polar and neutral lipid fractions in *longissimus* muscle from lambs fed graded levels of sunflower and linseed oils.

	Diets[1]				SEM	*P* values
	S	2SL	S2L	L		
18:0	0.61	0.63	0.66	0.63	0.020	0.557
18:1 isomers						
trans-6 + *trans*-7 + *trans*-8	0.28	0.32	0.35	0.32	0.020	0.155
trans-9	0.38	0.43	0.45	0.41	0.021	0.136
trans-10	0.25	0.30	0.32	0.29	0.024	0.185
trans-11	0.40^{a}	0.44^{ab}	0.50^{b}	0.43^{a}	0.022	0.022
trans-12	0.56^{a}	0.70^{b}	0.84^{c}	0.75^{bc}	0.037	<0.001
trans-13 + *trans*-14	0.54^{b}	0.63^{c}	0.80^{d}	0.44^{a}	0.032	<0.001
cis-9	0.40^{a}	0.51^{b}	0.49^{b}	0.62^{c}	0.027	<0.001
trans-15	0.38	0.44	0.50	0.47	0.033	0.069
cis-11	1.68^{a}	1.70^{a}	1.88^{b}	1.72^{a}	0.050	0.037
cis-12	1.78	1.88	2.02	1.59	0.133	0.183
cis-13	0.49^{ab}	0.41^{a}	0.60^{b}	0.82^{c}	0.054	<0.001
cis-14 + *trans*-16	0.54	0.55	0.55	0.43	0.032	0.058
cis-15	1.19	1.44	1.26	1.18	0.126	0.465
cis-16	1.88	2.21	2.49	1.59	0.232	0.066
18:2 non-conjugated isomers						
trans-11, *trans*-15	0.99	0.96	0.60	0.30	0.241	0.174
cis-9, *trans*-13 + *trans*-8, *cis*-12 [2]	0.97	0.69	0.54	0.58	0.226	0.533
trans-8, *cis*-13 + *cis*-9, *trans*-12 [3]	0.54	0.64	0.54	0.69	0.061	0.266
trans-9, *cis*-12	2.75^{d}	2.04^{c}	1.36^{b}	0.64^{a}	0.133	<0.001
trans-11, *cis*-15	0.40^{b}	0.29^{a}	0.31^{a}	0.28^{a}	0.030	0.036
cis-9, *cis*-12	5.28	5.59	6.01	5.98	0.217	0.078
cis-9, *cis*-15	NL[5]	NL[5]	0.58	0.85	0.490	0.731
cis-12, *cis*-15	-[6]	1.64	1.63	1.25	0.190	0.306
18:2 conjugated isomers						
trans-12, *trans*-14	0.50	0.61	0.65	0.62	0.046	0.121
trans-11, *trans*-13	0.75	0.80	0.77	0.67	0.059	0.494
trans-10, *trans*-12	0.19^{a}	0.29^{a}	0.63^{b}	0.73^{b}	0.073	<0.001
trans-9, *trans*-11	0.47^{a}	0.62^{ab}	0.75^{b}	0.72^{b}	0.062	0.017
trans-8, *trans*-10	0.38^{a}	0.73^{a}	1.29^{b}	1.50^{b}	0.178	<0.001
trans-7, *trans*-9	0.88	1.36	1.28	1.43	0.168	0.125
cis/trans-12, 14	0.60^{b}	0.25^{a}	0.42^{ab}	0.37^{a}	0.065	0.007
trans-11, *cis*-13	0.64	0.61	0.68	0.61	0.034	0.485
cis-11, *trans*-13	PL[7]	13.8	9.51	7.10	3.758	0.379
trans-10, *cis*-12	NL[5]	NL[5]	NL[5]	NL[5]		
cis-9, *trans*-11	0.48^{a}	0.55^{b}	0.56^{b}	0.65^{c}	0.020	<0.001
trans-7, *cis*-9	0.25^{a}	0.33^{b}	0.32^{b}	0.45^{c}	0.026	<0.001
18:3 isomers						
cis-9, *cis*-12, *cis*-15	1.86^{a}	2.40^{b}	2.78^{bc}	3.20^{c}	0.154	<0.001
partially conjugated unidentified [4]	-[6]	0.65	0.64	0.60	0.120	0.955
cis-9, *trans*-11, *cis*-15	-[6]	0.44^{a}	0.63^{b}	0.72^{b}	0.034	<0.001

[1] S - pelleted dehydrated lucerne with 6% of sunflower oil; 2SL - pelleted dehydrated lucerne with 4% of sunflower oil and 2% of linseed oil; S2L - pelleted dehydrated lucerne with 2% of sunflower oil 4% of linseed oil; L - pelleted dehydrated lucerne with 6% of linseed oil; [2] peak includes 18:2 *cis*-9, *trans*-13 + 18:2 *trans*-8, *cis*-12 + 17-*cyclo* (methyl 11-cyclohexylundecanoate); [3] peak includes 18:2 *trans*-8, *cis*-13 + 18:2 *cis*-9, *trans*-12; [4] Peak 5 of Figure 3.3; [5] Fatty acid only detected in neutral lipids; [6] Fatty acid not detected in polar and neutral lipid fractions; [7] Fatty acid only detected in polar lipids.

Selectivity index for 18:3*n*-3 also increased with dietary replacement of SO with LO, but remained above 1. Otherwise, SI of 18:2 *trans*-11, *cis*-15 and 18:2 *cis*-12, *trans*-14 decreased with dietary replacement of SO with LO, although they also remained below 1. Few 18:1 isomers (18:1 *trans*-11, 18:1 *cis*-11 and 18:1 *trans*-13 + *trans*-14) showed higher SI in the S2L diet than in S and L diets. The SI for 18:2 *trans*-9, *cis*-12 was lower than 1 only in the L diet, and for 18:2 *trans*-8, *trans*-10 and 18:2 *trans*-7, *trans*-9 it increased from values below 1 in the S diet to values above 1 in the L diet.

3.4 DISCUSSION

3.4.1 Effect of graded levels of sunflower and linseed oils on rumen biohydrogenation derived FA (BI)

Several studies report the effect of diet on intramuscular FA composition of PL and NL (Cooper *et al.*, 2004, Demirel *et al.*, 2004, Nuernberg *et al.*, 2005), as well as the C_{18} FA composition in total lipids (Nuernberg *et al.*, 2005, Bessa *et al.*, 2007) of lamb muscles, but data on the detailed composition of C_{18} FA BI in PL and NL are scarce. Although the FA composition in PL is less influenced by dietary factors than in NL (Wood *et al.*, 2008), we observed that the dietary replacement of SO with LO induced changes in PL C_{18} FA composition that followed the same general pattern observed in NL. This pattern is mostly determined by dietary availability of both 18:2*n*-6 (high in sunflower oil) and 18:3*n*-3 (high in linseed oil) and by their ruminal metabolism, as thoroughly discussed by Bessa *et al.* (2007). Briefly, the dietary replacement of SO with LO increased 18:3*n*-3, most of its BI, and decreased 18:2*n*-6 and some of its BI. The BI pattern changes with the replacement of SO with LO include a diversification of CLA isomers with a decrease in the relative proportion of 18:2 *cis*-9, *trans*-11 and an increase in several conjugated

isomers, particularly the 18:2 *trans*-11, *cis*-13 (from 3% to 25% of total CLA isomers in PL and 2% to 27% of total CLA isomers in NL, in the S and L diets respectively) and 18:2 *trans*-11, *trans*-13 (from 1% to 4% of total CLA isomers in PL and 0.7% to 4% of total CLA isomers in NL, in the S and L diets respectively), as previously reported (Kraft *et al.*, 2003, Collomb *et al.*, 2004, Loor *et al.*, 2004, Bessa *et al.*, 2007). Rumen BH pathways generate a complex pattern of BI that can be related to dietary oil intake. Nevertheless, the association of most individual BI exclusively to BH pathways of either 18:3*n*-3 or 18:2*n*-6 is not easy and needs further clarification. However, some BI seems to be exclusively derived from 18:3*n*-3 BH pathways because their concentrations highly increase with the replacement of SO by LO, as the 18:2 *cis*-9, *cis*-15, 18:2 *cis*-12, *cis*-15, 18:2 *trans*-11, *trans*-15, and the two partially conjugated 18:3 isomers that were only detected in lambs fed LO. The 18:3 *cis*-9, *trans*-11, *cis*-15 is well known as an intermediate of 18:3*n*-3 BH (Harfoot and Hazelwood, 1997), and the 18:2 *cis*-9, *cis*-15 was described as a minor intermediate of the 18:3*n*-3 BH by Kemp *et al*. (1975). Recently, the 18:2 *cis*-12, *cis*-15 was proposed as intermediate of 18:3*n*-3 BH (Bessa *et al.*, 2007). Present data confirm the previous data that reported the presence of 18:2 *trans*-11, *trans*-15 only in meat from lambs fed linseed oil (Jerónimo *et al.*, 2010a, chapter 4), suggesting that 18:2 *trans*-11, *trans*-15 is an intermediate of 18:3*n*-3 BH. Several 18:3 isomers, other than the established intermediate of 18:3*n*-3 BH, 18:3 *cis*-9, *trans*-11, *cis*-15, has been reported in milk fat (Destaillats *et al.*, 2005), beef (Plourde *et al.*, 2007) and duodenal flow of cows (Loor *et al.*, 2004) and associated to 18:3*n*-3 BH.

3.4.2 Deposition of C_{18} fatty acids in lipid fractions

The FA profile of a given tissue is expected to reflect the balance between FA incorporation (from either *de novo* synthesis or intestinal absorption) and mobilization. The extent of incorporation of individual positional *cis* and *trans* isomers into tissue lipids varies according to specific tissues and between the neutral and phospholipids in the same tissues (Wahle and James, 1993). So, the difference of isomeric FA level between tissues may reflect the preferential incorporation in particular lipid classes (Kinsella *et al.*, 1981). Also, different PL classes present distinct patterns of FA incorporation (Kramer *et al.*, 1998, Pérez-Palacios *et al.*, 2007). Moreover, it is well established that there is differential incorporation for different sn- positions within molecules like triacylglycerols and phospholipids (Wahle and James, 1993). Although data did not allow us to explore all the complexity involved, selectivity indices as defined here can be useful to give a crude indication of the preferential deposition of BI (mostly exogenous FA) in NL and PL in muscle. The preferential incorporation of PUFA, such as 18:2*n*-6, 18:3*n*-3 and LC-PUFA in PL has been widely described (Wood *et al.*, 2008), although detailed information on the distribution of most of the minor BI in NL and PL has not been reported. However, this information is expected to be important because it has been proposed that deleterious effects of some *trans* PUFA, and other isomeric FA, are due to their incorporation in membrane lipids, thus allowing possible competition with essential FA to FA elongation and desaturation systems (Wahle and James, 1993, Chardigny *et al.*, 2007). However, it no clear if the general pattern of distribution of these BI reported here will apply to monogastric species, including humans, and to other tissues.

Membrane fluidity and thus membrane function and cellular metabolism are mostly regulated by esterified FA of membranary phospholipids (Wahle, 1983, Spector and

Yorek, 1985). In the present experiment, the dietary replacement of SO with LO decreased the proportion of 18:2*n*-6 (-103 mg/g of total FA) and increased 18:3*n*-3 (+46 mg/g of total FA) and 18:1 *cis*-9 (+59 mg/g of total FA) in PL fraction, resulting in a fairly constant degree of unsaturation of C_{18} FA in membrane PL. Moreover, the proportion of *cis* PUFA with more than 3 double bonds in PL remained fairly constant (about 10%, data not shown) in spite of the marked substitution of *n*-6 by *n*-3 PUFA, induced by the dietary replacement of SO with LO, as discussed in the companion paper (Jerónimo *et al.*, 2009, chapter 2). This suggests that the homeoviscous adaptation mechanism of ovine muscle membrane is mainly regulated by selective deposition of C_{18} FA.

As expected, selectivity indices for 18:2*n*-6 and 18:3*n*-3 are well above 1 which indicates that both FA are preferentially incorporated in PL, although this is more pronounced for 18:2*n*-6 than for 18:3*n*-3 as previously pointed out by De Smet *et al*. (2004). Nevertheless, SI for 18:2*n*-6 (averaging 5.7) was well below those computed for ARA and EPA using data published in the companion paper (Jerónimo *et al.*, 2009, chapter 2) averaging 45.8 and 34.4, respectively. Moreover, selectivity indices for 18:0 and 18:1 *cis*-9 indicate that they are preferentially found in NL, which is consistent with the established knowledge. In contrast to 18:1 *cis*-9, all the other *cis* 18:1 FA, except the 18:1 *cis*-13, presented SI indicating preferential deposition in PL. The selective incorporation of 18:1 *cis*-12 into phospholipids (on sn-2 position) was already reported (Wahle and James, 1993). Calculations on the published data of PL and NL concentration of 18:1 *cis*-11 allow us to suggest that selective incorporation of 18:1 *cis*-11 in PL (SI > 1) is present in the skeletal muscle of lambs (Demirel *et al.*, 2004) and cattle (Choi *et al.*, 2000, Scollan *et al.*, 2003, Noci *et al.*, 2005, Costa *et al.*, 2006, Noci *et al.*, 2007a, Costa *et al.*,

2008, Warren *et al.*, 2008). The biological role or consequences of the selective incorporation of *cis* isomers of oleic acid in PL are not clear.

The two non-conjugated *cis*-*cis* 18:2 isomers of linoleic acid present in lamb muscle, particularly in lambs fed linseed oil, had distinct SI. The 18:2 *cis*-9, *cis*-15 was preferentially deposited in NL, but 18:2 *cis*-12, *cis*-15 was selectively deposited in PL. This BI is a methylene interrupted *cis*-*cis* FA (18:2*n*-3) and might be sensed as an essential FA by the mechanisms regulating PUFA selective incorporation in PL. The biological effects of this FA are unexplored. Almost all *trans* octadeca -enoic, -dienoic and -trienoic FA presented SI equal or lower than one, which indicates that they are not selectively incorporated in PL. When computing SI on data obtained by other authors, we also observed that *trans* 18:1 isomers (*trans* 18:1 total, 18:1 *trans*-9 and 18:1 *trans*-11) and the major CLA isomer (18:2 *cis*-9, *trans*-11) are selectively deposited in NL (SI < 1) of lamb muscle (Cooper *et al.*, 2004, Nuernberg *et al.*, 2005, Aurousseau *et al.*, 2007b) and beef (Scollan *et al.*, 2003, Noci *et al.*, 2005, Noci *et al.*, 2007a, Noci *et al.*, 2007b, Costa *et al.*, 2008, Warren *et al.*, 2008). The preferential incorporation of 18:1 *trans*-11 (Reichwald-Hacker *et al.*, 1979, Banni *et al.*, 2001, Kraft *et al.*, 2006) and 18:1 *trans*-12 (Kraft *et al.*, 2006) in NL had already been reported in rat studies. Moreover, tissue specific incorporation patterns of several *trans* 18:1 were reported in rats (Reichwald-Hacker *et al.*, 1979, Kraft *et al.*, 2006), where 18:1 *trans*-11 was preferentially incorporated in adipose tissue and ovaries, whereas isomers between 18:1 *trans*-12 and 18:1 *trans*-16 were preferentially incorporated in the lipids of the liver, heart and serum.

The preferential incorporation of 18:2 *cis*-9, *trans*-11 in NL fraction, as well as the tissue-specific incorporation patterns of CLA isomers in NL and PL fractions have been reported in the liver of rats (Yang *et al.*, 2002) or in the liver and heart (Warren *et al.*,

2008) of pigs (Kramer *et al.*, 1998). However, information on the distribution between NL and PL for most of the BI reported here is new. Overall, it seems that *trans* non-conjugated, and conjugated dienes and trienes are preferentially deposited in muscle NL. The most notable exception to preferential incorporation of *trans*-FA in NL was 18:2 *cis*-11, *trans*-13, that presented very high SI (≈ 10). This finding is consistent with the earlier report of Kramer *et al*. (1998) that observed greater deposition of 18:2 *cis*-11, *trans*-13 in the phospholipid diphosphatidylglycerol in cardiac muscle of pigs fed a synthetic CLA mixture containing this isomer (20% of total CLA). The reason for this exception is not clear. It can be hypothesized that 18:2 *cis*-11, *trans*-13 could mimic the 18:1 *cis*-11 pattern that is preferentially incorporated in PL. However, this does not explain the fact that SI for 18:2 *cis*-11, *trans*-13 (≈ 10) were much higher than the SI of 18:1 *cis*-11 (≈ 1.8).

The SI of 22 C_{18} FA were affected by the dietary replacement of SO with LO. However, for most of these FA, the magnitude of changes did not modify the biological interpretation of the preferential incorporation of these FA in NL or PL. SI of 18:2 *trans*-9, *cis*-12, 18:2 *trans*-8, *trans*-10 and 18:2 *trans*-7, *trans*-9 were highly modified by the diet, resulting in changes in its preferential deposition in lipid classes. The 18:2 *trans*-9, *cis*-12 eluted in a highly complex chromatogram region (Alves and Bessa, 2009), so we could not exclude a co-elution with other unidentified FA eventually related to 18:3*n*-3 BH pathways, resulting in modifications of computed SI. In fact, in a previous study Bessa *et al.* (2007) showed that 18:2 *trans*-9, *cis*-12 in intramuscular fat decreased with the dietary replacement of SO with LO as observed here in PL fraction but not in NL. The 18:2 *trans*-8, *trans*-10 and 18:2 *trans*-7, *trans*-9 are present in very small concentrations both in PL and NL, so differences observed in SI, although significant, are probably not biologically relevant.

Overall, the present data suggest that most of *trans* BI are preferentially deposited in the NL fraction of lamb intramuscular lipids which indicates that its potential for competitive interactions with elongation and desaturation metabolic pathways of essential FA is low.

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CHAPTER 4

EFFECT OF SODIUM BENTONITE AND VEGETABLE OIL BLEND SUPPLEMENTATION ON GROWTH, CARCASS QUALITY AND INTRAMUSCULAR FATTY ACID COMPOSITION OF LAMBS

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ABSTRACT

The effect of dietary sodium bentonite and a blend of sunflower and linseed oils at 1:2 (v/v) on growth, carcass and meat quality and FA composition of *longissimus dorsi* muscle of lambs was studied. Thirty-two Merino Branco lambs with initial live weights of 16.2 ± 2.93 kg were divided according to a completely randomized experimental design within a 2 × 2 factorial arrangement of treatments in order to evaluate effects of the vegetable oil blend supplementation (0 *vs.* 60 g/kg DM) and sodium bentonite inclusion in diets (0 *vs*. 20 g/kg DM). The basal diet consisted of pellets with 750 g dehydrated lucerne/kg DM and 250 g manioc/kg DM. The experimental period was 6 weeks. Bentonite affected neither daily live weight gain, DMI, nor carcass composition. However, bentonite decreased the *a** meat colour parameter (redness; $P = 0.004$). Oil supplementation affected neither daily live weight gain, nor DM intake. However, it increased fat proportion in chump and shoulder cuts ($P < 0.001$), as well as KKCF fat ($P < 0.001$) while it decreased muscle proportion in the dissected cuts ($P < 0.001$). Oil supplementation increased intramuscular fat ($P < 0.001$) and most meat FA. Polyunsaturated FA increased 23% with oil supplementation ($P = 0.007$), mostly by increasing proportions of *n*-3 PUFA and biohydrogenation derived PUFA. Oil supplementation decreased *n*-6 LC-PUFA ($P < 0.001$). The proportion of *n*-3 LC-PUFA was not affected by oil supplementation, so the increase in *n*-3 PUFA from 1.99 g/100 g of total FA to 4.23 g/100 g of total FA ($P < 0.001$) was mainly due to the increase of 18:3*n*-3 ($P < 0.001$). However, when expressed in mg/100g of meat, oil supplementation increased *n*-3 LC-PUFA concentration from 20 to 31 mg ($P < 0.001$). All BI increased with oil supplementation, except for 18:1 *cis*-11 which decreased, and 18:1 *cis*-13 and 18:2 *trans*-8, *cis*-10 which were unchanged. Conjugated linoleic acid increased with oil supplementation from 0.50 to 1.72 g/100g of total FA ($P < 0.001$). Bentonite did not affect most meat FA, although effects occurred on some BI. Bentonite increased 18:1 *trans*-11, but prevented the increase of 18:1 *trans*-10 in meat from oil supplemented lambs ($P < 0.001$). The 18:2 *trans*-11, *cis*-15, 18:2 *cis*-9, *cis*-15 and 18:3 *cis*-9, *trans*-11, *cis*-15 increased with dietary bentonite inclusion.

Keywords: lamb; carcass composition; fatty acids; sodium bentonite; linseed oil; sunflower oil

4.1 INTRODUCTION

In recent years, CLA and *n*-3 PUFA have received much attention due to their potential benefits for human health. Ruminant edible fat is highly saturated and strategies are needed to improve the FA profile. Supplementing ruminant diets with polyunsaturated lipids is an effective approach to decrease SFA in meats and increase meat CLA and *n*–3 PUFA (Sinclair, 2007).

Conjugated linoleic acid refer to the geometrical and positional isomers of 18:2*n*-6 with conjugated double bonds, and many studies suggest that specific CLA isomers exhibit anticarcinogenic, anti-adipogenic, anti-diabetogenic, anti-atherogenic and anti-inflammatory effects (Wahle *et al.*, 2004). Fat depots in ruminants are a rich source of CLA and, in particular, rumenic acid (18:2 *cis*-9, *trans*-11) (Schimd *et al.*, 2006). Rumenic acid is formed by ruminal BH of 18:2*n*-6 (Harfoot and Hazelwood, 1997) and by endogenous conversion of 18:1 *trans*-11 by stearoyl-CoA desaturase in tissues (Griinari *et al.*, 2000). Supplementation of lamb diets with lipid sources rich in 18:2*n*-6 or in 18:3*n*-3 increased CLA content in lamb muscle (Mir *et al.*, 2000, Wachira *et al.*, 2002, Cooper *et al.*, 2004, Szumacher-Strabel *et al.*, 2004, Bessa *et al.*, 2005). However, lipid sources rich in 18:2*n*-6 (*i.e.* sunflower oil) seem to be more effective in increasing 18:2 *cis*-9, *trans*-11 in lamb meat than lipid sources rich in 18:3*n*-3 (*i.e.* linseed oil) (Bessa *et al.*, 2007, Jerónimo *et al.*, 2009). Nevertheless, supplementation with lipid sources rich in 18:2*n*-6 systematically decreased *n*-3 PUFA content, thus increasing the *n*-6/*n*-3 ratio. *N*-3 PUFA, mainly *n*-3 LC-PUFA, as EPA and DHA are associated with anti-atherogenic, anti-thrombotic and anti-inflammatory effects (Givens *et al.*, 2006). Although the efficiency of 18:3*n*-3 conversion into *n*-3 LC-PUFA is low in lambs, supplementation of diets with lipids rich in 18:3*n*-3, as linseed or linseed oil, allows an increased *n*-3 LC-PUFA content in lamb muscle (Wachira *et al.*, 2002, Cooper *et al.*, 2004, Demirel *et al.*,

2004, Bessa *et al.*, 2007, Jerónimo *et al.*, 2009). Thus, blending sunflower and linseed oil is a good approach to simultaneously obtain higher levels of both *n*-3 LC-PUFA and CLA in lamb meat, as previously reported (Bessa *et al.*, 2007, Jerónimo *et al.*, 2009).

Dietary PUFA are extensively biohydrogenated in the rumen, thus resulting in a high level of SFA in meat, as well as in several unsaturated C_{18} FA, hereafter designated as BI. Protection of dietary PUFA from rumen metabolism can improve the nutritional quality of ruminant meat (Cooper *et al*., 2004). Rumen protection technologies that have emerged over the years were recently reviewed (Jenkins and Bridges Jr., 2007). The use of clays to adsorb oil in feed in order to decrease the interaction between oil and the rumen ecosystem has been attempted (Tamminga and Doreau, 1991), although without clear results on ruminal digestion (Van der Honning et al., 1983). More recently, Sinclair *et al*. (2005) used vermiculite as adsorbant of linseed oil and achieved partial protection of linseed oil FA in wethers.

We hypothesized that incorporation of clay in diets supplemented with PUFA rich oils might modify the pattern of rumen BH products by increasing escape of dietary PUFA from the rumen and change the profile of BI. Therefore, the present experiment was designed to explore effects of sodium bentonite and oil supplementation, and their interactions, on growth, carcass composition, meat colour and FA composition of the *longissimus dorsi* muscle, with emphasis on BI of Merino Branco lambs.

4.2 MATERIALS AND METHODS

4.2.1 Animal management and sampling procedures

Animal handling followed EU directive 86/609/EEC concerning animal care. Merino Branco ram lambs, thirty-two, were reared on pasture with dams until weaning, which occurred at about 60 days. At weaning, lambs were transported to UIPA-INRB where the study was completed. The initial live weight of lambs was 16.2 ± 2.93 kg (mean ± SD), and they were randomly distributed to eight pens which were randomly allocated to an experimental diet according to a completely randomized experimental design within a 2 × 2 factorial arrangement of treatments. The aim of this procedure was to evaluate effects of vegetable oil blend supplementation (*i.e.*, 0 g/kg DM *vs.* 60 g/kg DM) and sodium bentonite inclusion (*i.e.*, 0 g/kg DM *vs.* 20 g/kg DM) in diets. Two pens containing four lambs each were fed the same diet. The basal diet consisted of pellets containing 750 g dehydrated lucerne/kg DM and 250 g manioc/kg DM. The vegetable oil blend was sunflower and linseed oils in the proportion 1:2 (v/v). The target for oil inclusion was 60 g/kg on a DM basis, resulting in pellets with ether extract ranging between 67 and 73 g/kg DM. Diets were prepared in an industrial unit and oil was sprayed over the basal diet pellets in a 1000 kg capacity mixer. The chemical composition of diets is in **Table 4.1**.

During the study, lambs were housed and kept on a slatted floor. After an adaptation period of 7 days to experimental conditions, they were subjected to the study for 6 weeks. Feed was fed each morning at 1.10 of *ad libitum* intake calculated by a daily refusal weighing. Lambs were weighed weekly just before feeding.

Table 4. 1 Chemical composition of experimental diets.

	Diets[1]			
	C	CO	SB	SBO
g/kg DM				
Crude protein	117	112	116	108
Ether extract	21	67	20	73
Starch	221	210	215	206
NDF[2]	458	440	458	438
Ash	148	141	161	143
Gross energy (MJ/kg DM)	16.9	17.9	16.9	18.0
Fatty acid composition (g/100g total fatty acids)				
16:0	15.7	7.4	15.8	7.2
18:0	2.1	2.2	2.1	2.3
18:1 *cis*-9	19.4	19.5	19.2	19.4
18:2*n*-6	49.5	38.8	47.8	37.6
18:3*n*-3	10.0	31.5	9.4	32.7

[1] C – 750 g dehydrated lucerne/kg DM and 250 manioc/kg DM; CO – C with 60 g oil blend (sunflower oil and linseed oil - 1:2 v/v)/kg DM; SB – C with 20 g sodium bentonite/kg DM; SBO – C with 20 g sodium bentonite/kg DM and 60 g oil blend (sunflower oil and linseed oil - 1:2 v/v)/kg DM; [2] Neutral detergent fibre not assayed with a heat stable amylase and expressed inclusive of residual ash.

At the end of the study, lambs were transported to the experimental abattoir of the UIPA-INRB. After determining live weight, lambs were stunned and then exsanguinated. Carcasses were immediately weighed to obtain hot carcass weight, which was used to assess the dressing-out proportion. Carcasses were kept at 10 ºC for 24 h, and then chilled at 2 ºC until the third day after slaughter, when carcass traits were evaluated and meat samples collected. Kidney knob channel fat and kidneys were removed. Carcasses were split along the spine and left sides were separated into eight joints (Santos-Silva *et al.*, 2002b). Chumps and shoulders were dissected into muscle, subcutaneous and intermuscular fat and bone.

Samples of *longissimus dorsi* muscle were collected at the level of the 13th thoracic vertebra. The colour of the *longissimus* muscle was measured using a Minolta CR-300 chromometer (Konica Minolta, Portugal) in the *L** (lightness), *a** (redness) and *b**

(yellowness) system one hour after air exposure to allow blooming. After removing the epimysium, the *longissimus dorsi* was minced, vacuum packed, freeze-dried and stored at -80 ºC until lipid analysis.

4.2.2 Analytical procedures and calculation of variables

4.2.2.1 Feed analysis

Feed distributed to the lambs was sampled weekly (1 kg/wk/diet) and pooled. Ground samples (1 mm) were analyzed for ash (AOAC, 1990; #942.05), Kjeldahl N (AOAC, 1990; #954.01) and starch (Clegg, 1956). Neutral detergent fibre (NDF) was determined by the procedure of Van Soest *et al*. (1991). Neither sodium sulfite, nor α-amylase were added during NDF extraction, and NDF is expressed inclusive of residual ash. Diet gross energy content was measured using an adiabatic bomb calorimeter (Parr 1261, Parr Instrument Company, USA). The ether extract was determined by extracting the sample with petroleum ether using an automatic soxhlet extractor (Gerhardt Analytical Systems, Königswinter, Germany). Fatty acid methyl esters of feed lipids were prepared using one-step extraction transesterification with toluene and 17:0 as internal standard, according to the procedure of Sukhija and Palmquist (1988).

4.2.2.2 Lipid analysis

Intramuscular lipids were extracted by the Folch *et al*. (1957) method, using dichloromethane and methanol (2:1 v/v) instead of chloroform and methanol (2:1 v/v), as described by Carlson (Carlson, 1985). Fatty acids were transesterified using sodium methoxide in methanol followed by hydrochloric acid in methanol (1:1 v/v) as described

by Raes *et al*. (2001). Quantification of FAME used 19:0 as the internal standard. Fatty acid methyl esters were analysed using a HP6890A chromatograph (Hewlett–Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GC–FID) and fused silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 μm of film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA). Helium was the carrier gas and the injector split ratio was 1:50. The initial column temperature of 100 ºC was held for 15 min, increased to 150 ºC at 10 ºC/min and held for 5 min. Temperature was later increased to 158 ºC at 1 ºC/min and held for 30 min. Finally, it was increased to 200 ºC at a rate of 1ºC/min and maintained for 60 min. Injector and detector temperatures were 250 and 280 ºC, respectively. Fatty acids were identified by comparison with commercial FAME standard mixtures (Sigma and Supelco, St. Louis, MO, USA). When no commercial standards were available, elution profiles were compared with published chromatograms obtained with similar analytic conditions (Alves and Bessa, 2009). Moreover, identifications were also confirmed by gas chromatography-mass spectrometry (GC-MS) using a Varian Saturn 2200 system (Varian Inc., Walnut Creek, CA, USA) equipped with a CP-Sil 88 capillary column. For resolution and quantification of 18:1 *cis*-9, and both 18:1 *trans*-13 and 18:1 *trans*-14 that co-eluted in our GC–FID conditions, we proceeded as described by Jerónimo *el al*. (2009, chapter 2). The 20:3*n*–9 FA also co-eluted with the 18:3 *cis*-9, *trans*-11, *cis*-15 in our GC-FID conditions and its quantification and identification was conducted as described by Bessa *et al*. (2007). Methyl esters of CLA isomers were individually analysed by triple column silver-ion in series (ChromSpher 5 Lipids, 250 mm × 4.6 mm i.d. × 5 μm particle size, Chrompack, Bridgewater, NJ, USA), using a HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA), as described by Bessa *et al*. (2007).

4.2.3 Statistical analysis

This study was conducted as a 2 × 2 factorial design, where oil blend supplementation (O) and sodium bentonite (SB) were considered as main factors. The interaction between O and SB was also evaluated (O × SB). The experimental unit used for evaluation of DMI was the pen containing four lambs, whereas individual animals were considered as experimental units for other variables. The Shapiro-Wilk test was used in order to evaluate whether data followed a normal distribution. When not normally distributed ($P < 0.05$), data was Box-Cox transformed before further analysis. The SEM for transformed variables is in tables, although means are back-transformed. Data from intake and feed conversion ratio was analysed using GLM procedure of SAS with a model that included the main effects and their interaction. Other data was analysed using the MIXED procedure of SAS, considering oil and bentonite and their interaction as fixed effects and the pen as random effect. The covariance of measurements from lambs within each pen was considered in the model. Lambs were treated as repeated measure within pen and a compound symmetry covariance matrix was assumed.

4.3 RESULTS

4.3.1 Growth performance, carcass composition and meat colour

Treatment did not influence weight gain, DMI, feed conversion ratio, live slaughter weight, hot carcass weight or dressing proportion (**Table 4.2**). Inclusion of sodium bentonite in diets did not affect the tissue composition of chumps and shoulders, although oil supplementation decreased muscle proportion ($P < 0.001$) and muscle/bone ratio ($P = 0.008$) and increased fat (subcutaneous and intermuscular fat; $P < 0.001$). Bone proportion in chumps and shoulders was not affected by treatments, but KKCF increased

with oil supplementation ($P < 0.001$). The meat of lambs fed oil supplemented diets was lighter (L^*) and less red (a^*) than that of unsupplemented lambs. Bentonite reduced the a^* meat colour parameter.

Table 4. 2 Effect of sodium bentonite and oil supplementation on Merino Branco lambs growth, carcass composition and meat quality.

	Diets[1]				SEM	*P* values[2]		
	C	CO	SB	SBO		O	SB	O×SB
Initial live weight (kg)	18.5	15.4	15.9	15.0	2.93[3]			
Dry matter intake (g/d)	1317	1268	1283	1200	52.8	0.276	0.380	0.747
Average daily gain (g)	178	213	176	196	26.9	0.302	0.716	0.792
Feed conversion ratio	7.8	6.5	7.0	6.9	0.82	0.337	0.841	0.479
Live slaughter weight (kg)	24.6	24.7	24.1	22.4	0.77	0.314	0.080	0.221
Hot carcass weight (kg)	11.0	11.0	10.6	10.5	0.46	0.748	0.375	0.946
Dressing proportion [4]	0.45	0.44	0.44	0.47	0.108	0.364	0.457	0.137
Muscle proportion[5]	0.62	0.59	0.63	0.59	0.054	<0.001	0.446	0.160
Bone proportion[5]	0.20	0.21	0.21	0.21	0.062	0.476	0.908	0.299
Muscle/Bone ratio[5]	2.86	2.51	2.76	2.54	0.010	0.008	0.669	0.500
Total fat proportion[5, 6]	0.16	0.18	0.14	0.19	0.081	<0.001	0.585	0.106
KKCF proportion[7]	0.013	0.025	0.014	0.026	0.0159	<0.001	0.601	0.850
Colour[8]								
L^*	40.8	43.7	41.0	44.1	1.14	0.015	0.771	0.893
a^*	13.8	13.3	13.5	12.1	0.29	0.002	0.004	0.096
b^*	4.2	4.9	4.7	4.1	0.40	0.884	0.669	0.132

[1] C – 750 g dehydrated lucerne/kg DM and 250 manioc/kg DM; CO – C with 60 g oil blend (sunflower oil and linseed oil - 1:2 v/v)/kg DM; SB – C with 20 g sodium bentonite/kg DM; SBO – C with 20 g sodium bentonite/kg DM and 60 g oil blend (sunflower oil and linseed oil - 1:2 v/v)/kg DM; [2] O - Oil supplementation, SB – Sodium bentonite inclusion in diets; [3] Standard deviation; [4] Dressing proportion = (hot carcass weight / live slaughter weight); [5] Average of chump and shoulder; [6] Sum of subcutaneous and intermuscular fat; [7] kidney and knob channel fat; [8] L^* - lightness; a^* - redness; b^* - yellowness.

4.3.2 Intramuscular fat and total fatty acids

Intramuscular fat concentration increased with oil supplementation ($P < 0.001$) from 87.1 mg/g muscle DM in lambs fed diets with no oil to 119.5 mg/g muscle DM in oil supplemented lambs (**Table 4.3**). The same response occurred for IMFA concentration ($P < 0.001$) that increased from 68.2 mg/g muscle DM in lambs fed diets with no oil to 92.3 mg/g muscle DM in oil supplemented lambs.

4.3.2.1 General fatty acid composition

Dietary oil supplementation and bentonite inclusion in diets affected meat FA composition (**Table 4.3** and **4.4**), although betonite only affected some BI. Oil supplementation decreased the proportion of saturated FA 17:0, the branched-chain FA *iso*-16:0, *iso*-18:0, monounsaturated FA 17:1 *cis-8*, 17:1 *cis-9*, 18:1 *cis-9* and 20:1 *cis*-11 and increased the *anteiso*-17:0. Oil supplementation decreased ($P < 0.001$) 20:3*n*-6, ARA and 22:4*n*-6, while 18:2*n*-6 and 20:2*n*-6 were not affected. The muscle of lambs fed oil had 17% less ($P = 0.008$) total *n*-6 PUFA and 42% less ($P < 0.001$) *n*-6 LC-PUFA that unsupplemented lambs. Oil supplementation increased ($P < 0.001$) 18:3*n*-3, EPA, and total *n*-3 PUFA by 288%, 62% and 113%, respectively. However, DPA, DHA and total *n*-3 LC-PUFA were unaffected by oil supplementation. Total SFA and MUFA were not affected by treatments, although total PUFA increased 18% ($P = 0.007$) with oil supplementation. The *n*-6/*n*-3 ratio was higher in lambs fed basal diet with 20 g/kg DM of sodium bentonite than lambs fed the basal diet, and decreased with oil supplementation regardless of the basal diet (2.45).

Table 4. 3 Effect of sodium bentonite and oil supplementation on total fatty acid concentration (mg/g of fresh muscle) and composition (g/100 g total fatty acids) of *longissimus dorsi* from Merino Branco lambs.

	Diets[1]				SEM	*P* values[2]		
	C	CO	SB	SBO		O	SB	O×SB
Intramuscular fat[3]	20.0	23.3	17.2	25.1	<0.01	<0.001	0.237	0.220
Total fatty acids[3]	16.1	19.0	14.0	20.2	<0.01	<0.001	0.395	0.065
12:0	0.15	0.12	0.15	0.12	0.026	0.208	0.846	0.980
14:0	2.12	2.05	1.90	2.22	0.220	0.574	0.924	0.382
iso-15:0[3]	0.07	0.07	0.14	0.07	0.084	0.362	0.444	0.441
anteiso-15:0[3]	0.08	0.07	0.09	0.08	0.119	0.365	0.887	0.909
14:1 *cis*-9[3]	0.07	0.07	0.09	0.06	1.711	0.122	0.601	0.225
15:0	0.26	0.25	0.26	0.24	0.020	0.302	0.670	0.950
iso-16:0	0.11	0.09	0.12	0.09	0.011	0.030	0.534	0.910
16:0	19.6	18.9	19.8	19.6	0.44	0.263	0.306	0.528
iso-17:0	0.33	0.30	0.33	0.31	0.028	0.395	0.904	0.707
16:1 *cis*-9[3]	1.41	1.16	1.37	1.20	0.088	0.050	0.962	0.703
anteiso-17:0[3]	0.06	0.11	0.07	0.10	0.439	<0.001	0.437	0.146
17:0	0.91	0.73	0.88	0.65	0.045	<0.001	0.214	0.672
iso-18:0	0.14	0.09	0.13	0.09	0.007	<0.001	0.401	0.744
17:1 *cis*-8[3]	0.51	0.40	0.49	0.40	0.084	0.001	0.703	0.796
17:1 *cis*-9	0.61	0.46	0.63	0.48	0.036	<0.001	0.621	0.921
18:0	14.7	13.7	14.7	13.5	0.70	0.139	0.901	0.894
18:1 *cis*-9	33.7	26.4	32.5	25.6	1.28	<0.001	0.446	0.880
18:2*n*-6[3]	8.30	8.49	8.67	7.83	75.331	0.531	0.814	0.311
20:0	0.13	0.13	0.13	0.12	0.005	0.050	0.503	0.486
18:3*n*-3	0.70	2.72	0.65	2.56	0.295	<0.001	0.728	0.860
20:1 *cis*-11[3]	0.11	0.09	0.11	0.09	0.671	<0.001	0.256	0.676
20:3*n*-9	0.42	0.19	0.50	0.17	0.034	<0.001	0.392	0.145
20:2*n*-6	0.10	0.09	0.11	0.07	0.012	0.077	0.744	0.336
20:3*n*-6	0.27	0.19	0.30	0.18	0.025	<0.001	0.829	0.448
20:3*n*-3	0.04	0.06	0.07	0.06	0.008	0.840	0.282	0.291
20:4*n*-6	3.51	2.09	3.65	1.96	0.243	<0.001	0.990	0.581
20:5*n*-3	0.38	0.59	0.32	0.56	0.053	<0.001	0.361	0.714
22:4*n*-6[3]	0.25	0.14	0.34	0.12	0.109	<0.001	0.644	0.018
22:5*n*-3	0.70	0.73	0.69	0.73	0.070	0.577	0.974	0.967
22:6*n*-3	0.24	0.23	0.23	0.21	0.021	0.483	0.377	0.811
Other[4]	13.5	21.7	14.5	23.0	1.03	<0.001	0.286	0.888
n-6 PUFA[3, 5]	12.5	11.1	13.2	10.3	30.34	0.008	0.913	0.317
n-6 LC-PUFA[6]	4.14	2.57	4.43	2.39	0.311	<0.001	0.862	0.466
n-3 PUFA[7]	2.03	4.33	1.95	4.12	0.399	<0.001	0.717	0.878
n-3 LC-PUFA[8]	1.33	1.61	1.30	1.56	0.140	0.062	0.763	0.946
SFA	38.2	36.1	38.1	36.7	0.91	0.064	0.754	0.716
MUFA	40.8	39.5	40.0	39.5	1.00	0.365	0.678	0.708
PUFA[3]	16.0	20.1	16.3	19.4	0.08	0.007	0.966	0.662
n-6/*n*-3	6.18[b]	2.54[a]	6.72[c]	2.35[a]	0.156	<0.001	0.268	0.029

SFA - sum of saturated fatty acids; MUFA - sum of monounsaturated fatty acids; PUFA - sum of polyunsaturated fatty acids; [1] C – 750 g dehydrated lucerne/kg DM and 250 manioc/kg DM; CO – C with 60 g oil blend (sunflower oil and linseed oil - 1:2 v/v)/kg DM; SB – C with 20 g sodium bentonite/kg DM; SBO – C with 20 g sodium bentonite/kg DM and 60 g oil blend (sunflower oil and linseed oil - 1:2 v/v)/kg DM; [2] O - Oil supplementation, SB – Sodium bentonite inclusion in diets; [3] variables submitted to Box-Cox transformation; means presented are back-transformed values, although SEM is expressed in transformed scale; [4] For lambs fed diets with no oil, the sum of remaining areas (others) includes: dimethylacetals (about 3.5 g/100 g total FA), biohydrogenation intermediates (5.7 g/100 g total FA) and unidentified peaks (4.5 g/100 g total FA). For lambs fed diets with oil, the sum of remaining areas (others) includes: dimethylacetals (2.3 g/100 g total FA), biohydrogenation intermediates (16.1 g/100 g total FA) and unidentified peaks (3.5 g/100 g total FA); [5] *n*-6 PUFA = (18:2*n*-6 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6); [6] *n*-6 LC-PUFA = (20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6); [7] *n*-3 PUFA =(18:3*n*-3 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3); [8] *n*-3 LC-PUFA = (20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3).

Table 4. 4 Effect of sodium bentonite and oil supplementation on biohydrogenation intermediates (mg/100 g total fatty acids) of *longissimus dorsi* from Merino Branco lambs

	Diets[1]				SEM	*P* values[2]		
	C	CO	SB	SBO		O	SB	O×SB
18:1 isomers								
trans-6 + *trans*-7 + *trans*-8	198	348	228	369	13.7	<0.001	0.075	0.745
trans-9	203	357	221	363	13.0	<0.001	0.365	0.630
trans-10[3]	337[a]	802[c]	454[b]	554[b]	<0.1	<0.001	0.473	<0.001
trans-11[3]	747	2669	844	3831	0.1	<0.001	0.044	0.296
trans-12	283	764	355	744	28.2	<0.001	0.362	0.118
trans-13 + *trans*-14	366	1321	327	1304	87.6	<0.001	0.751	0.903
trans-15	118	359	120	341	8.3	<0.001	0.354	0.213
cis-11	1313	954	1321	885	26.6	<0.001	0.258	0.164
cis-12	365	1593	405	1672	104.5	<0.001	0.572	0.852
cis-13[3]	111	131	125	136	0.1	0.139	0.318	0.597
cis-14 + *trans*-16	185	394	210	394	22.1	<0.001	0.603	0.578
cis-15	53	263	65	283	21.8	<0.001	0.459	0.857
cis-16	75	172	96	171	19.0	<0.001	0.259	0.237
Total	4367	10936	4802	11644	570.7	<0.001	0.327	0.813
18:2 non-conjugated isomers								
trans-11, *trans*-15[3]	0[a]	102[b]	0[a]	129[c]	<0.1	<0.001	0.004	0.004
cis-9, trans-13 + trans-8, cis-12[4]	249	562	229	522	22.8	<0.001	0.201	0.682
trans-8, cis-13 + cis-9, trans-12[5]	172	312	171	295	15.0	<0.001	0.565	0.630
trans-9, *cis*-12	73	138	97	137	11.4	<0.001	0.326	0.283
trans-11, *cis*-15	87	1263	86	1506	61.1	<0.001	0.060	0.058
cis-9, *cis*-15[3]	76	119	93	145	0.1	<0.001	0.036	0.963
cis-12, *cis*-15	0	372	0	368	51.1	<0.001	0.965	0.965
Total	662	2957	667	3169	80.2	<0.001	0.189	0.209
18:2 conjugated isomers								
trans-12, *trans*-14	4	27	3	27	0.5	<0.001	0.436	0.415
trans-11, *trans*-13	7[a]	43[b]	6[a]	52[c]	1.3	<0.001	0.008	0.001
trans-10, *trans*-12[3]	4	8	4	9	<0.1	<0.001	0.032	0.979
trans-9, *trans*-11	10	28	10	31	0.6	<0.001	0.032	0.082
trans-8, *trans*-10[3]	4	4	3	6	<0.1	0.048	0.502	0.085
trans-7, *trans*-9[3]	3	4	3	5	<0.1	<0.001	0.061	0.588
cis/*trans*-12,14[3, 6]	4	32	4	26	0.2	<0.001	0.296	0.843
trans-11, *cis*-13[3, 7]	8	85	8	175	0.2	<0.001	0.116	0.060
cis-9, *trans*-11[3]	370	956	370	1308	0.1	<0.001	0.163	0.156
trans-8, cis-10	39	70	42	95	24.6	0.100	0.576	0.661
trans-7, *cis*-9[3]	36	69	38	62	<0.1	<0.001	0.710	0.197
Total	499	1543	497	1904	112.6	<0.001	0.124	0.120
Total 18:2 isomers	1161[a]	4500[b]	1164[a]	5073[c]	127.6	<0.001	0.034	0.035
18:3 isomers								
cis-9, *trans*-11, *cis*-15	0[a]	361[b]	0[a]	477[c]	5.7	<0.001	<0.001	<0.001

[1] C – 750 g dehydrated lucerne/kg DM and 250 manioc/kg DM; CO – C with 60 g oil blend (sunflower oil and linseed oil - 1:2 v/v)/kg DM; SB – C with 20 g sodium bentonite/kg DM; SBO – C with 20 g sodium bentonite/kg DM and 60 g oil blend (sunflower oil and linseed oil - 1:2 v/v)/kg DM; [2] O - Oil supplementation, SB – Sodium bentonite inclusion in diets; [3] variables submitted to Box-Cox transformation; means presented are back-transformed values, although SEM is expressed in transformed scale; [4] peak includes 18:2 *cis*-9, *trans*-13 + 18:2 *trans*-8, *cis*-12 + 17-*cyclo* (methyl 11-cyclohexylundecanoate); [5] peak includes 18:2 *trans*-8, *cis*-13 + 18:2 *cis*-9, *trans*-12; [6] peak includes 18:2 *cis*-12, *trans*-14 and 18:2 *trans*-12, *cis*-14; [7] peak includes a predominant 18:2 *trans*-11, *cis*-13 and a minor 18:2 *cis*-11, *trans*-13.

4.3.2.2 Biohydrogenation intermediates

The total of BI increased from 5.7 to 16.1 g/100g total FA with oil supplementation ($P < 0.001$). Oil supplementation increased ($P < 0.001$) all oleic acid isomers, except 18:1 *cis*-11 and 18:1 *cis*-13 (**Table 4.4**). The major oleic acid isomer in lambs fed diets with no oil was 18:1 *cis*-11 (decreasing ($P < 0.001$) from 23.2 g/100 g of total BI in oil unsupplemented lambs to 6.0 g/100 g in lambs fed oil), whereas in lambs fed oil, the major oleic acid isomer was 18:1 *trans*-11 (increasing ($P < 0.001$) from 14.1 g/100 g to 21.7 g/100 g total BI with oil supplementation). Bentonite inclusion in diets increased 18:1 *trans*-11 in meat ($P = 0.044$). An oil × sodium bentonite interaction occurred for 18:1 *trans*-10, in which bentonite increased ($P < 0.001$) 18:1 *trans*-10 in oil unsupplemented lambs, but prevented the increase induced by oil supplementation.

Oil supplementation increased ($P < 0.001$) all non-conjugated 18:2 BI, particularly 18:2 *trans*-11, *cis*-15. The 18:2 *trans*-11, *trans*-15 and 18:2 *cis*-12, *cis*-15 only occurred in lambs fed oil. Lambs fed the basal diet with sodium bentonite and oil (SBO diet) had a higher proportion of 18:2 *trans*-11, *trans*-15 than lambs fed the basal diet with oil (CO diet). Bentonite inclusion in diets increased 18:2 *cis*-9, *cis*-15 ($P = 0.036$).

In all diets, 18:2 *cis*-9, *trans*-11 was the predominant CLA isomer in muscle, ranging from 75 g/100 g total CLA in oil unsupplemented lambs to 70 g/100 g in lambs fed oil supplemented diets, and increasing 216% with oil supplementation ($P < 0.001$). In diets with no oil, the second and third major CLA isomers were 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9, comprising 8.1 and 7.5 g/100 g total CLA, respectively. When oil was added to the diet, the second major CLA isomer in muscle was 18:2 *trans*-11, *cis*-13 with 9.1 g/100 g total CLA, followed by the 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9, with 5.3 and 4.4 g/100 g total CLA, respectively. Inclusion of bentonite in diets increased ($P =$

0.032) 18:2 *trans*-10, *trans*-12 and 18:2 *trans*-9, *trans*-11 isomers. Lambs fed SBO had a higher proportion of 18:2 *trans*-11, *trans*-13 than lambs fed CO (O × SB, $P = 0.001$).

The conjugated triene 18:3 *cis*-9, *trans*-11, *cis*-15 was only detected in lambs fed oil, while lambs fed SBO showed a higher proportion of this BI than lambs fed CO (O × SB, $P < 0.001$).

4.4 DISCUSSION

4.4.1 Growth performance, carcass composition and meat colour

Overall, results indicate that the live weight gain of lambs was not affected by treatments, although oil supplementation changed the composition of the weight gain, slightly increasing fat deposition to the detriment of muscle. Reports on effects of bentonite on DMI and average daily live weight gain in lambs are inconsistent (Martin *et al.*, 1969, Ivan *et al.*, 1992, Walz *et al.*, 1998) and may vary according to initial live weight, basal diet and level of bentonite inclusion. An apparent decrease of muscle deposition (muscle proportion) is expected after fat accretion in dissected cuts. However, a decrease in muscle/bone ratio (computed as muscle weight/bone weight) indicates that muscle deposition was depressed in oil supplementation lambs. The increase in fat deposition with oil supplementation has been previously reported in lambs of the same breed fed similar diets (Santos-Silva *et al*., 2004). However, the decrease of the muscle/bone ratio with oil supplementation in our experiment was not observed in previous ones. Feed crude protein was much lower than in the previous studies, and thus lamb diets might have been protein deficient. Thus, the dilution of diet crude protein due to oil inclusion might accentuate protein scarcity, and hence decrease muscle growth. Moreover, increased energy intake in oil supplemented diets might increase fat deposition.

In our study, the effect of oil supplementation on meat colour contrasts with previous reports (Santos-Silva *et al.*, 2004, Bessa *et al.*, 2005), where supplementation of dehydrated lucerne with soybean oil did not modify *L** and *a** meat colour parameters. Manso *et al*. (2009) also reported that supplementation of concentrate based diets with hydrogenated vegetable fat or sunflower oil did not induce changes in meat colour. The increase of *L** and decrease of *a** parameter with oil supplementation in our study may be due to higher intramuscular fat content in this meat (Priolo *et al*., 2001). It is not clear why bentonite reduced the *a** colour parameter, but it may be linked to mineral availability in muscle (Mancini and Hunt, 2005). The effect of sodium bentonite on retention of several minerals was reported to increase retentions of magnesium and phosphorus and decrease retentions of calcium (Martin *et al.*, 1969, Huntington *et al.*, 1977).

4.4.2 Intramuscular fat and general fatty acid composition

Higher intramuscular fat and FA concentration in the muscle of lambs fed oil was consistent with carcass composition results indicating greater lipid deposition. The increase in intramuscular fat and FA concentration with lipid supplementation of forage based diets has been reported (Bessa *et al.*, 2005, Bessa *et al.*, 2007) although not always observed (Santos-Silva *et al*., 2004).

The FA composition was greatly modified by dietary oil supplementation, but generally remained unaffected by bentonite inclusion in the diet. The exception was in some BI. The oil supplementation effect on meat FA composition is most likely due to differences in the amount and profile of FA intake. However, the increase of IMFA (+47%) induced by oil supplementation must be considered in interpretation. The increase in IMFA is due

to an increase in triacylglicerols, and membranary polar lipid remains fairly constant (Wood *et al*., 2008). Incorporation of FA into polar lipids is under regulatory control in order to maintain proper membrane fluidity and function (Wahle, 1983, Spector and Yorek, 1985). The ratio of SFA to unsaturated FA is fairly constant, although differences within these FA have been observed (Raes *et al.*, 2004, Jerónimo *et al.*, 2009). Some FA, such as 18:2*n*-6, 18:3*n*-3, and *n*-6 and *n*-3 LC-PUFA are selectively incorporated into polar lipids, whereas triacylglicerols incorporate more SFA and 18:1 *cis*-9 (Raes *et al.*, 2004). Therefore, the increase of IMFA is expected to dilute LC-PUFA and 18:2*n*-6 and concentrate saturated FA, especially 16:0.

As expected, dietary oil supplementation increased total C_{18} FA, but did not decrease 16:0, the major hypercholesterolalemic FA, as has been frequently referred to in other reports (Bas *et al.*, 2007, Bessa *et al.*, 2007). In ruminants fed lipid supplemented diets, the decrease of 16:0 is generally explained by an inhibition of *de novo* synthesis by affluent exogenous FA, particularly PUFA (Chilliard, 1993). As explained above, the increase in IMFA for oil supplemented lambs would likely have resulted in increased 16:0 concentration. Moreover, in our experiment, the control level of 16:0 already seems to be depressed (below 20 g/100 g of total FA), suggesting that *de novo* FA synthesis might probably have been reduced because of a dietary energy deficiency.

The decrease of 18:1 *cis*-9 and 18:0 in muscle has been associated to dietary PUFA supplementation (Santos-Silva *et al.*, 2004, Boles *et al.*, 2005, Bessa *et al.*, 2007). The depression of 18:1 *cis*-9 might be due to a lack of 18:0 (substrate of Δ9-desaturase) resulting from incomplete BH (Bessa *et al*., 2007) or Δ9-desaturase inhibition due to high dietary levels of 18:3*n*-3 (Sinclair, 2007).

Oil supplementation increased total PUFA, mostly by increasing proportions of *n*-3 PUFA and biohydrogenation derived PUFA. This reflects both the low content of 18:3*n*-3 in the basal diet and the high content of 18:3*n*-3 in the oil blend. While the 18:2*n*-6 in meat would have been expected to increase with oil supplementation, it did not change. The 18:2*n*-6 shows a higher preferential deposition on phospholipids than 18:3*n*-3 (De Smet *et al.*, 2004). As stated above, oil supplementation increased IMFA, hence the neutral lipid fraction. However, membrane phospholipids levels remain fairly constant (Wood *et al.*, 2008). Therefore, the expected increase in 18:2*n*-6, due to oil supplementation was probably neutralized by dilution caused by increased IMFA.

Total *n*-3 PUFA increased, although total *n*-3 LC-PUFA were not affected by treatments. Since *n*-3 LC-PUFA are almost exclusively incorporated in PL, a dilution could be expected when IMFA increased. Thus, the concentration of *n*-3 LC-PUFA in PL must have increased in order to maintain the proportion in total FA profile. In fact, when expressed per 100 g of meat, the content of *n*-3 LC-PUFA increased ($P < 0.001$) from 20 mg in lambs fed diets with no oil to 31 mg *n*-3 LC-PUFA in lambs fed oil.

4.4.2.1 Biohydrogenation intermediates

Oil supplementation induced a major increase in all BI groups and the pattern in meat is consistent with responses observed in the sunflower/linseed oil blend supplementation of the lucerne based diet previously reported by our team and discussed by Bessa *et al*. (2007). Oil supplementation decreased 18:1 *cis*-11 and kept 18:1 *cis*-13 and 18:2 *trans*-8, *trans*-10 unchanged, reinforcing the suggestion that these FA may not be related to ruminal BH of PUFA (Bessa *et al*., 2007).

In our study, the concentration of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 in meat from lambs fed unsupplemented diets was particularly low (≤ 0.84 and 0.37 g/100 g of total FA, respectively), particularly when compared to previous experiments where dehydrated lucerne basal diets were fed (Bessa *et al.*, 2005, Bessa *et al.*, 2007). This might be due to the low 18:3*n*-3 content in the basal diet and/or to its relatively high starch content, as high starch diets promote changes in rumen BH pathways that favour production of 18:1 *trans*-10 instead of 18:1 *trans*-11 (Griinari and Bauman, 1999). Decreasing 18:1 *trans*-11 rumen outflow will result in lower 18:2 *cis*-9, *trans*-11 deposition in tissues, because most of it is derived from endogenous Δ9-desaturation (Palmquist *et al.*, 2004).

The 18:2 *trans*-11, *trans*-15 and 18:2 *cis*-12, *cis*-15, as well as 18:3 *cis*-9, *trans*-11, *cis*-15 were only detected in muscle of oil fed lambs. The 18:2 *cis*-12, *cis*-15 and 18:3 *cis*-9, *trans*-11, *cis*-15 were identified in lamb meat by Alves and Bessa (2007). While 18:3 *cis*-9, *trans*-11, *cis*-15 is well known as an intermediate of 18:3*n*-3 BH (Harfoot and Hazelwood, 1997), 18:2 *cis*-12, *cis*-15 has also recently been proposed as an intermediate of 18:3*n*-3 BH (Bessa *et al*., 2007). The 18:2 *trans*-11, *trans*-15 has been previously reported in milk (Shingfield *et al*., 2006), omasal digesta (Shingfield *et al*., 2008) and rumen fluid (Vlaeminck *et al*., 2008) of cows. As far as we know, this is the first report of its occurrence in lamb meat.

Use of clays as absorbing carriers of lipid supplements was proposed in order to allow an increase in FA intake without interfering with ruminal function (Tamminga and Doreau, 1991). As far as we know, the only report about the effect of this approach on ruminal BH of PUFA was that of Sinclair *et al*. (2005), who used vermiculite and observed partial protection of linseed oil FA, with a reduction of BI flowing to the duodenum of sheep. Our results suggest that an opposite effect of bentonite, with an increase of both 18:1

trans-10 and 18:1 *trans*-11 isomers occurred in meat. Moreover, some intermediates of 18:3*n*-3 BH were increased. Levels of bentonite incorporation were similar to those of vermiculite used by Sinclair *et al*. (2005). However, while they previously adsorbed oil into vermiculite, we pelleted the basal diet with bentonite and then oil was sprayed over the pellets. Therefore, differences between studies might be due to the technological processes of clay and oil incorporation, and/or to differences in oil adsorbent capacity between bentonite and vermiculite.

The TFA are potentially deleterious to human health and present recommendations point out an intake that is under 1 g/100 g of total energy intake (WHO, 2003). This increase may limit the usefulness of vegetable oil supplementation to increase the nutritional value of ruminant meat. Additional research is needed to increase 18:1 *trans*-11 and CLA content without major increases in other *trans* BI. Meat of ruminants fed concentrate diets contained a higher concentration of 18:1 *trans*-10 than ruminants fed forage (Sackmann *et al.*, 2003, Dannenberger *et al.*, 2004, Bessa *et al.*, 2005, Bessa *et al.*, 2008). Oil supplementation of diets containing high starch levels generally led to a drastic increase in meat 18:1 *trans*-10 to the detriment of 18:1 *trans*-11 and subsequent failure to increase 18:2 *cis*-9, *trans*-11 (Bessa *et al.*, 2005, Bessa *et al.*, 2008). The 18:1 *trans*-10 has been associated with detrimental effects on plasmatic lipid metabolism, whereas 18:1 *trans*-11 appears to be neutral (Bauchart *et al.*, 2007, Roy *et al.*, 2007). The effect of bentonite on preventing the increase of 18:1 *trans*-10 in oil supplemented diets suggests that it might mitigate the *trans*-10 shift of rumen BH pathways. Thus, it can be a promising approach to optimize the response to high concentrate diets supplemented with oils for meat FA pattern improvement.

4.5 CONCLUSIONS

Although oil supplementation did not affect growth performance, it induced a higher fat deposition and lower carcass muscle proportion. Oil supplementation increased intramuscular fat and greatly affected its FA composition, increasing *n*-3 PUFA and CLA contents. Bentonite effects were restricted to meat colour and to the content of some BI in meat. This clay increased 18:1 *trans*-11 and prevented the increase of 18:1 *trans*-10 in meat from oil supplemented lambs.

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CHAPTER 5

THE EFFECT OF GRAPE SEED EXTRACT, *CISTUS LADANIFER* L. AND VEGETABLE OIL SUPPLEMENTATION ON FATTY ACID COMPOSITION OF ABOMASAL DIGESTA AND INTRAMUSCULAR FAT OF LAMBS

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ABSTRACT

Thirty-six lambs were used in a 6 week experiment to evaluate the effect of vegetable oil blend supplementation (0 *vs.* 60 g/kg DM) and two dietary condensed tannin (CT) sources: grape seed extract (0 *vs.* 25 g/kg DM) and *Cistus ladanifer* L. (0 *vs.* 250 g/kg DM) on FA composition of abomasal digesta and intramuscular PL and NL. Grape seed extract did not affect FA profile of abomasal digesta nor muscle lipid fractions. *C. ladanifer* had a minor effect in lambs fed diets with no oil, but greatly changed the abomasal and muscle FA profiles in oil supplemented lambs. It decreased 18:0 and increased 18:1 *trans*-11 in abomasal digesta and increased 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 ($P = 0.062$) in muscle NL, resulting in an important enrichment of meat 18:2 *cis*-9, *trans*-11 when compared to other oil supplemented diets (19.2 *vs.* 41.7 mg/100 g muscle).

Keywords: lamb, abomasal digesta; meat; fatty acids; biohydrogenation intermediates; condensed tannins; oil supplementation

5.1 INTRODUCTION

The supplementation of ruminant diets with lipid sources rich in PUFA is an effective approach to improve the nutritional value of meat fat, through the decreasing of SFA and the enrichment in PUFA, including the health promoters CLA and *n*-3 PUFA (Scollan *et al.*, 2006, Sinclair, 2007). Supplementary C_{18} PUFA will be extensively metabolized by the rumen ecosystem, producing a complex pattern of isomeric C_{18} FA, mostly TFA, here after named BI. *Trans* FA have been associated with detrimental effects on human health (Hunter, 2006), although 18:1 *trans*-11, the precursor of 18:2 *cis*-9, *trans*-11 in tissues might be considered as a neutral or beneficial TFA (Field *et al.*, 2009).

Several factors modulate rumen BH of PUFA, including the amount and type of lipid supplements (Jenkins *et al.*, 2008) and basal diet (Bessa *et al.*, 2005). More recently, it has been suggested that condensed tannins (CT) might modulate rumen BH of PUFA (Khiaosa-Ard *et al.*, 2009, Vasta *et al.*, 2009a, Vasta *et al.*, 2009b, Vasta *et al.*, 2010b). Condensed tannins are plant secondary metabolites with astringency properties. *In vitro* (Khiaosa-Ard *et al.*, 2009, Vasta *et al.*, 2009a) and *in vivo* (Vasta *et al.*, 2009b, Vasta *et al.*, 2010b) studies showed that some CT sources are effective in the reduction of dietary PUFA ruminal BH, particularly in its last step, resulting in accumulation of 18:1 *trans*-11. Thus, CT supplementation could be a useful strategy to improve the nutritional value of ruminant fat. *Cistus ladanifer* L. was chosen due to its high tannin content and its abundance in marginal fields of Mediterranean countries (Dentinho *et al.*, 2005). Grape seed extract was a commercial available source of purified CT. Interactions between CT and vegetable oil supplementation are fairly unexploited. Therefore, the aim of the present study was to explore the effect of dietary CT sources (grape seed extract and leaves and soft stems of *Cistus ladanifer* L.), and oil supplementation, and their

interactions on lamb growth performance, carcass composition, ruminal biohydrogenation, as evaluated by FA composition of abomasal digesta and intramuscular fat of lambs.

5.2 MATERIALS AND METHODS

5.2.1 Animals, treatments and sample collection

Animal handling followed the EU directive 86/609/EEC concerning animal care. Thirty-six Merino Branco ram lambs were reared on pasture with dams until weaning approximately at 60 days. At weaning day, lambs were transported to the UIPA-INRB, where the trial was held. The average initial weight for lambs was 24.8 ± 1.55 kg (mean ± SD). Lambs (n=36) were randomly assigned to 12 pens; 3 lambs per pen and 2 pens per treatment, according to a completely randomized experimental design with a 3 × 2 factorial arrangement of treatments. The first factor was CT sources (with 3 levels; 1) no added CT source – control, 2) 25 g grape seed extract/kg DM, 3) 250 g *C. ladanifer* leaves and soft stems/kg DM) and the second factor was oil supplementation (with 2 levels: 0 g and 60 g oil blend/kg DM). This 3 × 2 factorial arrangement results in 6 diets: C, basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; CO, basal diet with 60 g oil blend/kg DM; GS, basal diet with 25 g grape seed extract/kg DM; GSO, basal diet with 25 g grape seed extract/kg DM and 60 g oil blend/kg DM; CL, basal diet with 250 g *C. ladanifer*/kg DM and CLO, basal diet with 250g *C. ladanifer*/kg DM and 60 g oil blend/kg DM. Grape seed (*Vitis vinifera* L.) extract contained 95% proanthocyanidines in DM (AHD international LLC, Atlanta, GA, USA). Leaves and soft stems of *C. ladanifer* shrubs were harvested in Portugal (39º30´36´´N/8º 19´00´´W) in March 2008, dried at room temperature, cut in small particles and milled to

a final particle size of 3 mm. The oil blend was composed of a mixture of sunflower and linseed oils in a proportion of 1:2 (v/v). Diets were prepared in an industrial unit and oil was sprayed over the pellets in a 1000 kg capacity mixer. The chemical composition of diets is present in **Table 5.1**.

Table 5. 1 Chemical composition of the experimental diets.

	Control		GSE		*C. ladanifer*	
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]
g/kg dry matter						
Crude Protein	149	143	138	132	150	128
Ether extract	18.6	68.7	20.7	67.8	34.1	87.9
Total fatty acids	14.2	66.0	14.5	66.7	24.4	78.4
NDF[7]	535	516	532	519	399	416
Total phenols	9.1	7.5	17.6	16.2	17.5	16.5
Grape seed condensed tannins[8]	0.95	0.70	14.9	13.3	-	-
Cistus ladanifer condensed tannins[9]	8.48	8.17	-	-	21.0	20.7
Fatty acid composition (g/100 g of total fatty acids)						
16:0	16.0	7.84	17.6	8.08	13.1	7.73
18:0	1.63	2.76	2.03	2.79	2.07	2.77
18:1 *cis*-9	16.0	19.1	15.7	18.8	17.6	18.6
18:2*n*-6	29.6	31.0	33.1	31.3	36.8	32.8
18:3*n*-3	11.0	29.9	14.0	29.9	8.52	29.4

[1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g *Cistus ladanifer*/kg DM; [6] Diet CLO - basal diet with 250 g *Cistus ladanifer*/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] Neutral detergent fibre; [8] Condensed tannins (CT) quantified using purified grape seed CT as standard; [8] Condensed tannins (CT) quantified using purified *Cistus ladanifer* CT as standard.

During the trial, lambs were housed and kept on a slatted floor. The trial started after an adaptation period of 7 days to experimental conditions and lasted for 6 weeks. Feed was offered every morning at the rate of 110% of *ad libitum* intake, a calculation based on daily refusal weighing. Animals were weighed weekly, just before feeding. At the end of the trial, lambs were transported to the experimental abattoir of the UIPA-INRB. After

determining live slaughter weight, lambs were stunned and slaughtered by exsanguination. Samples of abomasal digesta were collected immediately *post-mortem*, freeze-dried and stored at -80 ºC until lipid analysis. After preparation, carcasses were weighed to obtain hot carcass weight, which was used to determine dressing percentage. Carcasses were kept at 10 ºC for 24 h, and then chilled at 2 ºC until the third day after slaughter, at which time carcass traits were evaluated and meat samples collected. Kidney knob channel fat and kidneys were removed. Carcasses were split along the spine and left sides of the carcasses were separated into eight joints (Santos-Silva *et al.*, 2002b). Chumps and shoulders were dissected into muscle, subcutaneous and intermuscular fat and bone. Samples of *longissimus dorsi* muscle were collected at the level of the 13th thoracic vertebra. After removing the epimysium, muscle samples were minced with a food processor (3 × 5s), vacuum packed, freeze-dried and stored at -80 ºC until lipid analysis.

5.2.2 Lipid analysis of intramuscular fat and abomasal digesta

Intramuscular lipids were extracted using dichloromethane and methanol (2:1 v/v) and separated in PL and NL, by using a solid-phase extraction as previously described (Jerónimo *et al.*, 2009, chapter 2). The NL and PL fractions were transesterified with sodium methoxide in methanol, followed by hydrochloric acid in methanol (1:1 v/v) as described by Raes *et al*. (2001). Fatty acid methyl esters of abomasal digesta lipids were prepared using one-step extraction transesterification with toluene, according to the procedure reported by Sukhija and Palmquist (1988).

The quantification of muscle and abomasal lipid FAME was performed using 19:0 as internal standard. Fatty acid methyl esters were analyzed using HP6890A chromatograph

(Hewlett- Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GC-FID) and fused silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 µm film thickness; Chrompack, Varian Inc., Walnut Creek, CA). Helium was the carrier gas and the injector split ratio was 1:50. The initial column temperature of 100 ºC was held for 15 min, increased to 150 ºC at 10 ºC/min and held for 5 min, then increased to 158 ºC at 1 ºC/min and held for 15 min. Temperature was later increased to 175 ºC at 1 ºC/min and held for 10 min, and finally increased to 200 ºC at a rate of 1 ºC/min and maintained for 40 min. Injector and detector temperatures were 250 ºC and 280 ºC, respectively.

For the resolution of 18:1 *cis*-9 from both 18:1 *trans*-13 and 18:1 *trans*-14 (that co-eluted in our GC-FID conditions) a second temperature program was used. The initial temperature column of 70 ºC was held for 4 min, increased to 110 ºC at 8 ºC/min and then increased to 170 ºC at 5 ºC/min, held 10 min, and finally increased to 220 ºC at a rate of 4 ºC/min, and maintained for 25 min. Thus, the relative amounts of 18:1 *cis*-9 and 18:1 *trans*-13/14 were calculated from the second temperature program and applied to the area of the common peak identified in initial temperature program. Fatty acids were identified by comparison with commercial FAME standard mixtures (Sigma and Supelco, St. Louis, MO, USA). When no commercial standards were available, elution profiles were compared with published chromatograms obtained with similar analytic conditions (Alves and Bessa, 2009). Moreover, identifications were also confirmed by gas chromatography-mass spectrometry (GC-MS) using a Varian Saturn 2200 system (Varian Inc., Walnut Creek, CA, USA) equipped with a CP-Sil 88 capillary column.

The methyl esters of CLA isomers were individually analyzed by triple column silver-ion in series (ChromSpher 5 Lipids, 250 mm × 4.6 mm i.d. × 5 µm particle size, Chrompack, Bridgewater, NJ, USA), using an HPLC system (Agilent 1100 Series, Agilent

Technologies Inc., Palo Alto, CA, USA) equipped with autosampler and a diode array detector (DAD) adjusted at 233 nm. The mobile phase was 0.1% acetonitrile in *n*-hexane maintained at a flow rate of 1 mL/min, and injection volumes of 20 µl were used. The identification of the individual CLA isomers was achieved by comparison of their retention times with commercial and prepared standards, as well as with data published in the literature (Fritsche *et al.*, 2000). In GC analysis the main peak of 18:2 *cis*-9, *trans*-11 co-eluted with both 18:2 *trans*-7, *cis*-9 and 18:2 *trans*-8, *cis*-10. As proposed by Kraft *et al*. (2003), the he HPLC areas of 18:2 *cis*-9, *trans*-11, 18:2 *trans*-7, *cis*-9 and 18:2 *trans*-8, *cis*-10 were added and used to calculate three isomers peaks from GC chromatograms. The amounts of the other CLA isomers were calculated from their HPLC areas relative to the area of the main isomer 18:2 *cis*-9, *trans*-11 identified by GC.

5.2.3 Statistical analysis

This trial was conducted using a 3 × 2 factorial design, where the 2 factors were CT sources (CT, with 3 levels: control, grape seed extract and *C. ladanifer*) and vegetable oil blend supplementation (O, with 2 levels: 0 g and 60 g/kg DM). The interaction between CT sources and O was also evaluated (CT × O). The experimental unit used to evaluate DMI and FA intake was the pen (3 lambs), whereas individual animals were considered as experimental units for all other variables. The Shapiro-Wilk test was used to evaluate whether data followed a normal distribution. When not normally distributed ($P < 0.05$), data was Box-Cox transformed before further analysis. Data from intake and feed conversion ratio were analyzed using GLM procedure of SAS with a model that included the main effects and their interaction. Other data were analyzed using the MIXED procedure of SAS, considering oil and CT sources and their interaction as fixed effects and the pen as random effect. The covariance of measurements from lambs within each

pen was considered in the model. Lambs were treated as repeated measurements within the pen and a compound symmetry covariance matrix was assumed. Least squares means and SEM are presented in tables. For Box-Cox transformed variables the SEM is presented in tables, although means are back-transformed. Data presented in tables are the least square means obtained for each combination of factors levels (diets). Because, some variables did not present significant CT × O interactions, the least square means for main effects are presented in the text when needed. When only one level of CT source factor differs from the other two and these two are not significantly different, we only present the least square mean of the level that was different and the average of least square mean for other factor levels.

5.3 RESULTS

5.3.1 Feed intake

Dry matter intake was not affected by treatments, which averaged 1616 g DM/day (**Table 5.2)**. Dietary oil supplementation resulted in a significant increase of FA intake for lambs receiving the oil supplemented diets. Diets containing *C. ladanifer* increased ($P = 0.032$) total FA intake when compared to other diets. In spite of the great difference in grape seed extract and *C. ladanifer* dietary inclusions (25 *vs.* 250 g/kg DM), the enrichment of CT into diets was similar. The enrichment in grape seed CT, computed by the difference of CT concentration in grape seed diets and control diets (*i.e.* GS minus C and GSO minus CO) was 13.9 g/kg DM and 12.6 g/kg DM for GS and GSO diets, respectively. The enrichment in *C. ladanifer* CT, computed by the difference of CT concentration in *C. ladanifer* diets and control diets (*i.e.* CL minus C and CLO minus CO) were 12.5 g/kg for both CL and CLO diets.

Table 5. 2 Effect of dietary condensed tannin sources (Control, Grape Seed Extract-GSE and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on dry matter (DMI) and fatty acid intake (g/day) of lambs

	Control		GSE		*C. ladanifer*		SEM	*P* values[8]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
DMI	1704	1642	1701	1659	1467	1524	89.3	0.143	0.833	0.787
Fatty acid intake										
16:0	5.23	9.39	5.28	9.83	6.01	10.1	0.380	0.211	<0.001	0.814
18:0	0.53	3.30	0.61	3.39	0.95	3.62	0.107	0.032	<0.001	0.861
18:1 *cis*-9	5.24	22.9	4.70	22.9	8.11	24.3	0.740	0.033	<0.001	0.424
18:2*n*-6	9.68	37.1	9.91	38.1	16.8	42.9	1.319	0.005	<0.001	0.738
18:3*n*-3	3.61	35.8	4.20	36.4	3.89	38.5	1.114	0.437	<0.001	0.489
Total FA	24.5	108.8	24.9	111.1	36.6	120.5	3.64	0.032	<0.001	0.948

[1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g Cistus ladanifer/kg DM; [6] Diet CLO - basal diet with 250 g Cistus ladanifer/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] CT - Condensed tannin sources inclusion in diets, O - Oil supplementation.

5.3.2 Growth performance and carcass composition

Treatment did not influence ($P > 0.05$) average daily weight gain (279 g/d), Gain:Feed intake ratio (0.17) and live slaughter weight (36.2 kg) (**Table 5.3**). Oil supplementation increased ($P < 0.05$) hot carcass weight (15.2 *vs*. 16.6 kg), dressing percentage (41.4 *vs*. 43.9%) and accumulation of KKCF in carcass (1.80 *vs*. 2.48%). Dressing percentage was higher in lambs fed *C. ladanifer* diets ($P = 0.003$) than in lambs fed two other diets, which did not differ between each other (44.9 *vs*. 41.5%). When compared to other diets, feeding *C. ladanifer* resulted in a higher ($P = 0.020$) subcutaneous fat percentage (11.3 *vs*. 9.24 %) and lower ($P = 0.003$) muscle percentage (56.5 *vs*. 60.9%) in chump and shoulder cuts. Kidney and knob channel fat percentage also increased significantly with the inclusion of *C. ladanifer* in the diet ($P = 0.013$) when compared to other diets (2.58 *vs*. 1.92 %).

Table 5. 3 Effect of dietary condensed tannin sources (Control, Grape Seed Extract-GSE, and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on growth and carcass composition of lambs

	Control		GSE		*C. ladanifer*		SEM	*P* values[7]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
Initial live weight (kg)	24.6	24.9	24.4	24.3	24.3	26.4				
Average daily gain (g/d)	290	308	245	286	257	286	19.7	0.215	0.083	0.835
Gain:Feed intake ratio	0.17	0.19	0.14	0.17	0.17	0.19	0.012	0.192	0.106	0.785
Live slaughter weight (kg)	36.5	37.5	34.4	36.0	34.8	38.1	1.60	0.523	0.145	0.773
Hot carcass weight (kg)	15.5	16.6	14.5	15.5	15.6	17.8	0.82	0.127	0.044	0.739
Dressing percentage (%)[8]	41.1	43.1	40.2	41.7	42.9	47.0	1.08	0.003	0.009	0.470
Muscle (%)[9]	61.6	67.9	59.0	66.7	59.1	53.9	1.39	0.003	0.352	0.050
Bone (%)[9]	19.3	17.8	19.7	18.5	19.2	22.4	1.62	0.359	0.890	0.276
Muscle:Bone ratio[9]	3.25	3.54	3.03	3.35	3.11	2.75	0.219	0.134	0.642	0.227
Subcutaneous fat (%)[9]	9.20	8.60	9.28	9.83	10.0	12.5	0.808	0.020	0.235	0.183
Intermuscular fat (%)[9]	8.64	10.2	10.2	9.60	10.1	9.73	0.678	0.697	0.732	0.256
KKCF (%)[10]	1.61	2.12	1.63	2.32	2.17	2.99	0.237	0.013	0.002	0.799

[1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g *Cistus ladanifer*/kg DM; [6] Diet CLO - basal diet with 250 g *Cistus ladanifer*/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] CT - Condensed tannin sources inclusion in diets, O - Oil supplementation; [8] Dressing percentage (hot carcass weight x 100/live slaughter weight); [9] Average of chump and shoulder; [10] Kidney and knob channel fat.

5.3.3 Abomasal digesta fatty acids

Oil supplementation increased ($P < 0.001$) abomasal FA concentration from 33.4 to 119.2 mg/g DM (**Table 5.4**). Lambs fed *C. ladanifer* diets had a higher ($P < 0.001$) abomasal FA concentration than lambs fed control or grape seed diets (89.9 *vs.* 69.5 mg/g DM). Individual FA concentration (mg/g DM) of abomasal digesta was affected by oil supplementation and by the inclusion of CT sources in diets, mainly *C. ladanifer* (**Table 5.4**). Oil supplementation increased the concentrations of 15:0, 16:0, 17:0 and *iso*-16:0. The 16:0 also increased with *C. ladanifer* diets ($P < 0.001$). The 15:0 was higher ($P < 0.001$) in lambs fed control diets than in lambs fed *C. ladanifer* diets and was intermediate in grape seed diets. There was an interaction between oil and CT sources for *iso*-15:0 ($P = 0.045$), *anteiso*-15:0 ($P = 0.015$) and *iso*-17:0 ($P = 0.036$). Grape seed extract depressed *iso*-15:0, *anteiso*-15:0 and *iso*-17:0, but these responses were neutralized when oil was added. Oil supplementation did not affect the proportion of these FA in lambs fed *C. ladanifer* and the depressive effect of *C. ladanifer* was only present for *iso*-15:0, which was much lower in lambs fed CL diet than in lambs fed C diet. Oil supplementation of control diet decreased the concentration of *iso*-15:0, but did not affect *anteiso*-15:0 and *iso*-17:0.

Total C_{18} FA increased ($P < 0.001$) from 22.8 to 102 mg/g DM with oil supplementation (**Table 5.4**). Feeding *C. ladanifer* diets also resulted in a higher content of total C_{18} FA ($P = 0.002$) when compared to lambs fed control and grape seed diets (72.4 *vs.* 56.4 mg/g DM). Concentration of all C_{18} FA (expressed in mg/g DM) increased ($P < 0.01$) in abomasal digesta with oil supplementation, with the exception of 3 CLA isomers (18:2 *trans*-7, *trans*-9, 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9) which remained unchanged with oil (data not shown).

Table 5. 4 Effect of dietary condensed tannin sources (Control, Grape Seed Extract-GSE, and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on fatty acid concentration (mg/g dry matter) of abomasal digesta from lambs

	Control		GSE		*C. ladanifer*		SEM	*P* values[7]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
Total fatty acids	29.8	108.2	26.0	113.8	44.2	135.6	5.31	<0.001	<0.001	0.460
16:0	4.54	8.76	4.41	9.44	6.10	10.8	0.400	<0.001	<0.001	0.611
Odd- and branched-chain fatty acids										
iso-15:0	0.26^{c}	0.20^{ab}	0.20^{ab}	0.23^{bc}	0.15^{a}	0.17^{a}	0.020	0.008	0.704	0.045
anteiso-15:0	0.39^{b}	0.42^{b}	0.28^{a}	0.40^{b}	0.40^{b}	0.40^{b}	0.020	0.009	0.007	0.015
15:0	0.37	0.46	0.29	0.42	0.24	0.33	0.041	0.015	0.005	0.886
iso-16:0	0.13	0.14	0.10	0.17	0.09	0.11	0.018	0.078	0.020	0.245
iso-17:0[8]	0.10^{b}	0.09^{ab}	0.07^{a}	0.10^{b}	0.09^{ab}	0.09^{ab}	0.041	0.781	0.224	0.036
anteiso-17:0	0.07	0.08	0.06	0.07	0.07	0.08	0.012	0.524	0.500	0.999
17:0	0.24	0.31	0.19	0.30	0.21	0.28	0.021	0.260	<0.001	0.501
C_{18} fatty acids										
18:0	11.4	47.6	8.37	48.2	17.3	41.1	4.080	0.953	<0.001	0.140
18:1 *trans*-11	1.02	6.69	0.91	7.59	1.30	13.5	0.002	0.024	<0.001	0.448
18:1 *cis*-9	1.83	7.02	1.83	7.04	2.57	8.53	0.493	0.049	<0.001	0.683
18:2*n*-6	2.00	6.12	2.26	6.14	3.19	9.51	0.796	0.013	<0.001	0.259
18:2 *cis*-9, *trans*-11	0.03	0.61	0.02	0.46	0.07	0.40	0.060	0.346	<0.001	0.136
18:3*n*-3	0.80	4.57	0.90	4.68	0.90	7.67	0.832	0.121	<0.001	0.138
Total	19.4	92.5	16.5	97.2	29.5	115.3	4.48	0.002	<0.001	0.372
Others[9]	4.37	6.20	3.96	6.37	7.54	9.50	0.515	<0.001	<0.001	0.835

[1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g *Cistus ladanifer*/kg DM; [6] Diet CLO - basal diet with 250 g *Cistus ladanifer*/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] CT - Condensed tannin sources inclusion in diets, O - Oil supplementation; [8] Variables submitted to Box-Cox transformation; means presented are back-transformed values, although SEM is expressed in transformed scale. λ for *iso* 17:0 is 0.161; [9] The sum of the remaining area (others) includes unidentified peaks.

Table 5. 5 Effect of dietary condensed tannin sources (Control, Grape Seed Extract-GSE, and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on C_{18} fatty acid profile (g/100g of total C_{18} fatty acids) of abomasal digesta from lambs

	Control		GSE		*C. ladanifer*		SEM	*P* values[7]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
18:0	58.3[b]	50.6[b]	49.3[b]	49.4[b]	57.6[b]	36.7[a]	3.38	0.105	0.002	0.016
18:1 isomers										
trans-6 + *trans*-7 + *trans*-8	0.64	0.77	0.69	0.81	0.77	1.05	0.063	0.009	0.002	0.329
trans-9[8]	0.47	0.57	0.52	0.60	0.54	0.69	0.692	0.083	0.003	0.763
trans-10[8]	0.93	0.95	1.19	0.87	1.19	0.96	0.181	0.796	0.285	0.632
trans-11	5.43[a]	8.06[a]	5.96[a]	8.44[a]	4.88[a]	17.4[b]	1.866	0.051	<0.001	0.018
trans-12	0.94	1.86	1.08	2.05	1.52	2.16	0.126	0.007	<0.001	0.397
trans-13 + *trans*-14	2.05	4.48	2.44	4.74	2.52	4.23	0.467	0.778	<0.001	0.717
trans-15	0.86[a]	2.17[c]	0.86[a]	2.30[c]	1.17[a]	1.72[b]	0.151	0.671	<0.001	0.014
cis-9	9.56	7.71	11.4	7.31	9.01	7.41	0.671	0.237	<0.001	0.141
cis-11	2.00[c]	0.74[a]	2.35[d]	0.78[a]	1.27[b]	0.82[a]	0.107	<0.001	<0.001	<0.001
cis-12	0.55	1.39	0.65	1.81	0.95	2.44	0.248	0.024	<0.001	0.427
cis-13	0.07	0.13	0.10	0.14	0.09	0.18	0.021	0.289	<0.001	0.496
cis-14 + *trans*-16	1.05[a]	2.03[c]	1.09[a]	2.02[c]	1.32[b]	1.42[b]	0.076	0.038	<0.001	<0.001
cis-15[8]	0.16	0.60	0.19	0.82	0.16	0.53	0.183	0.139	<0.001	0.777
cis-16	0.18[a]	0.34[d]	0.22[ab]	0.38[d]	0.23[bc]	0.27[c]	0.018	0.041	<0.001	0.002
Total	25.0[a]	37.9[bc]	28.8[abc]	33.1[c]	25.7[ab]	42.5[d]	2.123	0.044	<0.001	0.018
18:2 non-conjugated isomers										
trans-11, *trans*-15	0.21	0.58	0.28	0.69	0.08	0.72	0.073	0.402	<0.001	0.151
cis-9, *trans*-13 + *trans*-8, *cis*-12[9]	0.44	0.35	0.64	0.41	0.69	0.44	0.142	0.472	0.107	0.842
trans-8, *cis*-13 + *cis*-9, *trans*-12[10]	0.09	0.09	0.13	0.09	0.10	0.07	0.012	0.088	0.072	0.415
trans-9, *cis*-12	0.13[ab]	0.18[c]	0.16[bc]	0.22[d]	0.12[a]	0.26[e]	0.012	0.005	<0.001	0.001
trans-11, *cis*-15	0.34[a]	1.56[b]	0.36[a]	1.93[d]	0.29[a]	1.73[c]	0.042	<0.001	<0.001	0.001
cis-9, *cis*-12	10.6	6.75	14.1	6.43	11.3	8.28	1.281	0.443	<0.001	0.172
cis-9, *cis*-15[8]	0.05	0.20	0.07	0.33	0.06	0.24	0.217	0.116	<0.001	0.806

cis-12, *cis*-15 [8]	0.07	0.25	0.08	0.47	0.05	0.32	0.329	0.067	<0.001	0.361
Total	11.9	9.98	15.9	10.6	12.7	12.3	1.358	0.245	0.033	0.207
18:2 conjugated isomers										
trans-12, *trans*-14[8]	0.04	0.12	0.04	0.11	0.04	0.16	0.542	0.533	<0.001	0.898
trans-11, *trans*-13[8]	0.03	0.19	0.03	0.17	0.06	0.18	0.171	0.034	<0.001	0.053
trans-10, *trans*-12	0.006	0.12	0.004	0.10	0.02	0.11	0.012	0.518	<0.001	0.648
trans-9, *trans*-11	0.05	0.12	0.05	0.10	0.09	0.12	0.013	0.088	<0.001	0.202
trans-8, *trans*-10	0.04	0.02	0.04	0.02	0.04	0.02	0.004	0.867	<0.001	0.926
trans-7, *trans*-9	0.02	0.004	0.02	0.005	0.02	0.004	0.097	0.862	<0.001	0.642
cis/*trans*-12,14[8, 11]	0.02^{ab}	0.04^{b}	0.01^{a}	0.05^{b}	0.04^{b}	0.03^{b}	0.903	0.289	0.005	0.017
cis/*trans*-11,13[12]	0.05	0.33	0.02	0.36	0.02	0.28	0.048	0.666	<0.001	0.717
trans-10, *c*12	0.04	0.09	0.04	0.06	0.05	0.09	0.019	0.541	0.018	0.750
cis-9, *trans*-11[8]	0.12^{a}	0.65^{d}	0.12^{a}	0.47^{cd}	0.22^{ab}	0.30^{bc}	0.212	0.787	<0.001	0.034
trans-8, *cis*-10[8]	0.02	0.004	0.02	0.006	0.02	0.004	0.130	0.360	<0.001	0.930
trans-7, *cis*-9	0^{a}	0.002^{b}	0^{a}	0^{a}	0.005^{c}	0.002^{b}	0.0006	<0.001	0.435	0.002
Total	0.45	1.71	0.72	1.46	0.68	1.59	0.123	0.275	<0.001	0.359
Total 18:2 isomers	12.4	11.7	16.3	12.1	13.4	13.9	1.388	0.290	0.217	0.227
18:3 isomers										
cis-9, *cis*-12, *cis*-15	4.20	5.07	5.55	4.91	3.16	6.69	0.854	0.786	0.085	0.066
cis-9, *cis*-11, *cis*-15	0.08^{a}	0.74^{c}	0.06^{a}	0.47^{b}	0.16^{a}	0.23^{a}	0.077	0.025	<0.001	0.003
Total	4.28	5.81	5.61	5.38	3.31	6.91	0.818	0.841	0.022	0.084
Total BI[13]	13.3^{a}	24.6^{b}	14.8^{a}	26.4^{b}	15.1^{a}	35.9^{c}	1.89	0.005	<0.001	0.031

[1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g *Cistus ladanifer*/kg DM; [6] Diet CLO - basal diet with 250 g *Cistus ladanifer*/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] CT - Condensed tannin sources inclusion in diets, O - Oil supplementation; [8] Variables submitted to Box-Cox transformation; means presented are back-transformed values, although SEM is expressed in transformed scale. Values of λ – 18:1 *trans*-9 = 0.057, 18:1 *trans*-10 = -0.697, 18:1 *cis*-15 = -0.254, 18:2 *cis*-9, *cis*-15 = -0.093, 18:2 *cis*-12, *cis*-15 = -0.295, 18:2 *trans*-12, *trans*-14 = -0.302, 18:2 *trans*-11, *trans*-13 = -0.004, 18:2 *cis*/*trans*-12,14 = -0.351, 18:2 *cis*-9, *trans*-11 = 0.116, 18:2 *trans*-8, *cis*-10 = 0.101; [9] Peak includes 18:2 *cis*-9, *trans*-13, 18:2 *trans*-8, *cis*-12 and 17-*cyclo* (methyl 11-cyclohexylundecanoate); [10] Peak includes 18:2 *trans*-8, *cis*-13 and 18:2 *cis*-9, *trans*-12; [11] Peak includes 18:2 *cis*-12, *trans*-14 and 18:2 *trans*-12, *cis*-14; [12] Peak includes 18:2 *cis*11, *trans*-13 and 18:2 *trans*-11, *cis*-13; [13] Total C_{18} biohydrogenation intermediates - total C_{18} fatty acids minus 18:0, 18:1 *cis*-9, 18:1 *cis*-11, 18:2*n*-6 and 18:3*n*-3.

For selected C_{18} FA displayed in **Table 5.4** no significant interactions between oil and CT sources were found ($P > 0.05$). However, *C. ladanifer* diets increased the concentration of 18:1 *trans*-11, 18:1 *cis*-9 and 18:2*n*-6, whereas grape seed diets had no effect.

The detailed profile of C_{18} FA (g/100 g of total C_{18} FA) in abomasal digesta is presented in **Table 5.5**. Stearic acid was the main C_{18} FA in abomasal digesta for all diets. In lambs fed diets with no oil, 14.4% of total C_{18} FA were BI, and oil supplementation increased this proportion to 25.5% in lambs fed control and grape seed diets and to 35.9% in lambs fed *C. ladanifer* diet ($P = 0.031$). For all diets, the major BI was 18:1 *trans*-11, which represented an average of 36.2% total BI.

Independently from the CT inclusion, oil supplementation increased the proportion of most of the C_{18} FA and decreased only six C_{18} FA (18:1 *cis*-9, 18:1 *cis*-11, 18:2*n*-6, 18:2 *trans*-8, *trans*-10, 18:2 *trans*-7, *trans*-9 and 18:2 *trans*-8, *cis*-10). Condensed tannin source *per se* had a minor effect on C_{18} FA profile, affecting only four FA (18:1 *trans*-6 – 8, 18:1 *trans*-12, 18:1 *cis*-12 and 18:2 *trans*-11, *trans*-13), although numerous interactions with oil supplementation were observed (12 FA). Adding oil to control and grape seed diets did not affect the proportions of 18:0 and 18:1 *trans*-11. However, when oil was added to *C. ladanifer* diet, 18:0 decreased 20.4% ($P = 0.016$) and 18:1 *trans*-11 increased 12.5% ($P = 0.018$). In contrast, oil supplementation increased 18:1 *cis*-16, 18:2 *cis*-9, *trans*-11 and 18:3 *cis*-9, *trans*-11, *cis*-15 and the unresolved 18:1*cis*-14 plus 18:1 *trans*-16 in lambs fed control and grape seed diets, but did not affect these FA when added to *C. ladanifer* diet.

5.3.4 Intramuscular fatty acids

In PL, lambs fed *C. ladanifer* diets had a higher (P = 0.006) FA concentration (5.27 mg/g fresh muscle; **Table 5.6**) than lambs fed control and grape seed diets (4.52 mg/g fresh muscle). Fatty acid concentration in NL fraction (**Table 5.8**) was higher (P = 0.048) in lambs fed CLO (25.6 mg/g fresh muscle) than in lambs fed other diets (15.8 mg/g fresh muscle). The general FA profile (g/100 g total FA) of PL and NL are presented in **Table 5.6** and **5.8**, respectively, whereas detailed C_{18} FA profile (mg/g total FA) of PL and NL are presented in **Table 5.7** and **5.9**, respectively.

5.3.4.1 Polar lipids

In lambs fed diets with no oil, the major FA in PL was 18:1 *cis*-9 followed by 18:2*n*-6 and 16:0. However, in lambs fed oil supplemented diets, the major FA in PL was 18:2*n*-6, followed by 18:1 *cis*-9 and 16:0. Oil supplementation decreased 16:0, 17:0, 14:1 *cis*-9, 16:1 *cis*-9, 17:1 *cis*-9, 20:1 *cis*-11, 20:3*n*-9, 20:3*n*-6, ARA, 22:4*n*-6, branched-chain FA, total MUFA and total *n*-6 LC-PUFA, while it increased 18:2*n*-6 and 18:3*n*-3, 20:3*n*-3, total PUFA and *n*-3 PUFA. Inclusion of *C. ladanifer* in diets increased 20:0, but decreased total SFA from 21.5 to 20.4% total FA. Significant interactions between oil supplementation and inclusion of CT sources in diets were found for minor FA and for *n*-6 PUFA sum (P = 0.036). Feeding the CL diet resulted in a higher accumulation of *n*-6 PUFA in PL fraction when compared to C diets. However, lambs fed GS diet had an intermediate value and differed neither from CL, nor C fed lambs. Oil supplementation increased *n*-6 PUFA in lambs fed control and grape seed diets, but had no effect in lambs fed *C. ladanifer* diet.

Table 5. 6 Effect of dietary condensed tannin sources (Control, Grape Seed Extract-GSE, and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on total fatty acid concentration (mg/g fresh muscle) and composition (g/100g of total fatty acids) of polar lipids in *longissimus dorsi* muscle from lambs

	Control		GSE		*C. ladanifer*		SEM	*P* values[7]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
Total fatty acids	4.67	4.02	4.63	4.75	5.46	5.09	0.259	0.006	0.170	0.344
12:0	0.06	0.02	0.07	0.06	0.09	0.10	0.024	0.096	0.506	0.612
14:0	0.50	0.38	0.46	0.38	0.64	0.55	0.128	0.362	0.369	0.985
iso-15:0	0.05	0.01	0.03	0.02	0.03	0.02	0.008	0.650	0.004	0.115
anteiso-15:0	0.05^{b}	0.02^{a}	0.05^{b}	0.04^{ab}	0.05^{b}	0.06^{b}	0.007	0.117	0.054	0.012
14:1 *cis*-9	0.12	0.11	0.13	0.11	0.13	0.12	0.005	0.343	0.007	0.106
15:0	0.18^{b}	0.17^{a}	0.18^{b}	0.13^{a}	0.16^{b}	0.16^{b}	0.008	0.118	0.006	0.016
iso-16:0[8]	0.09	0.07	0.08	0.06	0.07	0.07	0.093	0.121	<0.001	0.381
16:0	12.9	11.0	13.4	10.1	12.8	10.5	0.36	0.809	<0.001	0.170
iso-17:0	0.28	0.23	0.28	0.22	0.29	0.22	0.016	0.854	<0.001	0.985
anteiso-17:0[8]	0.13	0.08	0.12	0.07	0.12	0.30	0.030	0.380	<0.001	0.625
16:1 *cis*-9	0.69	0.26	0.62	0.28	0.67	0.35	0.042	0.403	<0.001	0.358
17:0	0.73^{c}	0.57^{b}	0.74^{c}	0.43^{a}	0.66^{c}	0.44^{a}	0.027	0.003	<0.001	0.020
17:1 *cis*-9	0.49	0.16	0.45	0.17	0.44	0.18	0.031	0.741	<0.001	0.520
Total C_{18}	56.2	60.4	54.7	59.5	55.4	60.1	0.40	0.021	<0.001	0.774
20:0	0.11	0.11	0.13	0.12	0.16	0.13	0.007	<0.001	0.033	0.493
20:1 *cis*-11	0.18	0.12	0.21	0.14	0.19	0.14	0.014	0.155	<0.001	0.869
20:2*n*-6[8]	0.12^{a}	0.15^{bc}	0.14^{ab}	0.14^{ab}	0.18^{c}	0.14^{ab}	0.280	0.079	0.956	0.013
20:3*n*-9	0.84	0.44	0.78	0.60	0.87	0.44	0.088	0.868	<0.001	0.348
22:0	0.03	0.05	0.05	0.05	0.05	0.06	0.008	0.109	0.087	0.204

20:3*n*-6[8]	0.67	0.58	0.74	0.60	0.76	0.56	0.045	0.400	<0.001	0.154
20:3*n*-3[8]	0.05	0.10	0.05	0.10	0.05	0.10	0.347	0.941	<0.001	0.836
20:4*n*-6	5.60	4.59	5.91	5.10	6.19	4.80	0.212	0.108	<0.001	0.391
20:5*n*-3[8]	1.67	2.08	1.79	2.19	1.58	2.10	0.328	0.854	0.055	0.970
22:4*n*-6[8]	0.39[c]	0.22[a]	0.39[c]	0.30[b]	0.45[d]	0.24[a]	0.041	0.045	<0.001	0.006
22:5*n*-3[8]	2.51	2.22	2.47	2.61	2.27	2.18	0.062	0.121	0.486	0.367
22:6*n*-3	0.59	0.54	0.59	0.64	0.54	0.56	0.048	0.379	0.827	0.642
Others[9]	15.0	15.4	15.5	16.1	15.2	15.9	0.399	0.338	0.095	0.963
SFA	21.2	22.0	21.0	21.7	20.8	20.1	0.38	0.011	0.365	0.114
MUFA	30.0	20.0	27.7	20.2	26.8	22.9	1.29	0.708	<0.001	0.080
PUFA	30.5	40.9	31.8	41.5	33.6	40.2	1.42	0.679	<0.001	0.372
n-6 PUFA[8, 10]	21.6[a]	28.5[d]	22.9[ab]	27.9[cd]	25.1[bc]	27.0[cd]	0.039	0.446	<0.001	0.036
n-6 LC-PUFA[11]	6.77	5.55	7.18	6.14	7.60	5.74	0.247	0.084	<0.001	0.238
n-3 PUFA[12]	7.03	10.0	7.04	10.8	6.30	10.3	0.537	0.486	<0.001	0.645
n-3 LC-PUFA[8, 13]	4.79	4.89	4.86	5.35	4.38	4.93	0.090	0.539	0.289	0.852

SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; [1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g *Cistus ladanifer*/kg DM; [6] Diet CLO - basal diet with 250 g *Cistus ladanifer*/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] CT - Condensed tannin sources inclusion in diets, O - Oil supplementation; [8] Variables submitted to Box-Cox transformation; means presented are back-transformed values, although SEM is expressed in transformed scale. Values of λ – *iso* 16:0 = -0.132, *anteiso* 17:0 = 0.506, 20:2*n*-6 = -0.748, 20:3*n*-6 = 0.207, 20:3*n*-3 = -0.539, 20:5*n*-3 = 1.320, 22:4*n*-6 = 0.236, 22:5*n*-3 and total *n*-6 PUFA = 0.010; [9] The sum of the remaining area (others) include about 11.6% of dimethylacetals and 3.87% unidentified peaks; [10] *n*-6 PUFA = (18:2*n*-6 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6); [11] *n*-6 LC-PUFA = (20:2*n*-6 + 20:3*n*-6 + 20:4*n*- 6 + 22:4*n*- 6); [12] *n*-3 PUFA = (18:3*n*-3 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3);[13] *n*-3 LC-PUFA = (20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3).

Table 5. 7 Effect of dietary condensed tannin sources (Control, Grape Seed Extract-GSE, and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on C_{18} fatty acid composition (mg/g total fatty acids) of polar lipids in *longissimus dorsi* muscle from lambs

	Control		GSE		*C. ladanifer*		SEM	*P* values[7]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
18:0[8]	101[a]	116[b]	98[a]	111[b]	98[a]	97[a]	0.02	<0.001	<0.001	0.001
18:1 isomers										
trans-6 + *trans*-7 + *trans*-8[8]	1.01	1.08	0.96	1.09	1.00	1.19	0.073	0.672	0.059	0.778
trans-9	1.02	1.26	0.87	1.29	1.07	1.56	0.133	0.201	0.002	0.623
trans-10[8]	1.10	1.58	0.86	1.46	1.48	1.71	0.153	0.138	0.021	0.458
trans-11[8]	4.91	10.4	4.92	12.9	5.01	24.9	0.082	0.235	<0.001	0.283
trans-12	2.24	4.54	1.86	4.39	2.64	5.15	0.247	0.015	<0.001	0.871
trans-13 + *trans*-14	2.47	8.36	2.57	8.06	3.20	6.75	0.053	0.689	<0.001	0.081
cis-9	239	116	206	123	221	119	12.2	0.584	<0.001	0.274
cis-11	22.3	19.4	20.2	17.2	18.9	16.0	0.758	<0.001	<0.001	0.998
cis-12	2.53	16.3	1.82	19.1	5.80	18.9	1.471	0.156	<0.001	0.336
cis-13	0.75	0.83	0.62	0.75	0.73	0.75	0.029	0.005	0.003	0.137
cis-14 + *trans*-16	1.63	2.86	1.56	2.98	1.81	2.85	0.100	0.710	<0.001	0.184
cis-15[8]	0.55[ab]	1.20[c]	0.46[a]	1.53[c]	0.66[b]	1.46[b]	0.083	0.078	<0.001	0.025
cis-16	0.51[ab]	0.62[b]	0.61[b]	0.45[a]	0.40[a]	0.53[ab]	0.059	0.236	0.564	0.037
Total	280	187	243	195	264	206	13.9	0.457	<0.001	0.258
18:2 non-conjugated isomers										
cis-9, *trans*-13 + *trans*-8, *cis*-12[9]	1.86	2.18	1.96	2.71	2.70	3.28	0.179	<0.001	<0.001	0.467
trans-8, *cis*-13 + *cis*-9, *trans*-12[10]	1.91[a]	2.60[b]	1.83[a]	3.05[b]	2.69[b]	2.86[b]	0.189	0.034	<0.001	0.036
trans-9, *cis-12*	1.09	1.22	1.12	1.15	1.33	1.32	0.078	0.036	0.441	0.658
trans-11, *cis*-15[8]	0.52	1.55	0.58	2.40	0.65	2.65	0.180	0.119	<0.001	0.569
cis-9, *cis*-12[8]	148	230	158	218	174	212	0.051	0.644	<0.001	0.059
cis-9, *cis*-15[8]	0.77	1.03	0.88	1.16	1.01	1.30	0.076	0.010	<0.001	0.914
cis-12, *cis*-15[8]	0	1.65	0	2.77	0	1.89	0.150	0.214	<0.001	0.214
Total[8]	154	240	164	231	182	226	0.05	0.470	<0.001	0.058

18:2 conjugated isomers										
trans-12, *trans*-14	0.03[a]	0.13[b]	0.02[a]	0.17[b]	0.03[a]	0.25[c]	0.020	0.015	<0.001	0.024
trans-11, *trans*-13[8]	0.07	0.29	0.05	0.32	0.05	0.23	0.246	0.523	<0.001	0.554
trans-10, *trans*-12	0.01	0.02	0.01	0.02	0.01	0.02	0.003	0.687	0.036	0.951
trans-9, *trans*-11	0.11	0.16	0.10	0.17	0.11	0.21	0.018	0.314	<0.001	0.368
trans-8, *trans*-10	0.03	0.03	0.02	0.02	0.02	0.02	0.004	0.212	0.921	0.632
trans-7, *trans*-9	0.04[b]	0.02[a]	0.03[ab]	0.02[a]	0.03[ab]	0.03[b]	0.004	0.276	0.405	0.049
cis-/*trans*-12,14[8, 11]	0.03	0.05	0.02	0.08	0.02	0.05	0.464	0.941	<0.001	0.299
cis-/*trans*-11,13[12]	0.18	0.76	0.15	1.23	0.16	1.02	0.105	0.142	<0.001	0.075
trans-10, *cis*-12	0.02	0.04	0.01	0.05	0.02	0.08	0.010	0.218	<0.001	0.336
cis-9, *trans*-11	3.86	5.14	3.59	5.87	3.71	7.94	0.799	0.228	<0.001	0.192
trans-8, *cis*-10	0.04	0.06	0.05	0.06	0.05	0.11	0.013	0.058	0.008	0.112
trans-7, *cis*-9	0.12	0.09	0.10	0.12	0.14	0.15	0.021	0.200	0.840	0.642
Total	4.55	6.81	4.15	8.14	4.36	10.2	0.948	0.249	<0.001	0.195
Total 18:2 isomers	159	249	168	240	188	236	8.4	0.529	<0.001	0.061
18:3 isomers										
cis-9, *cis*-12, *cis*-15	22.1	50.7	21.5	53.5	18.7	53.1	2.23	0.761	<0.001	0.434
cis-9, *trans*-11, *cis*-15	0	1.73	0	1.67	0	1.43	0.162	0.612	<0.001	0.612
Total[8]	21.8	52.3	21.0	55.7	18.1	54.7	0.09	0.558	<0.001	0.408
Total BI[13]	2.97	7.08	2.78	7.76	3.73	9.64	0.462	0.003	<0.001	0.173

[1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g *Cistus ladanifer*/kg DM; [6] Diet CLO - basal diet with 250 g *Cistus ladanifer*/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] CT - Condensed tannin sources inclusion in diets, O - Oil supplementation; [8] Variables submitted to Box-Cox transformation; means presented are back-transformed values, although SEM is expressed in transformed scale. Values of λ – 18:0, 18:1 *trans*-6 to 8, 18:2 *trans*-11, *cis*-15, 18:2 *cis*-9, *cis*-12, 18:2 *cis*-12, *cis*-15, total 18:2 non-conjugated isomers and total 18:3 isomers = 0.010, 18:1 *trans*-10 = -0.549, 18:1 *trans*-11 = -0.425, 18:1 *cis*-15 = -0.073, 18:2 *cis*-9, *cis*-15 = -0.369, 18:2 *trans*-11, *trans*-13 = -0.033, 18:2 *cis*/*trans*-12,14 = -0.157; [9] Peak includes 18:2 *cis*-9, *trans*-13, 18:2 *trans*-8, *cis*-12 and 17-*cyclo* (methyl 11-cyclohexylundecanoate); [10] Peak includes 18:2 *trans*-8, *cis*-13 and 18:2 *cis*-9, *trans*-12; [11] Peak includes 18:2 *cis*-12, *trans*-14 and 18:2 *trans*-12, *cis*-14; [12] Peak includes 18:2 *cis*11, *trans*-13 and 18:2 *trans*-11, *cis*-13; [13] Total C_{18} biohydrogenation intermediates - total C_{18} fatty acids minus 18:0, 18:1 *cis*-9, 18:1 *cis*-11, 18:2*n*-6 and 18:3*n*-3.

The total of C_{18} FA (**Table 5.7**) increased with oil supplementation ($P < 0.001$). Grape seed diets resulted in lower total C_{18} FA than control diets, whereas *C. ladanifer* diets presented an intermediate value ($P = 0.021$). Total BI increased with oil supplementation ($P < 0.001$), ranging from 5.7% total C_{18} FA in lambs fed diets with no oil to 13.6% in oil supplemented lambs. Lambs fed *C. ladanifer* diets also showed higher ($P = 0.003$) BI content in PL than lambs fed other diets (11.4 *vs.* 8.76% of total C_{18}). For all diets, 18:2 *cis*-9, *trans*-11 was the predominant CLA isomer, ranging from 86% total CLA in lambs fed diets with no oil to 74% in oil supplemented lambs.

Oil supplementation increased the proportion of most C_{18} FA, whereas it decreased only two (18:1 *cis*-9 and 18:1 *cis*-11). Condensed tannin source *per se* modified only six FA (18:1 *trans*-12, 18:1 *cis*-11, 18:1 *cis*-13, 18:2 *trans*-9, *cis*-12 and 18:2 *cis*-9, *cis*-15 and unresolved 18:2 *cis*-9, *trans*-13 plus 18:2 *trans*-8, *cis*-12). Some interactions between oil supplementation and CT sources were observed for C_{18} FA. The proportions of 18:0, 18:1 *cis*-15 and unresolved 18:2 *trans*-8, *cis*-13 plus 18:2 *cis*-9, *trans*-12 were not affected when oil was added to *C. ladanifer* diet, although an increase was observed when oil was added to control and grape seed diets. Oil supplementation resulted in a greater increase of 18:2 *trans*-12, *trans*-14 in lambs fed *C. ladanifer* than in lambs fed other diets. The 18:2 *cis*-12, *cis*-15 was only detected in lambs fed oil.

5.3.4.2 Neutral lipids

The major FA in NL were 18:1 *cis*-9, 16:0 and 18:0. Oil supplementation decreased 14:0, 16:0, 17:0, 20:0, *iso*-15:0, *iso*-16:0, *iso*-17:0, *anteiso*-17:0, 14:1 *cis*-9, 16:1 *cis*-9, 17:1 *cis*-9, ARA and total SFA, but increased 18:2*n*-6 and 18:3*n*-3, 20:3*n*-9, 20:3*n*-3, EPA, total PUFA, *n*-6 PUFA and *n*-3 PUFA. Lambs fed *C. ladanifer* diets had the lowest

concentration of *iso*-15:0 and *iso*-16:0, but the highest concentration of 16:1 *cis*-9 and 20:0. Inclusion of CT sources in diets decreased (P = 0.002) the *iso*-17:0 proportion.

Total C_{18} FA (**Table 5.8**) increased with oil supplementation in all diets. For diets with no oil, CT sources inclusion decreased total C_{18} FA when compared to control (P = 0.036). Total BI increased with oil supplementation (P < 0.001), representing 10% of total C_{18} FA in lambs fed diets with no oil and 21.6% in oil supplemented lambs.

Most C_{18} FA increased with oil supplementation (**Table 5.9**), while only 18:1 *cis*-9 decreased. Condensed tannin source *per se* had minor effects on C_{18} FA profile in NL fraction, affecting only three FA (18:1 *cis*-9, 18:2 *trans*-11, *trans*-13 and unresolved 18:2 *cis*-9, *trans*-13 plus 18:2 *trans*-8, *cis*-12). However, numerous interactions with oil supplementation were observed (14 FA). Oil supplementation decreased 18:1 *cis*-11 and increased 18:1 *trans*-10, 18:1 *cis*-16, 18:2 *trans*-10, *cis*-12 and unresolved 18:2 *trans*-8, *cis*-13 plus 18:2 *cis*-9, *trans*-12 when added to control and grape seed diets, although it did not affect these FA when added to *C. ladanifer* diets. Oil supplementation increased 18:1 *trans*-11 in all diets, but there was a greater increase in lambs fed *C. ladanifer* diet (P = 0.003). Conversely, supplementation of *C. ladanifer* diet with oil resulted in a smaller increase of 18:1 *trans*-12, 18:1 *trans*-15 and 18:1 *cis*-14 plus *trans*-16 than supplementation with oil in other diets. For all diets, 18:2 *cis*-9, *trans*-11 was the predominant CLA isomer, ranging from 81% total CLA in lambs fed diets with no oil to 75% in oil supplemented lambs. Oil supplementation resulted in an important increase (P < 0.001) of this CLA isomer in NL for all diets, although there was a tendency (CT × O, P = 0.062) for a greater increase in lambs fed CLO diet. The 18:2 *cis*-12, *cis*-15 and 18:3 *cis*-9, *trans*-11, *cis*-15 were only detected in lambs fed oil, although lambs fed GSO showed a higher proportion of 18:2 *cis*-12, *cis*-15 than lambs fed other diets with oil.

Table 5. 8 Effect of dietary condensed tannin sources (Control, Grape Seed Extract-GSE, and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on total fatty acid concentration (mg/g fresh muscle) and composition (g/100g of total fatty acids) of neutral lipids in *longissimus dorsi* muscle from lambs

	Control		GSE		*C. ladanifer*		SEM	*P* values[7]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
Total fatty acids	15.8[a]	16.2[a]	15.5[a]	17.0[a]	14.9[a]	25.6[b]	1.80	0.048	0.009	0.016
12:0[8]	0.33	0.39	0.48	0.31	0.34	0.30	0.098	0.398	0.235	0.085
14:0	3.91	3.69	4.29	3.54	4.63	3.47	0.198	0.456	<0.001	0.078
iso-15:0	0.12	0.10	0.11	0.10	0.09	0.08	0.005	<0.001	<0.001	0.724
anteiso-15:0	0.17	0.19	0.19	0.16	0.16	0.14	0.018	0.292	0.527	0.359
14:1 *cis*-9	0.10	0.09	0.11	0.08	0.13	0.09	0.007	0.319	<0.001	0.254
15:0	0.43	0.48	0.47	0.41	0.44	0.38	0.038	0.586	0.424	0.245
iso-16:0	0.16	0.16	0.17	0.14	0.14	0.11	0.008	<0.001	0.002	0.146
16:0[8]	21.2	20.6	25.9	20.5	27.0	22.0	0.029	0.059	<0.001	0.843
iso-17:0[8]	0.34	0.30	0.31	0.28	0.31	0.25	0.001	0.002	<0.001	0.680
anteiso-17:0	0.54	0.42	0.52	0.37	0.50	0.38	0.030	0.383	<0.001	0.838
16:1 *cis*-9	1.15	0.86	1.21	0.85	1.39	0.93	0.040	0.001	<0.001	0.109
17:0	1.07	0.90	1.05	0.86	1.00	0.83	0.049	0.328	<0.001	0.967
17:1 *cis*-9	0.43	0.32	0.43	0.29	0.45	0.29	0.017	0.527	<0.001	0.472
Total C_{18}	63.7[b]	68.8[c]	61.3[a]	68.7[c]	60.4[a]	68.0[c]	0.50	0.002	<0.001	0.036
20:0	0.14	0.12	0.17	0.14	0.28	0.18	0.023	<0.001	0.012	0.159
20:1 *cis*-11	0.12[bc]	0.09[a]	0.12[bc]	0.11[ab]	0.12[bc]	0.13[c]	0.006	0.018	0.275	0.016
20:2*n*-6[8]	0.05	0.05	0.05	0.04	0.05	0.05	0.204	0.321	0.078	0.246
20:3*n*-9[8]	0.05[a]	0.21[c]	0.05[a]	0.23[c]	0.05[a]	0.10[b]	0.084	<0.001	<0.001	<0.001

22:0	0.01	0.01	0.03	0.03	0.02	0.02	0.010	0.239	0.888	0.930
20:3*n*-6	0.05	0.03	0.06	0.04	0.04	0.05	0.011	0.644	0.425	0.520
20:3*n*-3	0.05	0.06	0.04	0.06	0.05	0.06	0.005	0.445	0.001	0.691
20:4*n*-6	0.18	0.16	0.19	0.16	0.17	0.15	0.014	0.405	0.039	0.812
20:5*n*-3	0.06	0.10	0.07	0.08	0.05	0.09	0.014	0.732	0.023	0.650
22:4*n*-6	0.03	0.07	0.01	0.01	0.02	0.01	0.031	0.349	0.663	0.649
22:5*n*-3	0.19	0.19	0.22	0.20	0.15	0.20	0.026	0.477	0.678	0.464
22:6*n*-3	0.06	0.07	0.06	0.07	0.05	0.06	0.015	0.728	0.416	0.969
Others[9]	1.49	2.06	2.54	2.54	2.08	2.16	0.330	0.088	0.436	0.655
SFA	48.8	43.0	49.0	43.3	49.8	42.5	1.08	0.961	<0.001	0.674
MUFA	42.7	42.4	41.3	41.7	41.0	43.6	0.66	0.295	0.096	0.257
PUFA	6.35	12.5	6.69	12.4	6.65	11.5	0.450	0.589	<0.001	0.414
n-6 PUFA[10]	2.75	4.52	2.93	4.11	3.13	3.78	0.236	0.743	<0.001	0.071
n-6 LC-PUFA[8, 11]	0.30	0.28	0.29	0.24	0.26	0.24	0.338	0.281	0.137	0.338
n-3 PUFA[12]	1.47	3.40	1.51	3.11	1.28	2.94	0.242	0.406	<0.001	0.773
n-3 LC-PUFA[8, 13]	0.36	0.42	0.18	0.40	0.39	0.36	0.077	0.524	0.138	0.682

SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; [1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g *Cistus ladanifer*/kg DM; [6] Diet CLO - basal diet with 250 g *Cistus ladanifer*/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] CT - Condensed tannin sources inclusion in diets, O - Oil supplementation; [8] Variables submitted to Box-Cox transformation; means presented are back-transformed values, although SEM is expressed in transformed scale. Values of λ – 12:0 = 0.292, 16:0 = 0.010, *iso* 17:0 = 2.786, 20:2*n*-6 = -0.225, 20:3*n*-9 = -0.363, total *n*-6 LC-PUFA = -1.003, *n*-3 LC-PUFA = 0.564; [9] The sum of the remaining area (others) includes unidentified peaks; [10] *n*-6 PUFA = (18:2*n*-6 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6); [11] *n*-6 LC-PUFA = (20:2*n*-6 + 20:3*n*-6 + 20:4*n*- 6 + 22:4*n*- 6); [12] *n*-3 PUFA = (18:3*n*-3 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3);[13] *n*-3 LC-PUFA = (20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3).

Table 5. 9 Effect of dietary condensed tannin sources (Control, Grape Seed Extract-GSE, and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on C_{18} fatty acid composition (mg/g total fatty acids) of neutral lipids in *longissimus dorsi* muscle from lambs

	Control		GSE		*C. ladanifer*		SEM	*P* values[7]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
18:0	176	166	164	172	159	152	6.48	0.063	0.549	0.352
18:1 isomers										
trans-6 + *trans*-7 + *trans*-8[8]	2.09	3.79	2.06	3.85	2.16	3.97	0.057	0.999	<0.001	0.715
trans-9	2.71	4.19	2.63	4.32	2.70	4.07	0.087	0.566	<0.001	0.177
trans-10[8]	3.18^{a}	6.96^{c}	3.07^{a}	5.27^{bc}	4.32^{b}	5.01^{bc}	0.045	0.308	<0.001	0.042
trans-11	18.0^{a}	39.7^{b}	20.1^{a}	43.5^{b}	18.6^{a}	69.8^{c}	4.30	0.004	<0.001	0.003
trans-12	3.31^{a}	8.62^{c}	2.96^{a}	8.70^{c}	3.76^{a}	6.61^{b}	0.434	0.192	<0.001	0.006
trans-13 + *trans*-14	5.63^{b}	16.7^{d}	4.82^{a}	17.9^{e}	5.59^{b}	12.2^{c}	0.265	<0.001	<0.001	<0.001
trans-15[8]	2.32^{a}	6.36^{c}	2.47^{a}	7.02^{c}	2.39^{a}	3.95^{b}	0.028	0.002	<0.001	0.004
cis-9	346	228	332	272	326	284	5.8	0.032	<0.001	0.264
cis-11	9.60^{c}	6.54^{a}	8.86^{c}	6.84^{ab}	7.67^{b}	7.24^{ab}	0.366	0.251	<0.001	0.005
cis-12	3.13^{ab}	9.72^{c}	2.35^{a}	13.2^{c}	3.96^{b}	10.2^{c}	0.406	0.011	<0.001	<0.001
cis-13	1.14	1.17	1.00	1.42	1.01	0.99	0.101	0.122	0.095	0.079
cis-14 + *trans*-16[8]	4.17^{a}	7.78^{c}	3.96^{a}	7.80^{c}	3.92^{a}	5.69^{b}	0.045	<0.001	<0.001	0.005
cis-15	1.78^{a}	3.50^{c}	1.70^{a}	4.44^{d}	1.52^{a}	2.95^{b}	0.165	<0.001	<0.001	0.001
cis-16	0.67^{a}	1.18^{c}	0.60^{a}	1.24^{c}	0.69^{ab}	0.87^{b}	0.064	0.054	<0.001	0.005
Total	404^{bc}	404^{bc}	388^{ab}	398^{ab}	384^{a}	418^{c}	6.5	0.249	0.012	0.048
18:2 non-conjugated isomers										
trans-11, *trans*-15	0.58	1.48	0.73	2.10	0.66	1.98	0.185	0.125	<0.001	0.393
cis-9, *trans*-13 + *trans*-8, *cis*- 12[9]	4.36	7.10	4.37	7.96	4.10	6.35	0.326	0.027	<0.001	0.139
trans-8, *cis*-13 + *cis*-9, *trans*-12[8, 10]	2.34^{a}	4.20^{c}	2.39^{a}	4.38^{c}	2.99^{ab}	3.28^{bc}	0.045	0.939	<0.001	0.017
trans-9, *cis*-12	0.61	1.10	0.61	1.31	0.64	1.15	0.065	0.252	<0.001	0.204
trans-11, *c*15	2.74	8.72	2.67	10.7	2.78	8.78	0.624	0.259	<0.001	0.204
cis-9, *cis*-12	24.4	42.0	26.4	38.6	28.7	35.3	2.12	0.843	<0.001	0.053
cis-9, *cis*-15[8]	0.79	1.44	0.59	1.82	0.58	1.23	0.147	0.239	<0.001	0.215

cis-12, *cis*-15[8]	0[a]	1.90[b]	0[a]	3.09[c]	0[a]	1.66[b]	0.126	0.049	<0.001	0.049
Total	36.0[a]	68.2[c]	37.8[a]	70.3[c]	41.4[a]	60.1[b]	2.35	0.378	<0.001	0.010
18:2 conjugated isomers										
trans-12, *trans*-14	0.17	0.48	0.16	0.52	0.15	0.50	0.015	0.497	<0.001	0.166
trans-11, *trans*-13[8]	0.28	0.68	0.23	0.68	0.21	0.42	0.130	0.040	<0.001	0.355
trans-10, *trans*-12[8]	0.03	0.08	0.03	0.07	0.04	0.06	0.094	0.645	<0.001	0.144
trans-9, *trans*-11[8]	0.21	0.40	0.23	0.37	0.21	0.50	0.081	0.185	<0.001	0.065
trans-8, *trans*-10	0.03	0.04	0.03	0.04	0.03	0.03	0.005	0.327	0.062	0.596
trans-7, *trans*-9	0.05	0.05	0.05	0.04	0.05	0.06	0.005	0.450	0.375	0.136
cis-/*trans*-12,14[8, 11]	0.09[a]	0.34[c]	0.07[a]	0.42[c]	0.07[a]	0.19[b]	0.186	0.012	<0.001	0.023
cis-/*trans*-11,13[12]	0.38	1.70	0.34	2.07	0.30	1.63	0.177	0.395	<0.001	0.435
trans-10, *cis*-12[8]	0.02[a]	0.10[c]	0.02[a]	0.06[bc]	0.03[b]	0.05[b]	0.857	0.397	<0.001	0.018
cis-9, *trans*-11	7.49	13.5	8.78	13.7	7.47	18.5	1.319	0.178	<0.001	0.062
trans-8, *cis*-0	0.23	0.28	0.20	0.32	0.16	0.33	0.044	0.958	0.004	0.378
trans-7, *cis*-9[8]	0.36	0.70	0.43	0.76	0.44	0.84	0.057	0.149	<0.001	0.793
Total[8]	9.27	18.1	10.4	18.9	8.59	22.3	0.125	0.775	<0.001	0.310
Total 18:2 isomers	45.3	86.6	48.4	89.3	50.6	83.3	2.51	0.504	<0.001	0.174
18:3 isomers										
cis-9, *cis*-12, *cis*-15	11.1	29.8	11.3	27.1	9.82	25.4	2.051	0.392	<0.001	0.697
cis-9, *ttrans*-11, *cis*-15	0.94	2.62	1.10	1.77	0.86	1.99	0.267	0.327	<0.001	0.182
Total	12.2	33.2	12.7	29.7	11.0	28.2	1.804	0.247	<0.001	0.481
Total BI[13]	6.07	13.8	6.05	15.3	6.46	15.7	0.523	0.188	<0.001	0.371

[1] Diet C - basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO - basal diet with 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [3] Diet GS - basal diet with 25 g grape seed extract/kg DM; [4] Diet GSO - basal diet with 25 g grape seed extract/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [5] Diet CL - basal diet with 250 g *Cistus ladanifer*/kg DM; [6] Diet CLO - basal diet with 250 g *Cistus ladanifer*/kg DM and 60 g oil blend (sunflower and linseed oils, 1:2 v/v)/kg DM; [7] CT - Condensed tannin sources inclusion in diets, O - Oil supplementation; [8] Variables submitted to Box-Cox transformation; means presented are back-transformed values, although SEM is expressed in transformed scale. Values of λ – 18:1 *trans*-6 to 8, 18:1 *cis*-14 + *trans*-16, 18:2 *cis*-12, *cis*-15, total 18:2 conjugated isomers = 0.010, 18:1 *trans*-10 = -0.594, 18:1 *trans*-15 = -0.650, 18:2 *trans*-8, *cis*-13 + *cis*-9, *trans*-12 = 0.626, 18:2 *trans*-11, *trans*-13 = 0.134, 18:2 *trans*-10, *trans*-12 = 0.072, 18:2 *trans*-9, *trans*-11 = -0.066, 18:2 *cis*/*trans*-12,14 = -0.195, 18:2 *trans*-10, *cis*-12 = -0.523, 18:2 *trans*-7, *cis*-9 = 0.944; [9] Peak includes 18:2 *cis*-9, *trans*-13, 18:2 *trans*-8, *cis*-12 and 17-*cyclo* (methyl 11-cyclohexylundecanoate); [10] Peak includes 18:2 *trans*-8, *cis*-13 and 18:2 *cis*-9, *trans*-12; [11] Peak includes 18:2 *cis*-12, *trans*-14 and 18:2 *trans*-12, *cis*-14; [12] Peak includes 18:2 *cis*11, *trans*-13 and 18:2 *trans*-11, *cis*-13; [13] Total C_{18} biohydrogenation intermediates - total C_{18} fatty acids minus 18:0, 18:1 *cis*-9, 18:1 *cis*-11, 18:2*n*-6 and 18:3*n*-3.

5.4 DISCUSSION

5.4.1 Growth performance and carcass composition

Lipid supplements are the richest energy containing feedstuffs, and thus their use in ruminant diets should theoretically increase growth performance. The effects of lipid supplementation on the growth performance of ruminants are inconsistent. Differences have been attributed to negative interactions of lipids with ruminal digestion of structural carbohydrates and to depressions in feed intake (Hess *et al.*, 2008). Previous works reported reductions of feed intake in lambs fed lipid supplemented diets coupled with no effect (Bessa *et al.*, 2005, Manso *et al.*, 2009) or reduction (Santos-Silva *et al.*, 2004) on lamb growth. In the present trial, DMI was not affected by oil supplementation, which might explain the tendency of average daily gain to increase (P = 0.083) and the significant enhancement in hot carcass weight. Higher fat deposition in carcass has been found in lambs fed diets supplemented with fat (Santos-Silva *et al.*, 2004). However, tissue composition of dissected cuts was not affected by oil supplementation, and only the percentage of KKCF in carcass increased.

Condensed tannins might have both adverse and beneficial effects in ruminants, depending on their chemical structure and concentration in diets (Waghorn, 2008). Thus, the reports on the effects of dietary CT on growth performance of lambs are inconsistent (Priolo *et al.*, 2005, Vasta *et al.*, 2009c, Vasta *et al.*, 2010b). Our results show that the inclusion of 25 g/kg DM of grape seed extract or 250 g/kg DM of *C. ladanifer* in diets did not affect growth performance. This is the first report on the effects of dietary inclusion of *C. ladanifer* on lamb growth performance. As far as we know, the effect of grape seed extract on lamb growth seems to be restricted to one trial (Schreurs *et al.*, 2007). These authors found that 33 g/day of grape seed extract, supplied as liquid supplement, reduced

weight gain but not carcass gain when basal diet was white clover, and had no effect when basal diet was perennial ryegrass. As the average daily intake of grape seed CT in our trial was approximately 24 g/day, no depression of growth performance was expected. *Cistus ladanifer* is a very abundant shrub in marginal fields of Mediterranean countries, but practically it is not used in direct grazing. This fact is probably due to its high content in anti-nutritional compounds, arising from the secondary metabolism of plants, such as CT and essential oils which are very abundant in a characteristic gum resin exuded by the plant, also known as labdanum (Dentinho *et al.*, 2005, Gomes *et al.*, 2005). Thus, we expected that the high incorporation of *C. ladanifer* in diets (250 g/kg DM) would result in greater depression in lamb growth performance. However, our results show that *C. ladanifer* may be successfully incorporated in lambs' diets without compromising animal performance, reinforcing the interest of its use in small ruminant nutrition. *Cistus ladanifer* affected the composition of the weight gain, increasing fat deposition probably due to the highest fat content in these diets.

5.4.2 Ruminal biohydrogenation

We did not obtain quantitative information on rumen outflow of C_{18} FA, thus definite conclusions on rumen biohydrogenation balance are not possible. Nevertheless, as lambs had similar feed intake and lipid supplements generally do not affect rumen fluid and particle passage ratio (Doreau *et al.*, 1993), large differences in digesta flow to abomasum are not expected. However, some caution is still needed because there is no information on the effects of grape seed extract and *C. ladanifer* on rumen outflow. The profile of C_{18} FA in abomasal digesta provides an insight on ruminal biohydrogenation pattern (*i.e.* relative distribution of substrates and products) allowing an evaluation of the effects of oil

supplementation and CT on modulation BH process. Modification of the BH pattern would reflect the metabolic pathways in use and microbial equilibriums in the rumen.

Dietary supplementation with oil rich in PUFA, by increasing the substrate availability for BH, is the most effective approach to increase BI outflow of rumen (Hess *et al.*, 2008) and their transfer to milk and deposition in tissues. As expected, oil supplementation increased the concentration (mg/g DM) of substrates (18:3*n*-3, 18:2*n*-6 and 18:1 *cis*-9), most BI and the main end product (18:0). Dramatic changes in BH pattern due to oil supplementation, with strong accumulation of 18:1 *trans*-11 and low concentrations of 18:0, have been reported *in vitro* (Fievez *et al.*, 2007). However, these changes are usually much less expressive *in vivo*, except when fish oil is used (Kim *et al.*, 2008). The changes in BH pattern, with increasing dietary PUFA concentration, have been explained by a putative toxic effect of PUFA on rumen bacteria, particularly on those catalyzing the last reductive step (Jenkins *et al.*, 2008). However, present data of BH pattern show that, although oil supplementation increased most BI, no effects of oil supplementation were observed for 18:0 and 18:1 *trans*-11, except in lambs fed CLO diet. This is due to a large variability of those FA proportions within each diet. Nevertheless, despite the type (rich in 18:3*n*-3) and amount (60 g/kg DM) of oil used, no dramatic changes in 18:0 were observed, which is not consistent with a toxic effect of PUFA on BH bacteria. The branched-chain FA have been proposed as markers for the rumen microbial ecosystem (Vlaeminck *et al.*, 2006). Only slight effects of oil supplementation were detected on branched-chain FA concentration in abomasal digesta, which is consistent with no general toxicity to rumen bacteria. Moreover, when rumen FA concentration is high, rumen bacteria tend to incorporate exogenous FA, decreasing the *de novo* FA synthesis (Vlaeminck *et al.*, 2006). Therefore, the lack of depression on branched-chain FA

concentration in the abomasal digesta might be indicative of higher microbial matter yield.

Our major objective was to test the ability of CT sources to differentially modulate the BH pattern in control and oil supplemented lambs. Grape seed CT extract (25 g/kg DM) had no effect on BH pattern, except for slight changes in minor non-conjugated 18:2 and conjugated 18:3. As far as we know, this is the first report on the effects of grape seed CT extract on ruminal BH. At the present stage, it is not clear whether a higher dose of grape seed CT could modify the BH pattern. Vasta *et al*. (2009b, 2010b) obtained significant responses for BH pattern with much higher doses of quebracho CT (ranging from 40 to 65 g/kg DM of CT supplied by *circa* de 100 g/kg DM quebracho powder). Even if no anti-nutritive effects of CT would manifest in such high doses, the dilution of nutrient content of diet and consequent low growth performance would be a major restriction for its practical application. In fact, the use of these high doses of quebracho in lamb diets resulted in decreased growth performance (Vasta *et al.*, 2009c, Vasta *et al.*, 2010b).

Cistus ladanifer had no major effects on BH pattern in lambs fed no oil, but greatly changed the BH pattern in oil supplementation lambs, with a depression of 18:0 and accumulation of 18:1 *trans*-11, without changing the 18:2 *cis*-9, *trans*-11. This indicates an inhibition of the last reductive step of BH, and is thus consistent with previous reports that used other CT sources (Khiaosa-Ard *et al.*, 2009, Vasta *et al.*, 2009a, Vasta *et al.*, 2009b, Vasta *et al.*, 2010b). Diets with *C. ladanifer* had higher C_{18} FA content, leading to a higher C_{18} FA intake and concentration in abomasal digesta. However, this fact should not explain the BH pattern modifications observed because, as discussed above, the increase of FA intake did not induce major changes in BH pattern.

The supply of CT through *C. ladanifer* diets was similar to that of grape seed diets. However, CT are heterogeneous compounds with quite variable structure and size, which is reflected by their reactivity and impact on digestion (Waghorn, 2008), as well on microbial ecosystem. It was been reported that the effect of tannins on microorganisms is species and tannin type dependent (Biolonska *et al.*, 2009, Selma *et al.*, 2009). Therefore, differential responses between grape seed extract and *C. ladanifer* on BH may be due to a different CT composition. Moreover, other secondary compounds in leaves and soft stems of *C. ladanifer* may be responsible for changes in the BI pattern. The *C. ladanifer* is an aromatic shrub that secretes abundant amounts of gum resin containing several flavonoids other than proanthocyanidins and terpenoids (Gomes *et al.*, 2005, Sosa *et al.*, 2005). Some of those compounds might be responsible for rumen BH effects and further studies using *C. ladanifer* extracts must be conducted in order to clarify.

The branched-chain FA concentration in abomasal digesta did not differ between lambs fed CL and CLO diets, suggesting that no general depression in bacterial biomass flow had occurred. Accumulation of *trans* octadecenoates in the rumen could be an adaptative response of rumen ecosystem to environmental stress stimuli, as suggested by Bessa *et al*. (2000), probably without involving major microbial community changes. Nevertheless, the mechanism responsible for oil × *C. ladanifer* interaction is not clear and further studies are needed.

5.4.3 Intramuscular lipid fractions

The total intramuscular FA content is determined mostly by the amount of FA in the NL fraction, while the level of FA in the PL fraction is considered to be fairly constant or slightly increased with degree of muscle fatness (Wood *et al.*, 2008). In general, lipid

supplementation in lamb diets had no effect or slightly increased intramuscular FA (Santos-Silva *et al.*, 2004, Bessa *et al.*, 2005, Boles *et al.*, 2005, Bessa *et al.*, 2007, Manso *et al.*, 2009, Jerónimo *et al.*, 2010a). In the present trial, oil supplementation did not change the intramuscular FA content, except in lambs fed *C. ladanifer*. Although CLO lambs had a higher FA intake than others, this does not seem to enough to explain the 60% increase in intramuscular NL. Once again, the reason is unclear as to what may be the explanation for the higher muscle lipogenic activity in CLO lambs. Nevertheless, the higher FA intramuscular deposition in these lambs could be related to changes in rumen BH pattern (less 18:0 and higher 18:1 *trans*-11). In fact, exogenous 18:0 inhibits the acetate incorporation into FA ovine adipose tissue *in vitro* as reported long ago by Vernon (1977). Therefore, a reduction in 18:0 availability, as suggested by our data, might contribute to stimulate *de novo* FA synthesis. It was recently reported that the supplementation of diets with 18:1 *trans*-11 reduced the relative abundance of hepatic FA synthesis enzymes in obese rats (Wang *et al.*, 2009). However, as far as we know, there are no studies regarding the effect of increased 18:1 *trans*-11 availability on muscular lipogenic regulation in ruminants. Vasta *et al*. (2009c) reported increased expression levels of Δ^9-desaturase protein in muscle of lambs fed fresh vetch supplemented with quebracho. Moreover, this dietary treatment also reduced 18:0 and increased 18:1 *trans*-11 levels in intramuscular fat.

As expected (Scollan *et al.*, 2006), the FA pattern of NL fraction was characterized by a high proportion of SFA and MUFA, whereas the PL fraction showed a high proportion of PUFA. The 18:2*n*-6, 18:3*n*-3 and LC-PUFA were preferentially deposited in PL fraction, but the 18:3*n*-3 was distributed more equally between NL and PL fractions, as previously reported (De Smet *et al.*, 2004). In opposition, 18:0, 18:1 *cis*-9 and most *trans* C_{18} FA,

including CLA isomers, were preferentially incorporated in NL. The PL are membrane components, so its FA composition is under regulatory control in order to maintain proper membrane fluidity and function. Therefore FA composition in PL is less influenced by dietary factors than NL (Scollan *et al.*, 2006). However, diet manipulation changed similarly the FA composition of both lipid fractions, reflecting the BH pattern observed in abomasal digesta although modulated by endogenous syntheses.

As suggested by FA concentration in abomasal digesta, oil supplementation increased 18:1 *trans*-11, 18:2 *cis*-9, *trans*-11 and most of the other BI in both PL and NL. The accumulation of CLA in intramuscular fat, as a response to the supplementation of diets with oil rich in 18:2*n*-6 and 18:3*n*-3, has previously been reported (Santos-Silva *et al.*, 2004, Bessa *et al.*, 2005, Boles *et al.*, 2005, Bessa *et al.*, 2007). However, oil supplementation of *C. ladanifer* diet resulted in a higher accumulation of 18:1 *trans*-11 in NL fraction than in lambs fed other diets. The 18:2 *cis*-9, *trans*-11 also tended ($P = 0.062$) to be highest in lambs fed CLO diet, mainly due to the increase of 18:1 *trans*-11 availability for endogenous desaturation, because its concentration in abomasal digesta was not superior than in other oil supplemented diets. This is fully consistent with the fact that 18:2 *cis*-9, *trans*-11 in tissues results mostly from the conversion of 18:1 *trans*-11 by endogenous Δ9-desaturase (Scollan *et al.*, 2006). The preferential deposition of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 into NL have been previously reported (Wood *et al.*, 2008). Lambs fed CLO diet had more NL in the muscle and higher concentration of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 in NL, resulting in a relevant enrichment of these FA in muscle compared to other oil supplemented diets (189.5 *vs.* 70.6 and 41.7 *vs.* 19.2 mg/100 g muscle for 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11, respectively). About 19% of dietary 18:1 *trans*-11 may be converted into 18:2 *cis*-9, *trans*-11 by Δ9-desaturase in humans (Turpeinen *et al.*, 2002). Therefore, when considering the 18:1 *trans*-11 content of CLO

lamb meat, the potential 18:2 *cis*-9, *trans*-11 supply would increase by 36 mg/100 g muscle, summing a total of 77.8 mg/100 g of muscle.

The dietary inclusion of lipid sources rich in 18:2*n*-6 and 18:3*n*-3 increased *n*-6 and *n*-3 PUFA content in PL and reduced SFA in NL. However, PUFA increase in muscle PL was caused exclusively by increased 18:2*n*-6 and 18:3*n*-3 deposition, because *n*-3 LC-PUFA were unchanged and the *n*-6 LC-PUFA decreased. This can be explained either by an inhibitory effect of *n*-6 PUFA and *n*-3 PUFA on Δ6- and Δ5-desaturase expression (Nakamura and Nara, 2002) or by competition between 18:2*n*-6 and 18:3*n*-3 for desaturation and elongation enzymes due to the preference of those enzymes for 18:3*n*-3 (Brenner, 1989). Recently, also was reported that supplementation of diet with linseed oil decreases the Δ6-desaturase protein level in cattle muscle (Herdmann *et al.*, 2010). These, or other, mechanisms regulating the incorporation of these highly FA unsaturated in membranes are probably linked to the homeostasis of membrane fluidity, but may constitute a limitation to dietary strategies designed for LC-PUFA enrichment of ruminant meat.

The increase of CLA content in lamb meat in response to supplementation of diets rich in forage with vegetable oils has been extensively shown (Santos-Silva *et al.*, 2004, Bessa *et al.*, 2005, Bessa *et al.*, 2007, Jerónimo *et al.*, 2010a). However, in the present study we found that inclusion of *C. ladanifer* in oil supplemented diets, but not of grape seed tannins, resulted in higher health benefiting FA content in lamb meat than only with oil supplementation. *Cistus ladanifer* did not compromise animal performance, which reinforces the interest of its use in association with vegetable oils in nutritional strategies in order to improve the nutritional value of lamb meat.

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CHAPTER 6

EFFECT OF DIETARY GRAPE SEED EXTRACT AND *CISTUS LADANIFER* L. IN COMBINATION WITH VEGETABLE OIL SUPPLEMENTATION ON OXIDATIVE STABILITY OF LAMB MEAT

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ABSTRACT

Thirty-six lambs were used in a 6 weeks experiment for evaluating the effect of two dietary CT sources: grape seed extract (0 *vs.* 2.5% DM) and *Cistus ladanifer* L. (0 *vs.* 25% DM) on lipid and colour stability of meat from lambs fed diets with or with no vegetable oil blend supplementation (0 *vs.* 6% DM). The effect of dietary CT sources on meat sensory properties was also evaluated. Meat antioxidant potential, determined after oxidation induction by a ferrous/hydrogen peroxide system, decreased with oil supplementation ($P < 0.001$), but inclusion of CT sources in diets protected the meat against lipids oxidation ($P = 0.036$). Meat colour was not affected by diets. Dietary CT sources did not change the sensory properties of meat. Thus, incorporation of these CT sources in diets seems to be a good approach to increase the antioxidant potential of meat without compromising its sensorial traits.

Keywords: lamb meat; grape seed extract; *Cistus ladanifer* L; oil supplementation; colour stability; lipid oxidation

6.1 INTRODUCTION

Ruminant meat is characterized by high contents of SFA and low levels of PUFA, which has been linked with an high cardiovascular diseases risk in humans (Givens, 2005). The supplementation of ruminant diets with lipid sources rich in PUFA is an effective means to improve the nutritional value of meat fat, decreasing the SFA and promoting the enrichment in PUFA, including the health enhancing FA, such as CLA and *n*-3 PUFA (Sinclair, 2007). However, the high content of PUFA in meat is associated with its increased susceptibility to oxidation (Morrissey *et al.*, 1998) and, thus meat quality deterioration.

The synthetic antioxidants are largely used in animal nutrition and food industry in order to improve the oxidative stability of foods. However, for satisfying the consumers on concern over food safety and toxicity of synthetic antioxidant, the interest in natural antioxidants in substitution of the synthetic ones has increased in recent years. Proanthocyanidins, also known as CT, are oligomers and polymers of flavanoid units linked by carbon-carbon bonds (Hagerman, 1998), arising from the secondary metabolism of plants. The effective antioxidant activity of CT sources, such as grape seed extract, has been reported when added to minced meat, including beef (Ahn *et al.*, 2002), pork (Carpenter *et al.*, 2007) and turkey (Lau and King, 2003). Feeding studies conducted with poultry also showed that dietary supplementation with grape seed and green tea extracts (Smet *et al.*, 2008), grape pomace concentrate (Brenes *et al.*, 2008) and high-tannin sorghum (Du *et al.*, 2002) limits lipid oxidation in meat. The inclusion of grape seed and peel extract directly in rumen of sheep was shown to be effective in reducing the susceptibility to lipid oxidation in plasma (Gladine *et al.*, 2007c). Although the effect of dietary CT sources on lipid oxidation in ruminant meat has been little explored, Luciano *et al*. (2009a) have reported that the inclusion of quebracho tannins in concentrate fed to

lambs did not affect lipid oxidation in *semimembranosus* muscle, although reduced the meat discoulouration.

Cistus ladanifer L. is a very abundant shrub in marginal fields of Mediterranean countries, with high contents of CT (Dentinho *et al.*, 2005). The antioxidant activity of *C. ladanifer* phenolic extract *in vitro* was reported recently (Andrade *et al.*, 2009), although the effect of dietary *C. ladanifer* on meat quality has yet not been explored. In addition, as far as we know the effect of dietary grape seed extract on lipid oxidation of ruminant meat has not been investigated. Thus, the main objective of the present study was to evaluate the effect of dietary grape seed extract or *C. ladanifer* on lipid oxidative and colour stability during storage of PUFA enriched lamb meat. We also investigated if these CT sources affected the sensorial properties of cooked meat.

6.2 MATERIALS AND METHODS

6.2.1. Animal and management

Details on diets and animal handling procedures have been reported elsewhere (Jerónimo *et al.*, 2010b, chapter 5; Vasta *et al.*, 2010a). Briefly, twenty-six Merino Branco ram lambs with 24.8 ± 1.55 kg (mean ± SD) of initial weight were randomly distributed to 12 pens which were allocated to one experimental diet according to a completely randomized experimental design with a 3 × 2 factorial arrangement of treatments. The first factor was the CT sources (with 3 levels; 1- no added CT source – control, 2- 2.5% DM of grape seed extract, 3- 25% DM of *C. ladanifer* leaves and soft stems) and the second factor was the oil supplementation (with 2 levels: 0% and 6% DM of oil blend supplementation). This 3 × 2 factorial arrangement results in 6 diets: C, basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; CO, basal diet with 6% DM of

oil blend; GS, basal diet with 2.5% DM of grape seed extract; GSO, basal diet with 2.5% DM of grape seed extract and 6% DM of oil blend; CL, basal diet with 25% DM of *C. ladanifer* and CLO, basal diet with 25% DM of *C. ladanifer* and 6% DM of oil blend. The oil blend was composed by a mixture of sunflower and linseed oils in a proportion of 1:2 (v/v). After 6 weeks of trial, the lambs were slaughtered in the experimental abattoir. Carcasses were kept at 10 ºC for 24 h, and then chilled at 2 ºC until the third day after slaughter.

6.2.2. Sample collection

Seventy-four hours after slaughter four samples of *longissimus dorsi* muscle of carcass left halves were collected. One sub-sample of muscle, after removing the epimysium, was minced, vacuum packed, freeze-dried and stored at -80 ºC until lipid analysis. Three sub-samples (1.5 cm thickness) were used to evaluate the lipid and colour stability during 0, 3 and 7 days of storage at 2 ºC in an illuminated cooler. At 0 day of storage the colour parameters were determined after 1 h of blooming and samples were vacuum packed. The other samples were individually placed on Styrofoam, over-wrapped with oxygen permeable film and displayed for 3 and 7 days. At the end of storage time, these samples were vacuum packed after determination the colour parameters. All samples were stored at -80 ºC until analysis. Seventy-four hours after slaughter loins and ribs of carcass right halves were collected and frozen at -20 ºC, until being used for sensorial analysis.

6.2.3. Lipid analysis

Intramuscular lipid extraction and FAME preparation are fully described by Jerónimo *et al*. (2010a, chapter 4). Briefly, intramuscular lipids were extracted using dichloromethane

and methanol (2:1 v/v) and FA were transesterified with sodium methoxide in methanol followed by hydrochloric acid in methanol (1:1 v/v). Quantification of muscle lipid FAME was done using 19:0 as internal standard. Fatty acid methyl esters were analysed using a HP6890A chromatograph (Hewlett–Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GC–FID) and fused silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 µm of film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA). Gas chromatography conditions and FA identification were the same as described in Jerónimo *et al*. (2010b, chapter 5).

6.2.4. Measurement of meat lipid oxidation after induction of oxidation in vitro

For the evaluation the meat lipid stability (antioxidant potential) it was followed the general procedure described by Mercier *et al*. (2004), which involves the induction of oxidation in meat homogenate by ferrous iron and hydrogen peroxide, followed by the measurement of oxidized lipids. Meat homogenates were prepared by homogenising 1 g tissue in 10 ml of sodium phosphate 100 mM (pH 7.0) using an Ultra-Turrax T25 homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany) for 1 min at 20 000 rpm. These homogenates were incubated with 100 µl of mixture of ferrous sulphate (0.5 mM) and hydrogen peroxide (1 mM) at 37 ºC water bath for 30 min. After incubation time, 2 mg of butylated hydroxytoluene (BHT) was added to homogenate for stopping the oxidation. Aliquots of 2 ml of homogenate were immediately frozen at -80 ºC until lipid oxidation measurement by thiobarbituric acid reactive substances (TBARS) as described by Lynch & Frei (1993). Briefly, homogenate samples (0.5 ml) were incubated with 0.25 ml of 1% (wt/v) 2-thiobarbituric acid in 50 mM of NaOH and 0.25 ml of 2.8% (wt/v) tricloroacetic acid in boiling water bath for 10 min. The pink chromogen was extracted with 2 ml of *n*-butanol and its absorbance measured at 535 nm in a UV/VIS

Spectrophotometer (Ultrospec III, Pharmacia LKB Biochrom Ltd., Cambridge, England). The 1,1,3,3 tetraethoxypropane standard curve was used for calculating the TBARS concentration and the results were expressed as mg of malonaldehyde (MDA)/kg of meat.

6.2.5. Meat colour measurement

Minolta CR-300 chromometer (Konica Minolta, Lisboa, Portugal) was used to measure meat colour coordinates, *L** (lightness), *a** (redness) and *b** (yellowness). Measurements were made using the C illuminant and 2 º standard observers. Hue angle (*H**) was calculated as $\tan^{-1}(b^*/a^*)$ x $(180/\pi)$ and colour saturation (chroma, *C**) as $(a^{*2} + b^{*2})^{1/2}$. Overall colour variation between each day of storage and the day 0 of measurements was calculated as $\Delta E^{*2}_{0-n} = (\Delta L^{*2}_{0-n} + \Delta a^{*2}_{0-n} + \Delta b^{*2}_{0-n})^{1/2}$. Where ΔL^{*2}_{0-n}, Δa^{*2}_{0-n} and Δb^{*2}_{0-n} are the differences between *L**, *a** and *b** measured at a day *n* of storage and their values at day 0.

6.2.6. Sensory analysis

For sensory analysis a panel of 30 regular consumers of lamb meat was used. A triangular test was conducted to evaluate if consumers could detect the inclusion of grape seed extract and *C. ladanifer* in lamb diets with or with no oil blend and if so, what their preference was. Therefore, the comparisons were performed on meat samples of lambs fed diets with no oil (i.e. C *vs.* GS and C *vs.* CL) and with oil (i.e. CO *vs.* GSO and CO *vs.* CLO). Frozen right joints (loins and ribs) were cut transversally to vertebral column in chops with about 2.5 cm of thickness. Twenty-four hours before sensory analysis sessions, chops were thawed at 0 ºC, cooked in an industrial grill, and served warm to the

consumer panel. Each panelist was offered simultaneously three chops, two corresponding to the same diet and one to the other. They were asked to identify the sample that was different from the other two (question 1) and to indicate the chop that tasted better (question 2). When analyzing the results, the answers to question 2 were considered only when the panelists answered correctly question 1.

6.2.7. Statistical analysis

This trial was conducted using a 3 × 2 factorial design, where the 2 factors were the CT sources (CT, with 3 levels: control, grape seed extract and *C. ladanifer*) and the vegetable oil blend supplementation (O, with 2 levels: 0% and 6%). The interaction between CT sources and O was also evaluated (CT × O). The individual animals were considered as experimental units. The Shapiro-Wilk test was used in order to evaluate whether data followed a normal distribution. When not normally distributed ($P < 0.05$) data was Box-Cox transformed before further analysis. Data of intramuscular FA composition were analysed using the MIXED procedure of SAS, considering the oil and CT sources and their interaction as fixed effects and the pen as random effect. The covariance of measurements from lambs within each pen was considered in the model. The colour and lipid oxidation were studied by repeated measure analysis of variance with the MIXED procedure of SAS with day of sampling as the repeated measurement. Last squares means and SEM are presented in tables. For Box-Cox transformed variables the SEM is presented in tables, although means are back-transformed. For sensory analysis results, a significance table for triangle tests was used to analyse the answers to question 1, and a significance table for pair tests to analyse the answers to question 2 (Roessler *et al.*, 1978). The level of statistical significance was set at $P < 0.05$ for main effects and at $P < 0.10$ for interactions.

6.3 RESULTS AND DISCUSSION

Intramuscular FA content and composition are presented in **Table 6.1**. Intramuscular FA content was higher ($P = 0.017$) in lambs fed CLO diet (30.7 mg/g of fresh muscle) than in meat from lambs fed other diets (20.6 mg/g of fresh muscle). As expected, supplementation of lamb diets with oils rich in 18:2*n*-6 and 18:3*n*-3 decreased SFA and increased PUFA, 18:1 *trans*-11 and CLA contents in meat (Bolte *et al.*, 2002, Bessa *et al.*, 2005, Bessa *et al.*, 2007, Jerónimo *et al.*, 2010a). An interaction between CT sources and oil was observed for several individual PUFA (18:2*n*-6, CLA, ARA and 22:6*n*-3) and for total PUFA and *n*-6 PUFA partial sums, in which meat from lambs fed CLO diet had lower PUFA content (17.1 % of total FA) than meat from other oil supplemented lambs (19.8 % of total FA). This is likely explained by higher intramuscular FA content in lambs fed CLO diet than in lambs fed other diets. This increase in intramuscular FA is due to higher level in triacylglicerols, while membranary PL remains fairly constant (Wood *et al*., 2008). Polyunsaturated FA are mainly incorporated in PL, whereas triacylglicerols incorporate more SFA and MUFA (Raes *et al.*, 2004). Thus, the lower content of PUFA in meat from lambs fed CLO diet than meat from lambs fed other diets with oil resulted probably by the dilution effect caused by the increase in IMFA. Moreover, meat from lambs fed CLO diet showed a higher 18:1 *trans*-11 and CLA than that from other oil supplemented lambs. The interaction of *C. ladanifer* with oil supplementation and its implications on ruminal BH and tissue lipid metabolism was thoroughly discussed in a companion paper, where detailed FA composition of both intramuscular polar and neutral lipid fractions were presented (Jerónimo *et al*., 2010b, chapter 5).

Table 6. 1 Effect of dietary condensed tannin sources (control, grape seed extract-GSE and *C. ladanifer*) and oil supplementation (0% and 6% of added oil in dry matter) on fatty acid concentration (mg/g fresh muscle) and composition (g/100g total fatty acids) of *longissimus dorsi* muscle from Merino Branco lambs.

	Control		GSE		*C. ladanifer*		SEM	*P* values[7]		
	0%[1]	6%[2]	0%[3]	6%[4]	0%[5]	6%[6]		CT	O	CT×O
Total FA	20.5[a]	20.2[a]	20.2[a]	21.8[a]	20.3[a]	30.7[b]	1.82	0.018	0.015	0.017
14:0	2.96	2.69	3.25	2.63	3.54	3.02	0.183	0.064	0.012	0.793
16:0	22.4	18.2	23.3	18.7	24.1	20.4	0.77	0.056	<0.001	0.828
16:1 *cis*-9	1.11	0.76	1.16	0.77	1.30	0.90	0.035	<0.001	<0.001	0.806
18:0	15.5	15.3	14.4	14.9	14.2	13.5	0.54	0.026	0.761	0.496
18:1 *trans*-11	1.43[a]	3.35[b]	1.55[a]	3.42[b]	1.55[a]	6.24[c]	0.383	<0.001	<0.001	0.001
18:1 *cis*-9[8]	32.3	24.8	30.3	23.5	29.6	25.1	0.03	0.084	<0.001	0.116
18:2*n*-6[8]	5.49[a]	8.68[c]	6.25[ab]	8.32[c]	6.72[b]	6.81[b]	0.065	0.595	<0.001	0.007
CLA[9]	0.68[a]	1.24[b]	0.76[a]	1.23[b]	0.69[a]	1.73[c]	0.114	0.081	<0.001	0.046
18:3*n*-3	1.34	3.52	1.33	3.50	1.18	3.17	0.188	0.344	<0.001	0.858
20:4*n*-6	1.45[b]	1.28[b]	1.77[c]	1.32[b]	1.76[c]	0.98[a]	0.097	0.137	<0.001	0.015
20:5*n*-3	0.47	0.62	0.56	0.72	0.49	0.49	0.047	0.013	0.012	0.165
22:5*n*-3	0.74	0.73	0.83	0.78	0.72	0.57	0.056	0.028	0.137	0.464
22:6*n*-3	0.17[b]	0.20[c]	0.21[c]	0.20[c]	0.17[b]	0.13[a]	0.009	<0.001	0.486	0.003
SFA	43.1	38.2	43.3	38.4	44.2	38.9	1.14	0.694	<0.001	0.979
PUFA	12.0[a]	19.6[c]	13.7[a]	20.0[c]	13.6[a]	17.1[b]	0.79	0.188	<0.001	0.043
n-6 PUFA[8, 10]	7.27[a]	10.3[c]	8.43[ab]	9.90[c]	8.89[bc]	8.00[ab]	0.065	0.442	0.017	0.007
n-6 LC-PUFA[11]	1.78[b]	1.58[a]	2.25[c]	1.62[ab]	2.20[c]	1.21[a]	0.134	0.122	<0.001	0.024
n-3 PUFA[12]	2.75	5.14	2.97	5.26	2.61	4.42	0.273	0.097	<0.001	0.546
n-3 LC-PUFA[13]	1.41	1.62	1.65	1.76	1.42	1.25	0.109	0.009	0.572	0.202

SFA - sum of saturated fatty acids; PUFA - sum of polyunsaturated fatty acids; [1] Diet C, basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; [2] Diet CO, basal diet with 6% DM of oil blend (sunflower and linseed oils, 1:2 v/v); [3] Diet GS, basal diet with 2.5% DM of grape seed extract; [4] Diet GSO, basal diet with 2.5% DM of grape seed extract and 6% DM of oil blend (sunflower and linseed oils, 1:2 v/v); [5] Diet CL, basal diet with 25% DM of *Cistus ladanifer*; [6] Diet CLO, basal diet with 25% DM of *Cistus ladanifer* and 6% DM of oil blend (sunflower and linseed oils, 1:2 v/v); [7] CT, condensed tannin sources inclusion in diets; O, oil supplementation; [8] variables submitted to Box-Cox transformation; means presented are back-transformed values but SEM is expressed in transformed scale; λ for 18:1*cis*-9, 18:2*n*-6 and *n* – 6 PUFA is 0.01; [9] Include 18:2 *cis*-9, *trans*-11, 18:2 *trans*-8, *cis*-10 and 18:2 *trans*-7, *cis*-9 isomers; [10] *n*-6 PUFA = (18:2*n*-6 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6); [11] *n*-6 LC-PUFA = (20:2*n*-6 + 20:3*n*-6 + 20:4*n*- 6 + 22:4*n*- 6); [12] *n*-3 PUFA = (18:3*n*-3 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3);[13] *n*-3 LC-PUFA = (20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3).

6.3.1. Meat lipid oxidation

As intended, meat from oil supplemented lambs had higher PUFA concentration than meat from oil unsupplemented lambs and, thus, can be expected to be more prone to lipid oxidation (Morrissey *et al*., 1998). It is well established that lipid oxidation decreases meat quality and acceptability by the consumers because it leads to colour deterioration and development of off-odours and off-flavours (Morrissey *et al*., 1998). Therefore, the susceptibility of PUFA to rapid oxidation might limit the nutritional strategies which aim at increasing PUFA concentration in meat. In present trial the lipid oxidation was measured after chemical oxidation by ferrous iron and hydrogen peroxide, allowing to determine the meat resistance against lipid oxidation in pro-oxidative conditions (Mercier *et al*., 2004).

As expected, lipid oxidation increased ($P < 0.001$) with storage time and dietary lipid supplementation (**Table 6.2**), reflecting both the reduction of meat capacity for resist against lipid oxidation during storage and the tendency of PUFA to oxidize. An interaction between oil supplementation and storage time ($P = 0.056$) was observed for lipid oxidation, in which at day 3 of storage the meat from oil supplemented lambs showed similar TBARS values to that from lambs fed diets with no oil stored after 7 days (**Figure 6.1**).

Table 6. 2 Effect of storage time, oil supplementation and inclusion of a condensed tannin sources on lipid oxidation (mg MDA/kg muscle) and surface colour parameters in raw *longissimus dorsi* muscle from Merino Branco lambs

	Lipid oxidation	L^*[1]	a^*[2]	b^*[3]	H^*[4]	C^*[5]	ΔE[6]
Effect Storage Time (T)							
0 days	2.01[a]	38.2[a]	14.5[b]	4.59[a]	17.4[a]	15.2[a]	
3 days	5.72[b]	39.9[b]	14.6[b]	8.68[b]	31.0[b]	17.0[c]	5.43
7 days	8.44[c]	39.7[b]	13.3[a]	9.06[c]	34.4[c]	16.1[b]	5.92
SEM	0.216	0.471	0.276	0.146	0.437	0.287	0.239
P values	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	0.003
Effect of oil supplementation (O)							
0%	4.45	39.4	14.5	7.36	27.1	16.5	6.09
6%[7]	6.29	39.1	13.7	7.52	28.1	15.7	5.25
SEM	0.225	0.597	0.289	0.156	0.421	0.299	0.321
P values	<0.001	0.750	0.052	0.470	0.101	0.070	0.070
Effect of dietary condensed tannin sources (CT)							
Control	6.00[b]	39.7	14.6	7.70	27.6	16.5	5.64
Grape seed extract	5.07[a]	39.0	13.7	7.24	27.7	16.7	5.89
Cistus ladanifer	5.10[a]	39.1	14.1	7.38	27.4	16.1	5.49
SEM	0.281	0.731	0.354	0.191	0.515	0.367	0.393
P values	0.036	0.802	0.214	0.223	0.932	0.202	0.765
Interactions			*P* values				
CT×O	0.461	0.928	0.736	0.749	0.796	0.675	0.222
O×T	0.056	0.154	0.401	0.561	0.729	0.445	0.272
CT×T	0.078	0.132	0.289	0.102	0.040	0.229	0.090
CT×O×T	0.476	0.202	0.385	0.744	0.915	0.441	0.857

[1] Lightness; [2] Redness; [3] Yellowness; [4] Hue angle; [5] Chroma; [6] Colour variation between each day of storage and the day 0; [7] Oil blend composed of sunflower and linseed oils in a 1:2 (v/v) proportion.

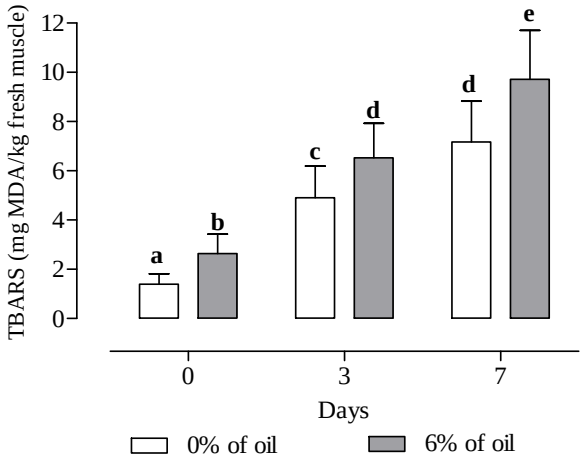

Figure 6. 1 Effect of oil supplementation and time of storage on TBARS values determined after oxidation induction in *longissimus dorsi* muscle.

Values are means, with SD represented by vertical bars. Values with different superscripts are significantly different ($P < 0.10$).

The inclusion of a CT sources in lamb's diets improved ($P < 0.036$) the meat resistance against induced lipid oxidation (**Table 6.2**). However, this protective effect only occurred after 3 days of storage ($P = 0.078$, **Figure 6.2**).

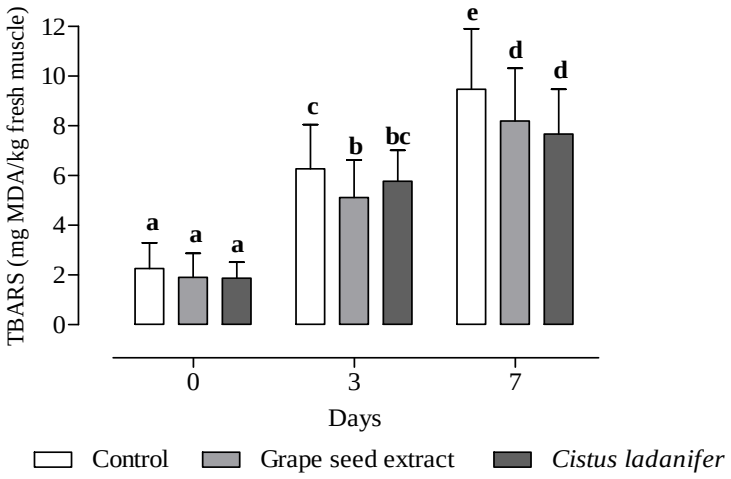

Figure 6. 2 Effect of inclusion the grape seed extract and *Cistus ladanifer* in diets and time of storage on TBARS values determined after oxidation induction in *longissimus dorsi* muscle.

Values are means, with SD represented by vertical bars. Values with different superscripts are significantly different ($P < 0.10$).

At day 3 of storage, the meat from lambs fed grape seed diets showed lower TBARS values than that from lambs fed control diets. Feeding *C. ladanifer* diets resulted in intermediate TBARS values between grape seed and control diets. Nevertheless, at day 7 of storage the grape seed extract and *C. ladanifer* were equally effective in protection against lipid oxidation. The antioxidant effect of dietary CT have been shown in poultry (Du *et al.*, 2002, Brenes *et al.*, 2008, Smet *et al.*, 2008) and rodents (Gladine *et al.*, 2007a, Gladine *et al.*, 2007b). However, information on potential antioxidant effect of dietary CT in ruminants seems to be restricted to a few studies. Gladine *et al*. (2007c) reported that inclusion of 10% DM of grape seed and peel extract directly into the rumen of sheep improved the antioxidant status and reduced the susceptibility to lipid oxidation of plasma measured by an oxidation induced assay. Luciano *et al*. (2009a) reported that inclusion of 8.9% DM of quebracho in the diet did not improve the lipid oxidative stability in lamb meat, measured as TBARS without oxidative induction in meat. Differences observed between methods using or not oxidative induction might be explained by the fact that although dietary CT increase the meat antioxidant capacity, this might be evident only when oxidative pressure exceeds the antioxidant capacity of control meats which might not be attained in normal meat storage conditions.

Despite the several studies that show that dietary CT sources have beneficial effects on oxidative stability of meat, their mechanisms of action remain to be established. The direct antioxidant activity of dietary CT would imply their absorption through the gastrointestinal tract and their transfer in tissues (Luciano *et al*., 2009a). However, the polymeric nature and high molecular weight of CT should limit their absorption and it is unlikely that oligomers larger than trimers could be absorbed in the small intestine in their native form (Manach *et al.*, 2004). Hydrolysis of CT polymers into compounds with low degree of polymerization or monomers would allow their absorption. Gladine *et al*.

(2007c) reported the presence of five different phenolic compounds in plasma, including epicatechin and unknown phenolic compounds in sheep that received grape seed and peel extract directly in the rumen. Considering the minor proportion of monomeric compounds presents in grape extract, these authors suggested that high level of epicatechin in sheep plasma resulted from the biodegradation of polymeric CT by rumen microorganisms. In fact, Terrill *et al*. (1994) showed that polymeric CT are poorly recovered after their transit though the rumen. However, Makkar *et al*. (1995) demonstrated that rumen microorganisms do not hydrolyse CT. Conversely, studies in rats and humans indicated that CT are not inert within the gut, but undergo structural modifications operated by the intestinal microflora (Déprez *et al.*, 2000, Abia and Fry, 2001). Nevertheless, the effect of dietary CT on meat oxidative stability may be indirect, through the interaction between CT with other antioxidants compounds or with pro-oxidants compounds present in meat. Gladine *et al*. (2007b) reported that rosemary and grape extract inclusion in rat diets increased significantly the vitamin E content in liver. Previous works showed that plant extracts, such as *Gymnema montanum*, grape and marigold extracts, increased the activity of antioxidant enzymes in kidney and liver of rats (Ananthan *et al.*, 2004, Gladine *et al.*, 2007b). However, in the present trial, nor vitamin E content neither, enzyme with antioxidant activity were determined.

In the present experiment we used the extract of grape seed that is composed mainly by proanthocyanidins (95%) and leaves and soft stems of *C. ladanifer* that contained secondary compounds, including several flavonoids other than proanthocyanidins and terpenoids (Gomes *et al.*, 2005, Sosa *et al.*, 2005). Several flavonoid and terpenoid compounds have been shown to have antioxidant properties (Harborne and Williams, 2000, Matkowski, 2008). Thus, the flavonoid and terpenoid compounds present in *C. ladanifer* also may also be responsible for the higher antioxidant capacity in the meat of

the lambs fed *C. ladanifer*. Further studies should be conducted to identify the compounds that contribute to *C. ladanifer* antioxidant activity.

6.3.2. Meat colour

Dietary oil supplementation and inclusion of a CT sources in diets did not affect the meat colour coordinates (*L**, *a** and *b**) but, as expected these parameters were affected by storage time (**Table 6.2**). Lightness (*L**) values increased during firsts 3 days of storage, but between day 3 and 7 of storage remained unchanged ($P < 0.001$). The yellowness (*b**) values increased over the 7 days of storage ($P < 0.001$). The redness (*a**) value is strongly correlate with myoglobin concentration (Pérez-Alvarez and Ferández-López, 2009) and its decrease has been used extensively as index the myoglobin oxidation. In the present trial, the redness (*a**) values were stable during firsts 3 days of storage, however decreased between day 3 and 7 of storage ($P = 0.003$), suggesting the occurrence of myoglobin oxidation after 3 days of storage. Meat from lambs fed oil tended to display lower *a** values ($P = 0.052$) than meat from oil unsupplemented lambs, suggesting a higher myoglobin oxidation in PUFA enriched meats. The changes in meat colour coordinates values during storage observed in present trial might affect negatively the sensory appreciation of meat by consumers (Insausti *et al.*, 2008).

The ΔE parameter is calculated by the combination of *L**, *a**, and *b** coordinates measured during different days of storage. Therefore, ΔE measures the overall variation of meat colour during storage (Mancini and Hunt, 2005). The ΔE values were 5.43 at day 3 and 5.92 at day 7 of storage, showing that meat colour variation occurred mainly between day 0 and 3 of storage. Nevertheless, the ΔE was higher at day 7 than at day 3 ($P = 0.003$). An increase of ΔE values is associated to meat discoloration. Abril *et al*. (2001)

reported that differences in ΔE values < 0.9 are not appreciable by visual assessment and therefore the overall change in colour, calculated as ΔE, occurred between day 3 and 7 of storage might not be perceived by consumers.

The intensity of the red colour (chroma, *C**) increased during firsts 3 days of storage, and decreased between day 3 and 7 of storage, indicating that at day 3 of storage the meat showed a more vivid colour (greater *C** values) than meat stored 0 and 7 days.

Hue angle (*H**) allows more realistic perspective on meat browning than single colour coordinates (Luciano *et al.*, 2009b). Independently from dietary treatment, *H** values increased (ranged from red to yellow) over the 7 days of storage, resulting from the decrease in *a** and the increase of *b**. We found an interaction between inclusion of a CT sources in diets and storage time ($P = 0.040$), which at 0 day (1 h of blooming) the *H** value was lower in meat of lambs fed grape seed extract diets than meat of lambs fed control diets, while meat of lambs fed *C. ladanifer* diets showed intermediate values (**Figure 6.3**), suggesting that dietary CT, especially grape seed extract has interfered with blooming development. However, after 3 and 7 days of storage this effect of the dietary CT sources on *H** values was not observed, which did not differ between dietary treatments. This response is contrasting with results reported by Luciano *et al*. (2009a), who found lower *H** values in minced meat from lambs fed diet supplemented with quebracho tannins after 7 and 11 days of storage as compared to meat from lambs fed diet without tannin supplementation.

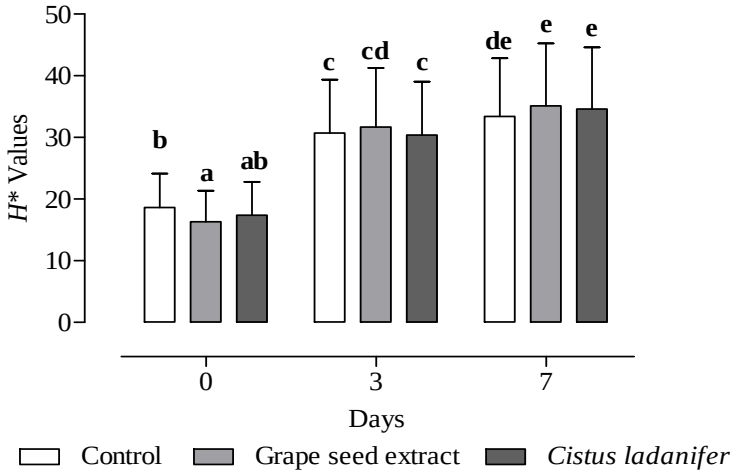

Figure 6. 3 Effect of inclusion the grape seed extract and *Cistus ladanifer* in diets on Hue angle (*H**) of *longissimus dorsi* muscle during storage time.

Values are means, with SD represented by vertical bars. Values with different superscripts are significantly different ($P < 0.10$).

It is widely accepted that lipid and myoglobin oxidation in meat are associated and, generally, both processes increase concurrently (Luciano *et al.*, 2009b). Previous works showed that colour and lipid stability of meat was not affected by PUFA enrichment in meat (Vastasever *et al.*, 2000, Ponnampalam *et al.*, 2001, Daly *et al.*, 2007) or by dietary CT sources (O'Gardy *et al.*, 2008). In contrast, Luciano *et al.* (2009a) showed that the inclusion of quebracho tannins in sheep diets improved meat colour stability during refrigerated storage, but did not affect the lipid stability. In the present study, the absence of effect of the dietary treatments on meat colour stability may suggest that in normal conditions (without induction of the oxidation) and in period of storage used in experiment (7 days) the oxidative pressure was not enough to causing the negative effects of oil supplementation and positive effects of inclusion of CT sources in diets on meat oxidative stability.

6.3.3. Sensory analysis

In a companion paper (Vasta *et al.*, 2010a), it was showed that *C. ladanifer* inclusion in lamb diets strongly affected meat volatile compounds profile, while grape seed extract inclusion had only minor effect on this profile. However, the consumers panel did not detect the effect of the grape seed extract and *C. ladanifer* L inclusion in diets on meat sensory proprieties (**Table 6.3**).

Table 6. 3 Results of sensory analysis by a consumer panel.

	Number of Panelists	D
C *vs*. GS	30	11(ns)
CO *vs*. GSO	30	13 (ns)
C *vs*. CL	30	13 (ns)
CO *vs*. CLO	30	14 (ns)

D, number of panellists that were able to distinguish the meats; ns, not significant effect ($P < 0.05$); Diet C, basal diet composed of 900 g dehydrated lucerne/kg DM and 100 g wheat bran/kg DM; Diet CO, basal diet with 6% DM of oil blend (sunflower and linseed oils, 1:2 v/v); Diet GS, basal diet with 2.5% DM of grape seed extract; Diet GSO, basal diet with 2.5% DM of grape seed extract and 6% DM of oil blend (sunflower and linseed oils, 1:2 v/v); Diet CL, basal diet with 25% DM of *Cistus ladanifer*; Diet CLO, basal diet with 25% DM of *Cistus ladanifer* and 6% DM of oil blend (sunflower and linseed oils, 1:2 v/v).

This result is in agreement with Priolo *et al.* (1998), who reported that trained panelist were unable to distinguish between meats from lambs fed diet with carob pulp (rich in CT) from meat of lambs fed control diet. In contrasts with these results, Priolo *et al*. (2009) and Schreurs *et al*. (2007) reported that lamb meat flavour and odour is affected by CT, when supplementing the diets with 10% of quebracho or supplied 33g/d of grape seed extract as liquid supplement, respectively. In these works, the dietary CT reduced the typical sheep meat odour and flavour. Our results showed that, at the levels of inclusion used, grape seed extract and *C. ladanifer* may be successfully used as supplements in lambs' diets without compromising the characteristic lamb meat sensory properties.

6.4 CONCLUSION

Dietary oil supplementation resulted in a beneficial effect on the FA composition of lamb meat but reduced the meat antioxidant potential. The increase of the lipid oxidation with oil supplementation was not reflected in meat colour stability during storage. Dietary grape seed extract and *C. ladanifer* did not improve the meat colour stability but protected the meat lipids against oxidation in lambs fed both diets with or with no oil. The sensory properties of lamb meat were not affected by the inclusion of grape seed extract and *C. ladanifer* in diets. Thus, the use of these CT sources in order to reduce the meat deterioration induced by lipid oxidation in ruminant meat enriched in PUFA, might be a good approach. Finally, further studies should be undertaken in order to elucidate the underlying mechanisms responsible for the oxidative stability of meat by CT.

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CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In recent years, consumer concern with health has leaded to an increasing interest on nutritional value of foods, especially in its FA composition. Consumption of ruminant products, by its FA composition has been associated with negative effects on human health. To meet the nutritional recommendations there is a large interest to improve the FA composition of ruminant fat. Thus, lipid metabolism in ruminants and factors influencing the FA composition of their products, particularly nutritional factors has been extensively studied. Dietary manipulation has shown to be effective means of improving the FA composition of ruminant meat, increasing its content in PUFA, such as *n*-3 PUFA and CLA and decreasing SFA (Scollan *et al.*, 2006, Sinclair, 2007). The studies reported in this thesis aimed to explore some nutritional approach to improve the nutritional value of lipid fraction from lamb meat.

In accordance with previous results obtained by our team (Bessa *et al.*, 2007), blend of sunflower oil and linseed oil showed to be a good approach to obtain simultaneously lamb meat enriched in CLA and *n*-3 LC-PUFA (chapter 2). Highest value of 18:2 *cis*-9, *trans*-11 was observed in meat of lambs fed diets with only sunflower oil, decreasing linearly by replacement of sunflower oil with linseed oil, while *n*-3 LC-PUFA increased only until 66% of oils replacement. The maximum concentration of both CLA and *n*-3 LC-PUFA in muscle (sum CLA and *n*-3 LC-PUFA) were estimated to be reached at 52% of replacement of sunflower oil with linseed oil. However, comparing the four experimental diets used in this trial the highest proportion of CLA and *n*-3 LC-PUFA (quadratic response, $P = 0.025$) was observed in lambs fed diets with blend of sunflower oil and linseed oil in proportion of 1:2 (v/v) (*i.e.* 66% of oils replacement). Facing this result, the blend of sunflower oil and linseed oil in proportion of 1:2 (v/v) was used in subsequent studies (chapter 4 to 6); showing to be an effective means to improving the nutritional value of lamb meat, increasing its content in PUFA, CLA and *n*-3 LC-PUFA.

However, supplementation of diets with this oil blend result in a small improvement in muscle *n*-3 LC-PUFA content, being generally limited to increase the EPA. The limited synthesis of *n*-3 LC-PUFA from dietary 18:3*n*-3 observed in this nutritional conditions can be explained either by an inhibitory effect of *n*-6 PUFA and *n*-3 PUFA on Δ6- Δ5-desatutase expression (Cho *et al.*, 1999a, Cho *et al.*, 1999b) or by competition between 18:2*n*-6 and 18:3*n*-3 for desaturation and elongation enzymes (Brenner, 1989). Moreover, increase of *n*-3 LC-PUFA in muscle lipids only until 66% of replacement of sunflower oil with linseed oil, and linear decrease the *n*-6 LC-PUFA with oils replacement observed in first study (chapter 2) suggests that 18:3*n*-3 might be more powerful in the down-regulation of the desaturases and elongases enzymes involved in the conversion to LC-PUFA, than 18:2*n*-6. The comparison of the effects of 18:3*n*-3 and 18:2*n*-6 on gene and protein expression, as well as activity of these enzymes has been little explored. Only recently, was reported that supplementation of cattle diet with 18:3*n*-3 (linseed oil) decreases the Δ6-desaturase protein abundance in cattle muscle (Herdmann *et al.*, 2010) when compared to control diet with higher proportion of 18:2*n*-6. This inhibition by dietary 18:3*n*-3 may contribute to lower synthesis of *n*-3 LC-PUFA, limiting thus the nutritional strategies to enrichment of *n*-3 LC-PUFA by 18:3*n*-3 dietary supply. Thus, it is need to known better the effect of dietary PUFA on these enzymes in ruminants, in order to increase the endogenous syntheses of health benefit FA, as well to optimize the levels of PUFA incorporation in diets. So, it would be important test *in vitro*, as preliminary study, the effect of graded increase of the 18:2*n*-6 and the 18:3*n*-3 on desaturases and elongases enzymes involved in the conversion to LC-PUFA (RNAm abundance, protein expression and activity) and FA profile. This could help to determine the levels of 18:2*n*-6 and 18:3*n*-3 from which the LC-PUFA synthesis is inhibited. Comparison between

effect of 18:2*n*-6 and 18:3*n*-3 on enzymes also could clarified if 18:3*n*-3 is more powerful in the down-regulation of enzymes involved in the conversion to LC-PUFA than 18:2*n*-6.

Additionally, the effect of dietary replacement of sunflower oil with linseed oil on FA composition of PL reported in chapter 2 suggest that there is a homeoviscous adaptation mechanisms that control the FA incorporation in membrane phospholipids. The dietary replacement of sunflower oil with linseed oil led to a substitution of 18:2*n*-6 (2 double bonds) by a pondered mixture of 18:3*n*-3 (3 double bonds) and 18:1 *cis*-9 (1 double bond) in polar lipids maintained fairly constant the degree of instauration of C_{18} FA in membrane polar lipids, probably in order to maintain proper membrane fluidity and function (Wahle, 1983, Spector and Yorek, 1985). As, PUFA are mainly deposited in PL, this metabolic regulatory mechanisms may constitute a limitation to dietary strategies designed for enrichment of ruminant meat in PUFA. Would be important to known the ceiling of PUFA incorporation in polar lipids.

Dietary replacement of sunflower oil with linseed oil changed the pattern of BI in intramuscular PL and NL fractions (chapter 3), inducing to strong diversification of BI, with decrease in relative proportion of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 and an increase in several BI related with 18:3*n*-3 BH pathway, such as 18:2 *trans*-11, *trans*-15 that was first time reported in lamb meat in this work (chapter 3, 4 and 5). Inclusion of oil blend in diets increased the most BI in abomasal digesta (chapter 5), and its deposition in muscle (chapter 4 and 5), including benefic 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 and several others TFA. It is proposed that deleterious effects of TFA are due to their incorporation in PL, competing with essential FA to desaturation and elgongation metabolic pathways (Wahle and James, 1993, Chardigny *et al.*, 2007). Thus, the distribution of C_{18} FA, with special attention on BI, between intramuscular PL and NL fractions, as well the effect of dietary replacement of sunflower oil with linseed oil on FA

distribution was evaluated (chapter 4). Selectivity index (SI), that was proposed for the fist time in this work to evaluate the selective deposition of C_{18} FA between lipid fractions, indicate that C_{18} FA are differentially incorporated in PL and NL. As expected the SI for 18:2*n*-6, 18:3*n*-3 and 18:1 *cis*-9 showed that both PUFA are preferentially incorporate in PL and 18:1 *cis*-9 in NL. Moreover, results also suggest that BI are differentially deposited between intramuscular lipid fractions, being *trans* C_{18} FA, including CLA preferentially deposited in NL and some *cis* isomers preferentially incorporate in PL. The preferentially deposition of *trans* BI in NL suggest that its potential for competitive interactions with elongation and desaturation metabolic pathways of essential FA might be low. However, this trial was initially not designed to evaluate the C_{18} FA deposition in PL and NL and further studies, possibly *in vitro* should be conducted to confirm the selective deposition of BI between PL and NL fractions reported here.

The TFA increase in meat with inclusion of vegetable oil rich in PUFA in diets may limit the strategies to improve the nutritional value of ruminant meat by supplementation of diets with these oils. Thus, research is need to increase the 18:1 *trans*-11 and CLA without major increase in other *trans* BI. In this context, the ability of sodium betonite and two condensed tannin sources (grape seed extract and leaves and soft stems of *Cistus ladanifer* L.) to modulate the ruminal BH in lambs fed control (with no oil) or oil blend supplemented diets was tested in present thesis (chapter 4 and 5). The effect of sodium bentonite inclusion in diets on lamb meat FA composition was limited to some BI (chapter 4). However, these effects are very promising; because sodium bentonite increased the 18:1 *trans*-11 and prevented the increase of 18:1 *trans*-10 in meat from lambs fed oil supplemented diets, suggesting that sodium bentonite limits the shift of ruminal BH pathways that favours the production 18:1*trans*-10 instead 18:1 *trans*-11

typically observed in high concentrate diets. Thus, sodium bentonite may be an effective approach to optimize the response of vegetable oil supplementation in concentrated based diets. Other levels of sodium bentonite inclusion in diets, as well the effect of sodium bentonite inclusion in concentrate based diets on ruminal BH and FA composition of ruminant meat would be interesting explore.

The two dietary condensed tannin sources tested, had a distinct effect on ruminal BH and FA composition of lamb meat, although the CT supply had been similar (Chapter 5). Grape seed extract inclusion of both diets with or with no oil had a minor effect on FA profile of abomasal digesta and muscle lipid fractions. *Cistus ladanifer* also no had major effects on BH pattern in lambs fed diets with no oil, but show to be able to inhibits the last step of BH in oil supplemented lambs, improving nutritional value of their meat. Inclusion of *Cistus ladaniner* in oil supplemented diets induced to accumulation of 18:1 *trans*-11 and depression of 18:0 in abomasal digesta and higher 18:1 *trans*-11 availability in tissues for endogenous synthesis resulted in higher deposition of 18:2 *cis*-9, *trans*-11 in muscle than other oil supplemented diets. This result suggests that inclusion of *Cistus ladanifer* in oil supplemented diets induced to changes in ruminal ecology, reducing the stearate producers' bacteria. *Cistus ladanifer* is composed by several secondary compounds in addition the condensed tannins, and is not clear that the effect of *Cistus ladanifer* in ruminal BH is caused by condensed tannins. Thus, in order to clarify this question further study using *Cistus ladanifer* extracts must be conducted.

Both sodium bentonite and *Cistus ladanifer* seem to change the ruminal population. However, the effect of sodium bentonite and *Cistus ladanifer* on microorganism involved in BH is not known and further work should be conducted to evaluate this. It would be important determine the eventual changes in rumen microbial communities from animals fed diets with sodium bentonite and *Cistus ladanifer* that could be related to changes in

BH. Moreover, also is important explored why sodium bentonite and *Cistus ladanifer* only changes the BH pattern in oil supplemented diets. So, the effect of the inclusion of sodium bentonite and *Cistus ladanifer* in oil supplemented diets on rumen microbial population also should be evaluated.

Globally, data reported here showed that oil blend supplementation; as well as inclusion of sodium bentonite and *Cistus ladanifer* in oil supplemented diets may be successfully used to improve the lamb meat nutritional value without compromising the animal performance. Oil blend supplementation increased the deposition of KKCF in carcass of lambs, but the effect on other carcass traits and tissue composition chumps and shoulders was inconsistent between studies (chapter 4 and 5). In contrast to sodium bentonite and grape seed extract, inclusion of *Cistus ladanifer* in diets affects the carcass and dissected cuts composition, increasing the its content in fat.

Although lamb meat enrichment in PUFA is considered benefit to human health, high content of PUFA in meat reduces its antioxidant potential (chapter 6), limiting the strategies to improve the nutritional value of meat. Results reported in chapter 6 showed that dietary grape seed extract and *Cistus ladanifer* are effective to improve the lamb meat resistance against lipid oxidation, suggesting that inclusion of these two condensed tannin sources in diets might be a good approach to prevent the lipid oxidation in meat, including meat enriched in PUFA. In addition, our results also showed that this approach not changes the characteristic lamb meat sensory properties. Nevertheless, in present work the lipid oxidation was measured after chemical oxidation, which allows evaluate the meat resistant against lipid oxidation in pro-oxidative conditions but not the real lipid oxidation. Thus, further work is required to evaluate the effect of these two dietary condensed tannin sources on meat lipid oxidative stability in real conditions. Additionally, the mechanisms responsible for increase the meat antioxidant capacity by

dietary condensed tannins sources is not known. The polymeric nature and high molecular weight of condensed tannins should limit their absorption, and the hydrolysis of polymeric condensed tannins in rumen, and absorption and deposition of their bioactive derivatives might be a possible explanation for higher oxidative stability observed in meat of lambs fed condensed tannin sources. Although some studies suggest that condensed tannins are hydrolysis in rumen its occurrence is not established. For elucidate if the condensed tannins are hydrolyse in rumen more studies are need. The occurrence of dietary condensed tannins and/or its derivatives in digesta, faeces, urine, plasma and in tissues, as muscle from ruminants fed condensed tannin sources also should be explored for elucidate the fate of the of dietary tannins in the organism. Some studies suggest that the effect of condensed tannins also might result the interaction between condensed tannins with other antioxidants compounds or with antioxidant enzymes present in meat. This possible interaction was not evaluated in present work, and further work should be conducted for determine the effect of grape seed extract and *Cistus ladanifer* inclusion in diets on activity of antioxidant enzymes and content of antioxidant compounds, as vitamin E.

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