UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

Influence of slaughter season, muscle type and gluconeogenesis precursors on intramuscular fat quality of ruminant meats

José Miguel Pestana Assunção

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TESE DE DOUTORAMENTO EM CIÊNCIAS VETERINÁRIAS

ESPECIALIDADE DE SEGURANÇA ALIMENTAR

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ABSTRACT

Influence of slaughter season, muscle type and gluconeogenesis precursors on intramuscular fat quality of ruminant meats

The consumption of animal fat has been associated with an increased incidence of chronic diseases, as cardiovascular disease, obesity and diabetes. Ruminant meats have high saturated fatty acids (SFA) content, potentially harmful to human health, but, on the other hand, are a good source of vaccenic acid (VA) and conjugated linoleic acid isomers, compounds related with human health benefits. The aim of this study was to determine the influence of slaughter season and muscle type on nutritional quality of intramuscular fat (IMF) in Mirandesa-PDO (Protected Designation of Origin) veal, Charneca-PDO beef and organic beef, assessing to the quality and status that may contribute to market differentiation. The results suggest that the IMF content of PDO veal, PDO beef and organic beef was slightly affected by the slaughter season but markedly changed by the muscle type. The meat analysed had a high nutritional value, with favourable ratios of *n*-6/*n*-3 and contents of *n*-3 PUFA and α-tocopherol.

Some studies indicate that increasing the stearoyl-CoA desaturase (SCD) expression and activity may significantly improve the nutritional quality of meat by decreasing the SFA content, while increasing rumenic acid content. This can be achieved by the administration of gluconeogenesis precursors, such as propylene glycol, which induces elevated propionate in the rumen and higher levels of blood insulin and glucose, resulting in the SCD activity elevation. Thus, the influence of dietary gluconeogenesis precursors (propylene glycol and calcium propionate mix; PP) and linseed oil on intramuscular fatty acid composition of lambs was assessed. Results suggest that gluconeogenesis precursors modify the biohydrogenation pathway and reduce the rumenic acid concentration. Linseed oil supplementation increased meat concentration of α-linolenic acid, as well as most of the C18 biohydrogenation intermediates, including VA and rumenic acids.

In conclusion, regarding the lipid composition of IMF, the studied PDO and organic meats have a high nutritional value, contributing to the consumer`s confidence on these products. The fatty acid profile of lamb's IMF was slightly affected by PP, without clear effects on insulinaemia and delta-9 desaturation, demanding future research on this subject.

Keywords: ruminant meats; fatty acids profile, lipid-soluble vitamins; nutritional value; propylene glycol; calcium propionate; linseed oil; biohydrogenation.

RESUMO

Influência da sazonalidade, do tipo de músculo e de precursores da neoglucogénese na qualidade da gordura intramuscular de carne de ruminantes

O aumento da incidência de doenças cardiovasculares, obesidade e diabetes, tem sido associado ao elevado consumo de gordura de origem animal. As carnes de ruminantes são ricas em ácidos gordos saturados potencialmente prejudiciais para a saúde mas, por outro lado, são também uma boa fonte de ácido vacénico e isómeros do ácido linoleico conjugado, compostos com propriedades benéficas para a saúde. Neste trabalho estudou-se o efeito da sazonalidade do abate e do tipo de músculo na qualidade da gordura intramuscular das carnes Mirandesa-DOP (Denominação de Origem Protegida) e Charneca-DOP, e da carne biológica, de modo a avaliar a qualidade e reputação alegada como fator diferenciador no mercado. Os resultados sugerem que a composição de ácidos gordos da gordura intramuscular das carnes analisadas foi influenciada ligeiramente pela sazonalidade do abate mas fortemente pelo tipo de músculo. Apesar desta variação, todas as carnes analisadas têm um elevado valor nutricional apresentando um índice favorável *n*-6/*n*-3 e teores elevados de PUFA *n*-3 e α-tocoferol. Alguns estudos indicam que o aumento da atividade da Δ-9 dessaturase pode melhorar a qualidade nutricional da carne, através da diminuição dos ácidos gordos saturados e do aumento do teor de ácido ruménico. Isto pode ser possível pela administração de precursores neoglucogénicos, tais como o propilenoglicol que induz o aumento do propionato no rúmen e aumenta os níveis sanguíneos de insulina e glucose, resultando no aumento da atividade da Δ-9 dessaturase. Assim foi avaliado o efeito da suplementação da dieta com propilenoglicol e propionato de cálcio, bem como óleo de linho, na composição lipídica da gordura intramuscular da carne de borrego. Os resultados sugerem que os precursores neoglucogénicos alteram a biohidrogenação ruminal e diminui o teor de ácido ruménico. A suplementação com óleo de linho aumenta o teor de ácido α-linolénico da carne, bem como a maioria dos C18 intermediários da biohidrogenação, incluindo os ácidos vacénico e ruménico. Em conclusão, a composição lipídica da gordura intramuscular das carnes de bovino DOP estudadas e da carne biológica apresenta um elevado valor nutricional, contribuindo para confiança do consumidor sobre estes produtos O perfil dos ácidos gordos da gordura intramuscular da carne de borrego é ligeiramente influenciado pelos precursores, sem efeitos claros na insulinemia e na Δ-9 dessaturase, exigindo-se estudos futuros sobre este assunto.

Palavras-chave: carne de ruminantes; perfil de ácidos gordos; vitaminas lipossolúveis; valor nutricional; propilenoglicol; propionato de cálcio; óleo de linho; biohidrogenação.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	Arachidonic acid, 20:4*n*-6
Ag^+-HPLC	Silver-ion high performance liquid chromatography
ALA	α-Linolenic acid, 18:3*n*-3
AOAC	Association of Official Analytical Chemists
BCFA	Branched chain fatty acids
BHT	Butylated hydroxytoluene
BI	Biohydrogenation intermediates
CIISA	Centro Interdisciplinar de Investigação em Sanidade Animal
CLA	Conjugated linoleic acid
CVD	Cardiovascular diseases
CoA	Coenzyme A
DAD	Diode array detector
DHA	Docosahexaenoic acid, 22:6*n*-3
DM	Dry matter
DPA	Docosapentaenoic acid, 22:5*n*-3
EC	European Commission
EEC	European Economic Community
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid, 20:5*n*-3
EU	European Union
FAO	Food and Agriculture Organization of United Nations
FAME	Fatty acid methyl esters
FID	Flame ionization detector
GC	Gas chromatography
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GIRM	Grupo de Investigação em Recursos Marinhos
GLM	General linear model
GPP	Gabinete de Planeamento e Políticas
HDL	High density lipoprotein
ID	Delta 9 desaturase index
IDRH	Instituto do Desenvolvimento Rural e Hidráulica
IM	Intramuscular
IMF	Intramuscular fat

INRB	Instituto Nacional de Recursos Biológicos
KKCF	Kidney and knob channel fat
LA	Linoleic acid, 18:2*n*-6
LC-PUFA	Long-chain polyunsaturated fatty acids
LDL	Low density lipoprotein
LL	L*ongissimus lomburum*
LSMEANS	Least square means
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
n	Number of experimental units
n.d	Not detected
NDF	Neutral detergent fibre
ns	Not significant
P	Probability
PDO	Protected Designation of Origin
PG	Propylene glycol
PGI	Protected Geographical Indication
PP	Propylene glycol and calcium propionate mix
PUFA	Polyunsaturated fatty acids
RDA	Recommended dietary allowance
SACN/COT	Scientific Advisory Committe on Nutrition/Committe on Toxicity
SAS	Statistical analysis system
SCD	Stearoyl-CoA desaturase
SF	Subcutaneous fat
SEM	Standard error of the mean
ST	S*emitendinosus*
TFA	*Trans* fatty acids
TSG	Traditional Specialties Guaranteed
UIPA	Unidade de Investigação em Produção Animal
UV	Ultraviolet
vs.	*Versus*
VA	Vaccenic acid, 18:1*t*11
VFA	Volatile fatty acids
WHC	Water holding capacity
WHO	World Health Organization

INTRODUCTION

The food that we consume today is scrutinized more now than it has ever been in the past in a whole variety of ways, such as: product composition, labelling concerns, health claims, product "naturalness", safety concerns and issues pertaining to the environment and sustainability (Troy & Kerry, 2010). Food fat plays an important role in the human diet. Fats are vital for a healthy body, provide energy, contribute to the absorption of fat-soluble vitamins and act as structural elements of cell membrane. On the other hand, a high fat intake has been associated with obesity, type 2 diabetes, cancer and cardiovascular disease (CVD). Animal fat contain a high proportion of saturated fatty acids (SFA) and is often the focus of attention when it comes to reducing the share of fat in the diet (Schmid, 2011). In recent years, fat and fatty acids have been considered as key nutrients that affect early growth and development, as well as, nutrition-related chronic diseases including CVD (Burlingame, Nishida, Uauy & Weisell, 2009). Particularly, meat is rich in proteins and poor in carbohydrates, and thus contributes to a low glycaemic index, which is assumed to have positive effects on human health (Biesalski & Nohr, 2009).

Fat content and fatty acid composition of meat are of major importance for consumers due to their importance for meat quality and nutritional value (Wood *et al.*, 2004). In fact, there has been an increased interest to understand the metabolism of dietary lipids and their role in human health and well-being (Wyness *et al.*, 2011). Human diets in industrialized countries are generally characterized by high levels of SFA, *n*-6 polyunsaturated fatty acids (PUFA) and *trans* fatty acids (TFA), and low levels of *n*-3 PUFA (Simopoulos, 2006). Recently, European Food Safety Authority (EFSA, 2010) has proposed that the reference intake for total dietary fat should range between 20-35% of energy and SFA and TFA intake should be as low as possible. Ruminant edible fats are characterized by high concentrations of SFA and TFA due to the extensive microbial biohydrogenation of dietary PUFA in the rumen.

Nutritionists recommend a higher intake of PUFA, especially *n*-3 PUFA at the expense of *n*-6 PUFA (British Department of Health, 1994). Recently, the results of Griffin (2008) reinforced the current recommendations of increasing α-linolenic acid (ALA, 18:3*n*-3) intake and decreasing linoleic acid (LA, 18:2*n*-6) intake in order to promote the endogenous synthesis of long chain (>C18) *n*-3 fatty acids. It is well known that the low PUFA/SFA and high *n*-6/*n*-3 ratios of some meats contribute to the imbalance in the fatty acid intake of today's consumers (Wood *et al.*, 2004).

Besides the beneficial effects of *n*-3 fatty acids on human health, conjugated linoleic acid (CLA) isomers have also attracted increased attention due to their health promoting biological properties (Aldai, Osoro, Barron & Najera, 2006). Ruminant fats are the richest natural dietary source of 18:2*cis*9,*trans*11 (*c*9,*t*11), which is the major conjugated linoleic acid (CLA) isomer, commonly known as rumenic acid (Kramer *et al.*, 1998). Finally, meat also provides from one-third to one half (Chizzolini, Zanardi, Dorigoni & Ghidini, 1999) of maximum recommended intake of cholesterol per day (300 mg) (American Heart Association, 2008).

European Union (EU) policies, with the objective of benefiting sustainable agriculture systems, have given meat obtained from regional cattle breeds the opportunity of being commercialised with a "certification of origin", that is known as Protected Designation of Origin (PDO). This is a consumer´s guarantee that meats produced in a specific, predetermined geographic region and animal breeds, according to defined rules approved by EU legislation, are expected to present unique quality and sensorial characteristics, especially associated with specific propriety of their lipid fraction. Despite its higher price, consumer demand for legally protected PDO bovine meat has increased in Portugal in the past few years (Costa *et al.*, 2011). Also, beside the contribution to landscape maintenance, as well as, nature preservation, pasture feeding system is desired by consumers.

Our group has characterized the influence of slaughter season and muscle type on the lipid composition and nutritional value of four Portuguese traditional meats: Carnalentejana-PDO beef (Alfaia *et al.*, 2006a), which is the most important commercial Portuguese-PDO beef, Mertolenga-PDO beef (Alfaia *et al.*, 2006b), the second most important commercial Portuguese-PDO beef, Barrosã-PDO veal (Alfaia *et al.*, 2007a), the second most important commercial Portuguese-PDO veal, and Arouquesa-PDO veal (Alfaia *et al.*, 2007b), the fourth most important commercial Portuguese-PDO veal. Despite this, detailed intramuscular fat (IMF) characterization of other important commercial Portuguese-PDO meats has not yet been published.

Seasonal differences in pasture nutritive value and availability have been reported to have an important influence on the IMF content and composition as well as underlined the importance of pre-weaning nutrition on the fatty acid composition including CLA isomers of meat (Santos, 2006; Aurousseau, Bauchart, Calichon, Micol & Priolo, 2004). It has been reported that fatty acid composition of intramuscular lipids fractions in other PDO meats were significantly affected by the slaughter season (Costa *et al.*, 2006; Alfaia *et al.*, 2006b).

It is well known that different muscles are composed by distinct proportions of fibre (oxidative or glycolytic), which may influence the IMF content (Hocquette *et al.*, 2010). Red muscle (type I) fibres, relative to white muscle (type II) fibres, have higher activity of oxidative enzymes and exhibit slower contraction. In contrast, white muscle fibres have tendency for glycolytic properties by consuming glucose instead of mitochondrial driven respiration, thus twitch fast (Lee *et al.*, 2010). Moreover, type I fibres contain a higher amount of lipids, some of which presumably serves as a source of aerobic metabolic fuel, and lower amounts of glycogen and glucose, than type IIB fibres (Klont, Brocks, & Eikelenboom, 1998).

Differences in the fatty acid composition of beef from animals reared predominantly on grain or forage diets have been widely reported (French *et al.*, 2000; Realini, Duckett, Brito, Rizza & De Mattos, 2004; Nuernberg *et al.*, 2005a; De la Fuente *et al.*, 2009). Meat from cattle produced under grass-based feeding systems is associated with more beneficial fatty acid profiles and lipid-soluble antioxidant vitamins contents (Daley, Abbott, Doyle, Nader & Larson, 2010).

Manipulation of animal diet composition appears to be a good strategy to enhance meat fatty acid composition by modifications on biohydrogenation. It is recognized that the oil supplementation affect ruminal fermentation, and biohydrogenation in particular, as has been widely studied (Bessa *et al.*, 2007; Jerónimo, Alves, Prates, Silva-Santos & Bessa, 2009). Other possibilities may be achieved by the administration of gluconeogenesis precursors such as propylene glycol (PG), calcium or sodium salts propionate, which induces elevated propionate in the rumen and higher levels of blood insulin and glucose (Christensen, Grummer, Rasmussen & Bertics, 1997). Daniel, Richards, Salter and Buttery (2004b) have shown that insulin increases the expression of stearoyl-CoA desaturase (SCD) and the synthesis of monounsaturated fatty acid (MUFA) from acetate. The up-regulation of the SCD activity through the administration of gluconeogenesis precursors can increase oleic and rumenic acids contents and improve the fatty acid profile in ruminant meats. However, in order to alter biohydrogenation and promote more health beneficial fatty acid outflow from the rumen, more research is needed.

Overall, this work is focused on the intramuscular lipids of ruminant meats, aiming to provide findings to explain the differences achieved on the different Portuguese production systems. Indeed, it is our goal to contribute to the knowledge on the research of bioactive lipids present

on ruminant meats aiming to clarify the added value of Portuguese traditional bovine meats. Additionally, the effect of gluconeogenesis precursors on the IMF composition in meat has been so far less investigated.

This thesis is structured in 6 chapters. The chapter 1 "Scientific background and objectives", introduces relevant concepts about lipid composition, nutritional quality and genetic and environmental factors that influenced IMF in meat. This chapter also approaches the propylene glycol and calcium propionate supplementation as a strategy to improve the nutritional value of ruminant fat. The objectives of this work are described at the end of this first section. The chapters 2 to 5 are based on scientific manuscripts, already published or accepted for publication to international peer reviewed journals. Like the manuscripts, each chapter is composed by an abstract, introduction, description of experimental procedures, results and discussion and conclusions.

The chapter 2 presents the influence of seasonal changes and muscle type on the nutritional value of IMF in Mirandesa-PDO veal. Chapter 3 describes the lipid composition and nutritional quality of IMF in Charneca-PDO beef. Chapter 4 reports the effects of slaughter season and muscle type on the fatty acid composition and nutritional value of IMF in organic beef. Chapter 5 explores effects of dietary gluconeogenesis precursors and linseed oil, and their interaction, on growth performance, rumen fermentation and intramuscular fatty acids of lambs.

Finally, chapter 6 intends to summarise and discuss in an integrated way the results obtained in each of the four previous chapters, the main conclusions and relevant perspectives for future research on this topic.

CHAPTER 1 | Scientific background and objectives

1.1 Production and consumption of ruminant meats

In the year of 2005, the total production of beef and veal in Portugal was about 118 000 carcass tons, which meant an increase of 25% compared to 1996 (Gabinete de Planeamento e Políticas, GPP, 2007). The dramatic decline in 1996 was due the bovine spongiform encephalopathy (BSE) and consequent consumer distrust. This crisis led to a significant drop in consumption and a reduction in demand as result of several measures taken to regulate the market, including early slaughter of calves and slaughter of >30 months old animals (GPP, 2007). In 2002, the cattle sector gradually came out of the animal health crises, but after 2005 the data available pointed out to a marked decline until today. Portugal is not self-sufficient in beef production (self-sufficiency ratio of 62% in 2005), with the EU being the major source of beef imports (Banovic, Grunert, Barreira & Fontes, 2009).

Recent figures on Portuguese beef consumption show an annual *per* capita consumption of approximately 18 kg, and 3 kg for sheep, values that are similar to the EU averages (Eurostat, 2011). Concerning public opinion, the red meat consumption has been associated with potential adverse health effects, as the increasing risk of CVD and colon cancer, together beef safety, stemming from the BSE outbreak, which might have resulted in a lack of consumer trust in red meat (Verbeke, Frewer, Scholderer & De Brabander, 2007). Fifteen years ago, Barreira and Duarte (1997) showed that prices and incomes have considerably determined beef consumption pattern in Portugal, highlighting the importance of convenience, a quality attribute, in this pattern. However, beef production with quality labels has increased in the last decade and accounts for approximately 3% of the total beef production in terms of slaughters approved for consumption (Banovic *et al.*, 2009). About beef labelling, Portuguese consumers perceive the region of production as a signal of enhanced quality, leading to better intrinsic attributes such as colour and fat, and consequently to higher expected beef eating quality (Banovic *et al.*, 2009). Banovic, Grunert, Barreira and Fontes (2010) suggested that consumers at the point of purchase preferred beef steaks that present information about the beef production (*e.g.* autochthonous breed, traditional methods, specific product characteristics). Concerning sensorial quality, the minimum amount of IMF to achieve acceptable consumer satisfaction is about 3% to 4% of fresh beef (Savell & Cross, 1986), and 5% of fresh sheep meat (Hopkins, Hegarty, Walker & Pethick, 2006).

1.1.1 PDO meats

The implementation of the European legislation (Council Regulation (EEC) nº 2081/92 of 14/7, which were repealed by PDO Council Regulation (EC) nº 510/2006 of 20/3) in order to protect and promote the edible products with PDO and Protected Geographical Indication (PGI) is an important contribution to the sustainability of the production chains. According to the Regulation (EC) nº 510/2006 of 20 March of 2006 designations of origin means the name of a region, a specific place or in exceptional cases, a country, used to describe an agricultural product or a foodstuff, which was originated in that region, specific place or country. These products present characteristics that are essentially, or exclusively, due to a particular geographical environment with its inherent natural and human factors, and the features of production, processing and preparation which take place in the defined geographical area. PDO products are regulated by the EU Regulation 2081/92, and pretend to be a way of valuing food products with a recognizable local identity. This legislation requires that producer's follow a "book of specifications" based on the argument of the authenticity protection of traditional products, as well as, the specificity of production and the use of traditional production methods.

Portugal is an important reservoir of genetic resources, being officially recognized 42 autochthonous breeds of which 15 are bovine breeds (Instituto do Desenvolvimento Rural e Hidráulica, IDRH, 2007). In Portugal, between 2006 and 2007, there were 13 bovine meats with protected names, 9 of which PDO (Arouquesa, Barrosã, Cachena da Peneda, Charneca, Marinhoa, Maronesa, Mertolenga Mirandesa, Carnalentejana), 3 PGI (Açores, Cruzado Lameiros do Barroso and Vitela de Lafões) and 1 Traditional Specialties Guaranteed (TSG) (Bovino Tradicional do Montado). These bovine PDO meats are distributed in limited regions of the north (Barrosã, Maronesa and Mirandesa), centre (Arouquesa, Marinhoa and Charneca) and south (Alentejana and Mertolenga) of Portugal. Between 2006 and 2007, corresponding approximately one year before of harvest in this study, the most important commercial Portuguese bovine PDO meats was Carnalentejana-PDO beef (average values of 60%, 1327.4 carcass tons), followed by Mirandesa-PDO veal (11.7%, 273.1 carcass tons), Barrosã-PDO veal (9.4%, 203.5 carcass tons), Maronesa-PDO beef (8.9%, 192.2 carcass tons) (IDRH, 2009). Relatively of Charneca-PDO beef represents 3.5 carcass tons (0.1%) of the production of meat PDO in 2005 (IDRH, 2007). In 1997, PDO beef production accounted for 1.3% of the total beef produced in Portugal and in 2005 this proportion increased to 2.7%, remaining in 2007 (IDRH, 2009).

In Europe, geographic origin labels, like PDO, PGI, and TSG, represent quality signals highly recognized by the consumer (Verbeke *et al.*, 2010). A well-functioning and reliable beef quality guarantee system, including eating quality parameters, can potentially meet current interests of European beef consumers (Verbeke *et al.*, 2010). Guaranteeing a consistent eating quality can not only increase consumers' satisfaction with beef products, but it can also lead to higher consumption rates and industry profitability, both highly desirable in the up-to-date scenario of high and global competition, financial and economic turmoil, and food price volatility (Verbeke *et al.*, 2010). However, for such a system to be successful, insights in consumer`s interest, scientific information need to be available to support the claimed quality and reputation.

Previous studies have shown that consumers are only moderately interested in beef traceability and origin as such (Verbeke & Ward, 2006; Verbeke & Roosen, 2009), whereas their interest in direct indications about beef healthiness and sensory quality might be considerably larger (Alfnes, Rickertsen & Ueland, 2008). In the last few years, traceability issues have grown in importance due to the consumers' increasing attention to food quality matters (Dalvit, De Marchi & Cassandro, 2007). In particular, traceability was defined by the European Regulation 178/2002 as ''the ability to trace and follow a food, feed, food producing animal or ingredients, through all stages of production and distribution''. Traceability of livestock products is an essential tool to safeguard public and animal health, and to valorise typical foods (Dalvit *et al*., 2007).

According to the PDO products specifications, in order to guarantee the product's authenticity and quality, consumer protection requirements facilitate the monitoring and complete traceability of both product and process. All the meat operations (processing and packaging) must take place within the specified geographical area, together with birth, rearing, fattening and slaughtering of the animals, and the cutting of the carcasses. Meanwhile, great efforts have been made to develop analytical tools to quantify specific compounds in the product or in the animal tissues that can act as tracers of the animal's feeding system and also give some insights into traceability of geographical origin (Luykx & van Ruth, 2008). The potential tracers in meat or animal tissues may come directly from the diet (direct markers), such as plant biomarkers (carotenoids, terpenes and phenolic compounds), or may be metabolic markers deriving from animal metabolism (indirect markers), like meat fatty acid composition.

Dias *et al.* (2008) used the fatty acid profile in order to differentiate the meat production sub-system (traditional and organic farming) of two Portuguese autochthonous bovine cattle breeds (Mirandesa and Barrosã). The authors concluded that the fatty acid composition, together with the PUFA/SFA and *n*-6/*n*-3 ratios, can be used as an effective tool to differentiate the breed and the production sub-system used. Alfaia *et al.* (2009) suggested that fatty acid composition is an effective parameter to discriminate ruminant feeding systems, including finishing periods on concentrate. According to Dannenberger *et al.* (2005), the differences found in conjugated linoleic acid (CLA) isomeric profiles may be explained by distinct grass intake, since it was shown that pasture feeding, compared to concentrate feeding, increases the proportion of the *t*11,*c*13, *t*11,*t*13 and *t*12,*t*14 CLA isomers, and decreases the percentage of the *t*7,*c*9 isomer, in beef lipids. Moreover, it is well known that the content of selected minerals and trace elements has been proposed to assure the geographic origin in food samples. Mono-elemental techniques have been successfully employed in food authentications, thus, stable-isotope ratio has been demonstrated to be good descriptor or different food products, providing unique and representative fingerprints that make it possible to differentiate between food samples of different PDO meats (Gonzalvez, Armenta & Guardia, 2009).

The preference of consumers for organic or traditional foods, as PDO meats, is increasing, since it is assumed that those products are healthier and their quality is above-average (Verbeke *et al.*, 2010). Following, it will be described the two PDO meats and the organic meat studied in the present work.

1.1.1.1 Mirandesa-PDO meat

Mirandesa cattle are a local meat breed from the Northeast region of Portugal, with an important role on the rural spaces maintenance, contributing to the fixation of the populations and the environment preservation (Galvão *et al.*, 2006). The geographic area covered by the protected denomination of origin of meat Mirandesa is limited to the parishes of council of Bragança, Macedo de Cavaleiros, Miranda do Douro, Mogadouro, Vimioso and Vinhais. This region is known as "Solar da Raça Mirandesa", as stated in the Commission Regulation nº. 1263/96 of 01/07 (EEC, for Mirandesa-PDO meat).

Mirandesa-PDO meat combines the specific genetic features of Mirandesa calves with the use of extensive natural resources in the north-eastern area of Portugal (Associação de Criadores

da Bovinos da Raça Mirandesa, ACBRM, 2007). The "Solar da Raça Mirandesa" possesses two different farm sizes, namely, farms with less than 8 cows, that have in average 14 hectare, and farms with more than 10 cows, which are the prevalent ones (ACBRM, 2007). Mirandesa cattle play an important role in these farms, where the animals are the only source of traction needed for land preparation and transportation of products. The births of the cows are distributed regularity throughout the year. During all year, cows remain outside for grazing while at night stay indoors (ACBRM, 2007). Calves are raised permanently indoors, nursed by their dams overnight (Galvão *et al.*, 2006). Cows graze natural pastures (herbaceous species) during spring and early summer and afterwards are fed with hays (of natural pasture or oat) and straws (oat, barley or wheat), complemented with local feeds like squash, potatoes, turnips, beetroots, regional maize fodder plant, rye forage and oat forage (Galvão *et al.*, 2006; Dias *et al.*, 2008).

Calves present at birth a mean live weight of 32-35 kg, remain with their mothers until weaning, usually at 7 months, and are slaughtered with a mean live weight of 132 kg (ACBRM, 2007). Commercially, Mirandesa PDO meat can be presented in two forms: 1) "carne de vitela" from animals (male or female) slaughtered between 5 and 9 months, and with an average weight carcass from 191 till 224 kg; and 2) "carne de novilho" from animals (male or female) slaughtered between 10 and 18 months, and with an average weight carcass from 298 till 380 kg (ACBRM, 2007).

1.1.1.2 Charneca-PDO meat

Charneca cattle are situated on a land parallel to the Tagus River, in the districts of Portalegre, Santarém, Setúbal, Évora, Beja and in Sado and Sorraia basins, typical areas of cork and holm oak (Associação de Criadores da Raça Preta, ACBRP, 2008). These pre-determined geographic regions, as well as the production system rules are well defined in the Council Regulation nº1495/02 of 22/08, the EEC for Charneca-PDO meat. These regions have Mediterranean climate, which is characterized by a hot and dry summer and a mild winter, with annual rainfall between 500 and 800 mm, mainly in autumn-winter (ACBRP, 2008). These climatic conditions contribute with annual variations of pasture production, as the growth period is very irregular. Since 15-35% of annual production is focused between the early rain (autumn) and February, the remaining 65-85% is obtained between March, when the grass is growing exponentially, and the end of growth season (late spring/early summer) (ACBRP, 2008).

During 4 and 6 months, when rainfall is negligible (summer), grass production is scarce, being dry and, consequently, with low nutritive value, low digestibility and low palatability. The breed is rustic and has a high capacity for survival in low feed availability. Generally, in winter/spring the animals feed with natural and/or improved pastures under holm and cork oak, which is usually referred to as "Montado", and pasture sown with seed mixtures based on oats and common vetch (ACBRP, 2008). During the summer or until there exist enough pasture; the animals feed stubble, hay and straws.

The production of Charneca young bulls is held on farms with average dimension of 50 and 100 cows. The reproduction season is calculated so that calves born in spring (period of grass abundance) (ACBRP, 2008). Calves are reared with their dams until weaning at 6-8 months of age. After weaning, calves that are not slaughtered going to rearing phase which has two distinct phases. In the first phase, between 9 and 18 months, the diet has more protein and fibre, feeding grazing pasture, cereals and dry forages (hay and straw).In the second phase, after 18 months, the diet is more energetic for finishing, animals are fed pasture, corn silage, grass hay and supplemented with concentrate feeds (ACBRP, 2008).

Commercially, Charneca PDO meat can be presented in two forms: 1) "carne de vitela" from animals (male or female) slaughtered between 6 and 12 months, and with an average weight carcass from 120 till 200 kg; and 2) "carne de novilho" from animals (male or female) slaughtered till 24-30 months, and with an average weight carcass from 200 till 400 kg (ACBRP, 2008).

1.1.2 Organic meats

In the last decade organic markets in the world have grown strongly (Cozzi *et al.*, 2010). The European market, which comprises more than 50% of the global revenues from organic products, has shown an estimated growth rate of 10-15% in the year 2005 and it is expected to be the fastest growing sector of the food industry in the next few years (Richter & Padel, 2007). In many European countries, organic animal derived foods, like drinking milk, dairy products and eggs, represent a significant segment of their total share. On the contrary, the market share of organic beef is still very low.

Price, product availability and quality are the three main reasons of the limited success of organic beef (Cozzi *et al.*, 2010). However, the provision of information about the benefits of organic farming on environment, animal welfare and health can increase consumer awareness and willingness to buy organic products (Napolitano *et al.*, 2010). In several European countries the average price of organic beef is 50% higher than the conventional product and it has often shown to exceed the consumer's willingness to pay (Nielsen & Thamsborg, 2005). In term of quantity, organic cattle represent a niche product since only 2% of the total European cattle population is raised according to organic systems (Eurostat, 2010).

1.1.2.1 Organic livestock production

Typically, organic meat production uses crossbred animals that are maintained according to a forage-based production system following the established guidelines for organic meat (Council Regulation no. 834/2007 of 28/07, EC). Based on the legislation, Napolitano *et al.* (2010) summarized the following criteria's for organic beef production: a) the cattle farming practices should ensure standards of animal welfare higher than those set by the current legislation by promoting grazing systems and the expression of species-specific natural behaviour; b) the use of pharmaceuticals should be markedly reduced; c) the use of genetic modified organisms (GMO) and chemicals for the production of animal feeds should be banned; and d) the stocking density per hectare should be low in order to reduce the impact of farming on the environment.

The guidelines determine that at least 95% of animal feed is forage or pasture (ingredients of agricultural origin are organic) and 5% is organic concentrates (Council Regulation no 834/2007 of 28/07, EC). Throughout the year, animals fed improved pasture (clover, alfalfa,

ryegrass tall fescue and others species), and native grasses. For the breeding purposes, the organic livestock shall be born and raised on organic holdings, while non-organically raised animals may be brought onto specific conditions (Council Regulation no. 834/2007 of 28/07, EC). The livestock shall have permanent access to open air areas, preferably pasture, whenever weather conditions and state of the ground allow this, unless restrictions and obligations related to the protection of human and animal health are imposed on the basis of Community legislation. The number of livestock shall be limited in order to minimize overgrazing, poaching of soil, erosion, or pollution caused animals or by the spreading of their manure (Council Regulation no. 834/2007 of 28/07, EC).

It is necessary to obtain feed for livestock from the holding where the animals are kept or from other organic holdings in the same region. The livestock shall be fed with organic feed that meets the animal`s nutritional requirements at the various stages of its development. Part of the ration may contain feed from holdings which are in conversion to organic farming (Council Regulation no. 834/2007 of 28/07, EC).

1.2 Fat content and fatty acid composition of ruminant meats

1.2.1 Dietary fat content and fatty acid profile

Fat appears in meat as adipose tissue. In addition to the adipose tissue under the skin (subcutaneous fat) and offal fat, it is visible as depot fat stored between muscles (intermuscular) and can be detected as marbling within the muscle (intramuscular) (Schmid, 2011). Lean beef has a low IMF content, typically 2-5%, and in many countries this is accepted as being ‘‘low in fat’’ (Scollan *et al.*, 2006). Marbling fat is an important meat quality trait in relation to juiciness, aroma and tenderness and is the fat depot of most interest in relation to fatty acid composition and human health (Scollan *et al.*, 2006). Meat and meat products have the reputation of being high-fat, but this is true only up to a point (Schmid, 2011). Red meat produced today is leaner and lower in fat content than that produced ten years ago (Higgs, 2000). Meat of lower fat content and higher muscle content has been obtained by means of breeding measures, changes in rearing (feed, environmental conditions), slaughter at a younger age, and leaner cuts of meat (Jakobsen, 1999; Honikel, 2006). Some of these conditions lead to lean red meat with low contents of both SFA and total fat (Li, Siriamornpun, Wahlqvist, Mann & Sinclair, 2005a; Williams, 2007). When trimmed of excess fat, commonly consumed cuts of beef and lamb were found to contain less than 5% of total fat

content (Enser *et al.*, 1998). The tendency is to focus on the production of edible lean with a minimum of excess visible fat, but the fact remains that fat in meat contributes to the eating quality of meat (Webb, 2006).

IMF mainly consists of triacylglycerols and phospholipids. The total IMF content generally depends on the amount of triacylglycerols, whereas the amount of phospholipids, as the building blocks of cell membranes, is relatively constant (Scollan *et al.*, 2006). The former is the main lipid component (>90%) of adipose tissue in mature animals (visible fat) and phospholipids are constituents of cell membranes that contribute with 10-40% of the total fatty acids in muscle (Wood *et al.*, 2008). Phospholipids possess a much higher concentration of PUFA than triacylglycerols. Hence, there is a strong relationship between IMF and the content of triacylglycerols which is mainly dependent on the degree of overall body fatness, breed and muscle type (Scollan *et al.*, 2006). The fatty acid content on phospholipids remain fairly constant but neutral lipid, with its high proportions of SFA and MUFA, increased markedly as total lipid increase (Wood *et al.*, 2008).

Fatty acids in meat are mainly of medium to long chain (LC) length, which means they have 12 to 22 carbon atoms in the molecule, with a basic structure of CH_3-$(CH_2)_n$-COOH. Small amounts of shorter chain length fatty acids are present in lamb fat (Wood *et al.*, 2008). Approximately 50% of the IMF of beef and lamb is made up of MUFA, primarily oleic acid (18:1*c*9), and of PUFA, predominantly the essential fatty acids LA and ALA (McAfee *et al.*, 2010).

A survey of European diets revealed that 21% of the total dietary fat intake comes from meat and meat products (Hulshof *et al.*, 1999). Between 30-50% of the fatty acids in meat fat are saturated (Schmid, 2011). The SFA occurring mainly in meat fat are myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0) (Schmid, 2011). Palmitic acid is generally the best represented, comprising approximately one-fourth to one-third of all fatty acids. Stearic acid comes next with a fatty acid share of approximately 10-20% of total SFA while the share of myristic acid is 3-6% of total SFA (Enser, Hallett, Hewitt, Fursery, & Wood, 1996; Diaz, Watkins, Li, Anderson & Campbell, 2005; Valsta, Tapanainen & Mannisto, 2005). Oleic acid not only comes top of the MUFA, but in overall is also the fatty acid most frequently found in meat fat (Schmid, 2011).

There are two essential fatty acids in human nutrition: ALA, a fatty acid that belongs to the *n*-3 family, and LA, a fatty acid that belongs to the *n*-6 family. The human body cannot synthesize these fatty acids and, for this reason, they must be obtained from food. These essential fatty acids can be converted to long chain polyunsaturated fatty acid (LC-PUFA) via a series of stepwise desaturations and elongations. Once eaten, the body converts ALA to eicosapentaenoic (EPA, 20:5*n*-3), docosapentaenoic (DPA, 22:5*n*-3) and docosahexaenoic (DHA, 22:6*n*-3) acids, albeit at low efficiency. Studies generally agree that whole body conversion of ALA to DHA is below 5% in humans, the majority of these LC fatty acids are consumed in the diet (Thomas, 2002). Lean tissue of red meat contains ALA and its elongation products *n*-3 LC-PUFA, EPA, DPA and DHA. One of the key enzymes in the metabolism of essential fatty acids and PUFA is Δ6-desaturase which recognizes and metabolizes LA and ALA producing γ-linolenic acid and octadecatetraenoic (stearidonic) acids, respectively (Russo, 2009). After Δ6-desaturation, a cycle of elongation and desaturation by Δ5-desaturase generates arachidonic acid (AA, 20:4*n*-6) and EPA, starting from LA and ALA, respectively (Russo, 2009). The obvious formation of 22:5*n*-6 and 22:6*n*-3 series by a further step of elongation and desaturation by a hypothetical Δ4-desaturase has been a matter of controversy, since this enzyme has been only identified in microalgae (Pereira, Leonard, Huang, Chuang & Mukerji, 2004).

In meat, the dominant PUFA are 18:2*n*-6 and 18:3*n*-3, representing approximately 2% of total triacylglycerol fatty acids (Scollan *et al.*, 2006). In phospholipids, the proportion of PUFA is much higher than in triacylglycerols, containing not only the essential fatty acids 18:2*n*-6 and 18:3*n*-3 but also their longer chain derivatives, such AA, EPA, DPA and DHA (Scollan *et al.*, 2006). There is competition between 18:2*n*-6 and 18:3*n*-3 for conversion to LC-PUFA because the enzymes are shared. Williams and Burdge (2006) suggested that 18:3*n*-3 is the preferred substrate but the presence of much more 18:2*n*-6 usually results in greater synthesis and deposition of LC-PUFA derived from this fatty acid.

Concentrations of *n*-3 LC-PUFA are recognizably higher within meat from animals fed a grass diet (Enser *et al.*, 1998; French *et al.*, 2000; Aurousseau *et al.*, 2004; Ponnampalam, Mann & Sinclair, 2006). Increasing *n*-3 contents in beef can be relevant to improving human supply with *n*-3 LC-PUFA (Razminowicz, Kreuzer & Scheeder, 2006). Studies which have discussed strategies to increase beneficial quantities of *n*-3 LC-PUFA within meat through grass feeding, or otherwise, have often referred that lowering the *n*-6/*n*-3 ratio of meat as desirable effect for the CVD risk in adults (Wood & Enser, 1997; Scollan *et al.*, 2006). In beef

and lamb, concentrations of *n*-3 LC-PUFA are lower than those within oily fish (Enser *et al.* 1996). Red meat is the main dietary source of DPA, which accumulates in mammals but not in oily fish (Givens & Gibbs, 2006). Meat contains lower concentration of AA (Smith, 2007). The PUFA/SFA ratio is approximately 0.11 in beef and 0.15 in lamb, and much lower than the desired dietary ratio of 0.4, owing to the degree of biohydrogenation of unsaturated fatty acids in the rumen (Scollan *et al.*, 2006). This ratio decreases with the rising on beef fat level (Barton, Marounek, Kudrna, Bures & Zahradkova, 2007). Ruminant meats usually have a more favourable *n*-6/*n*-3 ratio when compared with meat from monogastric animals, due to the relatively high levels of 18:3*n*-3 in pasture and forage. However, the usefulness of this ratio has recently been questioned because it undervalues the actual amounts of both *n*-3 and *n*-6 PUFA (Givens & Gibbs, 2008).

The fatty acids profile in ruminants` tissues are more complex than those in non-ruminants, containing higher proportions of TFA, fatty acids with an odd number of carbon atoms (arising from rumen-derived propionic acid rather than acetate as a precursor for fatty acid synthesis, *e.g.* 15:0 and 17:0), fatty acids with branched chains (with *iso*- or *anteiso*-structure) and fatty acids with conjugated double bonds (Wood *et al.*, 2008). These variations are the result of the actions of enzymes present in microorganisms in the rumen that degrade plant structures and dietary fatty acids, producing a wide range of products, some of which are absorbed in the small intestine and incorporated into the tissue lipids (Wood *et al.*, 2008). The odd and branched chain fatty acids (BCFA) are regarded as important compounds within microbial lipids (O'Kelly & Spiers, 1991) to maintain optimal fluidity of the microbial cell membrane. BCFA of bacterial origin can make up 1.0 to 3.0% of milk lipids (Alonso, Fontecha, Lozada, Fraga & Juárez, 1999) and carcass lipids (Bas & Morand-Fehr, 2000).

TFA are unsaturated fatty acids with at least a double bond in *trans* configuration or geometry, i.e. the two hydrogen atoms of the carbons adjacent to the double bond point to opposite directions which is the contrary from the double bond of *cis* configuration (Dutton, 1979). TFA occur naturally in dairy and other animal fats by biological hydrogenation in the rumen of cows and sheep, but they are originated mainly from the industrial process of catalytic hydrogenation of fats (Larqué, Zamora & Gil, 2001). They arise through partial hydrogenation and/or isomerisation of *cis*-unsaturated fatty acids from the feed by hydrogen produced during oxidation of substrates, with bacterial enzymes as catalysts (Fritsche & Steinhart, 1998a). Schmid (2011) reported that the concentration of TFA in beef was between 2.8 and 9.5% of fatty acid methyl esters (FAME), with similar values for lambs (4.3-9.2%

FAME), although the concentrations in pork and poultry were lower (0.2-2.2% FAME and 0.2-1.7% FAME respectively) (Schmid, 2011).

The vaccenic acid (VA, 18:1*t*11) is a positional and geometric isomer of oleic acid and is the predominant *trans* monoene in ruminant fats (50-80% of total *trans* content) (Lock *et al.*, 2004) and the IMF of bulls contain approximately 2.8-3.5% (Nuernberg *et al.*, 2005a). High concentrations of 18:1*t*10 has been observed in tissues of concentrate-fed ruminants, whereas 18:1*t*11 is consistently associated with forage feeding (Bessa *et al.*, 2006; Dugan *et al.*, 2007). Concerning other C18 *trans* FA, lean meat presented a 18:2*trans* concentration between 0.4-1.1% and 18:3*trans* between 0.2-04%, of total fatty acids, although with higher values being found in meat fat (Schmid, 2011).

1.2.1.1 Nutritional value and intake recommendations of fatty acids

Meat continues to be an important food group in the diet for many consumers, particularly in the developed countries. Recently, a number of epidemiological studies have associated red meat consumption with the development of two of the major chronic diseases in the Western World, as CVD and colon cancer (Cross *et al.*, 2007; Kontogianni, Panagiotakos, Pitsavos, Chrysohoou & Stefanadis, 2008). Constituents of red meat that have been proposed to be responsible for these associations include the fat content, fatty acid composition and the possible formation of carcinogenic compounds, such as heterocyclic amines, by cooking meat at high temperatures (Bingham, Hughes & Cross, 2002). Fat and LC fatty acids, whether in adipose tissue or muscle, contribute to important aspects of meat quality and are central to the nutritional and sensory values of meat (Webb & O`Neill, 2008).

Different studies have shown an increasing effect on blood cholesterol concentrations and an increased risk of CVD being important to reduce dietary saturated fatty acids, as 12:0, 14:0 and 16:0 (Rioux & Legrand, 2007; Schmid, 2011). Stearic acid has been shown to have no net impact on serum cholesterol concentrations in humans (Williamson, Foster, Stanner & Buttriss, 2005). It has been known that 14:0 increases cholesterol levels more potently than 16:0, being the most hypercholesterolaemic (Williamson *et al.*, 2005). Dietary recommendations to lower CVD risk have emphasized the need for a reduction in total fat and, in particular, the amount of SFA in the diet (Wyness *et al.*, 2011), despite 18:0 that have no effect on cholesterol levels.

In meat fat, the MUFA generally account for around 40-50% of the fat (Schimd, 2011). In addition, 30% of the fatty acid content in conventionally produced beef is composed of oleic acid (Whetsell, Rayburn & Lozier, 2003), a MUFA that elicits a cholesterol-lowering effect among other healthful attributes including a reduced risk of stroke and a significant decrease in both systolic and diastolic blood pressure in susceptible populations (Kris-Etherton, 1999).

n-3 LC-PUFA are widely recognized for their numerous effects on heart health, as improving platelet aggregation, vasodilatation and thrombotic tendency (Mann *et al.*, 2006; Siddiqui, Harvey & Zaloga, 2008). Recommendations for the intake of *n*-3 LC-PUFA are given by the health agencies and EFSA, because quantity and quality of dietary fat are strongly related to human health. Dietary daily intake of *n*-3 LC-PUFA in the United Kingdom, at 5.3-5.4% of total food energy, is still well below that recommended by the Scientific Advisory Committee on Nutrition (SACN)/Committee on Toxicity (COT) (2004), who proposed that the average consumption of *n*-3 LC-PUFA should increase from approximately 0.1 to 0.2 g/day. It is worth noting that although ALA is acknowledged as the parent molecule for the *n*-3 family of fatty acids, it was not included as an *n*-3 LC-PUFA by SACN/COT (2004) because of the reduced effectiveness of elongation/desaturation of ALA *in vivo* (Woods & Fearon, 2009).

However, there has been considerable debate amongst health professionals regarding recommendations for *n*-3 LC-PUFA intake. In 2008, the Technical Committee on Dietary Lipids of the International Life Sciences Institute North America concluded that the consistent evidence demonstrating a clear inverse relationship between EPA plus DHA consumption and CVD risk was sufficient to support a nutritionally achievable dietary recommended intake for EPA plus DHA between 250 and 500 mg/day (Harris *et al.*, 2009). The EFSA panel proposed to set an adequate intake of 250 mg for EPA plus DHA for adults based on cardiovascular considerations (EFSA, 2010). Nevertheless, Griffin (2008) established that the *n*-6/*n*-3 ratio was of no relevance to modify the risk of CVD. Stanley *et al.* (2007) outlined the main concerns about the use of this ratio: (i) the *n*-6/*n*-3 ratio makes no distinction between ALA and the metabolically more active EPA/DHA; (ii) all ratios suffer from the fact that the components can change in various directions or not change at all, to produce a higher or lower ratio; and (iii) there is the underlying hypothesis that the ratio is balancing the good and bad activities of *n*-3 and *n*-6 PUFA. Goyens, Spilker, Zock, Katan and Mensink (2006) reinforced the recommendations to increase consumption of pre-formed *n*-3 LC-PUFA and showed that the absolute amounts of dietary LA and ALA influenced the conversion of ALA *in vivo*. Brenna, Salem, Sinclair and Cunnane (2009) concurred with these findings, noting that the

conversion of ALA to LC-PUFA was reduced by high dietary ratios of LA/ALA. They proposed that *n*-3 LC-PUFA status could be improved by increasing dietary intake of *n*-3 LC-PUFA or by reducing intake of *n*-6 PUFA and that combining both strategies would be most effective. The balance of *n*-6 and *n*-3 PUFA is very important for the homeostasis and normal development, but these two families are not interconvertible, and are metabolically and functionally distinct, and often have important opposing physiological functions (Simopoulos, 2006). The AA and EPA are precursors of different classes of pro-inflammatory or anti-inflammatory eicosanoids, respectively, whose biological activities have been evoked to justify risks and benefits of PUFA consumption. Due of the increased amounts of *n*-6 PUFA in the Western diet, the eicosanoid metabolic products from AA, specifically prostaglandins, thromboxanes, leukotrienes, hydroxyl fatty acids and lipoxins, are formed in larger quantities than those formed from *n*-3 PUFA, specifically EPA. Thus, a diet rich in *n*-6 PUFA shifts the physiological state to one that is prothrombotic and pro-aggregatory with increases in blood viscosity, vasospasm and vasoconstriction and decrease in bleeding time (Simopoulos, 2006). Studies have shown that meat consumers have greater plasma concentrations of *n*-3 LC-PUFA than vegetarians (Li *et al.*, 1999; Rosell *et al.*, 2005; Mann *et al.*, 2006), but there is a lack of data to show whether consuming modest amounts of red meat can provide concentrations sufficient to produce biological effects (McAfee *et al.*, 2010).

The effects of ruminant TFA on lipoproteins and CVD are unclear (Brouwer, Anne, Wanders & Katan, 2010). Some epidemiological studies showed no association between ruminant TFA intake and CVD (Ascherio *et al.*, 1994; Pietinen *et al.*, 1997; Jakobsen, Overvad, Dyerberg & Heitmann, 2008), one showed a non-significant inverse association (Willet *et al.*, 1993) and another a non-significant positive association (Oomen *et al.*, 2001). Motard *et al.* (2008) found adverse effects of ruminant TFA high intakes but not of low intakes. Another study suggested that ruminant TFA produce higher low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels than industrial TFA in women, but not in men (Chardigny *et al.*, 2008). Recently, Brouwer and collaborators (2010) published data suggesting that all fatty acids with a double bond in the *trans* configuration raise the ratio of plasma LDL to HDL cholesterol, which is unfavourable for human health. EFSA (2010) evidenced that the intake of TFA in the EU has decreased considerably over recent years, owing to the reformulation of food products, *e.g.* fat spreads, sweet bakery products and fast food. The panel of EFSA (2010) concludes that TFA intake should be as low as possible within the context of a nutritionally adequate diet.

It is necessary to point out that not all TFA have been found to be detrimental for human health. It is also recognized that VA is the precursor for tissue synthesis of *c*9,*t*11 CLA isomer, a fatty acid with beneficial effects in both man and animals (Scollan *et al.*, 2006). Dietary 18:1*t*11 can be desaturated to rumenic acid by Δ9-desaturase (also known as SCD) in the tissues of ruminants (Griinari & Bauman, 2000). As VA is the precursor of rumenic acid, with well known positive effects on cancer prevention and inhibition, some authors consider it as neutral or even beneficial to human health (Prates & Bessa, 2009). However, Field, Blewett, Proctor and Vine (2009) provided a critical review of the beneficial effects of 18:1*t*11, but in many feeding studies the 18:1*t*11 results may be confounded by the beneficial effects of oleic acid that differs greatly between the test diets (Bassett *et al.*, 2009; Wang *et al.*, 2009).

1.2.2 Conjugated linoleic acid isomers profile in meat

CLA has been the subject of intensive research in the biomedical field. CLA is the acronym used for conjugated linoleic acid and refers to a family of geometrical and positional isomers of LA with two double bonds located on adjacent carbons, from positions 6,8 and 12,14. The geometric configuration of these double bond pairs can be *trans,trans, trans,cis, cis,trans* or *cis,cis.* Much of this awareness has been focused on the therapeutic potential of CLA, being the most studied biological isomers, the *c*9,*t*11 and the *t*10,*c*12 (Belury, 2002; Bhattacharya, Banu, Rahman, Causey & Fernandes, 2006). The predominant source of CLA for humans is through dietary intake (Herbel, McGuire, McGuire & Shultz, 1998). Twenty four different CLA isomers have been identified as occurring naturally in ruminant fat (Sehat *et al.*, 1998). The major CLA isomer found in food products is the *c*9,*t*11, accounting for more than 75-90% of CLA intake in the human diet (Kramer *et al.*, 1998; Bauman, Baumgard, Corl & Griinari, 2007).

The *c*9,*t*11 CLA isomer in food can be originated by one of two pathways: from the incomplete biohydrogenation of LA to stearic acid by rumen bacteria or from the Δ9-desaturation of 18:1*t*11 (a primary intermediate for ruminant biohydrogenation) in mammalian tissues (Kay, Mackle, Auldist, Thomson & Bauman, 2004) (Figure 1).

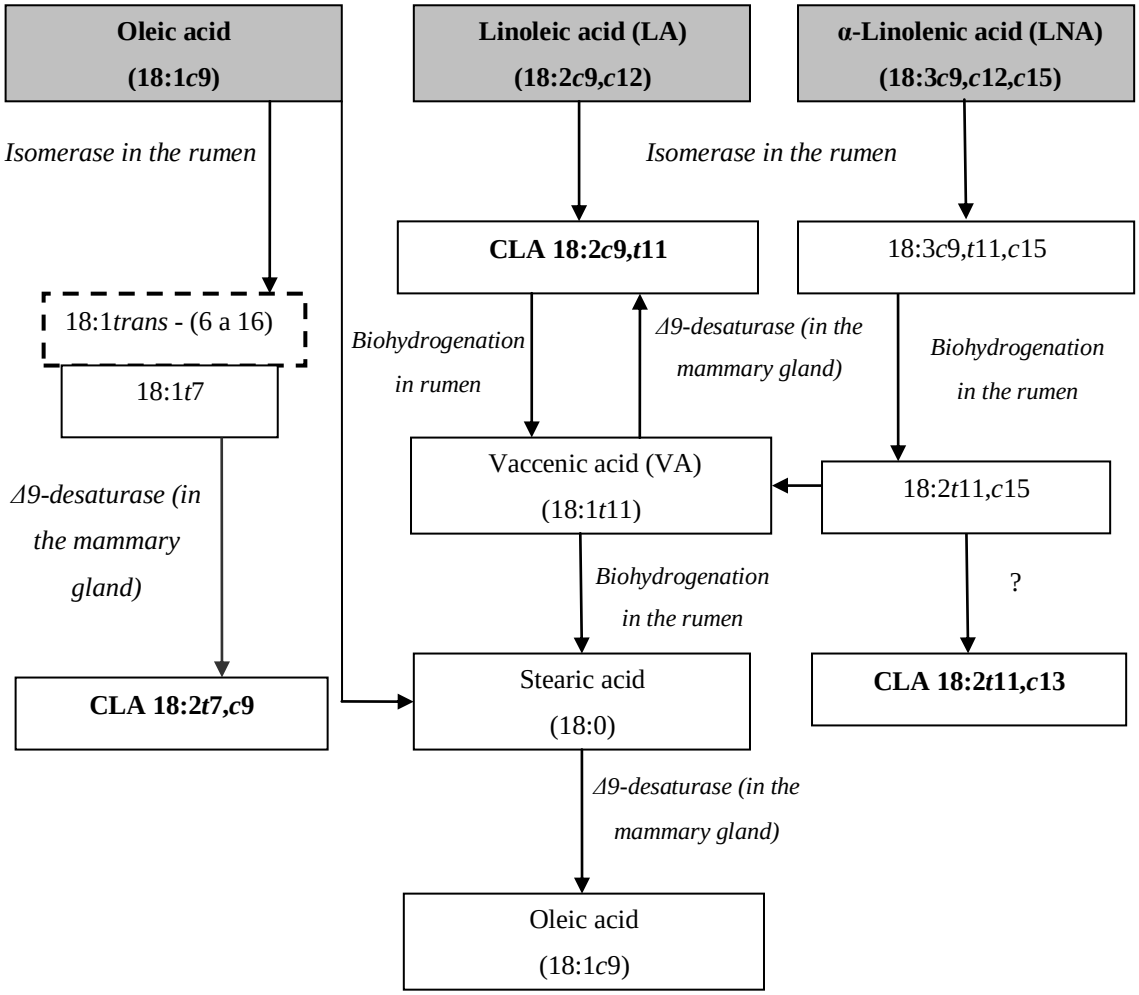

Figure 1 - Known metabolic pathways for the formation of CLA isomers (adapted from Collomb, Schmid, Sieber, Wechsler & Ryhänen, 2006).

The remaining consists of several minor CLA isomers of which *t*7,*c*9 isomer, in general, is the second most predominant one in ruminant fat, representing 3-16% of total CLA in milk fat (Yurawecz *et al.*, 1999) and 8-15% of total CLA in beef fat (Fritsche *et al.*, 2000). It has been suggested that *t*11,*c*13, *t*11,*t*13 and *t*12,*t*14 isomers are increased in meat from pasture-fed animals, becoming *t*11,*c*13 the second-most important isomer (Collomb, Sieber & Butikofer, 2004; Dannenberger *et al.*, 2005). Such a shift in the distribution pattern of CLA isomers in muscle tissues and subcutaneous fat was also observed in a study comparing bulls on a pasture or a concentrate diet. For bulls fed pasture, the *t*11,*c*13 isomer again became the second-most important isomer with a very high accumulation rate of up to a factor of 14.5, and the second- and third-highest accumulation rates were observed for the *t*11,*t*13 and *t*12,*t*14 isomers, with a factor of up to 7.1 (Dannenberger *et al.*, 2005). The *t*10,*c*12 isomer makes up less than 10% in natural food products (Toomey, McMonagle & Roche, 2006).

CLA can also be obtained commercially as a dietary supplement. Most of the CLA research has been focused on the two biological active conjugated isomers due to the fact that synthetic available CLA supplements consist of an equimolar mixture of *c*9,*t*11 and *t*10,*c*12 isomers (Pariza, Park & Cook, 2001). In commercial products, CLA isomers are present as free fatty acids, whereas in dietary sources are largely in the triacylglycerol form. Industrially, the production of CLA is carried out by chemical synthesis via alkaline-isomerisation of linoleic acid-enriched vegetable oils, such as safflower and sunflower oil (Kellersmann, Lehmann, Francke & Steinhart, 2006). These isomers are commercially available for research purposes, being produced at a low-temperature of crystallization reaching more than 90% of purity (Kellersmann *et al.*, 2006). This process yields a CLA mixture containing approximately 40% and 44% of *c*9,*t*11 and *t*10,*c*12, respectively (Pariza *et al.*, 2001). Although commercial CLA tends to contain the most physiological active isomers, several nutritional studies have been carried out with more complex mixtures. In fact, commercial preparations also contain approximately 4-10% of *t*9,*t*11 and *t*10,*t*12, as well as, trace amounts of other CLA isomers (Kennedy *et al.*, 2010).

The wide occurrence of CLA in common foods leads to different human dietary intakes depending on food selection and eating preferences (ruminant *vs*. non-ruminant sources and plant *vs*. animal sources) (O`Shea, Lawless, Staton & Devery, 1998). A multiplicity of factors can potentially influence CLA intake including the amount of total fat dairy and meat products consumed, and age, gender and physiological state of the animals (McGuire, McGuire, Ritzenthaler & Shultz, 1999).

Worldwide scientific interest in CLA was primarily encouraged by its identification as an anticarcionogenic compound isolated from grilled ground beef (Pariza & Hargraves, 1985; Ha Grimm & Pariza, 1987). In the 90´s, the National Academy of Sciences of the USA recognized CLA as the only fatty acid that unequivocally inhibits carcinogenesis in experimental animal models (National Research Council, 1996). It has been acknowledged that CLA inhibits the initiation and growth of breast, colorectal, prostate and skin cancers in animal models (Belury, 2002; Bhattacharya *et al.*, 2006; Cho *et al.*, 2006). Other health-related proprieties of CLA include anti-adipogenic (Terpstra *et al.*, 2003; Gaullier *et al.*, 2005), anti-inflammatory (Tricon *et al.*, 2004a) and anti-atherogenic (Toomey *et al.*, 2006; Franczyk-Zarow *et al.*, 2008) effects. However, if some reports describe health promising benefits, others reveal lipodistrophy, fatty liver and glucose intolerance, after CLA consumption (Park & Pariza, 2007).

The physiological effects produced by CLA isomers result, in some cases, from the independent actions of a single isomer, and, in other cases, from synergistic interactions involving both isomers with numerous metabolic signalling pathways (Pariza, Park, Xu, Ntambi & Kang, 2003). While both well known bioactive CLA isomers (*c*9,*t*11 and *t*10,*c*12) are equally effective in anticancer activity, the *t*10,*c*12 isomer is responsible for the anti-adipogenic effect. Moreover, antagonistic effects of *c*9,*t*11 and *t*10,*c*12 were described on blood lipids in healthy humans (Tricon *et al.*, 2004b). To date, most of the CLA research reports to biological activities of these two isomers. Yet, the effectiveness of the lesser-studied isomers would be of huge scientific value. A known biological effect of the *t*9,*t*11 isomer is the inhibition of the platelet aggregation (Li, Barnes, Butz, Bjorling & Cook, 2005b) together with an antiproliferative effect (Lai, Yin, Li, Zhao & Chen, 2005).

Recently, it was stated that the mixture of *t*8,*c*10 plus *c*9,*t*11 beneficially decreased triacylglycerols accumulation in 3T3-L1 cells, possibly due to the *t*8,*c*10 isomer, without lowering adiponectin levels, as described for the *t*10,*c*12 isomer (Joseph, Miller, McCleod & Jacques, 2009). In line with this, the same mixture decreased body fat in hamsters fed a hypercholesterolaemic diet (Joseph *et al.*, 2010). Moreover, it was also suggested that a mixture of *trans/trans* isomers has more anticarcinogenic effects than *c*9,*t*11 or *t*10,*c*12 alone (Islam *et al.*, 2010). The authors studied the inhibitory activity of *trans/trans* isomers in rat mammary tumorigenesis induced by N-methly-N-nitrosourea through the induction of apoptosis, in combination with the reduction of arachidonic acid metabolites.

Several steps have been developed to accuracy, completeness and quantitative determination of CLA analysis. Thus, sample preparation and extraction methods are critical since they can lead to destruction and/or incomplete extraction of lipids. The two established methods of lipid extraction from ruminant fat are based on the Folch method (Folch, Lees & Stanley, 1957) and Bligh and Dyer method (1959), or modifications thereof. Base-catalyzed methylations have the advantage that does not isomerize CLA (Cruz-Hernandez *et al.*, 2006).

Capillary gas-chromatography (GC) is by far the most common and usual method of fatty acid and CLA analysis. In the last 15 years there has been an encouraging move to use 100 m or more highly polar capillary columns due to many reports that demonstrated the potential of much improved separations (Yurawecz *et al.*, 1999). Although the GC separation of several CLA isomers even on 100 m capillary columns is incomplete and overestimates the major CLA isomer *c*9,*t*11 (*t*7,*c*9 and *t*8,*c*10 co-elutes) (Murru *et al.*, 2003) or misidentifies other

peaks in the CLA region such as 21:0 and 22:0 isomers (Roach, Mossoba, Yurawecz & Kramer, 2002). Many studies describe the separation and identification of CLA isomers by silver ion high performance liquid chromatography (Ag^+-HPLC) (Yurawecz & Morehouse 2001, Prates & Bessa, 2009). Ag^+-HPLC and GC are complementary techniques to identify most the CLA isomers, the combination of these two methods has been used to report improved analyses of dairy products, and meat. The complementary of new innovative techniques to GC, such as tandem-column Ag^+-HPLC, Fourier-transform infrared spectroscopy (FTIR) and near infrared transmittance spectroscopy (NIRS), attenuated total reflectance (ATR), mass spectrometry (MS) and carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR), have enabled complete and accurate assessment of all fatty acids and CLA isomers (Kramer *et al.*, 2004; Cruz-Hernandez *et al.*, 2006; Nuernberg Dannenberger, Ender & Nuernberg, 2007; Sierra *et al.*, 2008; Prates & Bessa, 2009).

For bovine meat, the CLA median value resulted from intramuscular (IM), intermuscular (IT) and subcutaneous (SF) fat, where the overall content of CLA in dissectible beef fat was formed using an average fat distribution ratio of 14% IM, 68% IT and 18% SF, reflecting the analysed mean fat contribution in three cattle genotypes (Aldai, Najera, Dugan, Celaya & Osoro, 2007). The origin of all other CLA isomers is ruminal biohydrogenation of dietary C18 PUFA (Collomb *et al.*, 2004) and make up a very small portion of total CLA (Bauman Corl & Peterson, 2003). As mammals do not have Δ12-desaturase enzyme, 18:1*t*11 cannot be transformed into *t*10,*c*12 isomer. So, the presence of this isomer in tissues will be exclusively from rumen output. Kramer *et al.* (1998) reported that *c*9,*t*11 isomer generally accumulates in higher amounts in tissue lipids, while *t*10,*c*12 isomer only in trace levels, depending on the nature of the diet (Ha, Storkson & Pariza, 1990; Parodi, 2003).

CLA isomers in ruminant meat are mainly incorporated as triacylglycerols in IMF and adipose tissue (Dannenberger *et al.*, 2004). It is well known that ruminant-derived foods are the primary source of CLA in the human diet, including milk (Collomb *et al.*, 2006; Rego *et al.*, 2009), dairy products (Martins *et al.*, 2007) and meat (Dannenberger *et al.*, 2005; Alfaia *et al.*, 2009). Ruminant meats are known to have markedly higher CLA contents compared to those from non-ruminant origin. CLA contents in meat and meat products were reviewed by Parodi (2003) and Schmid, Collomb, Sieber and Bee (2006). Highest CLA contents were found in lamb (4.3-19.0 mg/g fat) whereas in beef were slightly lower (1.2-10.0 mg/g fat), representing 0.5-2% of fatty acids. Since CLA is found in adipose fat, CLA concentrations are highly influenced by fat concentrations (Decker & Park, 2010). Dietary CLA from beef can

be increased by manipulation of animal diets and direct CLA addition to meats. CLA concentrations in ruminant meat can be influenced by diets containing oils or oilseeds high in PUFA (usually 18:2*n*-6 or 18:3*n*-3). These dietary practices can increase the *c*9,*t*11 isomer (Bessa *et al.*, 2007). In contrast, CLA contents in pork, chicken or meat from horses are usually lower than 2 mg/g fat. The CLA content of fish and marine products is negligible (Chin, Liu, Storkson, Ha & Pariza, 1992, Fritsche & Steinhart, 1998a). Furthermore, CLA contents vary substantially not only between species but also from animal to animal and within animal in different tissues. The feeding pattern is the most important factor in CLA contents both in meats, though diet does not necessarily increase CLA in all tissues (Shen *et al.*, 2007).

1.2.2.1 Nutritional value and intake recommendations of CLA isomers

Findings from studies of human CLA intake and cancer incidence are inconsistent as long as the only data availed come from epidemiological studies. One of the major limitations to achieve conclusive findings from these studies relies in the attempt to discriminate the effects of CLA through dietary surveys when a wide array of other compounds, such as LA, TFA, and SFA, found in the same food sources as CLA, may also interfere with cancer development (Kent & Muga, 2007). Until now, the exact mechanism of carcinogenesis modulation by CLA is not entirely understood, although it may be related to its anti-oxidative properties (Belury, 2002) or even to the induction of apoptotic cell death and cell-cycle regulation (Yamasaki, Miyazaki & Yamada, 2006). The replacement of PUFA by CLA in the plasma membrane composition may affect, for example, redox balance, eicosanoid metabolism (Park & Pariza, 2007) and signal transduction (Fritsche & Steinhart, 1998a). A significant number of reports have provided either direct or indirect evidences that CLA may interfere with prostaglandins production through a decrease in the supply of arachidonic acid precursor, and may improve insulin function with positive implications for type 2 diabetes, but in contradiction with some animal and human studies that have shown unsafe for diabetes (Ryder *et al.*, 2001).

Recent molecular assays have also indicated that CLA acts at least partially through its high affinity for ligand of peroxisome proliferator activated receptor-γ (PPAR-γ) which is a family of transcription factors known to affect gene expression involved in numerous pathways of lipid transport and metabolism (Kent & Muga, 2007). Furthermore, determining the ability of CLA isomers to influence glucose and lipid metabolism, as well as, markers of insulin sensitivity is imperative to understanding the role of CLA, and thus to aid in the management

of type 2 diabetes and other related conditions of insulin resistance (Lambert *et al.*, 2007; Diaz, Watkins, Li, Anderson & Campbell, 2008). Major biochemical actions of CLA associated with lipid metabolism are summarized in Table 1.

Table 1 - Major biochemical actions of CLA on lipid metabolism (adapted from Benjamin & Spener, 2009).

Biochemical action	*Experimental evidence*	*Ref.*
Preadipocyte proliferation	Proliferation inhibition	[1]
Preadipocyte differentiation	Human preadipocytes do not differentiate in the absence of a PPAR-γ ligand, like CLA	[2]
Fatty acid oxidation	Carnitine palmitoyltransferase activity increased by dietary CLA	[3,4]
Adipose tissue lipid synthesis	Inhibition of *de novo* lipogenesis through down regulation of acetyl-CoA carboxyxlase and fatty acid synthase	[5]
Lipolysis	Increased lipolysis and decreased fat	[6]
Energy expenditure	Increased of oxygen consumption and energy expenditure by *t*10,*c*12 CLA isomer	[7]
Stearoyl-CoA desaturase	Inhibition at protein or activity level, by *post*-translational modification	[8]
Plasma leptin	Decrease in serum leptin, a hormone regulating fat level	[9,10]
Apotopsis	Induction of apoptosis in adipocytes	[1]
Tumor necrosis factor-α	Increase the expression of TNF-α and low fat	[11]

[1]-Evans *et al.* (2000); [2]-McNeel *et al.* (2003); [3]-Brown *et al.* (2004); [4]-Close *et al.* (2007); [5]-Lin *et al.* (2004); [6]-Ostrowska *et al.* (2002); [7]-Wang *et al.* (2003); [8]-Belury *et al.* (2002); [9]-Perez-Matute *et al.* (2007); [10]-Rahman *et al.* (2001); [11]-Ryden *et al.* (2004).

Van Wijlen & Colombani (2010) analysed 23 studies on recorded CLA intake in humans. In 16 of them, food frequency questionnaires and/or diet records were used. Five were related to national nutrition surveys, and two stemmed from experimental diets. In one further study, the CLA intake was estimated based on recommendations for a balanced, normal daily diet (Van Wijlen & Colombani, 2010). The pooled median CLA intake of all 24 studies was 278 mg/day, not taking into account the gender and neglecting dietary record methods (Van Wijlen & Colombani, 2010). Depending on the country, the estimation of CLA daily intake varies from 15 to 1000 mg (Martins *et al.*, 2007), thus representing a wide range of consumption values. Fritsche and Steinhart (1998b) estimated that the mean dietary intake of

CLA in German population is 350 and 430 mg/day, for women and men, respectively, according to their habits of consumption. The ingestion of *c*9,*t*11 in the USA and England was estimated at 151 mg/day for American women, 212 mg/day for American men, and 97.5 mg/g for English people (Ritzenthaler *et al.*, 2001; Mushtaq, Mangiapane & Hunter, 2009). In some countries, the CLA consumption was also higher in males than in females for (for ***e.g*** France, 213 mg/day *vs.* 178 mg/day, respectively), data obtained by individual national food survey (Laloux, Du Chaffaut, Razanamahefa & Lafay, 2007).

Our research group has determined the CLA isomers intake based on the most consumed Portuguese CLA rich foods (milk, butter, yogurt, cheese, beef and lamb meat) and the contribution of these ruminant-derived foods on the daily intake of CLA isomers based on Portuguese consumption habits. The total CLA average intake estimated for the Portuguese population was 74 mg/day (Martins *et al.*, 2007). Wolff and Precht (2002) estimated the *c*9,*t*11 ingestion in 15 European countries, based on milk consumption data, obtaining higher intake values in North Europe and lower values in Mediterranean countries. According to them, France and Italy showed consumption average values close to those of EU and a similar daily intake was observed for Spain (140 mg), Greece and Portugal (150 mg). The difference from our values for Portugal might be explained by the distinct estimation methods and statistics sources used. However, on the basis of anticarcinogenic effects of CLA in rats, Parrish, Wiegand, Beitz, Ahm, Du and Trenkle (2003) proposed that a daily consumption between 0.8 and 3.0 g of CLA might provide a significant health effect to humans. While human studies have demonstrated beneficial effects of *c*9,*t*11 isomer at concentrations of >1.2 g of *c*9,*t*11/day (Tricon *et al.*, 2004a), the animal studies suggest that as little as 0.8 g of *c*9,*t*11/day may be sufficient for tumor inhibition (Watkins & Li, 2003). Moreover, the last authors found that the human equivalent CLA intake based on 0.1% dietary CLA given to rats is 0.72 g/day of CLA for a 70 kg person, adjusting for the difference in metabolic rate of human's *vs.* rats.

1.2.3 Cholesterol and lipid-soluble antioxidant vitamins

Red meat, regardless of feeding regimen, is nutrient dense and regarded as an important source of essential amino acids, vitamins A, B_6, B_{12}, D, E, and minerals, including iron, zinc and selenium (Williamson *et al.*, 2005, Biesalski, 2005). Along with these important nutrients, meat consumers also ingest a number of fats which are an important source of energy and facilitate the absorption of fat-soluble vitamins including A, D, E and K (Daley *et al.*, 2010).

Recently, EFSA (2010) established dietary reference values for fats but did not propose a reference on cholesterol intake since it depends on the intake of SFA. Furthermore, most dietary cholesterol is obtained from foods which are also significant sources of dietary SFA, *e.g*. meat products. Meat provides from one third to one half (Chizzolini *et al.*, 1999) of the maximum daily recommended cholesterol intake (300 mg) proposed by World Health Organization (WHO) (2003). Muchenje *et al.* (2009a) reported that the consumption of 200 g of beef represented cholesterol intakes range 73-83 mg from beef from natural pasture-based, respectively, which corresponds to less than 30% of the recommended maximum daily cholesterol intake (300 mg/day, WHO, 2003). Most notably, moderate consumption of lean red meat was found to lower total cholesterol, LDL cholesterol and triacylglycerides (Beauchesne-Rondeau, Gascon, Bergeron & Jacques, 2003) and to have no effect on markers of platelet aggregation (Li *et al.*, 1999) or oxidative stress (Hodgson, Burke, Beilin & Puddey, 2006). Rule, Macneil and Short (1997) emphasized that breed, nutrition, and sex do not affect the cholesterol concentration of bovine skeletal muscle. Thus, altering cholesterol concentration in muscle may require a marked redistribution of membrane fatty acids (Rule *et al.*, 1997). Also, beef muscles which contain predominantly, oxidative fibres with higher level of pigments, have been shown to have a higher concentration of cholesterol, comparatively to glycolytic fibres (Lynch, Keery, Buckley, Faustman & Morrissey, 1999). However, small but significant variations have been reported in cholesterol levels between muscle types (Costa *et al.*, 2008).

Several plant-derived compounds such as tocopherols, carotenoids, terpenes and phenolic compounds have been investigated as possible markers for milk or meat authentication (Prache, Cornu, Berdagué & Priolo, 2005). Carotenoids are a family of compounds that are synthesized by higher plants as natural plant pigments (Daley *et al.*, 2010). Xanthophylls, carotene and lycopene are responsible for yellow, orange and red colouring, respectively. Cattle produced under extensive grass-based production systems generally have carcass fat which is more yellow than their concentrate fed counterparts caused by carotenoids from the lush green forages (Daley *et al.*, 2010). Carotenes (mainly β-carotene) are precursors of retinol (vitamin A), a fat-soluble vitamin, that is important for normal vision, bone growth, reproduction, cell division and cell differentiation (Dunne, Monahan, O`Mara & Moloney, 2009).

The current recommended intake of vitamin A is 700 μg/day for females and 900 μg/day for males (Food and Nutrition Board of Institute of Medicine, 2001). In general, meats are not a good source of β-carotene or other carotenoids, with the exception of chicken (Decker & Park, 2010). The concentrations of β-carotene were 0.75 μg/g and 0.17 μg/g, for beef from pasture and grain-fed cattle, respectively (Insani *et al.*, 2007).

Vitamin E is also a fat-soluble vitamin that exists in eight natural compounds: α-, β-, γ- and δ-tocopherols, and α-, β-,γ - and Δ-tocotrienols, which differ in the identity and location of groups on their chromanol ring structure (Scherf, Machlin, Frye, Krautmann & Williams, 1996). Among all tocopherols, α-tocopherol is the form most commonly found and associated with vitamin E activity in animal tissues (Röhrle *et al.*, 2011). Vitamin E is recognized as a radical chain-breaking antioxidant although it has some limitations: 1) it requires another antioxidant to recycle it from the oxidized form and 2) it can act as a pro-oxidant when high doses are ingested (Mukai, Sawada, Kohno & Terao, 1993; Rietjens *et al.*, 2002). The current recommended intake of vitamin E is 12/15 mg/day (women/man) (Food and Nutrition Board of Institute of Medicine, 2001). Meats are an important dietary source of vitamin E with poultry and ground beef being the second and sixth most important sources of α-tocopherol, in men, while poultry is the third most important source, in women (Ma, Hampl & Betts, 2000). Furthermore, the concentration of natural α-tocopherol found in grain-fed beef ranged between 0.75 to 2.92 μg/g of muscle whereas pasture-fed beef ranges from 2.10 to 7.73 μg/g of tissue depending on the type of forage made available to the animals (Insani *et al.*, 2007; Descalzo & Sancho, 2008; De la Fuente *et al.*, 2009). α-tocopherol acts post-mortem to delay oxidative deterioration of the meat, a process by which myoglobin is converted into brown metamyoglobin, producing a darkened, brown appearance to the meat (Daley *et al.*, 2010). Several studies concluded that antioxidants in grass caused higher levels of vitamin E, improving the shelf life of the meat (Warren *et al.*, 2002; Yang, Lanari, Brewster & Tume, 2002).

1.3 Factors influencing fat content and fatty acid composition of ruminant meats

Muscle lipid content plays a key role in various quality traits of meat and depends on many factors including species, breed, genotype, growth rate, sex, age, muscle location and nutrition (Hocquette *et al.*, 2010). The amount of IMF and its fatty acid composition play major roles in the quality attributes of meats, including sensory properties and healthy considerations. It is generally assumed that IMF content positively influences sensory quality traits, including

flavour, juiciness and tenderness of meat, whereas a low amount of fat induces a less tasty meat (Hocquette *et al.*, 2010).

Biological mechanisms that explain the variability of IMF content differ between genetic and environmental factors (Hocquette *et al.*, 2010). IMF content varies between species, breeds and muscle types in the same breed (Hocquette *et al.*, 2010). Other factors are involved in the variation of IMF content in meat, including nutrition (Hocquette *et al.*, 2010), slaughter season (Alfaia *et al.*, 2007; Panjono, Kang, Lee & Lee, 2011), feeding system (Alfaia *et al.*, 2009) and farm altitude (Costa *et al.*, 2011). Recently, Warner, Greenwood, Pethick and Ferguson (2010) indicated that the interaction between genetic and environment may be a relatively important contributor to the phenotypic variance in meat quality traits. In addition, diets that promote glucose supply to the muscle might increase IMF deposition in ruminants (Hocquette *et al.*, 2010). This can be achieved by the administration of gluconeogenesis precursors, such as PG, which induces elevated propionate in the rumen and higher levels of blood insulin and glucose (Christensen *et al.*, 1997).

1.3.1 Genetic factors

Meat fatty acid composition is influenced by genetic factors, as the level of fatness (De Smet, Raes & Demeyer, 2004). Additionally, the fatty acid composition can be controlled by genes related to the lipid synthesis and fatty acid metabolism (Suzuki, Ishikawa, Arihara & Itoh, 2007). Genetic polymorphisms of Δ9-desaturase is one of the genes associated with fatty acid composition (Mannen, 2011). For example, this enzyme may contribute to the fatty acid compositional differences found between Japanese Black cattle and Holstein (Taniguchi *et al.*, 2004). Lipogenic genes have been postulated as good markers for IMF content or its composition, such as fatty acid synthase (FAS), sterol regulation element binding protein-1 (SREBP-1) and SCD-1 (Hoashi *et al.*, 2007). The key enzymes involved in SFA, MUFA and PUFA biosynthesis are acetyl-coenzymeA carboxylase (ACC), SDC and Δ6- desaturase, respectively (Herdmann, Nuernberg, Martin, Nuernberg & Doran, 2010).

Breed differences reflect discrepancies on gene expression or activities of enzymes involved in fatty acid synthesis, desaturation or chain elongation (Barton *et al.*, 2007). Different fatty acid compositions between breeds can often be explained by differences in the proportion of IMF, as the ratio PUFA/SFA. This ratio decreases with the increasing fat level of beef (Barton *et al.*, 2007) that depends on breed and nutrition.

IMF content varies from 3% to 11% in different muscles within a group of 25 Canada Aberdeen beef carcasses (Jeremiah, Dugan, Aalhus & Gibson, 2003). This may be explained by intrinsic biological patterns, of relative importance on oxidative and glycolytic metabolisms in muscle fibres (Hocquette *et al.*, 2000), and rearing factors, as commercial slaughter weight regarding to sexual maturity (Renand, Larzul, Bihan-Duval & Roy, 2003). Differences in muscle characteristics across bovine breeds can be associated not only with their fatness score but also with their biological type (rustic, beef or dairy) or with their geographical origin (Hocquette *et al.*, 2010). Within a given muscle, it is also known that oxidative fibres contain less phospholipid and more triacylglycerides (Hocquette *et al.*, 2010). As a consequence, differences in IMF content between species and muscles are often parallel to differences in muscle fibre types (Hocquette *et al.*, 2010). However, there is no strict association between IMF content and oxidative metabolism the muscle fibres (Hocquette *et al.*, 2010).

1.3.2 Environmental factors

It is well known that fatty acid composition of meat, and hence its nutritional value, can be manipulated by the diet (Enser *et al.*, 1998; Dannenberger *et al.*, 2004). The production system, including different diets, is responsible for much of the variation in the fatty acid of ruminants, lambs (Nuernberg *et al.*, 2005b) and cattle (Razminowicz *et al.*, 2006). Differences in the fatty acid composition of beef from animals reared predominantly on grain and grass-based diets have been widely reported (French *et al.*, 2000; Realini *et al.*, 2004; Nuernberg *et al.*, 2005a; Alfaia *et al.*, 2009).

Studies on suckling lambs found that the meat fat composition is mainly related to the fat composition of the milk they consume (Velasco, Cañeque, Lauzurica, Pérez & Huidobro, 2004). Before weaning, calves feed exclusively in milk. Analysis of the milk fatty acid profile revealed that the high content of the 16:0, 18:0 and 18:1*c*9 found in the milk, was partially transmitted to the unweaned class calves, whose beef had a higher content of these fatty acid than the weaned calves (Moreno *et al.*, 2006).

In monogastric animals, the quality of meat can be relatively easy improved by dietary manipulations, the dietary fatty acids can be directly incorporated in animal tissues (Nuernberg *et al.*, 2005a). However in ruminant's dietary manipulations of fatty acid composition proved to be difficult because of rumen biohydrogenation. The fatty acid

composition of ruminant edible fats is mostly determined by complex interactions between dietary factors and rumen metabolism. The Figure 2 showed biohydrogenation pathways in the rumen.

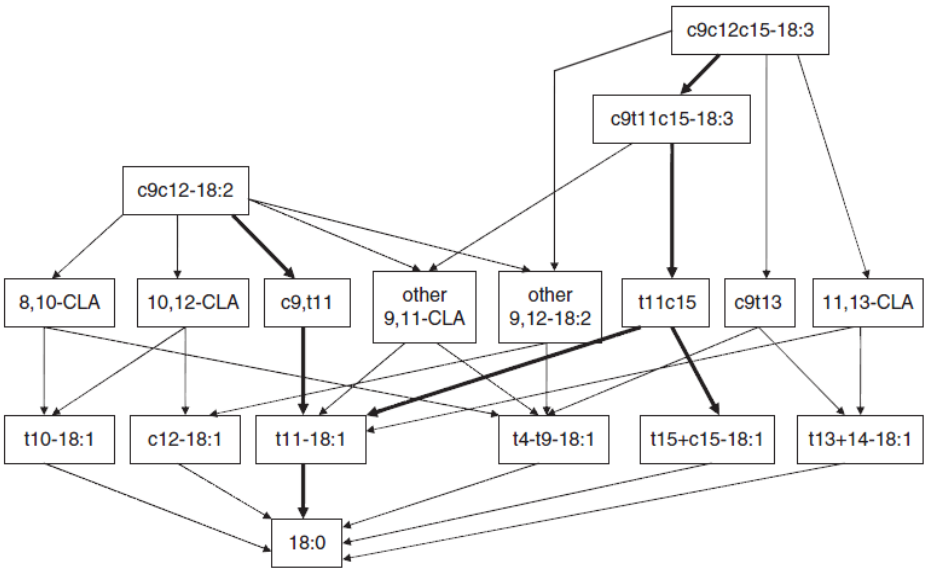

Figure 2 - Biohydrogenation pathways in the rumen (from Chilliard *et al.*, 2007).

Ruminants consume forages and cereal grains. In the rumen fermentation system, the cell wall and soluble carbohydrates of feedstuffs are degraded and fermented into volatile fatty acids. These fatty acids are shorter than 6 carbons long and are the principal energy source of these animals. Intake of diets containing more than 5% fat will inhibit rumen fermentation and are not recommended for ruminants (Dodson *et al.*, 2010). Lipids may be supplied in an animal's diet various forage sources, oil-rich cereals, oilseeds or fish oil, and until recently tallow was offered as a significant energy source (French *et al.*, 2000; Loor, Ueda, Ferlay, Chilliard & Doreau, 2005; Woods & Fearon, 2009). Feeding lipid sources rich in 18:2*n*-6 and 18:3*n*-3 will increase the *c*9,*t*11 isomer content of ruminant meat (Bessa *et al.*, 2005, 2007). However, feeding linseed oil (rich in 18:3*n*-3) seems to be less effective in the increase of *c*9,*t*11 isomer in muscle than sunflower oil (rich in 18:2*n*-6) (Bessa *et al.*, 2007).

Ruminants are generally supplied with unsaturated fatty acids from the forages and it has been well documented that animals consuming fresh grass pasture will have a more desirable fatty acid profile and higher antioxidant content (Daley *et al.*, 2010). In fact, grass intake increases the levels of fat soluble vitamins, mainly α-tocopherol and β-carotene in muscle tissues, which delay lipid oxidation despite the higher concentration of more oxidizable PUFA (Descalzo & Sancho, 2008). Grass is a good source of α-linolenic acid, although there can be variations due to maturity and variety (Boufaied *et al.*, 2003, Dierking, Kallenbach & Grun,

2010). Eriksson and Pickova (2007) indicate that this higher PUFA percentage in meat from pasture-fed bulls could result from the higher protection of fatty acids in fresh grass from the ruminal biohydrogenation, relative to that of grain or silage. The *n*-3 PUFA may be protected from biohydrogenation due to their association with thylakoid membranes of chloroplasts or protection by the cell walls (Wood & Enser, 1997). Moreover, this increase in meat PUFA percentage could also be due to the presence of secondary plant metabolites in spontaneous pastures that might inhibit microbial biohydrogenation activity within the rumen (Lourenço, Van Ranst, Vlaeminck, De Smet & Fievez, 2008). Forage feeding has been described as one of the best strategies to increase *n*-3 PUFA, 18:1*t*11 and *c*9,*t*11 isomer in ruminant milk and meat, partly because forages are a major source of 18:3*n*-3 and, to a lesser extent, of 18:2*n*-6 (Scollan *et al.*, 2006).

When the animals are fed with concentrates based on cereal grains (rich in LA) results in a increase of 18:1*t*10 and *t*10,*c*12 isomer in beef cattle. Dugan *et al.* (2007) reported that the major *trans* isomer in beef produced from a 73% barley grain diet is 18:1*t*10 (2.13% of total lipid) rather than 18:1*t*11 (0.77% of total lipid). This finding is not particularly favourable considering the data that would support a negative impact of 18:1*t*10 on LDL cholesterol and CVD (Dugan *et al.*, 2007). Concentrate diet increases the absorption of 18:1*c*9 and 18:2*n*-6, which are major fatty acids in cereal grains (Jenkins, 1994). However, it was reported that the high level of oleic acid observed in ruminant fed high grain diets might be explained by an increase in Δ9-desaturase activity, probably mediated by a higher production of insulin (Daniel, Wynn, Salter & Buttery, 2004a; Sinclair, 2007). Other works have reported that high levels of concentrate in the diet increases *n*-6/*n*-3 ratio of meat (Alfaia *et al.*, 2006a, Eriksson & Pickova, 2007).

The content and proportions of the individual CLA isomers in ruminant fat is strongly influenced by diet (Nuernberg *et al.*, 2007; Alfaia *et al.*, 2009). Several authors reported that diets containing proportionally high levels of α-linolenic acid, such as fresh grass, grass silage and concentrates containing linseed, resulted in high depositions of *c*9,*t*11 isomer in muscle (French *et al.*, 2000; Dannenberg *et al.*, 2005). Lorenzen *et al.* (2007) reported higher CLA contents, on an mg/kg fat basis, for fed pasture cattle than grain-fed cattle. Rumen pH may help to explain the apparent differences in CLA content between grain and grass-finished meat products (Daley *et al.*, 2010).

Geographic factors may also determine the fatty acid composition of meat as it determines the type of diet available. Recently, Panjono *et al.* (2011) indicated that bovine meat produced on high raising altitude (700 m) had lower SFA and higher MUFA concentrations than from low altitude (100 m). In Barrosã-PDO veal, Costa *et al.* (2011) observed that the farm location altitude slightly affected the fat composition, and still it depends on the muscle considered. In fact, differences on muscle composition from animals produced at distinct altitudes could be related with feed and suckling practices, namely suckling duration prior to slaughter (Moreno *et al.*, 2006). Differences on botanical diversity and availability of the pasture between altitudes could affect the fermentation processes in the rumen and therefore, the fatty acid composition of tissues and milk, including BCFA (Collomb *et al.*, 2008).

Climatic variations affect ruminant meat production in many ways, including direct action upon the animal, whereas other indirect actions are related to types and quantities of feed, life cycles of internal and external parasites and survival of disease organisms (Panjono *et al.*, 2011). Panjono *et al.* (2011) described that the different slaughter seasons between summer and winter affected fatty acid composition in Korean steer. Moreover, their results indicated that meat from summer had a healthier fatty acid composition than that from winter slaughter season. It is well known that slaughter season influence on the IMF composition in Portuguese traditional meats: Carnalentejana-PDO beef (Alfaia *et al.*, 2006a), Mertolenga-PDO beef (Alfaia *et al.*, 2006b), Barrosã-PDO veal (Alfaia *et al.*, 2007a) and Arouquesa-PDO veal (Alfaia *et al.*, 2007b). Alfaia *et al.* (2006b) reported that beef-PDO from June seems to have a fatty acid composition healthier than meat from October.

1.4 Gluconeogenesis precursors as a strategy to improve the nutritional value of ruminant fat

The improvement of beef fatty acid composition would be of direct benefit to human health and to the beef-producing industry (Herdmann *et al.*, 2010). The enhancement of the fatty acid profile of ruminant products can be achieved by three distinct approaches: (i) modification of fatty acid profile during meat or milk processing; (ii) modification through changes in animal diet and iii) animal genetic improvement (Higgs, 2000; Lourenço, Ramos-Morale & Wallace, 2010). Despite rumen microbial activity, increasing dietary PUFA intake enhances the PUFA content of ruminant meat and milk (Scollan *et al.*, 2006). 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 isomers and *n*-3 PUFA (Scollan *et al.*, 2006). Different nutritional strategies have been used, such as forage feeding, supply of vegetable oils or oilseeds, marine products or protected fat sources (Woods & Fearon, 2009). The lipid supplementation can lead to undesirable shifts in rumen microbial composition resulting in accumulation of *trans*-10 biohydrogenation intermediates (BI) (Lourenço *et al.*, 2010). For any dietary strategy to be useful, it must not compromise rumen fermentation and, concomitantly, dry matter intake and animal production and/or performance (Lourenço *et al.*, 2010).

Palmquist, Lock, Shingfield and Bauman (2005) reviewed the effects of nutrition on lipid metabolism in the rumen and resumed them into four main points, which are: i) the biohydrogenation of dietary PUFA is most extensive on high-forage diets based on grass silages; ii) diets containing high proportions of rapidly fermented carbohydrates, low amount of fibre, and/or plant oils or oilseeds promote a less extent biohydrogenation and the accumulation of 18:1 *trans* fatty acid; iii) the conversion of 18:1*trans* intermediates to 18:0 is inhibited more strongly by fish oil or marine lipids rich in 20:5*n*-3 and 22:6*n*-3 than by plant oils and oilseeds; and iv) modifications in the carbohydrate composition and lipid content of the diet markedly affect biohydrogenation fatty acid intermediate profile.

1.4.1 Effects of propionate sources in the diet

In ruminants, propionate serves as an energy source and may also act independently as metabolic mediator of nutritional status. It can contribute up to 60% of the substrate necessary for gluconeogenesis (Drackley, Overton & Douglas, 2001). Considerable energy has to be provided by diets in the form of propionic acid in the rumen or glucose in the intestine. Propionic acid can be supplied by carbohydrate fermentation in the rumen, but excess intake of readily fermented carbohydrate or starch could lead to acidosis and laminitis. Nevertheless, the increased ruminal propionate production from feeding high-concentrate diets or feed additives (*e.g.* monensin) has been associated with the improved animal performance (Liu *et al.*, 2009).

Forage diets generally promote high ruminal production of acetate relative to propionate. A number of studies have reported that addition of gluconeogenesis precursors, such as sodium or calcium salts of propionate and PG increased ruminal propionate production (Clapperton & Czerkawski, 1972; Harmon & Avery 1987; Veenhuizen, Russell & Young, 1988) and

decreased the ratio of acetate to propionate and ruminal pH (Villalba & Provenza 1997; Rigout, Hurtaud, Lemosquet, Bach & Rulquin, 2003). As early as the 1950s, drenching or feeding PG has been shown to effectively treat ketosis in dairy cows due to its glycogenic property (Johnson, 1954). Furthermore, PG is metabolized to propionate in the rumen but can also be converted to glucose via gluconeogenesis in the liver (Nielsen & Ingvartsen, 2004) (Figure 3).

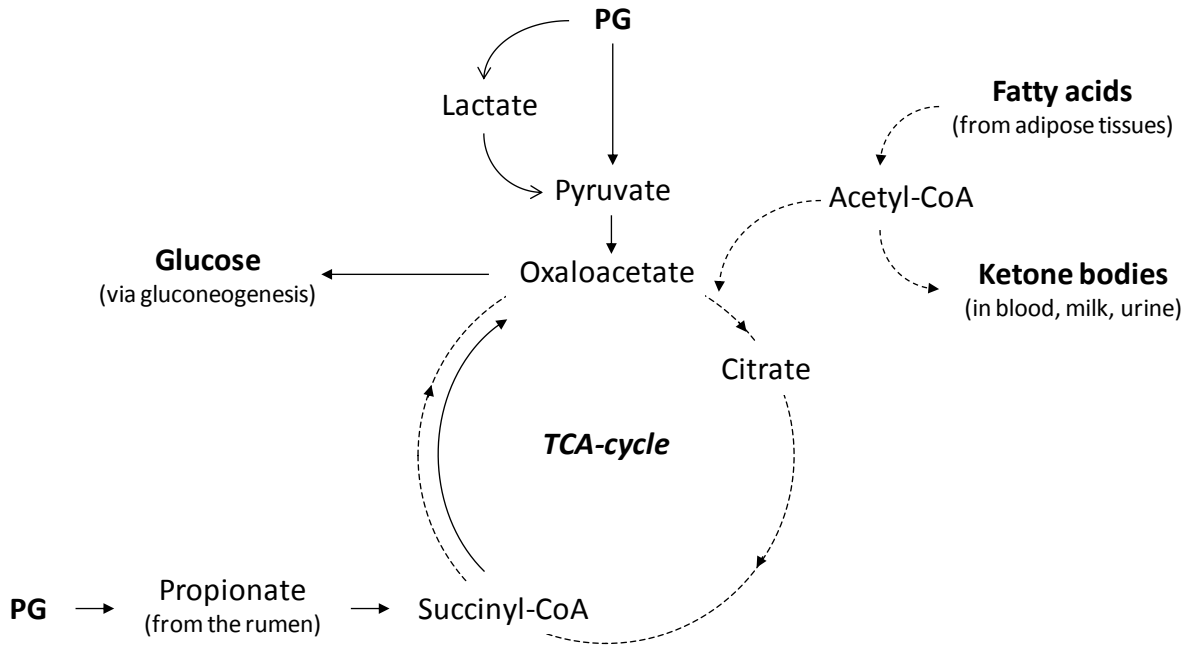

Figure 3 - Metabolism of propylene glycol (PG) and its interaction with the ketogenesis in the liver of cattle. Gluconeogenesis pathways are shown by the solid lines (Adapted from Nielsen & Ingvartsen, 2004).

The hepatic concentration of oxaloacetate is low in ketotic cows and is believed to be the key metabolite in determining whether acetyl-CoA (coenzyme A) enters the TCA-cycle (tricarboxylic acid cycle) or ketogenesis (Krebs, 1966). PG may prevent ketosis by increasing oxidation of acetyl-CoA in the TCA-cycle and by increasing the supply of glucose to the cow (Figure 3). Thus, increased production of glucose will stimulate insulin secretion from the pancreas, which decreases mobilization of fatty acids from adipose tissues and, hence, decreases the substrate for hepatic ketogenesis (Holtenius & Holtenius, 1996).

PG applied by feeding instead of routine oral drenching may be better accepted by dairy producers as a supplement to alleviate negative energy balance and, subsequently, prevent subclinical ketosis that often occurs during the first month of lactation (Chung, Martinez, Brown, Cassidy & Varga, 2009a). Periparturient supplementation of PG by incorporating it into the concentrate has been shown to decrease blood concentrations of non-esterified fatty

acid and β-hydroxybutyrate (Fisher, Erfle & Sauer, 1971; Hoedemaker *et al.*, 2004), and increase blood concentrations of glucose and insulin (Christensen *et al.*, 1997; Nielsen & Ingvartsen, 2004).

The major site for PG metabolism seems to be in the rumen by rumen microorganism rather than in the liver of ruminants (Kristensen *et al.*, 2002). PG is metabolized in the rumen rapidly as it has been shown to disappear from the rumen within 4 h of its infusion (Clapperton & Czerkawski, 1972; Kristensen & Raun, 2007), whereas its intermediary metabolism in the lactating cow is relatively slow (Kristensen *et al.*, 2002; Kristensen & Raun, 2007). Kristensen and Raun (2007) estimated that between 33% and 45% of PG infused into the rumen was metabolized to propanol and propionate within the rumen and 19% of the infused PG escaped rumen degradation and was taken up and oxidized to L-lactate in the liver. Propanol, from hepatic uptake, is metabolized to propionate and this propionate is partly released to the blood (Kristensen & Raun, 2007). The modes of action for PG and its metabolites to affect metabolism of cows, as proposed by Kristensen and Raun (2007), are by: (i) increasing supplies of L-lactate and propionate for hepatic gluconeogenesis and (ii) decreasing glucose utilization by peripheral tissues (mainly skeletal muscle and adipocytes) induced by increased concentrations of blood PG and propanol and/or a decreased ratio of ketogenic to glucogenic metabolites in the blood.

Insulin is involved in the coordination of nutrient partitioning through its central role in the regulation of glucose and energy homeostasis. Propionate and glucose are secretagogues for pancreatic release of insulin. Low-fibre diets result in increased rumen production of propionate (Sutton, 1985) and hepatic rates of gluconeogenesis (Annison, Bickerstaffe & Linzell, 1974). Daniel *et al.* (2004b) shown that insulin increases the expression of ovine SCD and synthesis of MUFA from acetate in cultured ovine adipose tissue explants. Consequently, Daniel *et al.* (2004a) showed that lambs fed concentrate had higher SCD gene expression and, consequently, higher oleic acid content in tissues than forage fed lambs. However, in spite of the downregulation of SCD on forage fed lambs, the rumenic acid content in tissues was higher than in concentrate fed lambs. Thus, it would be important to achieve simultaneously high availability of 18:1*t*11 and up-regulation of SCD in order to obtain high concentrations of rumenic acid in lamb meat. In forage fed lambs the availability of 18:1*t*11 for absorption is much higher than in concentrate fed lambs where the 18:1*t*10 predominates due to the changes in rumen biohydrogenation pathways (Griinari & Bauman, 1999). The most effective dietary approach to increase metabolic availability of 18:1*t*11 is supplementing high forage

diets with oils rich in PUFA (Bessa *et al.* 2000). As the rumenic acid is not an intermediate of the 18:3*n*-3 ruminal biohydrogenation (Harfoot and Hazlewood, 1997), the rumenic acid increase in tissues of supplemented ruminants with linseed oil (rich in 18:3*n*-3) is mainly due to the endogenous production through SCD desaturation of 18:1*t*11.

Kim *et al.* (2005) evaluated the efficacy of PG feeding once daily for 4 months, before slaughter at market live weights, in the increase of marbling scores in Korean native steers and the effect on related gene expression (leptin mRNA and IGF-1 mRNA). This study indicates that the amount of PG fed to steers was not sufficient to improve marbling score through ruminal propionate and insulin enhancement. The role of PG in fatty acid profile in ruminant meat remains to be further elucidated.

1.5 Research objectives

Ruminant products, such as meat and milk, constitute important foods for the human diet. The fatty acid composition of these animal products depends on many factors, such as diet, seasonal variation, muscle type and others. The overall aim of the current study was to evaluate the seasonal changes and muscle type effect on the nutritional value of ruminant meat from traditional and organic production systems by evaluating fatty acid composition, including CLA isomers, cholesterol and lipid-soluble antioxidant vitamins. Furthermore, the current knowledge clearly demonstrates that certain feed components (*e.g.* gluconeogenesis precursors) are necessary to positively influence CLA content in the meat fatty acids.

The specific objectives of the research can be outlined as follows:

- To characterize the lipid composition and nutritional quality of IMF in a traditional Portuguese bovine veal (Mirandesa-PDO), regarding the influence of slaughter season, (autumn and spring, with the least and the most abundant green pastures, respectively) and muscle type (*longissimus lumborum* and *semitendinosus* muscles, relatively red and white, respectively) on its lipid composition (chapter 2).
- To assess the effect of muscle type (*longissimus lumborum* and *semitendinous* muscles) in a Portuguese bovine beef (Charneca-PDO), characterizing the fatty acid composition and nutritional quality of IMF (chapter 3).
- To study the lipid composition and nutritional quality of intramuscular fat in organic meat (grass-based diet) regarding the influence of slaughter season (autumn and spring) with the least and the most abundant green pastures, respectively) and muscle type (*longissimus lumborum* and *semitendinosus* muscles) on its lipid composition (chapter 4).
- To determine the influence of dietary gluconeogenesis precursors that can be added to a forage diet supplemented with linseed oil without reducing 18:1*t*11 availability. It was also investigated if the dietary gluconeogenesis precursors enhanced the SCD desaturase activity resulting in increased oleic and rumenic acids contents in meat (chapter 5).
- To compare the results found for lipid composition and nutritional quality of IMF in these meats with other Portuguese PDO meats (chapter 6).

CHAPTER 2 | Seasonal changes and muscle type effect on the nutritional quality of intramuscular fat in Mirandesa-PDO veal

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Abstract

The influence of slaughter season and muscle type on the detailed fatty acid composition, including conjugated linoleic acid isomers, and contents of total cholesterol and lipid-soluble antioxidant vitamins (α-tocopherol and β-carotene) in Mirandesa-PDO veal was assessed. Mirandesa purebred calves (*n*=29) were raised in a traditional production semi-extensive system, slaughtered in late spring (June) or early autumn (October) and the *longissimus lumborum* and *semitendinosus* muscles were sampled for analysis. Although the lipid composition of PDO veal was only slightly affected by the slaughter season, it was markedly changed by the muscle type. However, PDO veal had values of pasture-fed cattle for lipid grass intake indicators, in both seasons and muscles. From a human health standpoint, IMF in Mirandesa-PDO veal has a high nutritional value throughout the year, with favourable ratios of *n-6*/*n-3* and contents of *n-3* PUFA and α-tocopherol, as a result of the beneficial effects of grass feeding.

Keywords: Veal; Fatty acids; CLA isomers; Cholesterol; α-Tocopherol; β-Carotene.

2.1 Introduction

The contribution of meat to dietary intake of fat and fatty acids has been of special concern for consumers and, consequently, for meat industry. Indeed, consumers are becoming more and more aware of the relationship between meat consumption, especially red meat, and the amount of SFA in human diet because of the possible raise of total and LDL-cholesterol, a risk factor for CVD (Hocquette *et al.*, 2010).

Thus, the Food and Agriculture Organization of United Nations (FAO), the WHO (Burlingame *et al.*, 2009) and, more recently, the EFSA (2010) reviewed the specific guidelines for fat and fatty acids for human intake. Among the recommendations, a reduction in the intake of SFA, TFA and cholesterol, as well as an increase in the intake of *n*-3 PUFA, in particular EPA and DHA, are particularly encouraged (Daley *et al.*, 2010). Additional health benefits from the consumption of meat lipids may be related with CLA isomers. CLA comprises a family of natural fatty acids found primarily in beef and dairy products. The major CLA isomer, the *cis*9,*trans*11 18:2 (*c*9,*t*11) commonly known as rumenic acid, mostly synthesized by ruminal bacterial biohydrogenation of dietary C18 PUFA or in tissues by Δ9 desaturation of 18:1*t*11 (Griinari & Bauman, 1999). CLA has been recognized to exert protective effects against a huge range of biological properties, including but not limited to effects on inflammation, lipid metabolism, carcinogenesis and atherosclerosis (Prates & Bessa, 2009).

Researches from different countries are trying to improve the lipid profile of meats offering consistently high meat quality (Razminowicz *et al.*, 2006; Muchenje *et al.*, 2009b). Several factors in beef production affect fatty acid composition, including breed and diet (Warren *et al.*, 2008). Beyond genetic effects, IMF content and composition of meat is mainly influenced by the feeding system, age, slaughter weight and duration of milk consumption. Dietary factors are often linked with a particular production system (Geay, Bauchart, Hocquette, & Culioli, 2001; Moreno *et al.*, 2006). Cattle produced under grass-based systems are associated with a more desirable fatty acid profile and higher antioxidant content (Daley *et al.*, 2010). It is well known that feeding forages to ruminants increases the *n*-3 PUFA and MUFA content in milk and meat as they are natural rich sources of 18:3*n*-3 than those receiving a cereal-based concentrate diet (Lourenço *et al.*, 2008).

Moreover, pasture intake increases the levels of fat-soluble vitamins, mainly α-tocopherol and β-carotene, in muscle tissues, which delay lipid oxidation despite the higher concentration of more oxidizable PUFA (Descalzo & Sancho, 2008). Meat also provides from one-third to one half (Chizzolini *et al.*, 1999) of the maximum recommended intake of cholesterol *per* day (300 mg) (American Heart Association, 2008). Pasture-based production systems are characterized by seasonal and geographic variations, due to the abundance and botanical diversity of pasture, and it is expected that meat lipids from these animals would reflect this variability.

In addition, the distinctive characteristics of muscle fibres can affect meat quality. In fact, muscles are composed by different proportions of muscle fibres types (oxidative or glycolytic), which may influence the IMF content (Hocquette *et al.*, 2010).

Local bovine breeds reared in Mediterranean areas have received a renewed interest due to the European Union policy of promoting and supporting the production of indigenous cattle well adapted to the environment being commercialised with a "certification of origin", for instance as PDO (Costa *et al.*, 2008). This is a consumer´s guarantee that the meat was produced in a specific, delimited geographic region and following the rules established (Commission Regulation, 1996). In Portugal, veal production has been based on local autochthonous breed produced under semi-extensive conditions. Mirandesa-PDO veal is the most important commercial Portuguese veal (247 carcass tons in 2007), obtained from Mirandesa purebred calves, produced in the northeast of Portugal, with an increasing demand by the consumers notwithstanding its higher price (IDRH, 2009; Costa *et al.*, 2011). Although this veal is produced throughout the year, the main slaughter seasons are spring and autumn. Nowadays, Mirandesa-PDO veal is of utmost importance regarding the commercialization of high quality meat, 5-10% is exported for EU countries and 90-95% is marketed in Portugal. Thus, the objective of the current study was to evaluate seasonal changes and carcass variation on the nutritional value of IMF in Mirandesa-PDO veal by assessing fatty acid composition, including CLA isomers, total cholesterol and lipid-soluble antioxidant vitamins.

2.2 Material and methods

2.2.1 Animals, management and sample collection

Mirandesa purebred calves (*n*=29) were maintained according to the traditional production pasture-based system under the Mirandesa-PDO product specifications. Mirandesa-PDO meat combines the specific genetic features of Mirandesa calves with the use of extensive natural resources in the north-eastern area of Portugal. Calves came from different representative private farms, and they are kept in barns, suckling their mothers until weaning (7 months of age) and then supplemented with farm products such as hay, straws (oat, barley or wheat) supplemented with local feeds like squash and potatoes (Galvão *et al.*, 2005; ACBRM, 2007) depending on the farm location) and concentrate (Table 2). However, the climatic environment differs with farm and can shorten the availability of grass and forage, so that it becomes necessary to increase the concentrated level (approximately 500 g daily) to finish the calves. After weaning, calves were maintained in the early-middle spring grass and slaughtered (Industrial Abattoir of Cachão, Mirandela, Portugal) in June 2008 (late spring sampling; *n*=14; mean ± standard error of age and live body weight were 8.8 ± 0.7 months and 135 ± 27 kg) or were raised on a late summer pasture until slaughtered in October 2008 (early autumn sampling; *n*=15; 8.5 ± 0.7 months and 132 ± 28 kg). After slaughter, carcasses were chilled at 7-10 °C for 48 h until sampling. Carcasses were classified using the SEUROP classification scales for conformation (P poor, O fair, R good, U very good, E excellent, and S superior) and for the fat cover (1 low, 2 slight, 3 average, 4 high, and 5 very high). Most of the carcasses were graded as O for conformation and 2 for fatness.

Meat samples were taken from the loin portion of *longissimus dorsi* (L4-L6 *longissimus lumborum* muscle, LL) and distal region of *semitendinosus* (ST) muscles of the left side of the carcasses. All meat samples were collected 2-3 days after slaughter (+1 ºC), ground using a food processor (3 × 5 s), vacuum packed and stored at –80 °C until required for analysis.

Table 2 - Ingredient (% feed), proximate (% feed) and fatty acid (% total fatty acids) composition of the concentrate feed from Mirandesa purebred calves reared according to Mirandesa-PDO veal specifications (*n*=2).

Ingredients	
Barley	44
Soybean meal	21
Corn	20
Rye	7
Mineral and premix	5
Molasses	3
Proximate composition	
Crude protein	16.9
Total fat	4.0
Crude fibre	4.4
Ashes	8.1
Fatty acid composition	
16:0	28.1
18:0	10.9
18:1	13.6
18:2*n*-6	38.6
18:3*n*-3	3.7

2.2.2 Lipid extraction and methylation

IMF was extracted from lyophilized (–60 ºC and 2.0 hPa, Edwards High Vacuum International, UK) meat samples for total lipid determination and for both fatty acid methyl esters (FAME) and CLA methyl esters profiles, using the procedures described previously by Alfaia *et al.* (2006). Total lipids were measured gravimetrically, in duplicate, by weighting the fatty residue obtained after solvent evaporation. Fatty acids and CLA isomers were converted to methyl esters by a sequential transesterification reaction with NaOH in anhydrous methanol (0.5 M), followed by HCl:methanol (1:1 v/v), at 50 ºC during 30 and 10 min, respectively, according to Raes, De Smet, & Demeyer (2004).

2.2.3 Determination of fatty acid composition

FAME were analysed by GC-FID (Gas chromatography- flame ionization detector) (chromatograph HP 6890; Hewlett–Packard, Avondale, PA, USA) using a fused-silica 100 m capillary column (CP-Sil 88^{TM}; 0.25 mm i.d., 0.2 µm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA), as described by Alves and Bessa (2009). Identification of common fatty acids was accomplished by comparison of sample peak retention times with those of FAME standard mixtures (Nu-Chek-Prep Inc., Elysian, MN, USA; Supelco Inc. Bellefonte, PA, USA) and by using published chromatograms obtained with similar analytic conditions

(Kramer, Hernandez, Cruz-Hernandez, Kraft, & Dugan, 2008). Structural analyses of some unknown peaks were conducted by GC-MS/MS technique, using a Varian Saturn 2200 system (Varian Inc.) equipped with a CP-Sil 88^{TM} capillary column (100 m × 0.25 mm i.d., 0.2 μm film thickness). Quantification of total FAME was done using nonadecanoic acid as internal standard. Results for each fatty acid were expressed as a percentage of the sum of detected fatty acids (total fatty acids).

2.2.4 Determination of individual CLA isomers

The methyl esters of CLA isomers were individually separated by triple silver-ion columns in series (ChromSpher 5 Lipids, 250 mm × 4.6 mm i.d., 5 μm particle size, Chrompack, Bridgewater, NJ, USA), using an high performance liquid chromatography system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA), according to the procedure reported previously by Alfaia *et al.* (2006). The identification of the individual CLA isomers was achieved by comparison of their retention times with commercial and prepared standards, as well as with values published in the literature (Prates & Bessa, 2009). Standards of CLA isomers (*c*9,*t*11, *t*10,*c*12, *c*11,*t*13, *c*9,c11 and *t*9,*t*11) were purchased from Matreya Inc. (Pleasant Gap, PA, USA) and Sigma Inc. (St. Louis, MO, USA) or prepared (*cis/trans* and *trans,trans* from carbons 7,9 to 12,14) by the procedure reported by Destaillats and Angers (2003). Total CLA content was calculated as the sum of its main isomer *c*9,*t*11 (plus *t*7,*c*9 and *t*8,*c*10) determined by GC-FID with the other identified minor isomers quantified by Ag^{+}-HPLC analysis (Kraft, Collomb, Mockel, Sieber, & Jahreis, 2003). The individual CLA isomers were expressed as a percentage of the sum of identified CLA isomers (% total CLA).

2.2.5 Determination of cholesterol, tocopherols and β-carotene

The simultaneous analysis of total cholesterol, tocopherols and β-carotene in meat was performed according to Prates, Quaresma, Bessa, Fontes and Alfaia (2006). After, the saponification of homogenized fresh meat samples (*ca* 750 mg) an aliquot of the *n*-hexane layer was filtered through a 0.45 μm hydrophobic membrane and injected in an HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, U.S.A.) using a normal-phase silica column (Zorbax RX-Sil, 250 mm × 4.6 mm i.d., 5 μm particle size, Agilent Technologies Inc., Palo Alto, CA, U.S.A.), with fluorescence detection for tocopherols (excitation wavelength of 295 nm and emission wavelength of 325 nm) and ultraviolet (UV)-visible photodiode array detection for cholesterol (202 nm) and β-carotene (450 nm) in series.

The contents of total cholesterol, tocopherols and β-carotene in meat were calculated, in duplicate for each muscle sample, based on the external standard technique from a standard curve of peak area *vs.* concentration using DL-α-, D-β-, D-γ- and D-δ-tocopherols (Calbiochem, Merck Biosciences, Darmstadt, Germany), all-*trans*–carotene and cholesterol standards (Sigma Chemical Co., St. Louis, MO, USA).

2.2.6 Statistical analysis

The statistical analysis was accomplished using the MIXED procedure of the Statistical Analysis Systems Institute, version 9.1 (SAS, 2004). The model considers the effects of slaughter season (late spring and early autumn), muscle type (LL and ST) and the interaction between slaughter season and muscle type. Since measurements on different muscles from the same animal are not independent observations, muscle type was treated as repeated measure on animal within slaughter season group as subject. Least square means were presented and compared using the LSMEANS/PDIFF option when interaction effect was significant ($P<0.05$). Means were reported as least square means with standard error of mean (SEM).

2.3. Results and discussion

2.3.1 Intramuscular fatty acid composition

The influence of slaughter season (late spring and early autumn) and muscle type (LL and ST muscles) on total lipids (mg/g muscle) and fatty acid composition (weight %) in Mirandesa-PDO veal is shown in Table 3. Mirandesa-PDO veal showed values of total lipids (0.7-1.8%) closer to those reported for meat from extensively (1.1-1.4%) rather than intensively (1.8-2.7%) produced cattle (Vestergaard *et al.*, 2000b), which is in agreement with the production system and may also outcome from genetic specificities of fat deposition (De Smet *et al.*, 2004) in Mirandesa breed calves. In contrast, the values of total lipids obtained for Barrosã-PDO veal (Alfaia *et al.*, 2007a) and Arouquesa-PDO veal (Alfaia *et al.*, 2007b) were higher (1.6-2.3% and 1.7-3.0%, respectively) compared to those of Mirandesa-PDO veal and closer to those of meat from intensively produced cattle. Based on the Food Advisory Committee (1990) criteria (<5% fat), Mirandesa-PDO veal is considered a lean meat. Total lipid content was higher ($P<0.001$) in LL muscle, compared with the ST muscle, but was not affected by the slaughter season. It is well known that IMF content varies among species, breeds and muscle type in the same breed (Hocquette *et al.*, 2010). According the former authors, other

factors are involved in the variation of IMF content in animals, including gender, age and feeding. Regarding muscle fibre composition, it is well established that lipid content is higher in red oxidative muscle fibres and the loin portion of LD muscle of cattle is relatively red and differently involved in the physical activity imposed by grazing in comparison with the ST muscle (Vestergaard, Oksbjerg, & Henckel, 2000a).

The major fatty acids in IMF were 16:0 (20-24%), 18:0 (11-15%) and 14:0 (1.6-3.5%) as SFA, which together comprise more than 40% of total FAME. Among MUFA, the most prevalent fatty acids were 18:1*c*9 and 16:1*c*9, contributing more than 86% and 7% of MUFA, respectively. Regarding PUFA, the predominant fatty acids were 18:2*n*-6 (LA, 4.3-10.2% of total FAME) followed by 20:4*n*-6 (1.4-4.4%). Our results are in agreement with mostly previously reported data (*e.g.* Eriksson, & Pickova, 2007; Muchenje *et al.*, 2009a). In addition, similar results were reported by Galvão *et al.* (2006) for a non-detailed fatty acid composition in Mirandesa-PDO veal, in spite of the slight differences between the three farms analysed.

Seasonal variations ($P<0.05$) in the fatty acid composition of Mirandesa-PDO veal were observed for 16:1*c*7 (in LL muscle), 17:1*c*9 (in LL muscle), 18:3*n*-3, 18:3*n*-6, 22:6*n*-3 and total *n*-3 PUFA. The percentages of total *n*-3 PUFA were higher ($P<0.001$) in meat from late spring than in that from early autumn. Mirandesa-PDO veal from early autumn had lower percentages ($P<0.01$) of ALA (18:3*n*-3) (0.44-0.91% of total FAME), in which grass lipids are rich (Alves, & Bessa, 2009), when compared with that from late spring (0.81-1.32%). The meat ALA values from late spring are close to those previously described for pasture-fed cattle (1.3%) (Realini *et al.*, 2004). The influence of slaughter season on fatty acid composition is not surprisingly if we consider the seasonal differences in pasture nutritive value and availability (Santos, 2006). In fact, the animals slaughtered in October were exposed to the less abundant and more mature late summer-early autumn pastures and suckled the milk produced during the summer pasture scarcity, while the calves slaughtered in June were exposed to the more abundant and less mature spring pastures and suckled the milk obtained during the late winter-spring richest pastures.

Table 3 - Total lipids (mg/g muscle), fatty acid composition (g/100 g total fatty acids) of *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of Mirandesa-PDO veal from late spring and early autumn.

	Spring		Autumn			Significance level		
	LL	ST	LL	ST	SEM	Season	Muscle	S×M
Total lipids	17.54	8.05	18.49	6.94	1.375	ns	***	ns
Fatty acid composition								
14:0	3.16	1.61	3.48	1.47	0.213	ns	***	ns
14:1*c*9	0.48	0.31	0.42	0.25	0.051	ns	***	ns
15:0	0.42^{a}	0.33^{b}	0.44^{a}	0.28^{b}	0.024	ns	***	*
15:0 *anteiso*	0.18^{a}	0.11^{b}	0.19^{a}	0.10^{a}	0.011	ns	***	*
15:0 *iso*	0.15	0.09	0.16	0.07	0.011	ns	***	ns
16:0	23.92	19.46	23.95	18.86	0.471	ns	***	ns
16:0 *iso*	0.17^{a}	0.13^{b}	0.16^{a}	0.10^{b}	0.009	ns	***	*
16:1*c*7	0.23^{b}	0.22^{b}	0.27^{a}	0.22^{b}	0.010	*	**	*
16:1*c*9	2.88	1.99	2.74	1.75	0.167	ns	***	ns
16:1*t*9	0.06	0.11	0.05	0.09	0.010	ns	***	ns
17:0	0.73	0.59	0.77	0.53	0.038	ns	***	ns
17:0 *anteiso*	0.58	0.37	0.59	0.33	0.029	ns	***	ns
17:0 *iso*	0.34	0.35	0.33	0.31	0.019	ns	ns	ns
17:1*c*9	0.17^{b}	0.54^{a}	0.54^{a}	0.45^{a}	0.032	***	***	***
18:0	14.16	11.27	14.76	11.59	0.382	ns	***	ns
18:1*c*9	30.85	22.17	31.44	22.99	0.875	ns	***	ns
18:1*c*12	0.21	0.20	0.21	0.15	0.015	ns	*	ns
18:1*c*13	0.20	0.15	0.16	0.09	0.023	ns	***	ns
18:1*c*15	0.06^{b}	0.05^{b}	0.09^{a}	0.04^{b}	0.008	ns	***	*
18:1*t*6+*t*8	0.11	0.09	0.08	0.09	0.011	ns	ns	ns
18:1*t*9	0.16	0.10	0.18	0.11	0.012	ns	***	ns
18:1*t*10	0.14	0.08	0.18	0.16	0.043	ns	ns	ns
18:1*t*11	0.65	0.44	0.80	0.39	0.065	ns	***	ns
18:1*t*12	0.17	0.15	0.12	0.15	0.021	ns	ns	ns
18:1*c*11+*t*15	1.77	1.89	1.70	1.74	0.069	ns	ns	ns
18:1*t*16+*c*14	0.17	0.11	0.18	0.10	0.012	ns	***	ns
18:2*c*9,*t*11†	0.30	0.23	0.31	0.19	0.026	ns	***	ns
18:2*t*11,*c*15	0.10	0.07	0.07	0.07	0.010	ns	ns	ns
18:2*n-6*	4.26	9.38	4.32	10.15	0.558	ns	***	ns
18:3*n-3*	0.81	1.32	0.44	0.91	0.096	**	***	ns
18:3*n-6*	0.01^{c}	0.01^{c}	0.04^{b}	0.10^{a}	0.004	***	***	***
18:3*c*9,*t*11,*c*15	0.13	0.28	0.12	0.33	0.028	ns	***	ns
20:0	0.09	0.10	0.10	0.10	0.009	ns	ns	ns
20:2*n*-6	0.07	0.13	0.06	0.11	0.008	ns	***	ns
20:4*n*-6	1.37	3.92	1.37	4.43	0.248	ns	***	ns
20:5*n*-3	0.42	1.40	0.31	1.32	0.128	ns	***	ns
22:0	0.32	0.85	0.33	1.02	0.056	ns	***	ns
22:4*n*-6	0.10^{c}	0.23^{b}	0.12^{c}	0.31^{a}	0.024	ns	***	*
22:5*n*-3	0.67	1.89	0.63	2.10	0.127	ns	***	ns
22:6*n*-3	1.97	2.83	0.09	0.43	0.211	***	**	ns
23:0	0.11	0.15	0.08	0.13	0.016	ns	**	ns

^{a,b,c} Least square means in the same row with different superscripts are significantly different (P<0.05); Significance: ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001; SEM, standard error of mean. The symbols used mean as follow: S×M, interaction between slaughter season (S) and muscle type (M); † This peak also includes minor amounts of the *t*7,*c*9 and *t*8,*c*10 CLA isomers.

Most of the fatty acids (26 in 41 identified) was influenced ($P<0.05$) by muscle type. The LL muscle, compared to the ST muscle, showed higher percentages of the individual SFA (except for 22:0 and 23:0) and total SFA proportion. Increases in dietary levels of saturated fat, particularly 12:0, 14:0 and 16:0, have been identified as the major dietary factors responsible for raising total and LDL-cholesterol concentrations in serum (Howell, McNamara, Tosca, Smith, & Gaines, 1997; Hu, Manson, & Willett, 2001). A significant source of variation on individual and total *cis* MUFA proportion among muscles was also detected.

Indeed, ST muscle had lower percentages of 14:1*c*9, 16:1*c*9, 18:1*c*9, 18:1*c*13 and 18:1*c*12. In addition, considering that *trans* 18:1 isomers are ruminal BI of C18 PUFA with biological relevance, the detailed composition of meat in these fatty acids could be of interest. Overall, LL muscle had higher ($P<0.001$) relative proportions of individual *trans* 18:1 (18:1*t*9, 18:1*t*11 and 18:1*t*16+*c*14) and total TFA but lower values of 16:1*t*9 compared to ST muscle. It is well known that grass-feeding promotes the accumulation of VA, 18:1*t*11, which can be converted to *c*9,*t*11 CLA isomer by delta9 desaturase in adipose tissue and mammary gland (Turpeinen *et al.*, 2002). In contrast, the major *trans* 18:1 isomer in grain-fed beef is 18:1*t*10, rather than 18:1*t*11, which has a negative impact on LDL-cholesterol and CVD (Dugan, Rollan, Aalhus, Aldai, & Kramer, 2008). The *c*9,*t*11 isomer, which co-elutes with minor amounts of the *t*7,*c*9 and *t*8,*c*10 isomers, was the only CLA isomer detected by GC-FID in all samples evaluated.

Regarding PUFA, the ST muscle also had higher values ($P<0.05$) of LC-PUFA compared to LD muscle, which are formed endogenously by the action of delta5 and delta6 desaturases and elongase from the precursors 18:2*n*-6 and 18:3*n*-3 (Wood *et al.*, 2008). Similar percentages of these families of fatty acids were reported for pasture-fed cattle (*e.g.* Realini *et al.*, 2004; Garcia *et al.*, 2008). The pattern obtained for the different partial sums of PUFA reflect the values described above for the major individual fatty acids of each group. The results for partial sums (% w/w) of intramuscular fatty acids in Mirandesa-PDO veal obtained in the two distinct slaughter seasons are presented in Table 4.

In fact, the ST muscle, from both slaughter seasons, had higher relative proportions of total PUFA, including *n-6* PUFA and *n-3* PUFA ($P<0.001$). Since SFA and PUFA are much more abundant in the triacylglycerol and phospholipid fractions, respectively (Wood *et al.*, 2004), the muscle type effect obtained for these partial sums of fatty acids reflects distinct triacylglycerol/phospholipid ratios between muscles, as a consequence of the different fat level (see Table 4). As fatness increases, increments in the content of SFA and MUFA are

larger than that of PUFA (De Smet *et al.*, 2004). These differences between muscles, as mentioned above, likely result from distinct percentages in muscle fibre type (Wood *et al.*, 2004), because LD muscle of cattle is relatively red (25%-31% of type I fibres) in comparison with ST muscle (17%-22% of type I fibres) (Vestergaard *et al.*, 2000a; Costa *et al.*, 2008).

Significant interactions between slaughter season and muscle type were observed for the percentage of some minor individual fatty acids (15:0 *anteiso*, 15:0, 16:0 *iso*, 16:1*c*7, 17:1*c*9, 18:1*c*15, 18:3*n*-6 and 22:4*n*-6). These interactions may result from modifications of muscle metabolic type caused by adaptations to the distinct grazing periods (summer and spring), which are associated with differences in fatty acid composition (Klont *et al.*, 1998). A significant interaction was also found for total BCFA. These BCFA of meat, together with the odd-chain fatty acids, are useful indicators of rumen activity.

The slaughter season of Mirandesa calves did not affect ($P>0.05$) the meat PUFA/SFA ratio. According to Realini *et al.* (2004), the PUFA/SFA ratio is generally increased with pasture feeding. Our values for PUFA/SFA ratio (0.17-0.63) are higher to those reported for IMF of pasture-fed Rubia Gallega breed calves (0.15-0.20) (Moreno *et al.*, 2006), as well as for Barrosã-PDO veal (Alfaia *et al.* 2007a) and Arouquesa-PDO veal (Alfaia *et al.* 2007b). In addition, Raes *et al.* (2004) suggested that this ratio is mainly influenced by genetics and much less by nutrition. These differences may be explained by the high variation in desaturase activity related with the anatomical location of the fat, as reported by Moibi and Christopherson (2001). The ST muscle had higher values of PUFA/SFA ratio for both slaughter seasons (0.59-0.63), and thus more favourable for human health, when compared with the LD muscle (0.17-0.23).

Regarding the *n*-6/*n*-3 ratio, the LL muscle from early autumn had higher values ($P<0.05$) than that from late spring and that ST muscle from both slaughter seasons. These values in meat from animals slaughtered in autumn are slightly higher than those reported for Barrosã-PDO veal (Alfaia *et al.*, 2007a) and Arouquesa-PDO veal (Alfaia *et al.*, 2007b). Moreover, the levels herein discussed for *n*-6/*n*-3 ratio (1.6-4.4) are higher than those obtained for LD muscle of pasture-fed Spanish Rubia Gallega breed calves (1.1-1.6) (Moreno *et al.*, 2006) and for grass-fed beef (2.8) (Leheska *et al.*, 2008). This ratio was consistently below 1.3 in silage-fed steers compared to those from concentrate-fed, which vary from 9 to 16 (Warren *et al.*, 2008). The use of cereals (rich in *n*-6 PUFA) in concentrate shifts meat fatty acid composition to increased *n*-6/*n*-3 ratio, when compared to animals produced on green pastures (rich in *n*-3

PUFA) (Nuernberg *et al.*, 2002). In grass-fed cattle, particularly with high IMF levels, it is apparently difficult to achieve a high PUFA/SFA ratio, whilst keeping *n*-6/*n*-3 low, probably due to a high degree of biohydrogenation of dietary PUFA, leading to SFA production in the rumen and absorption in the small intestine (Warren *et al.*, 2008).

The desaturation indices of SCD were calculated according to Malau-Aduli, Siebert, Bottema and Pitchford (1998) in order to estimate the activity of this enzyme (Table 4). Slaughter season of Mirandesa calves did not affect (P>0.05) the indices of SCD activity. On the contrary, a great difference in SCD activity was observed between the muscles analysed, except when estimated by the conversion of 18:1*t*11 into *c*9,*t*11 CLA isomer (ID18:1*t*11). The desaturation of 14:0 was higher (P<0.01) in the ST muscle, in contrast to the desaturation of 16:0 and 18:0, compared to the LL muscle. We speculate that this difference could be explained by genetic polymorphisms of the SCD between both muscles (Barton *et al.*, 2010).

Table 4 - Partial sums of fatty acids (% w/w) and related indices of *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of Mirandesa-PDO veal from late spring and early autumn.

	Spring		Autumn			Significance level		
	LL	**ST**	**LL**	**ST**	**SEM**	**Season**	**Muscle**	**S×M**
Partial sums								
SFA	42.9	34.37	43.91	33.99	0.678	ns	***	ns
MUFA	35.08	25.63	35.89	25.94	1.060	ns	***	ns
TFA	3.76	3.56	3.81	3.43	0.113	ns	***	ns
PUFA	9.67	21.1	7.38	19.87	1.002	ns	***	ns
BCFA	1.40[a]	1.04[b]	1.42[a]	0.91[b]	0.062	ns	***	*
n-6 PUFA	5.8	13.66	5.87	15.1	0.796	ns	***	ns
n-3 PUFA	3.87	7.44	1.48	4.77	0.407	***	***	ns
Unidentified	7.19	14.31	7.58	15.86	0.629	ns	***	ns
Fatty acid ratios								
PUFA/SFA	0.23	0.63	0.17	0.59	0.035	ns	***	ns
n-6/*n-3*	1.58[d]	1.96[c]	4.35[a]	3.37[b]	0.296	***	*	***
Indices of delta9 desaturase								
ID14:0	12.91	17.27	10.29	15.36	1.670	ns	**	ns
ID16:0	10.69	8.99	10.25	8.34	0.518	ns	***	ns
ID18:0	68.53	65.94	68.03	66.22	0.979	ns	***	ns
ID18:1*t*11	30.87	33.5	30.33	32.46	2.140	ns	ns	ns

[a,b,c,d] Least square means in the same row with different superscripts are significantly different (P<0.05); Significance: ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001; SEM, standard error of mean. The symbols used mean as follow: S×M, interaction between slaughter season (S) and muscle type (M); SFA = sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0; MUFA = sum of 14:1*c*9, 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*12, 18:1*c*13 and 18:1*c*15; TFA = sum of 16:1*t*9, 18:1*t*6+*t*8; 18:1*t*9, 18:1*t*10, 18:1*t*11, 18:1*t*12, 18:1*c*11,*t*15, 18:1*t*16+*c*14, 18:2*t*11,*c*15, 18:2*c*9,*t*11 and 18:3*c*9,*t*11,*c*15; PUFA = sum of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-3 and 22:6*n*-3; BCFA = sum of 15:0 *iso*, 15:1 *anteiso*, 16:0 *iso*, 17:0 *iso* and 17:0 *anteiso*; *n*-6 PUFA = sum of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:4*n*-6 and 22:4*n*-6; *n*-3 PUFA = sum of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3; PUFA/SFA = [(sum of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-3 and 22:6*n*-3)/(sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0)]; *n*-6/*n*-3 = *n*-6/*n*-3 ratio [(sum of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:4*n*-6 and 22:4*n*-6)/(sum of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3)]; Indices of delta9 desaturase activity: ID14:0=(14:1*c*9×100)/(14:0+14:1*c*9); ID16:0=(16:1*c*9×100)/(16:0+16:1*c*9); ID18:0=(18:1*c*9×100)/(18:0+18:*1c*9); ID18:1*t*11=(18:2*c*9,*t*11×100)/(18:1*t*11+18:2*c*9,*t*11).

2.3.2 Intramuscular CLA isomeric profile

Data on the contents and detailed isomeric profile of CLA in Mirandesa-PDO veal are displayed in Table 5. A significant interaction ($P<0.05$) between the slaughter season and muscle type was obtained for total CLA (mg/g muscle) and specific CLA (mg/g fat) contents. However, the LL muscle had higher ($P<0.05$) total CLA contents relative to the ST muscle in both slaughter seasons. The variation in total CLA contents is due to differences in IMF level among muscles (see Table 5), as showed by the positive correlations between these two parameters (correlation coefficients of 0.699 and 0.671 for LL and ST, respectively, $n=29$, $P<0.001$). The specific CLA contents varied from 1.6-3.2 mg/g fat, having the LL muscle from spring the highest ($P<0.05$) value. Previous works found specific CLA contents variation from 3.9 to 4.4 mg/g fat in meat from crossbred bullocks fed intensively with cereal-rich concentrates (Alfaia *et al.*, 2006) to relatively high values (6.8-8.9 mg/g fat) in meat from pasture-fed calves (Alfaia *et al.*, 2007a,b), which were raised on good quality pastures (relatively abundant green pastures) and suckled milk from mothers fed with those pastures.

Table 5 - Total (mg/g muscle) and specific (mg/g fat) CLA contents and its individual isomers (% total CLA) in *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of Mirandesa-PDO veal from late spring and early autumn.

	Spring		**Autumn**			**Significance level**		
	LL	**ST**	**LL**	**ST**	**SEM**	**Season**	**Muscle**	**S×M**
Total CLA	0.06[b]	0.02[c]	0.12[a]	0.01[c]	0.012	*	***	**
Specific CLA	3.21[a]	2.06[b]	1.58[b]	1.70[b]	0.269	***	ns	*
CLA isomers								
*t*12,*t*14	0.84	0.92	0.90	1.07	0.146	ns	ns	ns
*t*11,*t*13	2.24	2.48	1.83	1.49	0.249	**	ns	ns
*t*10,*t*12	0.73	1.13	0.85	1.05	0.195	ns	ns	ns
*t*9,*t*11	4.50	7.50	4.02	5.33	0.455	**	***	ns
*t*8,*t*10	1.43	2.92	1.29	2.13	0.286	*	**	ns
*t*7,*t*9	1.71	2.47	1.85	2.03	0.205	ns	*	ns
*t*6,*t*8	1.18[b]	0.88[b]	1.22[a,b]	1.59[a]	0.147	**	ns	**
Total *trans,trans*	12.63	18.30	11.97	14.69	1.097	*	**	ns
c/*t*12,14	0.66	0.81	0.68	0.60	0.117	ns	ns	ns
*t*11,*c*13	2.16	1.81	1.77	1.66	0.150	ns	ns	ns
*c*11,*t*13	0.76	0.70	0.60	0.94	0.123	ns	ns	ns
*t*10,*c*12	1.67	1.38	1.80	1.96	0.359	ns	ns	ns
*c*9,*t*11	74.35	66.38	74.50	71.62	1.992	ns	**	ns
*t*7,*c*9	6.42	4.52	7.54	7.25	0.853	*	ns	ns
Total *cis/trans*	86.03[a]	75.61[b]	86.90[a]	84.03[a]	1.337	**	***	**
Total *cis,cis* (*c*9,*c*11)	1.35	1.30	1.14	1.28	0.131	ns	ns	ns

[a,b,c] Least square means in the same row with different superscripts are significantly different ($P<0.05$); Significance: ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; SEM, standard error of mean. The symbols used mean as follow: S×M, interaction between slaughter season (S) and muscle type (M).

Realini *et al.* (2004) also reported that *longissimus dorsi* from grazing-based production systems had greater CLA contents (5.3 mg/g fat) than that obtained from concentrate-based production systems (2.5 mg/g fat). The values of specific CLA contents described in this work are closer to the values described for concentrate-fed cattle. However, the relatively high values of specific CLA contents in spring-slaughtered calves are in agreement with those reported by Lock and Garnsworthy (2003). The former authors observed that CLA percentages in milk fat varied throughout the year, with the highest values registered in the summer months (May to July) when, in the UK, cows receive fresh grass. A direct linear relationship between grass percentage in cattle diet and meat CLA content has been described by French *et al.* (2000), although the mechanism remains controversial. Several authors reported that diets containing proportionally high levels of 18:3*n*-3 fatty acid, such as fresh grass, grass silage and concentrate containing linseed, promotes an increased deposition of the *c*9,*t*11 CLA isomer in muscle (French *et al.*, 2000; Dannenberger *et al.*, 2005). Moreover, Daniel (2004a) proposed that the increased content of CLA in animals fed forage-based diets is associated with an increase in 18:1*t*11, which is the substrate of SCD in tissues. This seasonal variation on CLA content is related primarily to the seasonal change in 18:3*n*-3 content in grass lipids (Mel'uchová *et al.*, 2008). In addition, since the *c*9,*t*11 CLA isomer is predominantly deposited in the triacylglycerols, higher IMF levels are usually associated with higher CLA contents (Raes, De Smet, Balcaen, Claeys, & Demeyer, 2003).

The CLA isomeric distribution in Mirandesa-PDO veal from both seasons showed a clear predominance of the bioactive *c*9,*t*11 isomer (66-75%) followed in decreasing order by the *t*7,*c*9 (4.5-7.5%) and *t*9,*t*11 (4.0-7.5) isomers. Numerous studies mentioned the *t*7,*c*9 isomer as the second-most prevalent CLA isomer in meat from concentrated-fed animals (Bauman *et al.*, 2007). In contrast, the *t*11,*c*13, *t*11,*t*13, and *t*12,*t*14 CLA isomers increase in tissues lipids of pasture-fed bulls (Dannenberger *et al.*, 2005). Our data did not show these grass effects, except for the *t*11,*t*13 isomer. The differences found in CLA isomeric distribution might be explained by variations in grass intake. It was shown that pasture feeding compared with concentrate feeding mainly increases the proportion of the *t*11,*c*13 isomer (up to 18.5% of total CLA in LD muscle), with a decrease of the *t*7,*c*9 isomer (down to 4.1% of total CLA in LD muscle), while increasing the percentages of *t*11,*t*13 and *t*12,*t*14 isomers (Dannenberger *et al.*, 2005). The content of the other bioactive CLA isomer, *t*10,*c*12, was residual in all muscles analysed (1.4% to 2.0% of total CLA). The sum of *cis/trans* isomers contributed 84.0-87% of total CLA in autumn-slaughtered and 76-86% in spring-slaughtered calves,

while total *trans,trans* isomers contributed only 12-15% and 13-18% in meat from early autumn and late spring, respectively.

The slaughter season had an impact ($P<0.05$) on the percentages of four CLA isomers (*t*11,*t*13, *t*9,*t*11, *t*8,*t*10 and *t*7,*c*9) of the total 14 isomers detected in the IMF of Mirandesa-PDO veal. Slaughter season also affected ($P<0.05$) the sum of *trans,trans* CLA isomers. Moreover, ST muscle in both slaughter seasons had higher relative proportions of some individual CLA isomers (*t*9,*t*11, *t*8,*t*10 and *t*7,*t*9) and sum of *trans,trans* isomers than that from LL muscle, but lower proportions of the *c*9,*t*11 isomer. According to Garcia *et al.* (2008) such muscle differences in CLA concentration may indicate potential tissue differences in the activity of delta9 desaturase. Other authors emphasized the need to verify whether the different behaviour among muscles is related to their different metabolism and/or to a distinct tissue utilization of fatty acids (Serra *et al.*, 2009). Significant interactions ($P<0.01$) between the slaughter season and muscle type were observed only for the *t*6,*t*8 and total *cis/trans* CLA isomers. Dannenberger *et al.* (2005) found that pasture feeding compared to concentrate outcome changes in the distribution pattern and contents of individual CLA isomers between LD and ST muscles. According to those authors, although LD muscle from pasture-fed bulls has higher levels of the sum of CLA isomers, as well as of the *t*10,*c*12 isomer, with similar values of *c*9,*t*11 isomer, ST muscle depicts lower contents of *c*9,*t*11 and *t*10,*c*12 isomers, with no variation for the sum of CLA isomers. Our data did not show effects on the bioactive *t*10,*c*12 CLA isomer in LL and ST muscles from both seasons.

2.3.3 Contents of cholesterol, tocopherols and β-carotene in meat

Data on the total cholesterol, α-tocopherol and β-carotene contents in LL and ST muscles of Mirandesa-PDO veal obtained from late spring and early autumn are presented in Table 6.

Table 6 - Total cholesterol (mg/g muscle) and lipid-soluble antioxidant vitamins (α-tocopherol and β-carotene; µg/g muscle) in *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of Mirandesa-PDO veal from late spring and early autumn.

	Spring		**Autumn**			**Significance level**		
	LL	**ST**	**LL**	**ST**	**SEM**	**Season**	**Muscle**	**S×M**
Total cholesterol	0.38^{c}	0.45^{b}	0.57^{a}	0.41^{bc}	0.025	**	ns	***
α-Tocopherol	4.27	3.98	4.58	3.17	0.526	ns	*	ns
β-Carotene	0.06	0.04	0.05	0.03	0.014	ns	*	ns

[a,b,c] Least square means in the same row with different superscripts are significantly different ($P<0.05$); Significance: ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; SEM, standard error of mean. The symbols used mean as follow: S×M, interaction between slaughter season (S) and muscle type (M).

Significant interaction (P<0.05) between slaughter season and muscle type was observed for total cholesterol. This interaction may result from modifications of muscle metabolic types caused by adaptations to the distinct grazing periods (summer and spring), which are associated with differences in fatty acid composition (Klont *et al.*, 1998). In fact, differences in the cholesterol content on different muscles of the same animal species might be explained by differences in fibre composition (Chizzolini *et al.*, 1999). Total cholesterol content was the highest (P<0.05) in the LL muscle from autumn-slaughtered calves. The values for total cholesterol in Mirandesa-PDO veal are in accordance to those reviewed by Muchenje *et al.* (2009a) in beef raised on natural pasture (0.37-0.42 mg/g), as well as to those found by Prates *et al.* (2006) for Barrosã-PDO veal, obtained from calves slaughtered in early autumn (0.52 and 0.50 mg/g of cholesterol in LL and ST muscles, respectively) and by Costa *et al.* (2008) for Mertolenga-PDO beef. Moreover, the upper limit of the range (0.38-0.57 mg/g) reached in this study is similar to the cholesterol contents (0.57 mg/g) found by Leheska *et al.* (2008) in grass-fed strip steak.

Vitamin E is a natural fat-soluble vitamin with great antioxidant activity. Vitamin E is composed of eight homologues (α-, β-, λ- and δ-tocopherols, together with the corresponding tocotrienols), being α-tocopherol the most active form protecting cells against the effects of free radicals, which may contribute to the development of chronic diseases. α-Tocopherol was the only vitamin E homologue detected in Mirandesa-PDO veal. The prevalence of α-tocopherol in meat is well known and is due to the more than tenfold preference of the tocopherol-binding protein for α-tocopherol, relatively to γ-tocopherol, which is by far the most common vitamin E homologue in plant foods (Decker, Livisay, & Zhou, 2000). The results showed no significant differences (P>0.05) on the contents of α-tocopherol between spring- and autumn-slaughter seasons (Table 6). The LL muscle, relative to the ST muscle, had higher concentrations of α-tocopherol (P<0.05). The levels of α-tocopherol in Mirandesa-PDO veal (3.2-4.6 μg/g) are within the values reported for cattle produced under extensive grass-fed systems (2.1-7.7 μg/g) but much higher than those reported for meat derived from grain-fed cattle (0.75-2.92 μg/g) (reviewed by Daley *et al.*, 2010). Yang *et al.* (2002) observed that supplementation of pasture-fed cattle with vitamin E did not increase the levels of α-tocopherol (4.3-6.1 μg/g) in meat. The same authors concluded that it appears to have a limit for the accumulation of α-tocopherol in muscle tissues which in the *longissimus dorsi* should be around 7 μg/g. Our values of α-tocopherol in Mirandesa-PDO veal are similar to those reported by Prates *et al.* (2006) for Barrosã-PDO veal (3.3 and 3.6 μg/g for LL and ST

muscles, respectively), but higher to those found recently by Röhrle *et al.* (2011) for pasture-fed cattle (2.43-2.63 μg/g). Moreover, the huge range of α-tocopherol values in the *longissimus lumborum* of grazing cattle (3.7 and 7 μg/g) suggest that there may be a difference in the α-tocopherol levels found in pastures with different biomass composition (West, Young, & Agnew, 1997).

In addition to the α-tocopherol, pasture also supplies β-carotene, a pro-vitamin A compound and the predominant carotenoid form in meat and meat products (Mortensen & Skibsted, 2000). This carotenoid is also a lipid-soluble physiological antioxidant, with an important role in controlling oxidatively induced diseases, such as cancer and atherosclerosis (Decker *et al.*, 2000). Likewise, the contents of β-carotene in Mirandesa-PDO veal were not influenced ($P>0.05$) by slaughter season (Table 6). However, these values were different ($P<0.05$) between LL and ST muscles. In both seasons, the LL muscle showed higher contents of β-carotene (0.05-0.06 μg/g) than ST muscle (0.03-0.04 μg/g). The values of β-carotene obtained in this study are lower than those reviewed by Daley *et al.* (2010), who found >4 fold increases in β-carotene levels for grass-fed over the grain-fed beef counterparts. Nevertheless, muscle levels of β-carotene have shown a high variability among experiments. Descalzo and Sancho (2008) suggest that this could indicate that incorporation of β-carotene into muscle depends not only on dietary delivery and muscle type, but also on the individual uptake capacity.

2.3.4. Nutritional quality of intramuscular fat in Mirandesa-PDO veal

The recommended value for the PUFA/SFA ratio in the human diet is 0.45 or higher, and within PUFA, the *n*-6/*n*-3 ratio should not exceed 4.0 (British Department of Health, 1994). The values of PUFA/SFA ratio in Mirandesa-PDO veal (see Table 4) are inside the recommended dietary guideline in the ST muscle (0.59-0.63), which is favourable for human health, but below in the LL muscle (0.17-0.23). In addition, the *n*-6/*n*-3 PUFA ratio in the veal from both muscles and slaughter seasons are within the recommendation for the human diet, although slightly above in the LL muscle from early autumn (4.35). These results clearly result from the benefits of grass-fed on ruminant meat (Nuernberg *et al.*, 2005a). Both dietary ratios of fatty acids were widely used, in the last decade, to evaluate the nutritional value of fat for human consumption. Presently, the *n*-6/*n*-3 ratio has been the subject of much debate over its practical utility in optimizing the benefits of *n*-3 fatty acids (C18-22) on

cardiovascular health (Stanley *et al.*, 2007). Griffin (2008) reported that the absolute amounts of dietary LA and ALA are of relevance to the efficiency of conversion of ALA to EPA and DHA. Moreover, the conversion of ALA to *n*-3 LC-PUFA is reduced by high dietary ratios of LA/ALA (Brenna *et al.*, 2009). According to the former authors, the *n*-3 LC-PUFA status can be improved by increasing dietary intake of *n*-3 PUFA or by reducing the intake of *n*-6 PUFA and the combination of both strategies is likely to be the most effective measure. Hence, the absolute intake of *n*-3 LC-PUFA is more important than the *n*-6/*n*-3 ratio. In addition, EFSA (2010) proposed not to set specific values for the *n*-6/*n*-3 ratio but an adequate intake of 250 mg *per* day for EPA plus DHA. In fact, it was found that the consumption of these fatty acids in most Western populations is suboptimal for protection against the most prevalent chronic diseases.

Based on these recommendations and regarding *n*-3 PUFA, a daily consumption of 150 g of a fresh cut of Mirandesa-PDO veal, trimmed of all visible fat except the IMF, could provide 38-92 mg of *n*-3 PUFA, which represents a contribution of 4-22% to the coverage of recommended daily intake for EPA plus DHA. However, the predominant *n*-3 LC-PUFA in meat is DPA, an intermediate in the production of DHA from EPA, of which the functional and nutritional attributes are largely unknown.

Concerning CLA, EFSA (2010) proposes not to set any dietary value of reference for CLA since the optimal dietary intake of CLA remains to be established. On the basis of animal studies, some extrapolations suggested that a daily consumption of 0.8-3.0 g of CLA might promote human health benefits (Parrish *et al.*, 2003). Facing the above discussion, 150 g of Mirandesa-PDO veal would provide only 2-18 mg of CLA, which is less than 2% of the minimum amount required. However, the range of values proposed by the former authors symbolize a rough estimate because it is based on animal data extrapolation and, thus, should be interpreted carefully until consistent data are available in humans (Schmid *et al.*, 2006).

Looking upon cholesterol and fat-soluble vitamins, a daily consumption of 150 g of Mirandesa-PDO veal provides 57-86 mg of cholesterol, which corresponds to 19-29% of the maximum recommended daily cholesterol intake (<300 mg, American Heart Association, 2008).

In addition, the same portion of Mirandesa-PDO meat provides 0.4-0.7 mg of α-tocopherol and 3-5 μg of β-carotene, which represents 3-5% of the current recommended intake of

vitamin E (15 mg/day) and less than 1% of the recommended dietary allowance (RDA) for vitamin A (700 and 900 µg/day for women and men, respectively). While there is no RDA for β-carotene or other pro-vitamin A carotenoid, the Institute of Medicine of USA suggests that consuming 3 to 6 mg of β-carotene daily (equivalent to 833-1667 IU vitamin A) will maintain blood levels of β-carotene in the range associated with a low risk of chronic diseases (Food and Nutrition Board of Institute of Medicine, 2001).

2.4. Conclusions

Mirandesa-PDO veal only showed seasonal differences in the levels of some minor fatty acids and CLA isomers, in addition to slight differences in the sum of *n*-3 PUFA. In contrast, the lipid composition was much more influenced by the muscle type. The data indicate that the PDO veal has similar values to pasture-fed cattle, in both slaughter seasons, with regard to the percentage of several fatty acids, some partial sums of fatty acids, the *n*-6/*n*-3 ratio, some individual CLA isomers and the content of α-tocopherol (grass intake indicators). This might reflect the similar final effects of the different suckling and grazing periods on autumn and spring-slaughtered calves.

From a human nutrition perspective, IMF in Mirandesa-PDO veal has good human health related parameters in both slaughter seasons, since the *n*-6/*n*-3 ratio values are inside or very close to the recommended value for the human diet and the contents of *n*-3 PUFA and α-tocopherol are relatively high. In addition, the nutritional quality of ST muscle seems to be more favourable for human health, relative to the LL muscle, mainly due to the higher percentages of total *n*-3 PUFA. All together, the results indicate that Mirandesa-PDO veal IMF, as a result of the beneficial grass effects, is of high nutritional quality throughout the year.

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CHAPTER 3 | Lipid composition and nutritional quality of intramuscular fat in Charneca-PDO beef

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Abstract

This paper characterises the IMF from *longissimus lumborum* (LL, relatively red) and *semitendinous* (ST, relatively white) muscles of Charneca beef from young bulls reared according to the PDO specifications. The content of total lipids, total cholesterol, α-tocopherol and β-carotene, as well as the fatty acid composition, including the isomeric distribution of CLA, was assessed. Charneca young bulls (*n*=10) were raised on a semi-extensive production system, in which animals fed pasture plus concentrate during 15 months. The ST muscle was leaner and had higher percentages of PUFA, in contrast to the LL muscle, which presented higher percentages of SFA and MUFA. Thus, the ST muscle had a higher PUFA/SFA ratio than the LL muscle, although the ratio values of both muscles were inside the recommended figures for the human diet. In contrast, the contents of CLA isomers, total cholesterol, α-tocopherol and β-carotene, as well as the *n*-6/*n*-3 ratio, were not influenced by muscle type, thus suggesting no carcass variation for these compounds. In both muscles, the *n*-6/*n*-3 ratios were slightly above the dietary guideline for human diet and the contents of α-tocopherol were very high, indicating a good lipid stability of Charneca-PDO beef. Overall, the results suggest that IMF of Charneca-PDO beef has good human health related parameters, with small carcass variation, since the PUFA/SFA and *n-6*/*n-3* ratio values are inside or very close to the recommended figures for the human diet, and the content of α-tocopherol is very high.

Keywords: beef, fatty acids, CLA isomers, total cholesterol, α-tocopherol, β-carotene.

3.1 Introduction

A strong relationship between high-fat diets and cardiovascular diseases has been described, and the consumer's interest in the fat quality of food products has increased in the last decades (Scollan *et al.*, 2006). Indeed, the quantity and quality of dietary fats are strongly related to human health (EFSA, 2010). The EFSA (2010) reviewed the specific guidelines for fat and fatty acids for human intake. Among the recommendations, a reduction in the intake of SFA, TFA and cholesterol, as well as an increase in the intake of *n*-3 PUFA, are particularly encouraged (Daley *et al.*, 2010). In addition, the results of Griffin (2008) reinforced the current nutritional recommendations for increasing 18:3*n*-3 and decreasing linoleic acid (18:2*n*-6) intakes, in order to promote the endogenous synthesis of long chain (>C18) *n*-3 fatty acids. The beneficial effects of *n*-3 PUFA were recently reviewed by Gogus and Smith (2010).

Ruminants derived-foods are the richest natural dietary sources of CLA isomers, in which the 18:2*cis*9,*trans*11 (*c*9,*t*11, rumenic acid) is the predominant one (Kramer *et al.*, 1998). This isomer is mostly produced by ruminal biohydrogenation of dietary PUFA and by delta-9 desaturation of VA (18:1*t*11) in tissues (Griinari & Bauman, 1999). In fact, rumenic acid has been implicated on numerous health-promising properties, including potent anti-carcinogenic effects, as reviewed by Benjamin and Spener (2009). French *et al.* (2000) have shown that, compared with grass silage or concentrate, the inclusion of green pasture in the diets of steers increased the proportion of CLA deposition in beef.

Cattle produced under grass-based systems are associated with a more desirable fatty acid profile and higher antioxidant content (Daley *et al.*, 2010). The breed and management of cattle may affect fatty acid composition, as fatty acid composition is closely related to the fatness level (Barton *et al.*, 2007). It is well known that animals consuming fresh pastures have higher levels of *n*-3 PUFA and MUFA in their milk and meat than those receiving a cereal-based concentrate diet (Chilliard *et al.*, 2007; Lourenço *et al.*, 2008). Also, grass intake increases the levels of fat-soluble vitamins, mainly α-tocopherol and β-carotene, in muscle tissues, which delay lipid oxidation despite the higher concentration of more oxidisable PUFA (Descalzo & Sancho, 2008). Meat also provides from one-third to one half (Chizzolini *et al.*, 1999) of the maximum recommended intake of cholesterol *per* day (300 mg) (American Heart Association, 2008).

Red muscle (type I) fibres, relative to other muscle fibres, have higher activity of oxidative enzymes and exhibit slower contraction. In contrast, white muscle (type II) fibres have a tendency or glycolytic metabolism by consuming glucose instead of mitochondrial driven respiration, thus twitch fast (Lee, Joo & Ryu, 2010). Moreover, type I fibres contain a higher amount of lipids, some of which presumably serves as a source of aerobic metabolic fuel; they also contain lower amounts of glycogen and glucose than type IIB fibres (Klont *et al.*, 1998). It is well known that different muscles are composed by distinct proportions of fibres (oxidative or glycolytic), which may influence the IMF content (Hocquette *et al.*, 2010).

In order to promote sustainable agriculture systems, European Union policies have given the opportunity of commercialise meat from regional cattle breeds with a "certification of origin", known as PDO. This should be a consumer´s guarantee that the meat was produced in a specific, pre-determined geographic region and according to defined rules (Council Regulation nº 1495/02 of 22/08, EEC). Despite its higher price, consumer demand for legally protected PDO bovine meat has increased in Portugal in the past few years (Costa *et al.*, 2011). Charneca-PDO meat is a PDO beef obtained from a local autochthonous breed and produced under semi-extensive conditions, pasture-based supplemented with concentrate, in the southeast of Portugal (IDRH, 2007). The aim of this work was to characterise the carcass variation (from relatively red to relatively white muscles) in lipid composition and nutritional quality of IMF in Charneca-PDO beef, through the determination of fatty acid composition, including CLA isomeric distribution, and total lipids, total cholesterol, α-tocopherol and β-carotene contents.

3.2 Material and methods

3.2.1 Animals, management and sample collection

Ten Charneca purebred young bulls were maintained according to the semi-extensive production pasture-based system, following the rules established in the Charneca-PDO product specifications. The calves were reared with their dams until weaning at 7.5 months of age. After weaning, young bulls were raised on a semi-extensive production system, feeding grazing pasture plus 2-4 kg of concentrate *per* day (see concentrate composition on Table 7). On the last nine months, before slaughter, the animals were fed also corn silage and grass hay. The slaughter took place on the Regional Abattoir of Baixo Alentejo (Beja, Portugal), in November 2008 (mean ± standard error of age and live body weight were 20.6 ± 2.50 months

and 640 ± 29 kg, respectively). November is the month with the highest number of carcasses produced and, thus, the most commercially important period for this PDO meat. The carcass yield (carcass weight/live body weight) of the young bulls used in these experiments was approximately 50%. Meat samples were taken from the loin portion of the *longissimus dorsi* (L4-L6, *longissimus lumborum* muscle, LL) and from the distal region of *semitendinosus* (ST) muscles of young bulls. Comparing with ST muscle, LL muscle is relatively red and differently involved in the physical activity imposed by grazing (Vestergaard *et al.*, 2000a) All meat samples were collected 2-3 days after slaughter (+1 ºC), grounded using a food processor (3 × 5 s), vacuum packed and stored at -80 °C until required for analysis.

Table 7 - Ingredient (% feed), proximate (% dry matter) and fatty acid (% total fatty acids) composition of the concentrate from Charneca young bulls reared according to Charneca-PDO beef specifications.

Ingredients	
Corn	50.3
Palm kernel meal	12.0
Rape	12.0
Soybean meal	5.0
Barley	5.0
Sow	5.0
Soybean bark	4.1
Minerals and premix	3.3
Reed molasses	3.0
Urea	0.3
Proximate composition	
Gross energy (kJ/100g DM)	1876
Crude protein	17.6
Total fat	3.6
Crude fibre	12.0
Ash	6.3
Fatty acid composition	
14:0	4.7
16:0	25.7
18:0	3.0
18:1*c*9	23.1
18:2*n*-6	37.7
18:3*n*-3	3.8

3.2.2 Lipid extraction and methylation

For total lipids determination and for both FAME and CLA methyl esters profiles, IMF was extracted with dichloromethane/methanol (2:1 v/v) from lyophilized meat samples (*ca* 250 mg), using the procedures described previously by Alfaia *et al.* (2006a). All the extraction solvents contained 0.01% butylated hydroxytoluene (BHT) as antioxidant. Total lipids were

measured gravimetrically, in duplicate, by weighting the fatty residue obtained after solvent evaporation. Fatty acids and CLA isomers were converted to methyl esters by a sequential transesterification reaction with NaOH in anhydrous methanol (0.5 M) followed by HCl:methanol (1:1 v/v), at 50 °C during 30 and 10 min, respectively (Raes *et al.*, 2004). The same FAME solution was used for the analysis of both fatty acid composition and CLA profile, thus enabling the direct comparison of quantitative data and eliminating differences in sample preparation.

3.2.3 Determination of fatty acid composition

FAME were analysed using a HP6890A gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a GC-FID and a fused-silica capillary column (CPSil 88; 100 m × 0.25 mm i.d., 0.2 μm film thickness, Chrompack, Varian Inc., Walnut Creek, CA, USA), as described by Alves and Bessa (2009). Briefly, the initial oven temperature was 100 °C (held for 1 min), then increased to 150 °C by 50 °C/min (held for 20 min), then increased to 190 °C by 1 °C/min (held for 5 min), and finally increased to 200 °C by 1 °C/min (held for 35 min). Ultra pure helium was used as the carrier gas, at a flow rate of 1 mL/min, and the split ratio was 1:50. The injector and detector temperatures were maintained at 250 and 280 ºC, respectively. Identification of common fatty acids was accomplished by comparison of sample peak retention times with those of FAME standard mixtures (Nu-Chek-Prep Inc., Elysian, MN, USA; Supelco Inc. Bellefonte, PA, USA) and by using published chromatograms obtained with similar analytic conditions (Kramer *et al.*, 2008). Structural analyses of some unknown peaks were conducted by GC-MS/MS technique, using a Varian Saturn 2200 system (Varian Inc.) equipped with a CP-Sil 88™ capillary column (100 m × 0.25 mm i.d., 0.2 μm film thickness). Quantification of total FAME was done using nonadecanoic acid as internal standard. The results for each fatty acid were expressed as a percentage of the sum of detected fatty acids (% total fatty acids).

3.2.4 Determination of individual CLA isomers

Methyl esters of CLA isomers were individually separated by triple silver-ion columns in series (ChromSpher 5 Lipids; 250 mm × 4.6 mm i.d., 5 μm particle size, Chrompack, Bridgewater, NJ, USA), using an high performance liquid chromatography (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA). The HPLC was equipped with autosampler and diode array detector (DAD) adjusted to 233 nm. Chromatography conditions

were followed according to Alfaia *et al.* (2006a). The mobile phase was 0.1% acetonitrile in *n*-hexane, maintained at a flow rate of 1 mL/min, and the injection volume was 20 µL. The identification of individual CLA isomers was achieved by comparison of peak retention times with commercial and prepared standards, as well as, with values published in the literature (Prates & Bessa, 2009). In addition, the identity of each isomer was controlled by the typical ultraviolet spectra of CLA isomers from the DAD in the range from 190 to 360 nm, using the spectral analysis of Agilent ChemStation for LC 3D Systems rev. A.09.01 (Agilent, 2001). Standards of CLA isomers (*c*9,*t*11, *t*10,*c*12, *c*11,*t*13, *c*9,*c*11 and *t*9,*t*11) were purchased from Matreya Inc. (Pleasant Gap, PA, USA) and Sigma Inc. (St. Louis, MO, USA) or prepared (*cis/trans* and *trans,trans* from carbons 7,9 to 12,14) by the procedure reported by Destaillats and Angers (2003). Total CLA content was calculated from their HPLC area relative to the area of the main CLA isomer *c*9,*t*11 (plus *t*7,*c*9 and *t*8,*c*10) determined by GC-FID with the other minor isomers quantified by Ag^{+}-HPLC analysis (Kraft *et al.*, 2003; Cruz-Hernandez *et al.*, 2006). The individual CLA isomers were expressed as a percentage of the sum of identified CLA isomers (% total CLA).

3.2.5 Determination of cholesterol, tocopherols and β-carotene

The simultaneous analysis of total cholesterol, tocopherols and β-carotene in meat was performed according to Prates *et al.* (2006). Briefly, the saponification of homogenized fresh meat samples (*ca* 750 mg) was carried out with 0.2 g of L-ascorbic acid and 5.5 mL of saponification solution (11% w/v potassium hydroxide in a mixture of 55% v/v absolute ethanol and 45% v/v distilled water) in a shaking water bath (200 rpm) at +80 ºC for 15 min. After cooling, 1.5 mL of distilled water and 3 mL of *n*-hexane (containing 0.01% of BHT) were added and the tubes were vigorously vortexed for 2 min and centrifuged at 1500 *g* for 5 min. Afterwards, an aliquot of the *n*-hexane layer was filtered through a 0.45 µm hydrophobic membrane and injected in the HPLC system (Agilent 1100 Series). It was used a normal-phase silica column (Zorbax RX-Sil, 250 mm × 4.6 mm i.d., 5 µm particle size, Agilent Technologies Inc., Palo Alto, CA, U.S.A.), with fluorescence detection for tocopherols (excitation wavelength of 295 nm and emission wavelength of 325 nm) and UV-visible photodiode array detection for cholesterol (202 nm) and β-carotene (450 nm) in series. The solvent (1% v/v isopropanol in *n*-hexane) flow rate was 1 mL/min, the run last for 17 min and the temperature of the column oven was adjusted at 20 ºC. The contents of total cholesterol, tocopherols and β-carotene in meat were calculated, in duplicate for each sample, based on the external standard technique from a standard curve of peak area *vs.* concentration, using

DL-α-, D-β-, D-γ- and D-δ-tocopherols (Calbiochem, Merck Biosciences, Darmstadt, Germany), all-*trans*–carotene and cholesterol standards (Sigma Chemical Co., St. Louis, MO, USA).

3.2.6 Statistical analysis

Data were analysed using the PROC MIXED procedure of the Statistical Analysis Systems (SAS). The statistical model considered the muscle type (LL and ST) a repeated measure within the animal. Least squares means (LSMEANS), with the option PDIFF, were determined to compare groups. Differences were considered significant at a *P*-value below 0.05.

3.3 Results and discussion

3.3.1 Intramuscular fatty acid composition

The effect of muscle type on total lipids and fatty acid composition (weight %) of IMF in Charneca-PDO beef is shown in Table 8. Total lipid content ranged from 7 to 10 mg/g meat, thus being a lean meat, according to the criteria supplied by the Food Advisory Committee (1990) (fat content <50 mg/g). The higher content of total lipids ($P<0.05$) in LL muscle, relative to ST muscle, can be explained by differences in muscle fibre composition. It is well established that lipid content is higher in red oxidative muscle fibres (Enser *et al.*, 1998) and, in fact, the LL muscle is relatively red in comparison with the ST muscle (Vestergaard *et al.*, 2000a). Also, slightly higher values were obtained by Alfaia *et al.* (2009), using the same analytical methodology applied in this work, for total lipids of cattle fed on pasture with finishing on concentrate during 4 months (12 mg/g fat). With the same finishing conditions, De la Fuente *et al.* (2009) reported a higher IMF proportion (close to 30 mg/g). Charneca-PDO beef had lower total lipids when compared with those reported for meat from extensively produced cattle, 11-14 mg/g (Vestergaard *et al.*, 2000b). The intermediate levels of IMF reported here likely reflect the combination of animals fed with grazing pasture and cereal-based concentrates.

The muscle type affected 23 of the total 40 fatty acids analysed ($P<0.05$) in the IMF of Charneca-PDO beef. In both muscles, the predominant fatty acids were 16:0, 18:0, 18:1*c*9, 18:2*n*-6 and 20:4*n*-6. Saturated hypercholesterolemic fatty acids (14:0 and 16:0) and their monounsaturated derivatives (14:1*c*9 and 16:1*c*9) were affected ($P<0.01$) by the muscle type,

showing the ST muscle the lowest values, except for the 14:1*c*9. The oleic acid (18:1*c*9) was the most prevalent fatty acid in both muscle types, showing higher proportions in the LL muscle than in the ST muscle ($P<0.001$). Jenkins *et al.* (1994) reported that diets based on concentrate were expected to increase the absorption of oleic and linoleic (18:2*n*-6) acids, which are the major fatty acids in cereal grains. However, other factors might be involved in the increment of oleic acid concentration in meat (Daniel *et al.*, 2004a; Alfaia *et al.*, 2009). Daniel *et al.* (2004a) reported that the high content of oleic acid observed in ruminants that fed high grain diets might be explained by an increase in delta-9 desaturase activity, probably mediated by a higher insulin production.

Concerning PUFA, the ST muscle had close to 50% more 18:2*n*-6 comparing to the LL muscle ($P<0.01$). In contrast, no significant differences ($P>0.05$) between muscles were observed for the 18:3*n*-3 concentration. It is well known that grass pastures are a good source of 18:3*n*-3, being considered a very sensitive grass intake indicator (Wood *et al.*, 2008). The ST muscle showed higher values ($P<0.05$) of long chain C20-22 PUFA, like 20:4*n*-6, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3, than the LL muscle. These fatty acids are formed endogenously from 18:2*n*-6 and 18:3*n*-3 by the action of delta-5 and delta-6 desaturases and elongase (Realini *et al.*, 2004). *Trans* octadecenoates (18:1*trans*) are the major intermediates formed during rumen biohydrogenation of C18 PUFA (Bessa *et al.*, 2000). Apart from the 18:1*t*11 and 18:1*t*16+*c*14, the remaining 18:1*trans* fatty acids were unaffected ($P>0.05$) by the muscle type. It is known that cattle fed high concentrate diets (*i.e.* barley-based) increased the total *trans* fatty acids content in beef, notably the 18:1*t*10 isomer (Alfaia *et al.*, 2009). On the other hand, grass-feeding promotes an accumulation of VA, which can be desaturated to *c*9,*t*11 CLA isomer by delta-9 desaturase, in bovine adipose tissue and mammary gland (Griinari & Bauman, 2000).

Table 8 - Total lipids (mg/g muscle), fatty acid composition (% total fatty acids), of *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of Charneca-PDO beef.

	Muscles			
	LL	**ST**	**SEM**	**Significance**
Total lipids	10.38	6.64	0.74	***
Fatty acid composition				
14:0	1.68	0.78	0.116	***
14:1*c*9	0.25	0.55	0.033	***
15:0*iso*	0.18	0.18	0.018	ns
15:0*anteiso*	0.24	0.22	0.019	ns
15:0	0.38	0.26	0.029	***
16:0*iso*	0.20	0.18	0.020	ns
16:0	20.74	17.19	0.557	***
16:1*c*7	0.24	0.24	0.015	ns
16:1*c*9	1.76	1.25	0.101	**
16:1*t*9	0.22	0.37	0.036	*
17:0*iso*	0.38	0.36	0.015	ns
17:0*anteiso*	0.55	0.36	0.032	**
17:0	0.77	0.55	0.039	**
17:1*c*9	0.44	0.45	0.032	ns
18:0	17.73	15.41	0.509	**
18:1*c*9	26.34	20.44	0.924	***
18:1*c*12	0.25	0.32	0.020	*
18:1*c*13	0.13	0.03	0.017	***
18:1*c*15	0.14	0.10	0.031	ns
18:1*t*6+*t*8	0.15	0.20	0.027	ns
18:1*t*9	0.20	0.19	0.032	ns
18:1*t*10	0.24	0.22	0.024	ns
18:1*t*11	1.65	1.19	0.196	ns
18:1*t*12	0.65	0.28	0.264	ns
18:1*c*11+*t*15	1.95	2.09	0.065	ns
18:1*t*16+*c*14	0.26	0.23	0.020	ns
18:2*c*9,*t*11†	0.54	0.40	0.067	ns
18:2*t*11,*c*15	0.19	0.23	0.022	ns
18:2*n*-6	11.03	18.19	1.166	***
18:3*n*-3	1.79	2.64	0.367	ns
18:3*c*9,*t*11,*c*15	0.14	0.27	0.034	*
20:0	0.13	0.23	0.015	***
20:2*n*-6	0.12	0.22	0.018	**
20:4*n*-6	3.74	5.82	0.438	**
20:5*n*-3	0.74	1.18	0.108	**
22:0	0.76	1.23	0.110	**
22:4*n*-6	0.22	0.40	0.028	***
22:5*n*-3	1.16	1.97	0.132	***
22:6*n*-3	0.15	0.33	0.017	***
23:0	0.20	0.36	0.046	*

Significance: ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; SEM: standard error of the mean; means in the same row with different superscripts are significantly different ($P<0.05$). † This peak also includes minor amounts of the *t*7,*c*9 and *t*8,*c*10 CLA isomers.

The non-conjugated linoleic acid isomer 18:2*t*11,*c*15 is an intermediate of ALA (18:3*n*-3) biohydrogenation (Raes *et al.*, 2004) which was not affected ($P>0.05$) by muscle type. Concerning other minor isomers, the LL muscle, when compared to the ST muscle, had higher contents of 15:0, 17:0*anteiso*, 17:0 and 18:1*c*13, and lower values of 20:0, 20:2*n*-6, 22:0, 22:4*n*-6 and 23:0 ($P<0.05$). As already stated for the total lipids, the fatty acid differences found between muscles probably result from their distinct fibre type proportions (Costa *et al.*, 2008).

Regarding partial sums of fatty acids (Table 9), the observed patterns reflect the values described above for the major individual fatty acids of each group. The muscle type significantly affected ($P<0.05$) all partial sums of fatty acids with the exception of TFA. The LL muscle, comparing with the ST muscle, had higher relative proportions ($P<0.01$) of SFA, MUFA and BCFA, but lower percentages ($P<0.001$) of PUFA, *n*-6 PUFA and *n*-3 PUFA. Since SFA and PUFA are more abundant in triacylglycerol and phospholipid fractions, respectively (Scollan *et al.*, 2006), the muscle type effect for these partial sums of fatty acids likely reflect distinct triacylglycerol/phospholipid ratios between muscles, as consequence of different fat levels. While fatness increases, increments in the SFA content are larger than that of PUFA (De Smet *et al.*, 2004). These results are in full agreement with the relatively redness of LL muscle, in comparison with the ST muscle, which implies higher proportions of neutral lipids and thus higher percentages of SFA and MUFA (Wood *et al.*, 2008). Inversely, the ST muscle is relatively white, with higher proportions of phospholipids, which are rich in PUFA.

No muscle type effect ($P>0.05$) was observed for the meat *n*-6/*n*-3 ratio. In contrast, the LL muscle showed a lower PUFA/SFA ratio ($P<0.001$) than the ST muscle, which reflects the differences in PUFA and SFA between the muscles described above. According to the current nutritional recommendations, the PUFA/SFA ratio in human diets should be above 0.45 and, within the PUFA, the *n*-6/*n*-3 ratio should not exceed 4 (British Department of Health, 1994). This last index has been recently the topic of some debate. Stanley *et al.* (2007) proposed that it is more important to evaluate the total amounts of dietary PUFA than their respective ratio. Moreover, Brenna *et al.* (2009) proposed that *n*-3 PUFA status could be improved by increasing the *n*-3 PUFA intake or by reducing the *n*-6 PUFA intake, and that the combination of both strategies would be the most effective. In the present study, the PUFA/SFA ratios are within the recommended guidelines for the human diet for both muscles, but the *n*-6/*n*-3 ratios are slightly above 4.0 in both muscles (4.4-4.6).

Table 9 - Partial sums of fatty acids (% w/w) and related indices of *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of Charneca-PDO beef.

	Muscles			
	LL	**ST**	**SEM**	**Significance**
Partial sums				
SFA	42.39	36.01	0.802	***
MUFA	29.55	23.38	1.019	***
TFA	6.18	5.65	0.350	ns
PUFA	18.96	30.75	1.643	***
BCFA	1.55	1.30	0.064	*
n-6 PUFA	15.11	24.63	1.547	***
n-3 PUFA	3.86	6.12	0.575	*
Unidentified	1.36	2.91	0.263	***
Fatty acid ratios				
PUFA/SFA	0.46	0.86	0.052	***
n-6/*n*-3	4.41	4.58	0.643	ns

Significance: ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; SEM: standard error of the mean; means in the same row with different superscripts are significantly different ($P<0.05$). SFA = sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0; MUFA = sum of 14:1*c*9, 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*12, 18:1*c*13 and 18:1*c*15; TFA = sum of 16:1*t*9, 18:1*t*6+*t*8; 18:1*t*9, 18:1*t*10, 18:1*t*11, 18:1*t*12, 18:1*c*11,*t*15, 18:1*t*16+*c*14, 18:2*t*11,*c*15, 18:2*c*9,*t*11 and 18:3*c*9,*t*11,*c*15; PUFA = sum of 18:2*n*-6, 18:3*n*-3, 20:2*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-3 and 22:6*n*-3; BCFA = sum of 15:0*iso*, 15:1*anteiso*, 16:0*iso*, 17:0*iso* and 17:0*anteiso*; *n*-6 PUFA = sum of 18:2*n*-6, 20:2*n*-6, 20:4*n*-6 and 22:4*n*-6; *n*-3 PUFA = sum of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3; PUFA/SFA = [(sum of 18:2*n*-6, 18:3*n*-3, 20:2*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-3 and 22:6*n*-3)/(sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0)]; *n*-6/*n*-3 = *n*-6/*n*-3 ratio [(sum of 18:2*n*-6, 20:2*n*-6, 20:4*n*-6 and 22:4*n*-6)/(sum of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3)].

Warren et al. (2008) reported that the *n*-6/*n*-3 ratio was consistently below 1.3 in silage-fed steers, and ranged from 9 to 16, in those fed concentrate. In grass-fed cattle, especially with high IMF levels, it is apparently difficult to achieve a high PUFA/SFA ratio, whilst keeping *n*-6/*n*-3 low. The high degree of biohydrogenation of dietary PUFA leads to SFA production in the rumen and their absorption in the small intestine (Warren et al, 2008). Finally, comparing the nutritional quality of IMF reported so far for Portuguese PDO beef revealed that Charneca-PDO meat seems to have a favourable *n*-6/*n*-3 ratio, lower than that for Carnalentejana-PDO beef (10.0-13.7) and Mertolenga-PDO beef (6.8-14.9) (Alfaia *et al.*, 2006a; Alfaia *et al.*, 2006b). In contrast, these last two PDO-meats had lower values of PUFA/SFA ratio than those found in the present work. These differences may be explained by the finishing period of Alentejana and Mertolenga purebred bullocks on concentrate that diminishes the beneficial effects associated with grass intake. In the present study, the animals were maintained in grass pasture and were supplemented with small amounts of concentrate, which is reflected in the good nutritional quality of IMF in beef.

3.3.2 Intramuscular CLA isomeric profile

Detailed data on CLA isomers content present in IMF of LL and ST muscles from Charneca-PDO beef is displayed in Table 10. Total CLA content (mg/g muscle) had significant differences ($P<0.05$) between muscles. Specifically, the LL muscle had a higher total CLA content relatively to the ST muscle. However, since the specific CLA content (mg/g fat) was not affected ($P>0.05$) by the muscle type, the discrepancies found on the total CLA content may be completely explained by the differences on the total muscle lipids (see Table 8). The results reported here for specific CLA content (2.1-3.7 mg/g fat) were similar to those obtained from concentrate-fed animals reported by Realini *et al.* (2004). However, as far as we know, only a few studies have reported the effect of pasture *vs*. concentrate feeding on the detailed CLA isomeric profile of beef fat (Alfaia *et al.*, 2009), which is only achieved by high performance liquid chromatography using three silver-ion columns in series (Nuernberg *et al.*, 2002; Dannenberger *et al.*, 2005).

Table 10 - Total (mg/g muscle) and specific (mg/g fat) CLA contents and its individual isomers (% total CLA) in *longissimus lomborum* (LL) and *semitendinosus* (ST) muscles of Charneca-PDO beef.

	Muscles			
	LL	**ST**	**SEM**	**Significance**
Total CLA	0.04	0.01	0.007	*
Specific CLA	3.66	2.07	0.535	ns
CLA isomers				
*t*12,*t*14	1.23	1.51	0.210	ns
*t*11,*t*13	3.80	4.28	0.737	ns
*t*10,*t*12	0.98	1.71	0.258	ns
*t*9,*t*11	2.40	3.81	0.233	**
*t*8,*t*10	0.44	0.73	0.115	ns
*t*7,*t*9	1.58	0.93	0.246	ns
*t*6,*t*8	0.48	0.58	0.077	ns
Total *trans,trans*	10.91	13.80	1.128	ns
c/*t*1214	0.58	0.74	0.101	ns
*t*11,*c*13	5.17	5.22	0.651	ns
*c*11,*t*13	1.41	1.21	0.263	ns
*t*10,*c*12	2.59	1.55	0.482	ns
*c*9,*t*11	72.77	72.32	1.606	ns
*t*7,*c*9	5.73	4.54	0.552	ns
Total *cis*/*trans*	88.25	85.31	1.042	ns
Total *cis,cis* (*c*9,*c*11)	0.83	0.88	0.191	ns

Significance: ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; SEM, standard error of the mean; means in the same row with different superscripts are significantly different ($P<0.05$).

The muscle type had no impact on the 14 CLA isomers identified, except for the *t*9,*t*11 isomer, which suggests no muscle specific deposition of these compounds. In agreement with

this, in Carnalenteja-PDO beef, with the exception of *c*9,*t*11 isomer ($P<0.05$), no significant differences were observed between LL and ST muscles for individual CLA isomers (Alfaia *et al.*, 2006a). As expected, the CLA isomeric profile showed a clear predominance of the bioactive *c*9,*t*11 isomer, followed by the *t*7,*c*9 (in LL muscle) or *t*11,*c*13 (in ST muscle) isomers. The *t*7,*c*9 CLA isomer originates from the 18:1*t*7 (Collomb *et al.*, 2004) and is frequently recognized as the second-most predominant isomer in ruminant fat from concentrate-fed animals (Bauman *et al.*, 2007). In contrast, the *t*11,*c*13 isomer is the most sensitive CLA isomer grass intake indicator and the second-most prevalent isomer in ruminant fat from pasture-fed animals (Dannenberger *et al.*, 2005). In addition to this CLA isomer, the *t*11,*t*13 (the third most prevalent isomer in both muscles) and *t*12,*t*14 isomers are also sensitive grass intake indicators (Nuernberg *et al.*, 2002; Dannenberger *et al.*, 2005). Apart *c*9,*t*11 and *t*7,*c*9 isomers, all other CLA isomers are supposed to arise from ruminal microflora by biohydrogenation (Collomb *et al.*, 2004). The other bioactive CLA isomer, the *t*10,*c*12, appeared in residual amounts in both muscles (<2.6%). The sums of *trans,trans* and *cis/trans* (both *cis,trans* and *trans,cis*) CLA isomers reflect the profile of individual isomers, with relative high incorporations of *t*11,*t*13 and *t*9,*t*11 CLA isomers in the Charneca-PDO beef.

3.3.3 Contents of cholesterol, tocopherols and β-carotene in meat

The contents of total cholesterol, α-tocopherol and β-carotene did not show significant differences ($P>0.05$) between LL and ST muscles of Charneca-PDO beef (Table 11). Although differences in fibre type composition might be the most likely reason for changes in cholesterol content among muscles of the same animal species, in our case there were no significant differences between the two analysed muscles (Chizzolini *et al.*, 1999). The cholesterol contents found for Charneca-PDO meat (0.42-0.44 mg/g muscle) are close to those reported by Alfaia el al. (2006a, 2006b) for Carnalentejana-PDO and Mertolenga-PDO beef (0.42-0.48 mg/g muscle and 0.40-0.44 mg/g muscle, respectively). Slightly higher levels of cholesterol content were reported by Padre *et al.* (2007), 0.46-0.48 mg/g muscle, for beef from pasture-based production systems, and by Chizzolini *et al.* (1999), 0.49-0.54 mg/g muscle, for beef muscles.

The α-tocopherol was the only vitamin E homologue detected in Charneca-PDO beef, although with very high levels in both muscles (11.0-11.5 µg/g muscle). In fact, these values are much higher than those described for meats originated on pasture-fed cattle (4.4-5.8 µg/g)

and on grain-fed cattle receiving supra-nutritional doses of vitamin E (4.3-6.0 µg/g), which in turn are higher than those for meat derived from grain-fed cattle (1.8-2.4 µg/g) (Yang *et al.*, 2002). However, West *et al.* (1997) mentioned contents of α-tocopherol in the *longissimus lumborum* of pastured cattle varying between 3.7 and 7 µg/g, suggesting that there may be a difference in the α-tocopherol levels found in pastures with different biomass compositions. Thus, the values reported here suggest meat from animals grazed on good quality pastures.

Table 11 - Total cholesterol (mg/g muscle) and lipid-soluble antioxidant vitamins (µg/g muscle) in *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of Charneca-PDO beef.

	Muscles			
	LL	**ST**	**SEM**	**Significance**
Total cholesterol	0.44	0.42	0.014	ns
α-Tocopherol	11.0	11.5	1.830	ns
β-Carotene	0.15	0.15	0.042	ns

Significance: ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; SEM, standard error of the mean; means in the same row with different superscripts are significantly different ($P<0.05$).

Concerning carotenoids, β-carotene, a physiological antioxidant and a precursor of vitamin A, that causes some yellow colour to carcass fat, was the only detected in our meats (Lejeune, Peng, Boulengé, Larondelle & Van Hove, 2000). It is well known that, in cattle, β-carotene is essentially the only carotenoid absorbed at the level of the intestine and is, therefore, the predominant carotenoid form found in meat (Yang, Larsen & Tume, 1992; Yang *et al.*, 1993). Yang *et al.* (2002) described the β-carotene content (0.09-0.22 µg/g) in meat from cattle grazed on green pastures, which is rich in tocopherols and carotenoids. The contents of β-carotene found for Charneca-POD beef (0.15 µg/g muscle) are within the range described above.

3.3.4 Nutritional quality of intramuscular fat

A daily consumption of a 150 g steak, trimmed of all visible fat, except the IMF, provides, depending on the muscle, 1.0-1.6 g of total fat, 63-66 mg of cholesterol, 1.7 mg of α-tocopherol and 22.5 µg of β-carotene. Such scenario represents 21-22% of the maximum daily recommended for cholesterol (<300 mg/day) (American Heart Association, 2008), 11-12% of the RDA for α-tocopherol (12/15 mg/day) and 2.5%/3.2% (women/man) of the RDA for β-carotene (700/900 µg/day for women and men, respectively) (Food and Nutrition Board of Institute of Medicine, 2001).

EFSA (2010) proposes not to set any dietary value of reference or range for CLA. However, some extrapolations based on animal studies suggest that 0.8-3.0 g/day are needed to promote

human health benefits (Ip, Singh, Thompson & Scimera, 1994; Parrish *et al.*, 2003). Facing this range, 150 g of Charneca-PDO beef provides only 2.1-5.9 mg of total CLA, which is less than 0.7% of the minimum required to reach the potential beneficial effects. Notwithstanding, the range of values proposed by the former authors symbolize a rough estimative because it is based on animal data extrapolation and, thus should be interpreted carefully until consistent data are available in humans (Schmid *et al.*, 2006).

Regarding the *n*-3 PUFA, EFSA (2010) proposed a daily intake of 250 mg of EPA plus DHA for primary prevention of cardiovascular diseases. Charneca-PDO meat (150 g of steak) provides 56-60 mg of *n*-3 PUFA, thus representing between 5.2 and 6% of the recommended daily intake for EPA plus DHA.

3.4 Conclusions

Charneca-PDO beef showed significant differences between the two muscles analysed, LL (relatively red) and ST (relatively white), in total lipids and percentages of most of fatty acids. The ST muscle is leaner and has higher percentages of PUFA, in contrast to the LL muscle, which depicts higher percentages of SFA and MUFA. However, the contents of CLA isomers, total cholesterol, α-tocopherol and β-carotene were not affected by muscle type, thus suggesting no carcass variation for these compounds.

From a nutritional point of view, the ST muscle seems to be slightly healthier than the LL muscle because is leaner and possesses a higher PUFA/SFA ratio, although the ratio values of both muscles are inside the recommended figures for the human diet (>0.45). In addition, the *n*-6/*n*-3 ratios of both muscles are slightly above to the dietary guideline for human diet (<4). Finally, the high contents found for α-tocopherol suggest that Charneca-PDO beef has good lipid stability.

Taken together, this study indicates that IMF in Charneca-PDO beef has good human health related parameters, with small carcass variation, since the PUFA/SFA and *n-6*/*n-3* ratio values are inside or very close to the recommended values for the human diet, and the contents of α-tocopherol are relatively high. The high nutritional value of Charneca-PDO beef fat reflects the beneficial grass effects on meat lipids, which was only slightly attenuated by the cereal-based concentrate.

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CHAPTER 4 | Effect of slaughter season and muscle type on the fatty acid composition, including conjugated linoleic acid isomers, and nutritional value of intramuscular fat in organic beef

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Abstract

Consumers´ awareness regarding the intake of beef from organic origin is strongly associated with the beneficial outcomes to human health, environment and animal welfare. The effect of slaughter season and muscle type on the fatty acid composition, CLA isomeric profile, total cholesterol, α-tocopherol and β-carotene contents, and nutritional quality of IMF in organic beef (*n*=30) are herein reported for the first time. Organic beef showed a very low total lipid content with seasonal changes in the levels of some fatty acids, CLA isomers, *n*-6/*n*-3 PUFA ratio, total cholesterol and β-carotene. In addition, differences between *longissimus lumborum* (relatively red) and *semitendinosus* (relatively white) muscles were obtained for many fatty acids, specific CLA contents, many CLA isomers, and both PUFA/SFA and *n*-6/*n*-3 ratios. However, in spite of the seasonal and carcass variations, all organic meats analysed had values of beef similar to pasture-fed cattle. From a nutritional perspective, organic meat from both slaughter seasons seems to have high CLA contents, PUFA/SFA and *n*-6/*n*-3 indices within the recommended values for the human diet. In conclusion, the data indicate that IMF in organic meat has a high nutritional value throughout the year.

Keywords: organic beef, fatty acids, CLA isomers, cholesterol, fat-soluble vitamins

4.1 Introduction

Meat fatty acid composition has been studied for many years due to its implications to human health (Woods *et al.*, 2004). Currently, nutritionists recommend not only limiting fat intake but also to consume higher amounts of PUFA, especially those of the *n*-3 family, rather than those from the *n*-6 series (Simopoulos, 2002). In addition, CLA is a group of minor fatty acids occurring naturally in ruminant-derived foods with a multitude of beneficial health effects (Williams, 2000). The most common strategy to produce meat animals, in particular beef cattle, is based on the intake of high levels of concentrate diets, rich in cereals, at least in the finishing period (Dierking *et al.*, 2010). However, it is well documented that animals consuming fresh pastures have higher contents of unsaturated fatty acids in their milk and meat than those receiving a cereal-based concentrate diet (Chilliard *et al.*, 2007; Lourenço *et al.*, 2008). In fact, grass is a good natural source of *n*-3 PUFA, mainly ALA, (18:3*n*-3), although variations occur regarding its maturity and floristic diversity (Woods & Fearon, 2009). In addition, it is also well known that meat from pasture fed cattle is rich in vitamin E, as well as in other natural antioxidants, such as carotenoids (Nuernberger *et al.*, 2005a). Thus, meat from cattle produced under grass-based systems combines the potential beneficial fatty acid profiles and lipid-soluble antioxidant vitamins contents (Daley *et al.*, 2010).

Consumer's studies continue to show that expectations concerning health effects or organic food are the strongest motives for consumers to buy organic products (Huber, Rembialkowska, Srednicka, Bugel & Van der Vijver, 2011). There is also an increase on consumer´s awareness with the environmental impact and animal welfare benefits of organic foods (Brennan, Gallagher & McEachern, 2003; Zanoli *et al.*, 2004). In addition, commercial crossbred bovines, reared in pasture-based systems, have been progressively reintroduced in Portuguese dietary habits, as a result of public perception of chemical residues safety (Rodrigues, Pinto de Andrade & Várzea, 1998) and absence of animal genetic modifications (Napolitano *et al.*, 2010).

The scientific information available to support the nutritional quality of organic beef, mainly dependent on its lipid composition, is scarce. In addition, few works have been conducted to assess seasonal changes on lipid composition of organic beef. Therefore, the aim of the current study was to determine the influence of slaughter season and muscle type on the lipid content, fatty acid composition, including detailed CLA isomeric profile, and total cholesterol,

α-tocopherol and β-carotene contents of organic meat. Moreover, the seasonal and carcass variations of nutritional quality of IMF in organic beef were assessed.

4.2 Material and methods

4.2.1 Animals, diets and meat samples

Limousin × Charolais crossbred young bulls (*n*=30) were maintained according to a traditional production pasture-based system following the established guidelines for organic meat (Council Regulation no. 834/2007 of 28/07, EC). Calves were reared with their dams until weaning at 9 months of age. After weaning, young bulls were raised in an extensive grazing system for fourteen months (Alentejo, South of Portugal). Young bulls were maintained on pasture lands until slaughter in June 2008 (which corresponds to late spring sampling; *n*=15, 23.1±0.9 months, 209±7.5 kg cold carcass weight). The remaining animals were maintained on pasture lands and slaughtered in November 2008 (which corresponds to early autumn sampling; *n*=15, 22±0.8 months, 206±9.1 kg cold carcass weight). The time on pasture was equal in both seasons. Animals fed improved pasture, including clover, alfalfa, ryegrass and tall fescue, as well as native grasses throughout of the year. The predominant species were ryegrass and clover. Naturally, in early autumn the green pastures were less abundant than in late spring. Both slaughter periods were carried out under commercial processing conditions (STEC Abattoir, Montijo, Portugal). Meat samples were taken from the loin portion of *longissimus dorsi* (L4-L6 *longissimus lumborum* muscle, LL) and distal region of *semitendinosus* (ST) muscles on the left side of the carcass. All meat samples were collected 2-3 days after slaughter (1 ºC), ground using a food processor (3 × 5 s), vacuum packed and stored at -80 °C until required for analysis.

4.2.2 Lipid extraction and methylation

IMF was extracted with dichloromethane/methanol (2:1, v/v) from lyophilised (-60 °C and 2.0 hPa; Edwards High Vacuum International, UK) meat samples (*ca* 250 mg), for total lipid determination and for both FAME and CLA methyl esters profiles, using the procedures described previously by Alfaia *et al.* (2006a). All the extraction solvents contained 0.01% BHT as an antioxidant. Total lipids were measured gravimetrically, in duplicate, by weighting the fatty residue obtained after solvent evaporation. Fatty acids and CLA isomers were converted to methyl esters by a combined transesterification procedure with NaOH in

anhydrous methanol (0.5 M), followed by HCl:methanol (1:1, v/v), at 50 °C during 30 and 10 min, respectively, according to Raes *et al.* (2004). The same FAME solution was used for the analysis of both fatty acid composition and CLA isomeric profile, enabling the direct comparison of quantitative data and eliminating differences in sample preparation.

4.2.3 Determination of fatty acid composition

FAME were analysed by GC-FID (chromatograph HP 6890; Hewlett–Packard, Avondale, PA, USA) using a fused-silica 100 m capillary column (CP-Sil 88^{TM}; 0.25 mm i.d., 0.2 µm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA), as described by Alves and Bessa (2009). Briefly, the initial oven temperature was 100 °C (held for 1 min), then increased by 50 °C per min to 150 °C (held for 20 min), then increased by 1 °C per min to 190 °C (held for 5 min), and finally increased by 1 °C per min to 200 °C (held for 35 min). Ultra pure helium was used as the carrier gas, at a flow rate of 1 mL/min, and the split ratio was 1:50. The injector and detector temperatures were maintained at 250 and 280 ºC, respectively. The identification of common fatty acids was accomplished by comparison of sample peak retention times with those of FAME standard mixtures (Nu-Chek-Prep Inc., Elysian, MN, USA; Supelco Inc. Bellefonte, PA, USA) and by using published chromatograms obtained with similar analytic conditions (Kramer *et al.*, 2008). Structural analyses of some unknown peaks were conducted by GC-MS/MS technique, using a Varian Saturn 2200 system (Varian Inc.) equipped with a CP-Sil 88^{TM} capillary column (100 m × 0.25 mm i.d., 0.2 µm film thickness). The quantification of total FAME was done using nonadecanoic acid (19:0) as internal standard. Results for each fatty acid were expressed as g/kg of total fatty acids.

4.2.4 Determination of individual CLA isomers

The methyl esters of CLA isomers were individually separated by triple silver-ion columns in series (ChromSpher 5 Lipids, 250 mm × 4.6 mm i.d., 5 µm particle size, Chrompack, Bridgewater, NJ, USA), using an high performance liquid chromatography system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA), according to the procedure reported previously by Alfaia *et al.* (2006a). Briefly, the mobile phase was 0.1% acetonitrile in *n*-hexane, at a flow rate of 1 mL/min, the DAD was adjusted to 233 nm and volumes of 20 µL were injected by the autosampler. The identification of the individual CLA isomers was achieved by the comparison of their retention times with commercial and prepared standards, as well as with values published in the literature (Fritsche, Rumsey, Yurawecz, Ku &

Fristsche, 2001; Prates & Bessa, 2009). In addition, the identity of each isomer was controlled by the typical ultraviolet spectra of CLA isomers from the DAD in the range from 190 to 360 nm, using the spectral analysis of Agilent Chemstation for LC 3D Systems rev. A.09.01. Standards of CLA isomers (*c*9,*t*11, *t*10,*c*12, *c*11,*t*13, *c*9,*c*11 and *t*9,*t*11) were purchased from Matreya Inc. (Pleasant Gap, PA, USA) and Sigma Inc. (St. Louis, MO, USA) or prepared (*cis/trans* and *trans,trans* from carbons 7,9 to 12,14) by the procedure reported by Destaillats and Angers (2003). Total CLA content was calculated as the sum of its main isomer *c*9,*t*11 (plus *t*7,*c*9 and *t*8,*c*10) determined by GC-FID with the other minor isomers quantified by Ag^{+}-HPLC analysis (Kraft *et al.*, 2003; Cruz-Hernandez *et al.* 2006). The individual CLA isomers were expressed as g/kg of total fatty acids.

4.2.5 Determination of cholesterol, tocopherols and β-carotene

The simultaneous analysis of cholesterol, tocopherols and β-carotene in meat was performed according to Prates *et al.* (2006). Briefly, the saponification of homogenised fresh meat samples (*ca* 750 mg) was carried out with 0.2 g L-ascorbic acid and 5.5 mL saponification solution containing 11% w/v potassium hydroxide, in a mixture of 55% v/v absolute ethanol and 45% v/v distilled water, in a shaking water bath at 80 °C for 15 min. After cooling, 1.5 mL of distilled water and 3 mL of 25 μg/mL BHT solution in *n*-hexane were added, then the samples were vigorously vortexed for 2 min and centrifuged at 1500 *g* for 5 min. Afterwards, an aliquot of the *n*-hexane layer was filtered through a 0.45 μm hydrophobic membrane and injected in an HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, U.S.A) using a normal-phase silica column (Zorbax RX-Sil, 250 mm × 4.6 mm i.d., 5 μm particle size, Agilent Technologies Inc., Palo Alto, CA, USA), with fluorescence detection for tocopherols (excitation and emission wavelengths of 295 nm and 325 nm, respectively) and UV-Vis photodiode array detection for cholesterol (202 nm) and β-carotene (450 nm) in series. The solvent was 1% v/v isopropanol in *n*-hexane, the flow rate was 1 mL/min, the run lasted for 17 min and the temperature of the column was adjusted at 20 °C. The contents of total cholesterol, tocopherols and β-carotene in meat were calculated, in duplicate for each muscle sampled, based on the external standard technique from a standard curve of peak area *vs.* concentration, using DL-α-, D-β-, D-γ- and D-δ-tocopherols (Calbiochem, Merck Biosciences, Darmstadt, Germany), all-*trans*–carotene and cholesterol as standards (Sigma Chemical Co., St. Louis, MO, USA).

4.2.6 Statistical analysis

Data were analysed using the MIXED procedure of the Statistical Analysis Systems software package, version 9.1 (SAS Institute, Cary, NC, USA). The model considers the effect of slaughter season (late spring *versus* early autumn), muscle type (LL and ST muscles) and the interaction between slaughter season and muscle type (S×M). Each animal from the slaughter season was considered as the subject and the muscle type as repeated measures. Least square means were presented and compared using the PDIFF option, when the interaction effect was significant ($P<0.05$).

4.3. Results and discussion

4.3.1 Intramuscular fatty acid composition

In autumn, total lipid content was higher ($P<0.05$) in LL muscle than in ST muscle, in contrast to spring, in which both muscles had similar values ($P>0.05$) (Table 12). This interaction between muscle type and slaughter season can be explained by modifications in muscle fibre composition throughout the year due to the seasonal changes in the physical activity of animals. Comparing to ST muscle, LL muscle is relatively red and differently involved in the physical activity imposed by grazing (Vestergaard *et al.*, 2000a).The lipid content is higher in red oxidative muscle fibres (Enser *et al.*, 1998) and, as explained above, the LL muscle of cattle is relatively red in comparison to the ST muscle. Organic beef had lower values of total lipids (6.3-7.2 g/kg) when compared with those reported for the meat from extensively (11-14 g/kg) produced cattle (Vestergaard *et al.*, 2000b). In addition, higher values were also obtained by Alfaia *et al.* (2009), using the same analytical methodology than the one used in this work, for total lipids of cattle fed on pasture (10 g/kg). The levels of IMF reported here, which is in close agreement with the ones described above for the cold carcass weights, can be explained by the low energy content of pastures and the absence of concentrate finishing period (Alfaia *et al.*, 2009). These values suggest low levels of muscle triacylglycerols since the values of phospholipids, although dependent on the muscle fibre composition, are fairly constant in the same muscle fibre type (Alasnier, Remignon & Gandemer, 1996). In fact, the content of fatty acids from phospholipids (mainly PUFA) remain fairly constant but those from neutral lipids, with their high proportions of SFA and MUFA, increase markedly as total lipids increase (Wood *et al.*, 2008).

Table 12 - Total lipids (g/kg muscle), fatty acid composition (g/kg total fatty acids), partial sums of fatty acids and nutritional indices of *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of organic meat from late spring and early autumn.

	Spring		Autumn			Significance level		
	LL	ST	LL	ST	SEM	Season	Muscle	S × M
Total lipids	6.56[a,b]	6.92[a,b]	7.18[a]	6.31[b]	0.410	ns	***	*
Fatty acid composition								
14:0	7.88	4.24	8.38	4.81	0.699	ns	***	ns
14:1*c*9	1.21[a]	0.55[b]	1.21[a]	0.01[c]	0.118	ns	***	**
15:0*iso*	1.23	0.84	0.95	0.83	0.072	ns	***	ns
15:0*anteiso*	2.06	1.55	1.49	1.03	0.100	***	***	ns
15:0	4.41	3.58	3.29	2.90	0.147	***	***	ns
16:0*iso*	1.42	1.21	1.22	1.21	0.073	ns	ns	ns
16:0	183[a]	156[b]	191[a]	177[a]	3.008	**	***	**
16:1*c*7	3.31	2.30	2.36	2.31	0.305	ns	ns	ns
16:1*c*9	10.9	9.21	13.3	10.5	0.757	ns	***	ns
16:1*t*9	4.62[a]	4.69[a]	3.17[b]	4.55[b]	0.314	ns	***	***
17:0*iso*	4.40[a]	3.90[b]	3.22[c]	3.17[c]	0.127	***	***	*
17:0*anteiso*	3.70	2.59	3.47	2.57	0.210	ns	***	ns
17:0	7.92	6.65	7.28	5.88	0.256	*	***	ns
17:1*c*9	6.45	6.47	6.08	5.69	0.258	ns	ns	ns
18:0	156[a]	153[a]	146[a]	127[b]	2.626	***	***	***
18:1*c*9	198	178	186	181	6.975	ns	***	ns
18:1*c*12	1.63	1.59	2.08	1.82	0.153	*	ns	ns
18:1*c*13	0.88	0.80	1.08	1.10	0.102	*	ns	ns
18:1*c*15	0.86	0.89	0.92	0.87	0.070	ns	ns	ns
18:1*t*6+*t*8	1.06	0.93	1.28	1.02	0.080	ns	**	ns
18:1*t*9	0.97	0.83	1.48	1.09	0.090	***	***	ns
18:1*t*10	0.89[c]	0.75[c]	5.75[a]	4.76[b]	0.672	***	**	*
18:1*t*11	16.4	13.0	17.9	10.8	1.290	ns	***	ns
18:1*t*12	2.38[a]	1.95[b]	2.55[a]	2.57[a]	0.139	*	*	*
18:1*c*11+*t*15	16.7[c]	18.0[b]	20.1[a]	19.0[b]	0.450	**	ns	***
18:1*t*16+*c*14	1.96	1.81	1.81	1.40	0.093	**	**	ns
18:2*c*9,*t*11 †	5.90	5.13	5.54	5.26	0.312	ns	ns	ns
18:2*t*11,*c*15	1.96	1.41	1.98	1.14	0.159	ns	***	ns
18:2*n*-6	124	148	133	158	6.297	ns	***	ns
18:3*n*-3	52.2	61.9	35.3	42.8	2.410	***	***	ns
18:3*n*-6	1.15[b]	1.18[b]	0.99[b]	1.43[a]	0.078	ns	**	*
18:3*c*9,*t*11,*c*15	3.40	4.48	2.90	3.79	0.212	*	***	ns
20:0	1.33	1.41	1.18	1.16	0.053	**	ns	ns
20:2*n*-6	1.67	1.29	1.19	1.61	0.176	ns	ns	ns
20:4*n*-6	41.6[b]	47.3[b]	43.4[b]	57.7[a]	2.793	ns	***	*
20:5*n*-3	21.6	27.6	21.7	31.1	1.222	ns	***	ns
22:0	8.45	11.8	10.1	13.9	0.569	**	***	ns
22:4*n*-6	2.14	2.55	2.20	2.96	0.158	ns	***	ns
22:5*n*-3	27.8	34.8	26.7	36.2	1.335	ns	***	ns
22:6*n*-3	2.23	2.64	3.04	3.76	0.266	**	*	ns
23:0	2.85	3.64	3.01	3.97	0.240	ns	**	ns

[a,b,c]Least square means in the same row with different superscripts are significantly different ($P<0.05$); Significance: ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; SEM, standard error of the mean. The symbols used mean as follow: S × M, interaction between slaughter season (S) and muscle type (M); † This peak also includes minor amounts of the *t*7,*c*9 and *t*8,*c*10 CLA isomers.

As the majority of the beef produced in Europe, the analysed organic beef can be considered a lean meat, according to the Food Advisory Committee (1990) criteria (<5 g/kg fat).

Data on the fatty acid composition (g/kg total fatty acids) of IMF in organic beef from late spring and early autumn are presented in Table 12. In both seasons, the predominant fatty acids in IMF were 16:0, 18:0, 18:1*c*9, 18:2*n*-6, 18:3*n*-3 and 20:4*n*-6. Similar results were reported for cattle in numerous studies (Eriksson & Pickova, 2007; Alfaia *et al.*, 2009; Muchenje *et al.*, 2009a; Dierking *et al.*, 2010). Among these, only 18:1*c*9 and 18:2*n*-6 were not affected by seasonal variations ($P>0.05$). Nevertheless, 12 of the 41 fatty acids analysed were affected ($P<0.05$) by this variable. Regarding the minor fatty acids, organic meat from autumn had higher contents of 18:1*c*12, 18:1*c*13, 18:1*t*6+*t*8, 18:1*t*12, 22:0 and 22:6*n*-3, but lower contents of 15:0*anteiso*, 15:0, 17:0*iso*, 18:1*t*16+*c*14, 18:3*c*9,*t*11,*c*15 and 20:0, relative to meat from spring. It is well known that beef fatty acid composition is influenced by both diet and season (Wood *et al.*, 2008). The botanic diversity of pastures influence fatty acid metabolism in the rumen through the presence of secondary metabolites in plants (Lourenço *et al.*, 2010). Meat from young bulls from late spring had higher ALA contents ($P<0.001$) when compared to that from early autumn. ALA is a very sensitive grass intake indicator due to its presence in large amounts in grass lipids (Wood *et al.*, 2008). This result was expected and can be explained by the fact that young bulls raised during late spring were exposed to more abundant pastures than animals raised on early autumn. The contents of ALA in meat from spring are higher than the ones previously found for meat from pasture-fed cattle (Eriksson & Pickova, 2007; Muchenje *et al.*, 2009a) but lower than those reported by Alfaia *et al.* (2009) for pasture-fed bulls.

The muscle type had a high influence on the fatty acid profile, affecting 21 of the 41 fatty acids analysed. The LL muscle, relatively to the ST muscle, had higher contents of 14:0, 15:0*iso*, 15:0*anteiso*, 15:0, 16:0, 16:1*c*9, 17:0*iso*, 17:0*anteiso*, 17:0, 18:1*t*12, 18:1*t*16+*c*14, 18:2*t*11,*c*15, but lower contents of 18:2*n*-6, 18:3*n*-3, 18:3*c*9,*t*11,*c*15, 20:4*n*-6, 20:5*n*-3, 22:0, 22:4*n*-6, 22:5*n*-3 and 23:0. These differences likely result from variations in muscle fibre composition because the lipid content, which affects directly fatty acid composition, is higher in red oxidative muscle fibres (Enser *et al.*, 1998).

The results for partial sums of intramuscular fatty acids (g/kg total fatty acids) in organic beef obtained in two distinct slaughter seasons are presented in Table 13.

Table 13 - Partial sums of fatty acids (g/kg total fatty acids) of *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of organic beef from late spring and early autumn.

	Spring		Autumn			Significance level		
	LL	**ST**	**LL**	**ST**	**SEM**	**Season**	**Muscle**	**S × M**
Partial sums								
SFA	373	341	370	337	5.362	ns	***	ns
MUFA	223	198	243	203	7.840	ns	***	ns
TFA	56.2^{b}	53.0^{b}	64.4^{a}	55.5^{b}	2.132	*	***	*
PUFA	274	327	267	335	11.78	ns	***	ns
BCFA	12.8	10.1	10.4	8.81	0.445	**	***	ns
n-6 PUFA	170	200	181	222	8.063	*	***	ns
n-3 PUFA	104	127	86.7	114	4.415	**	***	ns
Unidentified	60.8	71.7	45.2	60.4	2.786	***	***	ns
Fatty acid ratios								
PUFA/SFA	0.74	0.94	0.74	1.00	0.044	ns	***	ns
n-6/*n*-3	1.65	1.59	2.10	1.96	0.052	***	***	ns

[a,b,c]Least square means in the same row with different superscripts are significantly different (*P*<0.05*);* Significance: ns, *P*>0.05; *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001; SEM, standard error of the mean. The symbols used mean as follow: S × M, interaction between slaughter season (S) and muscle type (M); SFA = sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0; MUFA = sum of 14:1*c*9, 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*12, 18:1*c*13 and 18:1*c*15; TFA = sum of 16:1*t*9, 18:*1t6+t8*, 18:1*t*9, 18:1*t*10, 18:1*t*11, 18:1*t*12, 18:1*c*11,*t*15, 18:1*t*16+*c*14, 18:2*t*11,*c*15, 18:2*c*9,*t*11 and 18:3*c*9,*t*11,*c*15; PUFA = sum of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-3 and 22:6*n*-3; BCFA = sum of 15:0*iso*, 15:0*anteiso*, 16:0*iso*, 17:0*iso* and 17:0*anteiso*; *n*-6 PUFA = sum of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:4*n*-6 and 22:4*n*-6; *n*-3 PUFA= sum of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3; PUFA/SFA = [(sum of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-3 and 22:6*n*-3)/(sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0)]; *n*-6/*n*-3 = *n*-6/*n*-3 ratio [(sum of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:4*n*-6 and 22:*4n*-6)/(sum of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3)].

SFA and PUFA in meat were influenced by muscle type, with higher and lower values for the LL muscle, respectively. The slaughter season had no influence on these fatty acid sums. Knowing that SFA are mainly located in the triacylglycerols and PUFA in the phospholipids, the muscle effect is largely explained by the higher triacylglycerol/phospholipid ratio in the relatively red LL muscle when compared to the relatively white ST muscle. Furthermore, both season and muscle type affected the values of BCFA, *n*-6 PUFA and *n*-3 PUFA in meat. In agreement with our results, Rule, Broughton, Shellito & Maiorano (2002) and Alfaia *et al.* (2007) reported higher proportions of PUFA, including the sums of *n*-3 and *n*-6 in the ST muscle than in the LL muscle. However, distinct grass and pasture plants produce different PUFA concentrations in meat due to distinct levels of individual PUFA and variations in the way the feed is processed in the rumen (Wood *et al.*, 2008).

Significant interactions between slaughter season and muscle type were observed for TFA sum, and for the 16:0, 14:1*c*9, 16:1*t*9, 17:0*iso*, 18:0, 18:1*c*9, 18:1*t*10, 18:1*t*11, 18:1*t*12, 18:1*c*11+*t*15, 18:3*n*-6 and 20:4*n*-6 fatty acids. These interactions may result from modifications of muscle metabolic type caused by adaptations to distinct season periods (Klont *et al.*, 1998). Grass-feeding systems promote the accumulation of 18:1*t*11 fatty acid in beef, which can be desaturated to 18:2*c*9,*t*11 by Δ9-desaturase enzyme in bovine adipose tissue and mammary gland (Griinari *et al.*, 2000). The 18:1*t*11 fatty acid is the precursor of

18:2*c*9,*t*11 CLA isomer, the major CLA isomer in ruminant-derived products (Lock *et al.*, 2004). Alfaia *et al.* (2009) reported similar values for 18:1*t*11 fatty acid in meat from bulls fed with grass but Leheska *et al.* (2008) obtained higher values in beef from grass-fed cattle. Alfaia *et al.* (2009) also reported, in muscle from pasture-fed cattle, the presence of higher contents of the non-conjugated linoleic acid isomer 18:2*t*11,*c*15, an intermediate of ALA biohydrogenation in the rumen (Bessa *et al.*, 2007), comparing to our study.

The ratios of PUFA/SFA and *n*-6/*n*-3, which are indices widely used to evaluate the nutritional value of fat for human consumption, are defined and presented in Table 13. A season effect was observed for the *n*-6/*n*-3 ratio ($P<0.001$) but not for the PUFA/SFA ratio ($P>0.05$). According to the current nutritional recommendations, the PUFA/SFA ratio in human diet should be above 0.45 and, concerning PUFA, the *n*-6/*n*-3 ratio should not exceed 4.0 (British Department of Health, 1994). However, this last index has been recently subject of controversy (Griffin, 2008). Concomitantly, Stanley *et al.* (2007) have proposed that it is more important to evaluate the total amount of dietary PUFA than their respective ratio. Moreover, Brenna *et al.* (2009) proposed that the *n*-3 PUFA status could be improved by increasing the dietary intake of *n*-3 PUFA or by reducing the intake of *n*-6 PUFA and that combining both strategies would be the most effective nutritional measure. In this study, the PUFA/SFA and *n*-6/*n*-3 ratios in organic meat are within the recommended values for human diet, which is favourable, for both slaughter seasons and muscle types. These desirable values clearly result from the benefits of grass feed on ruminant meat, as stated by Nuernberg *et al.* (2005a). Comparing to available data, our values for the *n*-6/*n*-3 ratio are close to those reported for meat from grass-fed cattle (Enser *et al.*, 1998; Eriksson & Pickova, 2007; Leheska *et al.*, 2008; Alfaia *et al.*, 2009). It is well established that in meat from cattle fed with concentrates based on cereals (rich in *n*-6 PUFA) the *n*-6/*n*-3 ratio shifts to higher values when compared to animals produced on green pastures (rich in *n*-3 PUFA) (Nuernberger *et al.*, 2002). Our meat values for the PUFA/SFA ratio meet the recommended guidelines for the human diet throughout the year. In fact, several studies described that the PUFA/SFA index is generally increased with pasture feeding (French *et al.*, 2000; Realini *et al.*, 2004; Wood *et al.*, 2009).

4.3.2 Intramuscular CLA isomeric profile

Data regarding the contents (g/kg muscle and g/kg fat) and isomeric profile of CLA (g/kg total fatty acids) in the IMF of organic meat are displayed in Table 14. Total and specific CLA contents in organic beef did not show significant differences ($P>0.05$) when slaughter seasons were compared. In contrast, the LL muscle had higher total and specific CLA contents than the ST muscle ($P<0.001$). The results reported here for specific CLA content (2.0-3.2 g/kg fat) were lower than those reported by Realini *et al.* (2004) and Alfaia *et al.* (2009) and in the LL muscle from grazing beef (5.3 and 5.1 g/kg fat, respectively). However, a direct linear relationship between grass percentage in cattle diet and meat CLA content has been described by French *et al.* (2000), although the mechanism remains controversial.

The slaughter season influenced the content of *t*11,*t*13, *t*11,*c*13 *t*10,*c*12 and *t*7,*c*9 CLA isomers in organic beef. In addition, the muscle type had impact on *t*11,*t*13, *t*11,*c*13 *c*9,t11 and *t*7,*c*9 CLA isomers. Only 3 of the 14 CLA isomers analysed were affected by the interaction between slaughter season and muscle type, *t*9,*t*11, *t*8,*t*10 and *c*11,*t*13, and the sum of total *trans,trans* CLA isomers. The higher *c*9,*t*11 CLA content in the LL muscle of organic beef may be explained by the higher contents of 18:1*t*11 in this muscle, which is the substrate of Δ9-desaturase, relative to the ST muscle, as proposed by Daniel *et al.*, (2004a). The CLA isomeric profile showed a clear predominance of the bioactive *c*9,*t*11 isomer in both muscle types and slaughter seasons (68-73%). Several authors reported that diets containing proportionally high levels of ALA, such as fresh grass, grass silage and concentrate containing linseed, resulted in an increased deposition of the *c*9,*t*11 CLA isomer in the muscle (French *et al.*, 2000; Dannenberger *et al.*, 2005). The *t*11,*c*13 isomer, the second-most prevalent CLA isomer in the organic meat reported here, was higher in the meat from spring than in that from autumn. In addition, the *t*11,*t*13 (the third most prevalent isomers) and *t*12,*t*14 CLA isomers are also sensitive grass intake indicators (Nuernberg *et al.*, 2002; Dannenberger *et al.*, 2005). In line with this, the high content of these isomers in our meat reflects the exposition to abundant availability and quality of pasture from spring-slaughtered animals, relative to that of autumn-slaughtered animals. According to Dannenberger *et al.* (2005), the variations found in CLA isomeric profile might be explained by differences in grass intake because pasture feeding increases some CLA isomers (the sensitive grass intake indicators) and decreases the *t*7,*c*9 CLA isomer in beef lipids. The LL muscle in early autumn had higher contents of the *t*7,*c*9 CLA isomer, relative to the other meats, which is originated from the 18:1*t*7 (Collomb *et al.*, 2004), and is frequently recognized as the second-most

predominant isomer in ruminant fat from concentrate-fed animals (Bauman *et al.*, 2007). All other CLA isomers are supposed to arise from ruminal microflora by biohydrogenation (Collomb *et al.*, 2004). The other bioactive CLA isomer, the *t*10,*c*12, appeared in residual amounts in all meats (<2.6% of total CLA). This isomer was influenced by the slaughter season, displaying higher contents in meat from early autumn relative to that from late spring, which is in agreement with the results described by Alfaia *et al.* (2006a).

Table 14 - Total (g/kg muscle) and specific (g/kg fat) CLA contents and its individual isomers (g/kg total fatty acids) in *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of organic meat from late spring and early autumn.

	Spring		**Autumn**			**Significance level**		
	LL	**ST**	**LL**	**ST**	**SEM**	**Season**	**Muscle**	**S × M**
Total CLA	0.02	0.01	0.02	0.01	0.002	ns	***	ns
Specific CLA	3.18	1.97	2.77	2.09	0.333	ns	***	ns
CLA isomers								
*t*12,*t*14	0.10	0.06	0.06	0.04	0.018	ns	ns	ns
*t*11,*t*13	0.30	0.25	0.20	0.12	0.028	**	ns	ns
*t*10,*t*12	0.04	0.04	0.05	0.06	0.008	ns	**	ns
*t*9,*t*11	0.24^{b}	0.29^{a}	0.19^{b}	0.07^{c}	0.016	***	ns	***
*t*8,*t*10	0.05^{b}	0.04^{b}	0.11^{a}	0.03^{b}	0.009	ns	***	***
*t*7,*t*9	0.05	0.04	0.04	0.04	0.008	ns	ns	ns
*t*6,*t*8	0.04	0.03	0.02	0.02	0.006	*	ns	ns
Total *trans*,*trans*	0.81^{a}	0.74^{a}	0.66^{a}	0.37^{b}	0.049	***	ns	**
c/*t*12,14	0.03	0.02	0.04	0.05	0.009	ns	ns	ns
*t*11,*c*13	0.38^{a}	0.30^{a}	0.29^{b}	0.21^{c}	0.025	***	***	**
*c*11,*t*13	0.08^{a}	0.07^{a}	0.04^{b}	0.07^{a}	0.006	ns	***	*
*t*10,*c*12	0.07	0.06	0.11	0.10	0.011	***	ns	ns
*c*9,*t*11	3.61	2.81	3.32	2.81	0.221	ns	***	ns
*t*7,*c*9	0.13	0.10	0.26	0.17	0.021	***	*	ns
Total *cis*/*trans*	4.29	3.36	4.07	3.41	0.251	ns	***	ns
Total *cis*,*cis* (*c*9,*c*11)	0.03	0.04	0.03	0.05	0.005	ns	**	ns

[a,b,c]Least square means in the same row with different superscripts are significantly different (*P*<0.05*);* Significance: ns, *P*>0.05; *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001; SEM, standard error of the mean. The symbols used mean as follow: S × M, interaction between slaughter season (S) and muscle type (M).

4.3.3 Contents of cholesterol, tocopherols and β-carotene in meat

Data on the contents of total cholesterol, α-tocopherol and β-carotene in organic meat are presented in Table 15. Total cholesterol content was higher in meat from spring-slaughtered young bulls than in that from autumn-slaughtered animals (*P*<0.001). This effect is in line with results reported by Alfaia *et al.* (2006b). In contrast, the muscle type had no influence on total cholesterol contents. The levels of total cholesterol in organic beef (0.36-0.42 g/kg muscle) were similar to those found by Muchenje *et al.* (2009a) in beef raised on natural pasture (0.37-0.42 g/kg muscle). However, Leheska *et al.* (2008) found higher values of cholesterol (0.57 g/kg muscle) in strip steak from grass-fed animals, in contrast to Padre *et al.*

(2007), who reported lower levels (0.46-0.48 g/kg muscle) in beef from pasture-based production systems.

Table 15 - Total cholesterol (g/kg muscle) and lipid-soluble antioxidant vitamins (α-tocopherol and β-carotene; mg/kg muscle) in *longissimus lumborum* (LL) and *semitendinosus* (ST) muscles of organic beef from late spring and early autumn.

	Spring		**Autumn**			**Significance level**		
	LL	**ST**	**LL**	**ST**	**SEM**	**Season**	**Muscle**	**S × M**
Total cholesterol	0.37	0.36	0.41	0.42	0.013	***	ns	ns
***α*-Tocopherol**	5.28	5.43	5.45	5.48	0.530	ns	ns	ns
***β*-Carotene**	0.10	0.09	0.09	0.06	0.010	**	ns	ns

Significance: ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; SEM, standard error of the mean. The symbols used mean as follow: S × M, interaction between slaughter season (S) and muscle type (M).

The slaughter season and muscle type did not affect ($P>0.05$) α-tocopherol contents in organic meat. The levels of α-tocopherol in beef, ranging from 5.28 to 5.48 mg/kg muscle, were higher than the values reported by Prates *et al.* (2006) (3.30-3.90 mg/kg muscle), Costa *et al.* (2008) (1.30-2.80 mg/kg muscle) and, more recently, by Röhrle *et al.* (2011) (2.43-2.63 mg/kg muscle). It was reported by some authors that pasture feeding increases the accumulation of α-tocopherol in the muscle tissue due to the higher contents of this lipid-soluble antioxidant vitamin in grass relative to concentrate feeds (Yang *et al.* 2002; Descalzo *et al.* 2005).

β-carotene content was only influenced by the slaughter season, with higher levels in organic beef from late spring than in that from early autumn. The contents of β-carotene, depending on the muscle and slaughter season considered (0.06-0.10 mg/kg), reached the lower limit described for meat from cattle grazed on green pastures (0.09-0.22 mg/kg), which are naturally rich in tocopherols and carotenoids (Yang *et al.*, 2002).

4.3.4. Nutritional quality of intramuscular fat

A daily consumption of 150 g of steak from the organic meat reported here, trimmed of all visible fat with the exception of IMF, provides 54-62 mg of cholesterol, 0.8 mg of α-tocopherol and 8.5-14.5 μg of β-carotene. Such values represent 18-21% of the maximum daily recommended for cholesterol (<300 mg/day) (American Heart Association, 2008), 5.3-5.5 % of the RDA for α-tocopherol (15 mg/day) and only 1.2-2.1%/1.0-1.6% (women/men) of the recommended dietary allowance for β-carotene (700/900 μg/day for women and men, respectively) (Food and Nutrition Board of Institute of Medicine, 2001).

The EFSA (2010) proposed not to set any dietary value of reference or range for CLA intake. However, some extrapolations based on animal studies suggest that 0.8-3.0 g/day are needed to promote human health benefits (Ip *et al.*, 1994; Parrish *et al.*, 2003). Facing this range, 150 g of the organic beef described here provides only 2.1-3.0 mg of total CLA, i.e., less than 0.4% of the minimum required to reach the beneficial effects. Notwithstanding, the range of values proposed by Ip and colleagues represents only approximate values with potential beneficial effects to humans because it was based on animal data extrapolation. Hence, it should be interpreted with caution until experimental human consistent findings are available (Schmid *et al.*, 2006).

Regarding the *n*-3 PUFA, the EFSA (2010) proposed an intake of 250 mg *per* day of EPA plus DHA for primary prevention of CVD. This organic meat (150 g of steak) provides 23.5-33.0 mg of EPA plus DHA, thus representing from 9.4 to 13.2% of the recommended daily intake for EPA plus DHA.

4.4. Conclusions

The data indicate that organic beef is a lean meat with seasonal differences in the levels of some fatty acids (12 in a total of 41), CLA isomers (4 in a total of 14), *n*-6/*n*-3 PUFA ratio, total cholesterol and β-carotene. In addition, significant differences were obtained between LL (relatively red) and ST (relatively white) muscles for 21 fatty acids, specific CLA content, 4 CLA isomers and both PUFA/SFA and *n*-6/*n*-3 ratios. In spite of the seasonal and carcass variations, organic beef seems to have values of meat similar to pasture-fed cattle, due to the contents of some individual fatty acids and CLA isomers (sensitive grass intake indicators), and to the contents of lipid-soluble antioxidant vitamins (α-tocopherol and β-carotene).

From a nutritional point of view, organic meat from both slaughter seasons and muscles has health related parameters since the content of the *c*9,*t*11 CLA isomer is high and the PUFA/SFA and *n*-6/*n*-3 indices are within the recommended values for the human diet. Based on the synergistic antioxidant effect between α-tocopherol and β-carotene, these findings suggest that organic beef seems to have high stability towards lipid oxidation. Taken together, the data indicate that organic meat IMF, as a result of the beneficial effects of grass feeding on the characteristics of the meat lipids, has high nutritional quality throughout the year.

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CHAPTER 5 | Influence of dietary gluconeogenesis precursors and linseed oil on growth performance, rumen fermentation and intramuscular fatty acids of lambs

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Abstract

This experiment was conducted to test the hypothesis that adding gluconeogenesis precursors (propylene glycol and calcium propionate mix) to a high-forage and high-oil diet would enrich lamb meat in 18:2*c*9,*t*11 (rumenic acid), through both the maintenance of high level of 18:1*t*11 in rumen outflow and an increase in its endogenous conversion to rumenic acid. To test this hypothesis, the effect of inclusion of a propylene glycol and calcium propionate mix (PP) (0 g/kg *vs.*50 g/kg dry matter, DM) and linseed oil supplementation (0 g/kg *vs.* 60 g/kg DM) in diets was evaluated, during 6 weeks, on thirty-six Merino Branco lambs with initial live weights of 27.3±3.52 kg (randomized 2×2 factorial design). PP did not affect DM intake, average daily gain and carcass traits, except for an increase ($P<0.05$) of subcutaneous fat proportion of chump and shoulders. Serum insulin concentration was not affected by treatments, although PP tended ($P=0.09$) to decrease serum glucose concentration. Linseed oil supplementation increased 18:3*n*-3 as most of the C18 BI, including 18:1*t*11 and rumenic acid. PP attenuated the strong increase of 18:1*t*11 induced by linseed oil supplementation and tended to reduced ($P=0.054$) the 18:1*t*11/18:1*t*10 ratio in meat. The SCD activity, estimated by the ratio of catalysed FA, was depressed by oil supplementation but not by PP, except for the 16:1*c*9/(16:1*c*9+16:0) index. Contrarily to our working hypothesis, the PP reduced the rumenic acid concentration.

Keywords: lamb, propylene glycol, calcium propionate, linseed oil, stearoyl CoA desaturase, fatty acids

5.1 Introduction

Ruminant meat lipids are highly saturated and, therefore, its consumption has been discouraged supporting the nutritional guidelines from national and international public health agencies (WHO, 2003). Extensive hydrogenation of ingested PUFA by rumen microorganisms is the major determinant of high content of SFA in ruminant fats. However, ruminal biohydrogenation of PUFA involves both isomerisation and reduction steps leading to the formation of BI, including conjugated-dienes, *trans*-monoenes and SFA, which are subsequently absorbed and available for incorporation into muscle lipids (Jenkins, Wallace, Moate & Mosley, 2008).

The nutritional quality of lipids in lamb meat may be enhanced by dietary manipulation strategies envisaging minimise the extension of the biohydrogenation of ingested PUFA in the rumen (Bessa *et al.*, 2000). The stearic (18:0) and VA (18:1*t*11) acids are the main products of rumen biohydrogenation, and both can be desaturated by the SCD to oleic (18:1*c*9) and rumenic (18:2*c*9,*t*11) acids, respectively (Griinari & Bauman, 1999). Rumenic acid, which derives either directly from rumen biohydrogenation or indirectly by endogenous desaturation of 18:1*t*11, has been associated with numerous health-promoting properties, including potent anti-carcinogenic effects, as reviewed by Benjamin and Spener (2009). Thus, increasing the SCD gene expression and activity may significantly improve the nutritional quality of lamb meat by decreasing the SFA content, while increasing both the oleic acid, and 18:2*c*9,*t*11 contents. Daniel *et al.* (2004b) have shown that insulin increases the expression of SCD and the synthesis of MUFA from acetate, in cultured adipose tissue from ovine explants. In addition, Daniel *et al.* (2004a) showed that concentrate-fed lambs had higher SCD gene expression and, consequently, higher oleic acid content in tissues than forage-fed lambs. However, in spite of down-regulation of SCD in forage-fed lambs, the rumenic acid content in tissues was higher than in concentrate-fed lambs. In forage-fed lambs, the availability of 18:1*t*11 for absorption is much higher than in concentrate-fed lambs, where the 18:1*t*10 predominates due to changes in rumen biohydrogenation pathways (Griinari & Bauman, 1999). Thus, it would be important to achieve simultaneously high availability of 18:1*t*11 and up-regulation of SCD in order to obtain high concentrations of rumenic acid in lamb meat. The most effective dietary approach to increase metabolic availability of 18:1*t*11 is supplementing high forage diets with oils rich in PUFA (Bessa *et al.* 2000). As the rumenic acid is not an intermediate of ALA (18:3*n*-3) ruminal biohydrogenation (Harfoot & Hazlewood, 1997), the increase of rumenic acid in tissues observed in ruminants

supplemented with linseed oil (rich in 18:3*n*-3) are mainly due to endogenous production by SCD desaturation of 18:1*t*11.

We hypothesized that increasing dietary availability of gluconeogenesis precursors, like propylene glycol (1,2-propanediol) and propionate salts, would up-regulate the SCD activity without inducing changes in rumen biohydrogenation (*trans*-10 shift) that could compromise the 18:1*t*11 availability. The supplementation of PG to cows has been shown to increase serum insulin and glucose concentrations (Christensen *et al.*, 1997; Nielson & Ingvartsen, 2004) and, thus, can simulate the effects of concentrate feeding. Moreover, PG and propionate salts are readily converted in the rumen to propionic acid (Kristensen *et al.*, 2002), which constitutes an end product of rumen fermentation. So, a major impact on rumen microbial ecology is not anticipated. In line with this, the objectives of the current experiment are: 1) to test if dietary gluconeogenesis precursors (mix of propylene glycol and calcium propionate, PP) can be added to a forage diet supplemented with linseed oil without reducing 18:1*t*11 availability; 2) to test if the dietary gluconeogenesis precursors enhance the SCD desaturase activity resulting in increased oleic and rumenic acids contents in lamb meat.

5.2 Material and methods

5.2.1 Animals and experimental design

The animal handling protocol followed the EU directive 86/609/EEC concerning animal care. Thirty-six Merino Branco ram lambs were reared on pasture with their dams until weaning at about 90 days of age. At the weaning day, lambs were transported to the Unidade de Investigação em Produção Animal - Instituto Nacional de Recursos Biológicos (UIPA-INRB; Vale de Santarém, Portugal) where the trial was conducted. The average initial live weight of lambs was 27.3±3.52 kg. Animals were randomly assigned to 12 pens of 3 lambs each. Three pens were randomly assigned to one of four experimental treatments: pelleted dehydrated lucerne (L); pelleted dehydrated lucerne with 50 g/kg of a propylene glycol and calcium propionate mix (Lupro-mixGlycol75, BASF, Spain) (LPP); pelleted dehydrated lucerne with 60 g/kg of linseed oil (LO); pelleted dehydrated lucerne with 60 g/kg of linseed oil and 50 g/kg of a propylene glycol and calcium propionate mix (LOPP). Oil supplemented diets were prepared by mixing the oil with ground dehydrated lucerne and then pelleted with a 6 mm matrix sieve. The target for oil inclusion was 6 g/100 g on dry matter (DM) basis, resulting in pellets with ether extract of 80 g/kg of DM. For LPP and LOPP groups, diets were

supplemented on top with 50 g/kg of Lupro-mixGlycol75 (BASF, Spain), and the added amount was adjusted daily, according to feed intake. The Lupro-mixGlycol75 (PP) composition was 500 g/kg of propylene glycol, 250 g/kg of calcium propionate and 250 g/kg of excipients. The chemical composition of dietary treatments is presented in Table 16. The experiment started after one week of adaptation. Feed was fed each morning at 1.10 of *ad libitum* intake calculated by a daily refusal weighing. Lambs stayed on the trail for 6 weeks and were, throughout the trial, weighted weekly just before feeding.

Table 16 - Chemical (g/kg DM) and fatty acid composition (g/100g total fatty acids) of the experimental diets.

	Diets[a]	
	L	**LO**
Chemical composition		
Crude protein	148	140
Ether extract	23	80
NDF[b]	472	451
Starch	0.02	0.03
Ash	113	103
Gross energy (MJ/kg DM)	17.5	18.7
Fatty acid composition		
16:0	19.4	7.7
18:0	3.0	2.9
18:1*c*9	3.9	13.4
18:2*n*-6	18.3	18.0
18:3*n*-3	32.1	50.7

[a] L - dehydrated lucerne; LO - dehydrated lucerne with 60 g of linseed oil/kg of DM;
[b] NDF - Neutral detergent fibre not assayed with a heat stable amylase and expressed inclusive of residual ash.

5.2.2 Slaughter, carcass measurement and sampling

At the end of trial, animals were transported to the experimental abattoir of the UIPA-INRB. After weighing to obtain the slaughter live weight, lambs were sent to the experimental abattoir where they were stunned and slaughtered by exsanguination. Carcasses were immediately weighed to obtain the hot carcass weight. Once the stomach was removed from the carcass, approximately 50 ml of ruminal fluid was collected by sampling digesta from the central region of the rumen. Carcasses were kept at 10 ºC for 24 h and were later chilled at 0 ºC until the third day after slaughter. The kidney knob channel fat (KKCF) and kidneys were removed and the carcasses were split along the spine. The left sides of the carcasses were separated in eight joints (Santos-Silva, Mandes & Bessa, 2002). Chumps and shoulders were dissected into muscle, subcutaneous fat and bone. With a Minolta CR-300 chromometer (Konica Minolta, Portugal) it was possible to determine the colour parameters of muscle using the L* (lightness), a* (redness) and b* (yellowness) system, at the level of the left 13th

thoracic vertebra, after 1 h of air exposure to allow blooming. After removing the epimysium, samples from the left *longissimus dorsi* muscle were collected to determine water holding capacity (WHC) on the same day. WHC was determined according to the method described by Santos-Silva *et al.* (2002). The remaining portions of *longissimus dorsi* were minced, vacuum packed, freeze-dried and stored at -80ºC until further analysis.

5.2.3 Feed, rumen and blood samples analysis

Representative samples of feeds were collected weekly (1 kg/week/diet) and pooled. Ground samples (1 mm) were analysed for ash (AOAC, 1990; method 942.05), Kjeldahl nitrogen (AOAC, 1990; method 954.01) and starch (Clegg, 1956). Neutral detergent fibre (NDF) was determined by the procedure of Van Soest, Robertson and Lewis (1991). Neither sodium sulfite nor α-amylase was added during NDF extraction. NDF was expressed inclusive of residual ash. Diet gross energy content was measured using an adiabatic bomb calorimeter (Parr 1261, Parr Instrument Company, USA). Ether extract was determined by extracting the sample with petroleum ether, using an automatic soxhlet extractor (Gerhardt Analytical Systems, Königswinter, Germany).

Immediately after slaughter, the pH in rumen was measured using a pH meter (pH100, VWR, Germany). Samples of rumen fluid for volatile fatty acids (VFA) determination were stabilized with 0.5 ml of saturated mercury (II) chloride (20 µl/ml rumen fluid) and stored at -20°C until analysis. Rumen VFA were determined by gas-liquid chromatography using a chromatograph Agilent HP6890 (Agilent Technology Inc., Palo Alto, CA, USA) equipped with a flame ionization detector and a Permabond-FFAP capillary column (100 m × 0.25 mm i.d., 0.20 µm film thickness; Macherey-Nagel, Duren, Germany).

Three days before the end of the trial, blood samples were collected approximately 12 h after the last feeding from the jugular vein. Whole blood samples were centrifuged at 1500 g for 15 min at 4 ºC to harvest the serum. Serum samples were stored at -20 ºC until analysed. Serum glucose was analysed through a diagnostic test kit (Roche Diagnostics, Mannheim, Germany), using a Modular Hitachi Analytical System (Roche Diagnostics). Finally, serum insulin was determined using the Mercodia Ovine Insulin ELISA kit (Mercodia AB, Sweden).

5.2.4 Feed and muscle lipid analysis

FAME of feed lipids were prepared using one-step extraction transesterification with toluene as solvent and heptadecanoic acid (17:0) as internal standard, according to the procedure reported by Sukhija and Palmquist (1988). Intramuscular lipids were extracted from *longissumis dorsi* (approximately 250 mg), after lyophilisation (-60 ºC and 2.0 hPa) for total lipid, fatty acid and CLA determination. Total lipids were extracted by the method of Folch *et al.* (1957), using dichloromethane and methanol (2:1 v/v). 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.* (2004). Quantification of muscle FAME was done using nonadecanoic acid (19:0) as internal standard. FAME were analysed using a gas-chromatograph HP6890A (Hewlett-Packard, Avondale, PA. USA), equipped with a flame-ionization detector and fused silica CP-Sil 88 capillary column (100 m × 0.25 mm i.d., 0.20 µm 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, 100 ºC, was held for 15 min, increased to 150 ºC at 10 ºC/min and held for 5 min. Then, temperature 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. The identification of peaks was accomplished by comparison of sample peak retention times with those of 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 analytical conditions (Alves & Bessa, 2009).

The CLA methyl esters were individually analysed by triple silver ion columns in series (ChromoSpher 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 an autosampler and DAD adjusted to 233 nm. The mobile phase was 0.1% acetronitrile in *n*-hexane, maintained at a flow rate of 1 ml/min, and an injection volume of 20 µl was used. The identification of CLA isomers was achieved by spectral analysis and by comparison of their retention times with commercial standards, as well as with data published in the literature (Fritsche *et al.*, 2001). Amounts of the CLA isomers were calculated from their silver-ion HPLC areas relative to the area of the main CLA isomer, 18:2*c*9,*t*11, identified by GC (which co-eluted with *t*7,*c*9 and *t*8,*c*10 CLA isomers), as described by Kraft *et al.* (2003). The results for each fatty acid were expressed as a percentage of the total fatty acid, assuming direct proportionality between GC peak area and FAME

weight. The indices of the SCD desaturase activity (ID14, ID16, ID18, and ID18:1*t*11) were calculated according to Malau-Aduli *et al.* (1998).

5.2.5 Statistical analysis

This trial was conducted using a 2×2 factorial design, considering oil supplementation (O) and propylene glycol and calcium propionate mix (PP) as main factors and its respective interaction (O×PP). The experimental unit used for DM intake evaluation was the pen (with 3 animals), whereas the experimental unit used for the other variables was the individual animal. Data from feed intake and feed conversion ratio were analysed using the GLM procedure of SAS (2008) by using a model with the main effects and its interaction. The other parameters were analysed using the MIXED procedure of SAS (SAS Institute Inc., 2009) considering oil and PP and its 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. Simple linear regression between 18:1*t11* and 18:2*c*9,*t*11 were conducted using the Proc REG from SAS (SAS Institute Inc., 2009).

5.3 Results

5.3.1 Growth performance, carcass composition and meat colour

Lambs initial weight, average daily gain and slaughter live weight did not differ between treatments ($P>0.05$) (Table 17). The DM intake was decreased ($P<0.001$) by oil supplementation, but not by PP. Dressing, KKCF, and subcutaneous fat proportions increased, whereas muscle proportion decreased with oil supplementation ($P<0.001$). A significant interaction between oil supplementation and PP was observed for muscle/bone ratio. Meat colour parameters and WHC were not affected by the dietary treatment.

5.3.2 Ruminal fluid pH, volatile fatty acid concentration and blood metabolites

Means of ruminal fluid pH and total VFA concentration were 7.0 and 35.7 mmol/L, respectively, and were not affected by the dietary treatments (Table 18).

Table 17 - Influence of gluconeogenesis precursors (propylene glycol and calcium propionate mix) and oil supplementation (O) on Merino Branco lambs growth, carcass composition and meat quality.

	Diets[a]				SEM	Effects		
	L	LO	LPP	LOPP		O	PP	O×PP
Initial live weight (kg)	25.9	26.9	27.8	28.8	3.52[b]	-	-	-
Dry matter intake (g/d)	1718	1523	1653	1516	25.6	<0.001	0.167	0.267
Average daily gain (g)	287	266	253	243	24.6	0.385	0.112	0.740
Live slaughter weight (kg)	37.6	37.8	38.2	38.7	1.59	0.758	0.499	0.872
Hot carcass weight (kg)	16.0	17.0	16.6	18.0	0.71	0.030	0.152	0.602
Dressing proportion[c]	0.43	0.45	0.43	0.47	0.01	<0.001	0.071	0.296
KKCF proportion[d]	0.013	0.022	0.013	0.024	0.0018	<0.001	0.586	0.338
Chump and shouder								
Muscle [e]	0.60	0.58	0.60	0.57	0.009	<0.001	0.325	0.787
Subcutaneous fat [e]	0.066	0.067	0.067	0.084	0.0059	0.046	0.046	0.060
Muscle/bone ratio[e]	2.82[a]	2.57[b]	2.64[b]	2.69[b]	0.01	0.709	0.153	0.033
Meat colour[f]								
L*	39.0	39.4	38.4	37.2	1.25	0.653	0.127	0.372
a*	15.9	15.2	14.7	15.9	0.86	0.666	0.677	0.118
b*	6.1	5.8	6.1	5.7	0.60	0.420	0.994	0.994
WHC proportion	0.32	0.34	0.33	0.33	0.02	0.492	0.748	0.339

[a] L - dehydrated lucerne; LO - dehydrated lucerne with 60 g of linseed oil/kg of DM; LPP – dehydrated lucerne with 50 g of PP/kg of DM; LOPP – dehydrated lucerne with 50 g of PP and 60 g of linseed oil/kg of DM. [b] Standard deviation. [c] Dressing proportion = HCW/LSW. [d] KKCF, kidney and knob channel fat. [e] Average of proportions of chump and shoulder dissection. [f] L* - lightness; a* - redness; b* - yellowness. WHC, water holding capacity.

Table 18 - Influence of dietary propylene glycol and calcium propionate mix (PP) and linseed oil supplementation (O) on rumen fluid parameters and serum metabolites of lambs.

	Diets[a]				SEM	Effects		
	L	LO	LPP	LOPP		O	PP	O×PP
Ruminal measurements								
pH	7.0	6.9	7.1	7.0	0.11	0.155	0.327	0.866
Total VFA (mmol/L)	38.1	40.8	28.1	35.6	8.85	0.426	0.223	0.704
VFA (mol/100 mol)								
Acetate	74.9	76.4	74.0	73.6	3.47	0.818	0.451	0.701
Propionate	14.6	13.9	15.7	15.1	1.94	0.546	0.414	0.993
Isobutyrate	1.6	1.3	1.8	1.8	0.42	0.584	0.240	0.717
Butyrate	6.1	5.9	5.2	6.13	0.88	0.578	0.571	0.374
Isovalerate	2.0	1.6	2.4	2.4	0.61	0.680	0.187	0.647
Valerate	0.81	0.78	0.90	1.01	0.19	0.770	0.240	0.602
Acetate/Propionate ratio	5.3	5.7	5.0	5.0	0.94	0.716	0.429	0.759
Serum metabolites								
Glucose (mg/dl)	98.1	103.9	97.1	95.5	3.84	0.446	0.089	0.176
Insulin (µg/l)	0.61	0.29	0.35	0.37	0.157	0.208	0.417	0.133

[a] L - dehydrated lucerne; LO - dehydrated lucerne with 60 g of linseed oil/kg of DM; LPP – dehydrated lucerne with 50 g of PP/kg of DM; LOPP – dehydrated lucerne with 50 g of PPM and 60 g of linseed oil/kg of DM.

Dietary supplementation with PP did not change serum insulin concentration, although PP tended (P<0.10) to decrease serum glucose concentration.

5.3.3 Fatty acid composition of *longissimus dorsi* muscle

The effect of dietary treatment on the general fatty acids profile and partial sums of fatty acids in the *longissimus dorsi* muscle are presented in Table 19, whereas the detailed composition of C18 FA is presented in Table 20. The IMF (total lipids) was similar among groups. The oil supplementation had a strong effect on the fatty acid profile (affecting 44 in a total of 48 fatty acids, most of them C18). In addition, PP supplementation induced only a slight effect on fatty acids composition, decreasing (P<0.05) the 16:1*c*7, 20:3*n*-9, 18:2*t*11,*t*13 and increasing (P<0.05) 17:0 and 22:4*n*-6 fatty acids. Also, few significant interactions between oil supplementation and PP were observed. In all treatments, the major fatty acids were 16:0, 18:0, 18:1*c*9 and 18:2*n*-6, which together represented about 70% of total fatty acids. Oil supplementation increased (P<0.001) total C18 FA, from 61.2 to 65.3% of total fatty acids, whereas decreased (P<0.001) 16:0 from 22.1 to 19.9% of total fatty acids. Additionally, oil supplementation also decreased most of the *cis*-9 MUFA and some of the very long-chain PUFA. Nevertheless, oil supplementation decreased (P<0.001) the percentage of total SFA mostly by increasing (P<0.001) the percentage of total TFA. The inclusion of PP in the diet attenuated the large increase of total TFA induced by oil supplementation (O×PP, P<0.001) and, consequently, tended (O×PP, P=0.091) to alleviate the decrease of total SFA induced by oil supplementation. Total PUFA were unaffected by oil supplementation, although the sum of *n*-3 PUFA was increased (P<0.001), due to the 18:3*n*-3 contribution. The oil supplementation increased (P<0.039) the proportion of 15:0*anteiso* but decreased (P<0.05) the 17:0*iso* and *anteiso*, whereas the other BCFA were not affected. Indices of Δ-9 desaturase activity, estimated by fatty acid ratios (ID16:0, ID18:0, ID18:1*t*11), were decreased by oil supplementation (P<0.01), whereas PP increased (P<0.05) ID16:0 index.

The oil supplementation increased most of the C18 BI in meat but not the rumen end product 18:0 (Table 20). For some of these C18 BI, PP attenuated (O×PP, P<0.05, 18:1*t*11, co-eluted 18:1*t*6 to *t*8, 18:2*t*9,*t*11, 18:2*t*11,*c*13, 18:2*t*10,*c*12 and 18:3*c*9,*t*11,*c*15) or tended to attenuate (O×PP, P<0.10, 18:2*c*9,*t*11,18:2*t*7,*t*9 and co-eluted 18:2*c*9,*t*13 and 17:0 *cyclo*) the effects of oil supplementation.

Table 19 - Effect of propylene glycol and calcium propionate mix (PP) and oil supplementation (O) on total lipids (mg/g muscle) and composition (g/100 g total fatty acids) of *longissimus dorsi* muscle from lambs.

	Diets[a]				SEM	Effects		
	L	LO	LPP	LOPP		O	PP	O×PP
Total lipids (mg/g muscle)	16.7	16.8	17.2	18.5	1.52	0.535	0.337	0.590
Fatty acids (FA)								
12:0	0.15	0.16	0.14	0.18	0.023	0.289	0.763	0.473
14:0	2.42	2.45	2.39	2.68	0.184	0.400	0.584	0.487
15:0*iso*	0.10	0.12	0.08	0.09	0.318	0.318	0.234	0.646
15:0*anteiso*	0.12	0.13	0.09	0.13	0.013	0.039	0.294	0.294
14:1*c*9	0.13	0.15	0.14	0.18	0.011	0.023	0.127	0.382
15:0	0.31	0.31	0.32	0.34	0.008	0.142	0.077	0.204
16:0*iso*	0.14	0.14	0.12	0.15	0.011	0.228	0.578	0.217
16:0	22.4	19.6	21.7	20.1	0.383	<0.001	0.862	0.130
16:1*t*9	0.18[b]	0.24[a]	0.23[a]	0.19[b]	0.017	0.672	0.738	0.010
17:0*iso*	0.35	0.26	0.34	0.30	0.026	0.021	0.533	0.332
17:0*anteiso*	0.41	0.33	0.37	0.33	0.017	0.002	0.258	0.235
16:1*c*7	0.30	0.32	0.27	0.29	0.012	0.164	0.024	0.956
16:1*c*9	1.09	0.88	1.21	0.96	0.060	0.001	0.115	0.777
17:0	1.06	0.71	1.11	0.83	0.032	<0.001	0.012	0.336
17:1*c*9	0.53	0.34	0.62	0.35	0.032	<0.001	0.117	0.220
Total C18 FA	61.6	65.9	60.8	64.7	0.612	<0.001	0.119	0.777
20:0	0.02	0.41	0.03	0.40	0.024	0.287	0.334	0.869
20:1*c*11	0.10	0.12	0.11	0.09	0.017	0.972	0.714	0.159
20:2*n*-6	0.05	0.01	0.07	0.02	0.009	<0.001	0.096	0.739
20:3*n*-9	0.21	0.15	0.08	0.12	0.034	0.691	0.023	0.204
22:0	0.20	0.19	0.22	0.17	0.015	0.059	0.949	0.177
20:3*n*-3	0.12	0.20	0.10	0.10	0.059	0.450	0.296	0.532
20:4*n*-6	1.90	1.59	2.14	1.81	0.185	0.099	0.230	0.970
20:5*n*-3	0.58	0.59	0.68	0.56	0.052	0.334	0.488	0.225
22:4*n*-6	0.09	0.06	0.13	0.08	0.012	0.005	0.035	0.592
22:5*n*-3	0.81	0.68	0.88	0.66	0.061	0.009	0.689	0.506
22:6*n*-3	0.29	0.25	0.39	0.26	0.032	0.018	0.096	0.179
Other FA[b]	6.85	8.25	6.32	6.39	0.721	0.163	0.229	0.478
Partial sums								
∑SFA	41.9	38.3	40.4	38.9	0.57	<0.001	0.421	0.091
∑*cis*-MUFA	36.0	30.6	35.5	31.7	0.92	<0.001	0.774	0.397
∑TFA	5.20[a]	13.20[c]	5.52[a]	11.22[b]	0.161	<0.001	<0.001	<0.001
∑PUFA	11.7	13.0	12.5	13.1	0.65	0.148	0.444	0.596
∑*n*-3 PUFA	4.05	5.42	4.28	4.96	0.284	0.001	0.686	0.253
∑*n*-6 PUFA	7.25	6.80	8.02	7.48	0.455	0.285	0.123	0.925
Delta 9 desaturase indices								
ID14:0[c]	5.24	6.00	5.24	6.23	0.560	0.183	0.693	0.994
ID16:0[d]	4.63	4.23	5.26	4.56	0.205	0.013	0.028	0.454
ID18:0[e]	68.6	64.5	69.2	66.2	0.99	0.002	0.260	0.587
ID18:1*t*11[f]	30.9	23.7	32.4	24.9	1.33	<0.001	0.334	0.890
18:1*t*11/18:1*t*10	4.94	7.94	3.45	6.31	1.079	<0.001	0.054	0.934

[a] L - dehydrated lucerne; LO - dehydrated lucerne with 60 g of linseed oil/kg of DM; LPP – dehydrated lucerne with 50 g of PP/kg of DM; LOPP – dehydrated lucerne with 50 g of PP and 60 g of linseed oil/kg of DM. [b] The sum of the remaining area (others) includes: dimethylacetals (about 2.8 g/100 g total FA) and unidentified peaks (4.1 g/100 g total FA). [c] ID14 = 14:1*c*9×100/14:0+14:1*c*9. [d] ID16= 16:1*c*9×100/16:0+16:1*c*9. [e] ID18= 18:1*c*9×100/18:0+18:1*c*9. [f] ID18:1*t*11= 18:2*c*9,*t*11×100/18:1*t*11+18:2*c*9,*t*11.

Table 20 - Effect of propylene glycol and calcium propionate mix (PP) and oil supplementation (O) on C18 fatty acids (mg/g total fatty acids) of *longissimus dorsi* muscle from lambs.

	Diets[a]					**Effects**		
	L	**LO**	**LPP**	**LOPP**	**SEM**	**O**	**PP**	**O×PP**
18:0	152	148	144	145	5.1	0.606	0.123	0.533
18:1 isomers								
*t*6 to *t*8	1.79[a]	3.41[c]	1.88[a]	3.12[b]	0.014	<0.001	0.213	0.032
*t*9	2.16	3.38	2.18	3.19	0.174	<0.001	0.508	0.393
*t*10	2.81	5.63	4.05	6.30	0.955	<0.001	0.171	0.674
*t*11	12.50[a]	44.30[c]	11.08[a]	34.58[b]	1.913	<0.001	<0.001	<0.001
*t*12	2.01	5.56	1.91	4.45	0.674	<0.001	0.216	0.297
*c*9	333	272	326	284	12.4	<0.001	0.808	0.279
*c*11+ *t*15	11.76[ab]	12.81[b]	12.81[b]	11.61[a]	0.679	0.867	0.878	0.028
*c*12	2.11	7.78	2.41	7.72	0.691	<0.001	0.804	0.708
*c*13	0.73	1.71	0.85	1.54	0.241	<0.001	0.880	0.415
*t*16 + *c*14	2.73	5.75	2.45	5.00	0.494	<0.001	0.154	0.510
*c*15	1.58	5.84	1.46	4.72	0.721	<0.001	0.234	0.335
Total	373	368	367	366	13.2	0.731	0.645	0.806
18:2 non-conjugated isomers								
*t*9,*t*12	1.39	2.54	1.65	2.34	0.252	<0.001	0.882	0.211
*t*8,*c*12	1.53	2.95	1.63	2.60	0.217	<0.001	0.429	0.158
*c*9,*t*13+ *t*8,*c*12 17-*cyclo*[b]	0.29	1.40	0.39	1.06	0.150	<0.001	0.269	0.051
*t*8,*c*13 + *c*9,*t*12[c]	1.86	3.40	2.22	3.55	0.336	<0.001	0.289	0.656
*t*9,*c*12	0.21	1.33	0.433	0.81	0.267	<0.001	0.445	0.062
*t*11,*c*15	2.56	18.28	2.56	15.73	1.213	<0.001	0.152	0.150
*c*9,*c*12	52.02	51.37	56.91	55.62	3.779	0.721	0.100	0.905
*c*9,*c*15	1.22	1.94	0.99	1.71	0.344	0.001	0.360	0.995
*c*12,*c*15	0.19	4.12	0.30	4.02	0.706	<0.001	0.997	0.836
Total	61	87	67	87	3.9	<0.001	0.304	0.324
18:2 conjugated isomers (CLA)								
*t*12,*t*14	0.10	0.11	0.11	0.11	0.027	0.119	0.314	0.506
*t*11,*t*13	0.14	0.09	0.11	0.07	0.014	<0.001	0.024	0.618
*t*10,*t*12	0.08	0.66	0.08	0.45	0.093	<0.001	0.147	0.131
*t*9,*t*11	0.19[a]	1.27[c]	0.23[a]	0.90[b]	0.112	<0.001	0.050	0.018
*t*8,*t*10	0.05	0.17	0.05	0.10	0.037	0.003	0.227	0.198
*t*7,*t*9	0.09	0.50	0.12	0.38	0.058	<0.001	0.252	0.072
*t*6,*t*8	0.02	0.07	0.04	0.05	0.022	0.062	0.943	0.160
c/*t*12,14[d]	0.07	0.50	0.08	0.38	0.072	<0.001	0.254	0.206
*t*11,*c*13	0.39[a]	6.67[c]	0.42[a]	4.39[b]	0.343	<0.001	0.0001	<0.001
*c*11,*t*13	0.04	n.d	0.02	n.d				
*t*10,*c*12	0.04[a]	0.33[c]	0.05[a]	0.15[b]	0.038	<0.001	0.005	0.003
*c*9,*t*11	5.52	13.23	5.18	11.35	0.596	<0.001	0.015	0.079
*t*7,*c*9	0.48	0.89	0.51	0.87	0.076	<0.001	0.959	0.618
*c*9,*c*11	0.04	0.04	0.06	0.04	0.027	0.531	0.732	0.531
Total	7.25[a]	24.53[c]	7.02[a]	19.24[b]	0.657	<0.001	<0.001	<0.001
Total 18:2	68	111	74	107	4.0	<0.001	0.942	0.073
18:3 isomers								
*c*9,*c*12,*c*15	22.57	37.00	22.36	33.77	2.723	<0.001	0.381	0.439
*c*9,*t*11,*c*15	1.10[a]	5.68[c]	2.47[b]	4.59[c]	0.687	<0.001	0.777	0.018
Total	23.7	42.7	24.8	38.3	3.07	<0.001	0.475	0.219
Total BI[e]	7.43[a]	22.21[b]	7.86[a]	19.24[b]	1.469	<0.001	0.118	0.040

[a] L - dehydrated lucerne; LO - dehydrated lucerne with 60 g of linseed oil/kg of DM; LPP – dehydrated lucerne with 50 g of PP/kg of DM; LOPP – dehydrated lucerne with 50 g of PP and 60 g of linseed oil/kg of DM. [b] Peak includes 18:2*c*9,*t*13+18:2*t*8,*c*12+17-cyclo(methyl-11-cyclohexylundecanoate). [c] Peak includes 18:2*t*8,*c*13 + 18:2*c*9,*t*12. [d] Peak includes 18:2*c*12,*t*14 and 18:2*t*12*c*14. [e] Total C18 biohydrogenation intermediates, total C18 fatty acids minus 18:0, 18:1*c*9, 18:1*c*11, 18:2*c*9,*c*12 and 18:3*c*9,*c*12,*c15*. n.d – not detected

5.4 Discussion

The main objective of this experiment was to test if high forage basal diets supplemented with gluconeogenesis precursors would simultaneously maintain a high 18:1*t*11 rumen output, which is characteristic in lambs fed high forage diets, and up-regulate the SCD activity, which is characteristic in lambs fed high starch diets, thus resulting in increased concentrations of 18:1*c*9 and 18:2*c*9,*t*11 in meat.

Average daily gain, carcass traits and meat colour were in accordance to results obtained with the same breed finished on similar conditions, including the effects of oil supplementation (Bessa *et al.*, 2007; Jerónimo *et al.*, 2009; Jerónimo *et al.*, 2010). The on top supplementation with 50 g/kg of PP did not affect growth performance and carcass composition, which is in agreement with the results described by Kim, Choi and Myung (2005) in Korean native steers fed concentrate with 2.5 ml/kg body weight $(BW)^{0.75}$ of PG.

The effect of PP on rumen metabolism were evaluated through VFA concentration and molar proportions in rumen contents collected *post-mortem* and indirectly through deposition of BI in muscle lipids. Although it is well established that both dietary PG and calcium propionate increase propionate and decrease acetate molar proportions in the rumen (Shingfield, Jaakkola & Huhtanen, 2002; Kim *et al.*, 2005), we did not observe any effect of PP on VFA concentration and molar proportions in the rumen content. The rumen collection method used by us, *post-mortem*, and overall experimental conditions might explain why we could not detect changes in VFA molar proportions. Animals had permanent access to feed until being transported to the experimental abattoir where the slaughter proceeded during several hours. So, the time between last meal eaten and rumen content sampling differ between animals. This could have resulted in an increase of ruminal VFA variability that, ultimately, prevented the detection of any significant effect.

Nevertheless, the increase of ruminal propionate concentration after dietary calcium propionate supplementation is straightforward, as calcium salts dissociate easily in the rumen. Thus, the molar proportions of other VFA would be diluted by the exogenous propionate. However, it is not clear if the dietary intake of propionate salt affects rumen metabolism and microbial populations. In addition, PG is quickly converted to propionate and other metabolites (Czerkawski, Platkova & Breckenridge, 1984). These authors indicate that PG added to *in vitro* semi-continuous fermentation system had only a small effect on digestion of

basal diet. However, it reduced the output of acetate and butyrate, mainly through a shift towards an increased production of 2-methylbutyric acid. Recently, Chibisa, Gonzo and Mutsvangwa (2009) did not detect any negative effects of top dressing PG supplementation on rumen microbial protein production in dairy cows, as evaluated by purine derivative excretion. These former authors suggest that top dressing PG supplementation have no major negative effects on rumen microbial population.

Our results on BI in meat suggest that a small but detectable effect in biohydrogenation pathways had occurred. Usually, a diet rich in sugar and starch, thus low in fibre, induce a high propiogenic rumen environment which can result in a shift from 18:1*t*11 to 18:1*t*10 as the major BI, with implications on milk fat synthesis and on 18:2*c*9,*t*11 deposition in tissues (Bauman & Griinari, 2003; Bessa *et al.*, 2005). In the PP fed lambs, it was detected a trend (P=0.054) for a reduction on 18:1*t*11/18:1*t*10 ratio, mainly due to low 18:1*t*11 levels. The relationship between meat 18:1*t*11 and 18:2*c*9,*t*11 proportions across treatments was very high (r^2=*0.91*, n=36, P<0.001) suggesting that the small proportion of 18:2*c*9,*t*11 found in PP fed lambs resulted from a low metabolic availability of 18:1*t*11.

We did not evaluate the rumen biohydrogenation directly, but the pattern of BI in meat suggests that addition of 50 g/kg of PP interfered with rumen metabolism, resulting in lower 18:1*t*11 production. Although most of C18 FA was not individually affected by PP, the increase of total *trans* isomers as result of oil supplementation was strongly attenuated by PP. Also, 18:2*c*9,*c*12 (linoleic acid) tended (P=0.10) to increase in meat from PP fed lambs. The effect of PP on rumen biohydrogenation pathways, as perceived by meat BI pattern, was not anticipated and should be better exploited.

The other reason for using PP supplementation was to establish a more glucogenic nutrient profile available for absorption which could promote a more insulinemic metabolic environment in an attempt to increase the SCD activity. It is well established that both PG and propionate supplementation increase the availability of propionate for liver gluconeogenesis (Harmon & Avery, 1987; Veenhuizen *et al.*, 1988; Nielsen & Ingvartsen, 2004). In the same manner, PG administration in dairy cows increases insulin status (Studer *et al.*, 1993; Grummer *et al.*, 1994; Christensen *et al.*, 1997). Daniel *et al.* (2004a) identified insulin as a major key factor in SCD up-regulation in ruminant tissue. SCD is the enzyme that catalyses the conversion of 18:1*t*11 into 18:2*c*9,*t*11. So, we anticipated that the 18:2*c*9,*t*11 concentrations in the muscle of lambs fed PP would be increased. The 18:2*c*9,*t*11 could also

be originated directly from the rumen, through isomerisation of 18:2*c*9,*c*12, but not from 18:3*c*9,*c*12,*c*15. Thus, we used linseed oil to minimise the availability of 18:2*c*9,*t*11 directly produced in the rumen.

In the present study, the concentration of 18:2*c*9,*t*11 in the muscle did not increase after PP administration, probably due to the lower availability of 18:1*t*11, as discussed above. Lower 18:1*t*11 in the rumen outflow would result in less substrate to SCD and, thus, a lower deposition of 18:2*c*9,*t*11 in tissues, because most of it derives from endogenous delta-9 desaturation (Palmquist, St-Pierre & McClure, 2004).

Despite the fact that it was not detected any effect on serum insulin concentration, it cannot be excluded that animals were in a higher insulimemic status. Actually, blood was sampled before the morning feeding, but as animals were fed *ad libitum*, feed was in fact always available to them. The increase in serum glucose and insulin concentrations occurs shortly after PG feeding (Nielsen & Ingvartsen, 2004). Christensen *et al.* (1997) reported that PG supplements stimulate an increase in plasma insulin concentration within 1.5 h after administration. Much less information is available on the effects of gluconeogenesis precursors incorporated into feed and fed *ad libitum* on plasma insulin concentration. Chung *et al.* (2009b) and Chung, Girard and Varga (2009c) observed no changes in plasma insulin concentration after top dressing supplementation of dry PG to early lactation dairy cows, although they found reduced levels of beta-hydroxibutirate. This suggests a reduced lipid mobilization from adipose tissue, and thus a higher insulinemic status. We observed a trend to lower glucose serum concentration in PP fed lambs. Thus, the metabolic availability of gluconeogenesis precursors should be higher in PP fed lambs, the lower glucose serum concentrations may reflect a higher insulinemic status.

We expected an increase of SCD activity in PP fed lambs. It should be reflected on SCD desaturation indices, calculated from product/substrate pairs, as suggested by Malau-Aduli *et al.* (1998) and Kelsey, Corl, Collier and Bauman (2003). However, the desaturation indices did not suggest any major effects of PP on SCD activity, although the ID16:0 increased. Conversely, the desaturation indices were clearly decreased by oil supplementation, which is fully consistent with other reports (Bas, Berthelot, Pottier & Normand, 2007; Manso, Bodas, Castro, Jimeno & Mantecon, 2009).

The lack of effects for PP dietary inclusion on SCD activity might be explained by the high dietary levels of 18:3*n*-3, either in basal or in linseed oil supplemented diets. Daniel *et al.* (2004a) suggested that 18:3*n*-3 presented in forages might be held responsible for SCD down-regulation observed in lambs fed forage *vs*. concentrate. Our results reinforce this explanation as increasing dietary availability of gluconeogenesis precursors (and consequent insulinemic response) was probably insufficient to suppress the down-regulation effects of 18:3*n*-3 dietary availability.

Linseed oil supplementation to a forage basal diet induced a strong effect on muscle fatty acid composition, increasing 18:3*n*-3, most of the C18 BI, particularly 18:1*t*11, 18:1*c*15, 18:1*t*11,*c*15, 18:2*c*12,*c*15, 18:2*c*9,*t*11, 18:2*t*11,*c*13 and 18:3*c*9,*t*11,*c*15 fatty acids. However, linseed oil supplementation showed a decrease on both 18:1*c*9 and long chain *n*-3 PUFA, which are fatty acids nutritionally desirable. These results are fully consistent with previous experiments and have been extensively discussed in Bessa *et al.* (2007) and Jerónimo *et al.* (2009).

5.5 Conclusion

The data presented here suggest that the addition of 5% propylene glycol and calcium propionate mix to lamb diets modulates the response to linseed oil supplementation, inducing changes in muscle fatty acid, including the decrease of 18:1*t*11 fatty acid and 18:1*t*11/18:1*t*10 ratio. This change in fatty acids composition is most likely explained by modifications on rumen biohydrogenation pathways. Moreover, these results also indicate that the increased availability of gluconeogenesis precursors, and the consequent putative insulinemic response, are insufficient to mitigate the inhibitory effects of forage and linseed oil feeding on the SCD activity.

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CHAPTER 6 | General discussion, conclusions and future perspectives

6.1 Effect of slaughter season

As described previously, we have considered meat from different production systems, namely, PDO veal from the northeast (Mirandesa-PDO), PDO beef from animals reared in the the centre south under a semi-extensive production system (Charneca-PDO,) and organic beef from the south (extensive production system) of Portugal to carry out our studies. These PDO meats were primarily selected by their commercial relevance in the national market and the year of harvest was 2008, following the work of our research group on PDO meats quality.

The feeding of these breeds is based on natural resources and is affected by the soil and weather conditions. The traditional Portuguese extensive production systems are sustained and based on the use of local available resources - indigenous cattle breeds and natural or seeded forages. The application of semi-extensive and extensive systems means a correct and appropriate land use taking into account the specific climate of each region. Therefore, edapho-climatic conditions have an indirect influence on the production and quality of meat, as long as they affect the quantity and quality of available pasture and forage. However, the climatic environment differs with region and can shorten the availability of pasture and forage, so that it becomes necessary to increase the concentrated level to finish the calves (Costa *et al.*, 2011).

The weather in Portugal is generally classified as Mediterranean. However, in the north and in the centre there is a significant Atlantic influence, especially near the coast. Contrarily, in the south (Alentejo) there is a Continental influence (Rodrigues *et al.*, 1998). Temperatures vary from the coast to the interior. Near the coast, there is low diurnal temperature variation and high air humidity, while in the interior there is more extreme daily temperature fluctuation and low air humidity. Rainfall is higher in the north and lower in the south but the evolution of precipitation is characterized by a large interannual variability, with no clear trend in its annual values (Rodrigues *et al.*, 1998).

While in the north and centre of Portugal it is possible to produce green forage on non-irrigated land during a significant part of the year, and in “lameiros” during almost all year, in the south the grazing period for high quality pasture is very short. In the seeded or spontaneous non-irrigated pastures, pasture vegetative growth decreases and even ceases

during the coldest part of the year (Rodrigues *et al.*, 1998). At the end of winter temperatures rise and, as there is a high availability of water in the soil, create ideal conditions for fast vegetative growth. At the end of spring, as temperatures increase and soil water content declines, the vegetative cycle is completed, dry matter content increases rapidly and digestibility declines. Generally, the feeding of these animals is based on the use of natural or improved pasture, hay, cereal straw, crop residues and cereal grain. In the north, cows graze natural pastures (herbaceous species) during spring and early summer and afterwards are fed with hays (of natural pasture or oat) and straws (oat, barley or wheat), complemented with local feeds like squash or potatoes (Galvão *et al.*, 2006). In the south, livestock are fed under an extensive grazing system based on pastures under holm and cork oak, which is usually referred to as "Montado" (Alfaia *et al.*, 2006a). Supplementation with cereals and dry forages (hay and straw) is provided during periods of feed scarcity (Alfaia *et al.*, 2006a). The animals are finished on commercial concentrate.

In the case of organic production, the livestock is maintained permanently in pasture or forage, being 95% of animals feed, and the remaining 5% is organic concentrate (Council Regulation no. 834/2007 of 28/07, EC). Organic foods (including meat) are produced according to strictly defined standards in order to satisfy the expectations of consumers concerned with various aspects of food quality. Therefore, the provision of information about the benefits of organic farming on environment, animal welfare and health can increase consumer awareness and willingness to buy organic products.

Pasture-based production system and semi-extensive production are characterized by seasonal and geographic variations, due to the abundance and botanical diversity of pasture, and it is expected that meat lipids from these animals would reflect this variability. In general, data indicate that IMF of the studied meats has a high nutritional value throughout the year. The effect of slaughter season on the lipid composition of IMF of the studied traditional bovine meat (Mirandesa-PDO) and organic beef was small, mainly influencing the levels of 18:3*n*-3, *t*11,*t*13 CLA isomer and total cholesterol.

The small influence of slaughter season on meat fatty acids is surprising if we consider the well known seasonal differences in pasture nutritive value and availability. It appears that the different suckling and grazing periods between spring-slaughtered and autumn-slaughtered bovine result in similar final effects. Despite this small difference in slaughter season, discrimination between meats from ruminants finished on concentrate or pasture can be easily

achieved through differences in meat fatty acid profile. In the present study, meat from late spring had higher ALA contents when compared to that from early autumn. ALA is a very sensitive grass intake indicator due to its presence in large amounts in grass lipids (Woods *et al.*, 2008). This result was expected and can be explained by the fact that animals raised during late spring were exposed to more abundant pastures than animals raised in early autumn. In addition, the *t*11,*t*13 CLA isomer, also a sensitive grass intake indicator (Dannerberger *et al.*, 2005), was higher in the meat from spring than from autumn. The high content of this isomer in our meats reflects the exposition to abundant availability and quality of pasture from spring-slaughtered animals, relative to that of autumn-slaughtered animals.

Total cholesterol content in organic beef was higher in meat from autumn-slaughtered bulls than in that from spring-slaughtered animals. In several studies, the main reported factors that influence cholesterol content are muscle type (due to the fibre type) and the total lipid content (less triacylglycerols, more cholesterol). However, in our case these two variables do not explain the results. We believe that, apart from a significant statistical result, the biological meaning of the season effect on cholesterol is reduced. Significant interaction between slaughter season and muscle type was observed for total cholesterol in Mirandesa-PDO. This interaction may result from modifications of muscle metabolic types caused by adaptations to the distinct grazing periods (summer and spring), which are associated with differences in fatty acid composition (Klont *et al.*, 1998).

6.2 Effect of muscle type

The effect of muscle type, and thus carcass varation, on the fatty acid profile of PDO and organic meats is markedly revealed. The LL muscle, compared to the ST muscle, showed higher total lipids, total SFA, total MUFA, and lower total PUFA, including *n*-3 and *n*-6 PUFA. These differences between LL and ST muscles, may result from distinct percentages of muscle fibre type (Woods *et al.*, 2004), because LL muscle of cattle is relatively red (25-31% of type I fibres) in comparison with the ST muscle (17-22% of type I fibres) (Vestergaard *et al.*, 2000a; Costa *et al.*, 2008). In LL muscle, the increase of IMF increments the triacylglycerol/phospholipid ratio, and, consequently, SFA and MUFA contents become larger than that of total PUFA (De Smet *et al.*, 2004). The ST muscle has higher proportions of phospholipids, which are rich in PUFA and PUFA/SFA ratio than the LL muscle (Wood *et al.*, 2008).

The variations in total and specific CLA contents are due to differences in IMF levels among muscles. According to Gracia *et al.* (2008) such muscle differences in CLA concentration may indicate potential tissue differences in the activity of SCD. Other authors emphasized the need to verify whether the different behavior among muscles is related to their different metabolism and/or to a distinct tissue utilization of fatty acids (Serra *et al.*, 2009).

The contents of total cholesterol, α-tocopherol and β-carotene did not show significant differences between LL and ST muscles from the analysed meats except from Mirandesa-PDO. In fact, the LL muscle from Mirandesa-PDO had higher total cholesterol, α-tocopherol and β-carotene than the ST muscle. Lynch *et al.* (1999) suggested that beef muscles which contains predominantly oxidative fibres (LL) with higher level of pigments, have a higher concentration of cholesterol and α-tocopherol, comparatively to glycolytic counterparts (ST). Oxidative fibres have a higher capillary density and, therefore, also high capillary supply of α-tocopherol, as well as a higher number of mitochondria, in which the α-tocopherol accumulates (Costa *et al.*, 2008). Differences found in β-carotene might be explained by different incorporations depending not only on dietary delivery and muscle type, but also on the individual uptake capacity (Descalzo & Sancho, 2008).

6.3 Nutritional value of PDO meat and organic beef

Our research group has characterized previously the lipid composition and nutritional value of four Portuguese traditional meats: Carnalentejana-PDO beef (a large meat breed from the south of Portugal), Mertolenga-PDO beef (a small meat breed from the south of Portugal), Barrosã-PDO veal (from the north of Portugal), and Arouquesa-PDO veal (from the centre of Portugal). In line with this, the present work intended to continue the lipid composition and nutritional value characterization of bovine breeds PDO.

In an attempt to integrate the nutritional value results for the reported meats in this thesis with other PDO meats and extensively produced beef, Table 21 presents a summary of lipid composition and related health indices comprising both slaughter seasons and muscles studied, based on a daily consumption of a 150 g steak.

Table 21 - Summary of the nutritional value for Portuguese PDO meats, organic beef and intensively produced beef on a basis of a daily consumption of a 150 g steak [1].

	Meats analysed in this study			***Meats analysed by Alfaia et al. (2006a,b and 2007a,b)***				
	Mirandesa veal	**Charneca beef**	**Organic beef**	**Barrosã veal**	**Arouquesa veal**	**Carnalentejana beef**	**Mertolenga beef**	**Intensively produced beef**
Total lipids (g/150 g)	1.0-2.8	1.0-1.6	0.95-1.1	2.4-3.5	2.6-4.0	1.6-3.3	1.8-2.7	1.3-2.2
Total cholesterol (mg/150 g)	57-86	63-66	54-63	63-84	73-79	63-73	60-75	53-55
Partial sum of fatty acids (mg/150 g)								
SFA	356-1224	360-669	319-401	933-1413	966-1944	558-1257	609-1071	340-880
MUFA	274-1013	234-471	192-265	903-1336	957-1744	624-1221	543-919	385-766
TFA	36-106	59-97	52-70	66-106	42-121	48-108	51-127	39-93
BCFA	10-40	13-25	8-13	-	-	-	-	-
PUFA	192-248	275-305	268-337	223-340	288-354	300-403	373-411	339-342
n-3 PUFA	38-92	56-60	91-131	57-85	96-114	24-51	25-75	16-21
n-3 LC-PUFA	26-73	30-34	51-67	37-60	58-83	19-48	15-41	11-17
n-6 PUFA	141-159	218-245	166-210	165-256	192-238	276-358	306-378	318-325
n-6 LC-PUFA	36-50	58-64	45-59	54-82	75-88	84-136	74-103	90-102
CLA isomers (*mg/150 g*)								
Total CLA	3-12	3-8	4-5	16-30	18-39	6-15	4-10	3-9
*c*9,*t*11	2-9	4-8	2-3	13-25	13-30	6-12	3-7	1-6
*t*10,*c*12	0.05-0.19	0.05-0.14	0.06-0.12	0.06-0.17	0.11-0.21	0.12-0.29	0.08-0.23	0.12-0.36
Fat-soluble vitamins (µg/150 g)								
α-tocopherol	475-687	1639-1738	792-822	418-588	690-1021	207-339	480-693	-
β-carotene	5-9	20-25	9-14	9-16	30-46	3-13	3-15	-
Nutritional ratios and n-3 LC-PUFA								
PUFA/SFA	0.2-0.6	0.5-0.9	0.7-1.0	0.2-0.3	0.2-0.3	0.3-0.7	0.4-0.7	0.4-0.8
n-6/*n*-3	1.6-4.4	4.4-4.6	1.6-2.1	3	2	10-14	7-15	17-20
EPA+DHA (*mg/150 g*)	10-56	13-15	24-33	14-24	18-35	6-28	3-8	2-6

[1] Values are presented as a minimum-maximum range among LL and ST muscles. SFA = sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0; MUFA = sum of 14:1c9, 16:1c7, 16:1c9, 17:1c9, 18:1c9, 18:1c12, 18:1c13 and 18:1c15; TFA = sum of 16:1t9, 18:1t6+t8; 18:1t9, 18:1t10, 18:1t11, 18:1t12, 18:1c11,t15, 18:1t16+c14, 18:2t11,c15, 18:2c9,t11 and 18:3c9,t11,c15; BCFA = sum of 15:0 iso, 15:1 anteiso, 16:0 iso, 17:0 iso and 17:0 anteiso; PUFA = sum of 18:2n-6, 18:3n-6, 18:3n-3, 20:2n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3 and 22:6n-3; *n*-3 PUFA = sum of 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3; *n*-3 LC-PUFA= sum of 20:5n-3, 22:5n-3 and 22:6n-3; *n*-6 PUFA = sum of 18:2n-6,18:3n-6, 20:2n-6, 20:4n-6 and 22:4n-6; *n*-6 LC- PUFA= sum of 20:2n-6, 20:4n-6 and 22:4n-6; PUFA/SFA = polyunsaturated/saturated ratio [(sum of 18:2n-6, 18:3n-6, 18:3n-3, 20:2n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3 and 22:6n-3)/(sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0)]; n6/n-3 = n-6/n-3 ratio [(sum of 18:2n-6, 18:3n-6, 20:2n-6, 20:4n-6 and 22:4n-6)/(sum of 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3)]; EPA+DHA = 20:5n-3+22:6n-3.

Alentejana, Mertolenga and Charneca PDO meats are obtained from purebred young bulls (16-24 months) produced in the south of Portugal according to a semi-extensive grazing system and finished on concentrate (3-9 months). Barrosã, Mirandesa and Arouquesa PDO meats have their origins on purebred calves (6-9 months) reared under extensive grazing systems, fed with farm products and suckling from cows grazed on natural pastures.

The data show that Mirandesa-PDO veal seems to have typical pasture-fed characteristics, based on values of *n*-3 PUFA, total CLA and *c*9,*t*11 isomer, *n*-6/*n*-3 ratio, and α-tocopherol. In contrast, Charneca-PDO beef have intermediate values between meat from grain-fed and pasture-fed cattle, looking at the content of total lipids, *n*-6 PUFA, total CLA and *t*10,*c*12 isomer, *n*-6/*n*-3 ratio, which can be explained by the semi-extensive production system of Charneca-PDO animals. The use of cereals (rich in *n*-6 PUFA) in concentrates shifts the meat fatty acid composition to an increase of *n*-6/*n*-3 ratio and *t*10,*c*12 isomer when compared to animals raised on pasture. The results of organic beef showed typical pasture-fed characteristics (based on the CLA isomers and *n*-3/*n*-6 ratio values), although these values were lower comparing to Mirandesa-PDO beef. This fact may reflect the leanness of the beef, as well as the quality and quantity of pasture, usually poor in the south of Portugal (Alentejo). The levels of IMF reported for organic beef are fully consistent with the low cold carcass weights for the age of slaughter, which can be explained by the low energy content of pastures and the absence of concentrate finishing period (Alfaia *et al.*, 2009). These values suggest low levels of muscle triacylglycerols since the values of phospholipids, although dependent on the muscle fibre composition, are fairly constant in the same muscle fibre type (Alasnier *et al.*, 1996). As the majority of the beef produced in Europe, the analysed meat is considered a lean meat, according to the Food Advisory Committee (1990) criteria (<5 % fat). Normally, pasture-based beef tends to have lower fat content, an important consideration for those consumers interested in decreasing overall fat consumption. However, it is generally accepted that very low levels of IMF lead to dry and less-tasty meat (Hocquette *et al.*, 2010). The minimum amount of IMF to achieve acceptable consumer satisfaction is about 3% to 4% of lipids for fresh beef (Savell & Cross, 1986), and 5% of lipids for fresh sheep meat (Hopkins *et al.*, 2006).

Based on a daily consumption of a 150 g steak, trimmed of all visible fat, except the IMF, PDO and organic meats provide 54-86 mg of cholesterol which corresponds to 18-29% of the maximum recommended daily cholesterol intake (<300 mg, American Heart Association, 2008). The values for total cholesterol in meat studied are similar to others PDO meats.

Normally, when increases total lipids in IMF increases total cholesterol (Muchenje *et al.*, 2009b), and this finding was also found in the present study.

Organic beef had higher *n*-3 PUFA content than PDO meats. It is well documented that animals consuming pastures have higher contents of *n*-3 PUFA, mainly ALA, as grass is a good source of this fatty acid, although variations occur regarding its maturity and floristic diversity (Woods & Fearon, 2009).

The values for total CLA and *c*9,*t*11 CLA isomer in Mirandesa PDO was higher than that of Charneca PDO and organic beef. Among PDO meats, the total CLA content and *c*9,*t*11 CLA isomer varies a lot, this fluctuation may result from the different feeding systems and different IMF levels. Higher IMF levels are usually associated with higher total CLA contents and the *c*9,*t*11 CLA isomer is predominantly deposited in the triacylglycerols (Raes *et al.*, 2003).

Concerning CLA dietary intake, EFSA (2010) proposes not to set any reference value since the optimal dietary intake of CLA remains to be established. On the basis of animal studies, some extrapolations suggested that a daily consumption of 0.8-3.0 g of CLA might promote human health benefits (Parrish *et al.*, 2003). Facing the discussion, 150 g of PDO meats and organic beef would provide only 3-12 mg of CLA, which is around 1.5 % of the minimum amount proposed, similarly to the other PDO meats, with exception of Arouquesa veal (5% of the minimum amount required). However, the range of values proposed by the former authors symbolize a rough estimative because it is based on animal data extrapolation and, thus, should be interpreted carefully until consistent data are available in humans (Schmid *et al.*, 2006).

Charneca-PDO beef showed higher α-tocopherol and β-carotene concentrations than those found for other PDO meats. In fact, the values are much higher than those described for meats originated on pasture-fed cattle (660-870 µg/150 g) and grain-fed cattle receiving supra-nutritional doses of vitamin E (645-900 µg/150 g), which in turn are higher than those for meat derived from grain-fed cattle (270-360 µg/150 g) (Yang *et al.*, 2002). However, West *et al.* (1997) mentioned contents of α-tocopherol in the *longissimus lumborum* of pastured cattle varying between 555 and 1050 µg/150 g, suggesting that there may be a difference in the α-tocopherol levels found in pastures with different biomass compositions. Regarding β-carotene, in Charneca PDO, beef the values are similar to those from cattle grazed on a good green pasture (14-33 µg/150 g) (Yang *et al.*, 2002), although the animals fed pasture plus

concentrate during 15 months. Thus, the values reported for α-tocopherol and β-carotene here suggest meat from animals grazed on good quality pastures.

A 150 g portion of the PDO meats and organic beef analysed provides 0.5-1.7 mg of α-tocopherol and 5-25 μg of β-carotene, which represents 3-11% of the current recommended intake of vitamin E (12/15 mg/day) and only 0.6-3.6%/0.5-2.8% (women/men) of the recommended dietary allowance for β-carotene (700/900 μg/day for women and men, respectively).

According to the current nutritional recommendations, the PUFA/SFA ratio in the human diets should be above 0.45 (British Department of Health, 1994). In the present study, the values of PUFA/SFA ratio in PDO meats and organic beef are inside the recommended dietary guideline. However, organic beef showed a higher ratio (0.7-1.0) comparing to other meats. In fact, several studies described that the PUFA/SFA index is generally increased with pasture feeding (Realini *et al.*, 2004; Alfaia *et al.*, 2009).

The *n*-6/*n*-3 ratio in PDO meats and organic beef were inside close to the recommended figures for the human diet, <4 (British Department of Health, 1994). Compared to available data, our values for *n*-6/*n*-3 ratio are close to those from pasture feeding cattle. Other studies have reported that high levels of concentrate in the diet increase the *n*-6/*n*-3 ratio of meat (Eriksson & Pickova, 2007; Alfaia *et al.*, 2009), whilst the increase of pasture intake decrease this ratio (Nuernberg *et al.*, 2003). Presently, the *n*-6/*n*-3 ratio has been the subject of much debate over its practical utility in optimizing the benefits of *n*-3 LC-PUFA (C18-22) on cardiovascular health (Stanley *et al.*, 2007). Griffin (2008) reported that the absolute amounts of dietary LA and ALA are of relevance to the efficiency of conversion of ALA to EPA and DHA. Hence, the absolute intake of *n*-3 LC-PUFA is more important than the *n*-6/n-*3* ratio. In addition, EFSA (2010) proposed not to set specific values for the *n*-6/*n*-3 ratio but an adequate intake of 250 mg *per* day for EPA plus DHA. PDO meats and organic beef provide 10-56 mg of EPA+DHA, thus representing 4 to 22% of the recommended daily intake for EPA plus DHA.

In summary, IMF composition in PDO veal (Arouquesa, Barrosã and Mirandesa) and organic beef have a fatty acid profile from typical pasture-fed systems (high levels of *n*-3 PUFA and *c*9,*t*11 CLA isomers), while in PDO beef (Alentejana, Mertolenga and Charneca) the values are close to those obtained in animals raised on concentrates. Therefore, the provision of

information about the benefits of organic beef and PDO meats, respects on environment, animal welfare and health can increase consumer awareness and willingness to buy these products.

6.4 Influence of dietary gluconeogenesis precursors

Manipulation of animal diet composition appears to be a good strategy to modulate rumen biohydrogenation. However, in order to alter biohydrogenation and promote more health beneficial fatty acid outflow from the rumen more research is needed. The investigation presented in this thesis was designed to address specific issues and provide new insights in this area of research.

SFA present in ruminant fats are originated either by *de novo* synthesis of SFA or by the rumen biohydrogenation of dietary fatty acids, being the latter the most important. Dietary lipids entering the rumen are rapidly and extensively hydrolysed to non-esterified fatty acid and the PUFA, mainly α-linolenic and linoleic acids, are hydrogenated to stearic acid (Jenkins *et al.*, 2008). During this process, *i.e.* biohydrogenation, several BI are formed, among which CLA and VA are of particular interest. VA also exhibits beneficial health effects on its own or by its endogenously Δ9-desaturation to *c*9,*t*11 CLA isomer in tissues (Lock *et al.*, 2004; Field *et al.*, 2009).

Ruminant milk, meat and dairy products are a considerably part of most diets in developed and developing countries, particularly in Western diets. Thus, fatty acid profile of these products has a strong impact in human's diet, and possibly on human health. Improving fatty acid profile to meet nutritional recommendations is possible and, in fact, appears to be the best strategy to improve human fatty acid intake without consumers' effort or altering their consumption habits.

Meat fatty acid profile are closely related, and dependent of lipid metabolism in the rumen, namely of biohydrogenation (Demeyer & Doreau, 1999). Many are the factors affecting biohydrogenation in the rumen (Bessa *et al.*, 2000; Palmquist *et al.*, 2005), such as pH (Van Nevel & Demeyer, 1996) forage to concentrate ratio (French *et al.*, 2000) oil supplementation (Bessa *et al.*, 2007; Jerónimo *et al.*, 2009) and ionophores (Fellner, Sauer & Kramer, 1997).

The experiment in Chapter 5 was designed to address the effects of gluconeogenesis precursors and oil supplementation (linseed oil) on fatty acid composition and biohydrogenation profiles of lamb meat. It was hypothesized that adding gluconeogenesis precursors (PP) to a high-forage high-oil diet would simultaneous maintains the high 18:1*t*11 rumen outflow and increase the 18:1*t*11 endogenous conversion to rumenic acid by insulin mediated upregulation of delta-9 desaturase, resulting in lamb meat enriched in this fatty acid. In fact, propylene glycol and calcium propionate mix (PP) to lamb diets did modulate the meat fatty acid response to linseed oil supplementation, but in a direction not desired. In fact, the decrease of 18:1*t*11 fatty acid and *trans*-11/*trans*-10 ratio and consequently lack of positive response on 18:2*c*9,*t*11 in the muscle. Moreover it was not detected any effect on serum insulin concentration, it cannot be excluded that animals were in a higher insulinaemic status.

Daniel *et al.* (2004b) have shown that insulin increases the expression of SCD and the synthesis of MUFA from acetate, in cultured adipose tissue from ovine explants. The desaturation indices did not suggest any major effects of PP on SCD activity. Results suggested a lack of effects for PP dietary inclusion on SCD activity which, might be explained by the high dietary levels of 18:3*n*-3, either in basal or in linseed oil supplemented diets. Our results reinforce this explanation as increasing dietary availability of gluconeogenesis precursors (and consequent insulinaemic response) was probably insufficient to suppress the down-regulation effects of 18:3*n*-3 dietary availability. In fact, linseed oil supplementation increased 18:3*n*-3 as most of C18 BI, including 18:1*t*11 and rumenic acid. In the scientific literature, there are few studies on the gluconeogenesis precursor's effects in growth animals, and further studies are needed. Our hypothesis in this study failed, however can be improved in a next study, through several blood samples collections after PP intake throughout the day and evaluating the concentration of serum glucose and insulin. In addition, it is important to quantify the SDC mRNA expression and correlate it with the amount of rumenic and oleic acids in meat lamb.

6.5 General conclusions

The slaughter season seems to have only a slight influence on IMF lipid composition of Mirandesa-PDO veal and organic beef, which was not expected due to seasonal differences in pasture nutritive value and its availability. Mirandesa-PDO veal seems to have small seasonal differences in the levels of fatty acids, namely some minor fatty acids, CLA isomers and *n*-3

PUFA sum. Regarding organic beef, slaughter season seems to affect the levels of some fatty acids, CLA isomers, *n*-6/*n*-3 PUFA ratio, total cholesterol and β-carotene.

The results indicate that muscle type has a strong effect on the IMF quality of the analysed meats, which is likely due to the different fibre type proportions (oxidative or glycolytic) in LL and ST muscles. The lipid composition of Mirandesa-PDO veal, Charneca-PDO and organic beef seem to be markedly influenced by the muscle type, affecting most of the fatty acids.

Comparing the lipid composition of IMF of the studied meats with other Portuguese PDO meats and intensively produced beef, Mirandesa-PDO veal and organic beef have similar fatty acid profiles with Arouquesa and Barrosã veal, while IMF of Charneca-PDO beef are within the values reported for Alentejana and Mertolenga beef.

In general, the present work support that beef from pasture-fed animals has a good nutritional quality (mainly due to the higher levels of *n*-3 PUFA, CLA, α-tocopherol and β-carotene) and *n*-6/*n*-3 ratio values are inside or very close to the recommended figures for the human diet. Based on these values, the lipid composition of IMF of the studied traditional bovine meats and organic beef depicts a high nutritional value regarding seasonal and muscle variations, reinforcing the consumer`s confidence to buy these kind of products.

Regarding the gluconeogenesis precursors effects, the data suggest that the administration of PP does not seem to be an effective strategy to improve IMF in lamb meat, as PP reduces the rumenic acid. In addition, oil supplementation increases IMF and greatly affects its fatty acid composition, increasing *n*-3 PUFA and CLA contents.

6.6 Future perspectives

Apart from seasonal and muscle effects, future studies should address other factors such as sex, age and slaughter weight that may influence PDO and organic meat fat composition. Biochemical and molecular genetic studies of these meats should be encouraged to unravel the mechanisms responsible for differences in the metabolism and incorporation of specific fatty acids in meat.

The experiments of diets enriched with lipid sources (oilseeds, marine algae, novel feeds, *e.g.* lupin and hemp) aid to enhance these potential health-promoting fatty acids present in ruminant fats (Woods & Fearon, 2009). However, the effect of nutritional strategies on IMF requires a better understanding on the metabolic regulation of fatty acid fluxes (Hocquette *et al.*, 2010), assessing to fatty acid composition and protein expression of key lipogenic enzymes in cattle tissues.

Additionally, differential expression of adipogenic factors (*e.g.* nuclear transcription factors, such as PPAR) is known to play a key role on regulating multiple responsive pathways involved in fat development and lipid metabolism in cattle adipocytes (Taniguchi *et al.*, 2008). Although the adipogenic mechanism is extremely complicated some genes have been elucidated and confirmed to be associated with fatty acid composition in ruminants.

Further analysis is needed to investigate the key enzymes involved in SFA, MUFA and PUFA biosynthesis, such as acetyl-CoA carboxylase, SCD and delta-6 desaturase. There is limited information on the tissue distribution and regulation of these lipid metabolic enzymes in cattle, which have more been extensively studied in other species, including pigs (Herdmann *et al.*, 2010).

A future study to better understand the effects of PG should be delineated by using hyperinsulinemic-euglycemic clamps. This method consists of repeated measurement of both insulin and glucose, which strengthens the validity of the findings and reduces the risk of counter-regulatory hormones influencing the results. Endogenous insulin secretion and glucose production are suppressed by a continuous exogenous insulin infusion, while blood glucose concentration is maintained at normal physiological level by regulating the glucose infusion rate. This approach could be used to evaluate the insulin concentration after PG administration and its effect on meat fatty acids.

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