Universitat de Lleida

Assessing the role of the stearoyl-CoA desaturase gene on the fatty acid profile of pork

Eliana Henriquez Rodriguez

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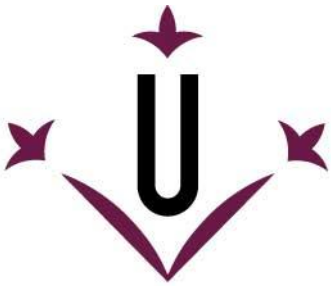

Universitat de Lleida

TESI DOCTORAL

Avaluació de l'efecte del gen estearoil-CoA desaturasa en el perfil d'àcids grassos de la carn de porc

Eliana Henriquez Rodriguez

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida

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Assessing the role of the stearoyl-CoA desaturase gene on the fatty acid profile of pork

By Eliana Henriquez Rodriguez

This Thesis has been submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Agricultural and Food Science and Technology, International mention, at the University of Lleida.

Thesis supervisor:
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INDEX

INDEX OF FIGURES AND TABLES

Introduction

Chapter IV

Chapter V

Chapter VI

Chapter VII

SUMMARY

This PhD dissertation is framed on a line of research aimed at improving pork quality and, particularly, intramuscular fat (IMF) and monounsaturated fatty acid (MUFA) content as two of the main traits affecting nutritional and sensorial pork attributes. Several strategies have been investigated to enhance IMF and MUFA without increasing the rest of fat depots, but one of the most promising approaches is to find out genetic markers specifically associated to them. There is a variant in the promoter of the stearoyl-CoA desaturase (*SCD*) gene (*AY487830:g.2228T>C* used as tag single nucleotide polymorphism) that specifically enhances MUFA. This polymorphism localizes in the core sequence of a putative retinoic acid response element. The primary objective of the thesis was to assess the impact of this *SCD* polymorphism in different production and commercial scenarios. The thesis comprises four studies. The first one was intended to show whether the effect of this *SCD* polymorphism is maintained at different market carcass weights. The second and third study examined whether the effect of the *SCD* polymorphism is still evident in dry-cured products from purebred Duroc and Duroc-sired Iberian crossbreds, respectively. The fourth study investigated the impact of carotenoid intake as a source of dietary retinoic acid on IMF and MUFA in pigs from opposite genotypes at the *SCD* gene polymorphism. Four experiments, one per objective, were designed. The first consisted of a series of 1-4 repeated samples of m. longissimus thoracis and subcutaneous fat of 214 Duroc barrows collected at 160, 180, 210 and at 220 days of age. The second and third experiments were based, respectively, on 125 dry-cured hams from purebred Duroc pigs (53 traced throughout curation and 72 randomly sampled) and on 74 dry-cured hams from Duroc × Iberian pigs (taken from sliced trays randomly purchased from the same supplier). Dry-cured hams were from barrows and gilts. The fourth experiment consisted of 32 Duroc barrows which were allocated in a 2 x 2 split-plot design consisting of two finishing diets (from 165 to 195 days of age) differing in pro-vitamin A carotenoid content and the two *SCD* homozygotes. The diets were identical except the corn line used in the feed. The carotenoid-rich diet was formulated with 20% of a carotenoid-fortified corn while the carotenoid-restricted diet used instead 20% of its near isogenic line, which did not contain pro-vitamin A carotenoids. The positive effect of the T allele at the *SCD* gene on fat desaturation and MUFA content was confirmed throughout the growing-finishing period and after the curing process on both purebred Duroc and Duroc × Iberian dry-cured hams. A strong relationship between MUFA in green and dry-cured samples was found, with TT pigs being more effective in retaining increased MUFA in green hams until the end of the curing period. Moreover, the *SCD* polymorphism had a greater impact on MUFA than using hams from barrows instead of gilts. The results of the last experiment indicated that pigs fed with the carotenoid-rich diet had 2.8-fold more retinoic acid and 4.5-fold more *SCD* gene expression in liver, around one fifth less fat and MUFA in liver and one third less IMF in m. gluteus medius. The TT genotype at the *SCD* gene increased MUFA in all tissues. Liver fat and MUFA content declined non-linearly with liver all-trans retinoic acid, suggesting a saturation point at relatively low all-trans retinoic acid content. The results obtained support that a pro-vitamin A carotenoid restricted diet at finishing and the TT genotype at the *SCD* gene complement well each other to simultaneously increase IMF and MUFA without increasing total fat content. The leptin receptor (*LEPR*) *NM_001024587:g.1987C>T* polymorphism was also segregating in Duroc, with the T allele positively affecting IMF and the saturated fatty acid (SFA) content. Selection for the *SCD* T allele, particularly in combination with selection for the *LEPR* C allele, is confirmed as a good strategy to enhance the MUFA/SFA ratio and therefore to produce healthier meat.

RESUMEN

La presente tesis doctoral se enmarca en una línea de investigación dirigida a mejorar la calidad de la carne cerdo y, en particular, su contenido de grasa intramuscular (IMF) y ácidos grasos monoinsaturados (MUFA) al ser dos de los principales caracteres que afectan atributos tanto nutricionales como organolépticos de la carne. A la fecha, diversas estrategias han sido investigadas para mejorar IMF y MUFA sin aumentar el resto de depósitos grasos de la canal, siendo uno de los enfoques más prometedores el encontrar marcadores genéticos asociados específicamente a dichos caracteres. Existe una variante en el promotor del gen de la estearoil-CoA desaturasa (*SCD*) (*AY487830: g.2228T>C* ha sido utilizado como polimorfismo de un solo nucleótido de referencia) que mejora MUFA. Este polimorfismo se localiza en el núcleo de la secuencia de un elemento de respuesta putativo al ácido retinoico. El objetivo principal de la tesis fue evaluar el impacto del polimorfismo *SCD* en diferentes escenarios productivos y comerciales. La tesis está compuesta por cuatro estudios. El primero tuvo como objetivo evaluar si el efecto del polimorfismo *SCD* se mantiene a diferentes pesos comerciales. El segundo y tercer estudio examinaron si el efecto del polimorfismo *SCD* es evidente en jamones curados de raza pura Duroc y de Ibéricos cruzados con Duroc. El cuarto estudio investigó el impacto de la ingesta de carotenoides, como fuente de ácido retinoico en la dieta, sobre IMF y MUFA en cerdos de genotipos opuestos para el polimorfismo *SCD*. Para cada objetivo se diseñó un experimento. El primero consistió en un muestreo repetido (de 1 a 4) de m. longissimus thoracis y grasa subcutánea a las edades de 160, 180, 210 y 220 días en 214 machos castrados Duroc. El segundo y tercer experimento se basaron en 125 jamones curados de cerdos Duroc de raza pura (53 rastreados a lo largo de la curación y 72 muestreados al azar) y en 74 jamones curados Duroc × Ibérico (tomados de bandejas loncheadas compradas aleatoriamente del mismo proveedor), respectivamente. Los jamones curados provenían de machos castrados y hembras. El cuarto experimento consistió en 32 machos castrados Duroc que fueron asignados a un diseño split-plot 2 x 2 consistente en dos dietas de engorde (de 165 a 195 días de edad) que diferían en el contenido de carotenoides precursores de vitamina A y los dos genotipos homocigotos del gen *SCD*. Las dietas fueron idénticas excepto en la línea de maíz usada en la formulación. La dieta rica en carotenoides se formuló con 20% de maíz fortificado con carotenoides, mientras que la dieta restringida en carotenoides contuvo en su lugar un 20% de una línea casi-isogénica que no contenía carotenoides precursores de vitamina A. El efecto positivo del alelo T del gen *SCD* sobre la desaturación de la grasa y el contenido de MUFA se confirmó a lo largo del período de crecimiento-engorde así como al final del proceso de curado, tanto en los jamones de raza pura Duroc como en los Duroc × Ibérico. Se encontró una fuerte correlación en el contenido de MUFA entre muestras frescas y curadas, mientras que el genotipo TT fue más efectivo reteniendo el aumento de MUFA observado en los jamones frescos hasta el final del período de curación. Por otra parte, el polimorfismo *SCD* tuvo un mayor impacto sobre MUFA que el uso de jamones de machos castrados en lugar de hembras. Los resultados del último experimento mostraron que los cerdos alimentados con la dieta rica en carotenoides tuvieron 2.8 veces más ácido retinoico y 4.5 veces más expresión del gen *SCD* en hígado, alrededor de un quinto menos grasa y MUFA en el hígado, y un tercio menos de IMF en el m. gluteus medius. El genotipo TT del gen *SCD* aumentó MUFA en todos los tejidos. La grasa hepática y el contenido de MUFA disminuyeron de forma no lineal con el ácido retinoico hepático, sugiriendo un punto de saturación a un nivel relativamente bajo de ácido retinoico. Los resultados obtenidos evidencian que los efectos de una dieta restringida en carotenoides precursores de vitamina A durante el engorde y del genotipo TT del gen *SCD* se complementan bien para aumentar simultáneamente IMF y MUFA sin alterar el contenido total de grasa. El polimorfismo del gen del receptor de la leptina (*LEPR*) *NM_001024587:g.1987C>T* también segrega en esta población Duroc, con el alelo T afectando positivamente IMF y el contenido de ácidos grasos saturados (SFA). La selección del alelo T del gen *SCD* en combinación con la selección del alelo C de *LEPR* se confirma como una buena estrategia para aumentar el ratio MUFA / SFA y en consecuencia producir carne más saludable.

RESUM

Aquesta tesi doctoral s'emmarca en una línia d'investigació dirigida a millorar la qualitat de la carn de porc i, principalment, el greix intramuscular (IMF) i els àcids grassos monoinsaturats (MUFA), per ser dos dels principals trets que afecten els atributs nutricionals i sensorials del porc. S'han investigat diverses estratègies per millorar IMF i MUFA sense augmentar la resta dels dipòsits de greix, i una de les més prometedores és la de trobar marcadors genètics associats específicament a ells. Hi ha una variant en el promotor del gen de la estearoil-CoA desaturasa (*SCD*) que millora específicament MUFA (*AY487830: g.2228T> C*, polimorfisme d'un sol nucleòtid utilitzat com a marcador de referència). Aquest polimorfisme es localitza en la seqüència central d'un putatiu element de resposta a l'àcid retinoic. L'objectiu principal de la tesi va ser avaluar l'impacte del polimorfisme *SCD* en diferents escenaris de producció i comercials. La tesi consta de quatre estudis. El primer tenia per objectiu mostrar si l'efecte del polimorfisme *SCD* es manté a diferents pesos comercials de la canal. El segon i tercer estudi es dedicaren a examinar si l'efecte del polimorfisme *SCD* és fa encara evident en productes curats de raça pura Duroc i en els seus creuaments amb Ibèric, respectivament. En el quart estudi es va investigar l'impacte de la ingesta de carotenoides, com a font d'àcid retinoic en la dieta, sobre IMF i MUFA en porcs de genotips oposats en el polimorfisme del gen *SCD*. Es van dissenyar quatre experiments, un per cada objectiu. El primer experiment consistí en un a sèrie de fins a 4 mostres repetides de m. longissimus thoracis i de greix subcutani presses a 160, 180, 210 i en 220 dies d'edat en 214 mascles castrats Duroc. El segon i tercer experiments es van basar, respectivament, en 125 pernils curats de porcs Duroc de pura raça (53 traçats des de fres fins el final de la curació i 72 escollits a l'atzar) i en 74 pernils curats procedents d'un encreuament Duroc × Ibèric (presos de safates de pernil llescat comprades a l'atzar del mateix proveïdor). Els pernils curats provenien de mascles castrats i femelles. El quart experiment va consistir en 32 mascles castrats Duroc disposats segons un disseny split-plot 2 x 2, amb dues dietes de finalització (des de 165 a 195 dies d'edat) que diferien en el contingut de provitamina A carotenoide i els dos homozigots *SCD*. Les dietes van ser idèntiques, excepte en la línia genètica de blat de moro utilitzat en el pinso. La dieta rica en carotenoides es va formular amb un 20% de blat de moro fortificat amb carotenoides, mentre que la dieta control va incloure en el seu lloc un 20% de blat de moro d'una línia quasi isogènica sense carotenoides precursors de provitamina A. Els resultats obtinguts confirmen que l'efecte positiu de l'al·lel T del gen *SCD* sobre la dessaturació del greix i el contingut de MUFA es manté durant tot el període de creixement i d'acabat i al final del procés de curació, tant en els pernils Duroc com en els Duroc × Ibèric. S'ha posat en evidencia una forta relació entre el contingut de MUFA en el pernil fresc i curat, de tal manera que els porcs TT són més eficaços retenint els MUFA fins al final del període de curació. D'altra banda, el polimorfisme *SCD* ha tingut un major impacte sobre MUFA que la utilització de porcs castrats en lloc de femelles per la producció de pernil. Els porcs alimentats amb una dieta enriquida en carotenoides mostraren 2.8 vegades més àcid retinoic i 4.5 vegades més expressió del gen *SCD* en fetge, aproximadament un cinquè menys de greix i MUFA en el fetge i un terç menys de IMF en m. gluteus medius. El genotip TT del gen *SCD* ocasionà un augment de MUFA en tots els teixits. El greix del fetge i el contingut de MUFA disminuïren de forma no lineal amb l'àcid holo-trans retinoic del fetge, el que suggereix l'existència d'un punt de saturació a un nivell relativament baix d'àcid holo-trans retinoic. Els resultats obtinguts han posat de manifest que els efectes d'una dieta restringida en carotenoides precursors de la vitamina A i el genotip TT del gen *SCD* es complementen bé per augmentar simultàniament IMF i MUFA sense variar el contingut de greix total. S'ha observat que el polimorfisme *NM_001024587: g.1987C> T* del gen del receptor de la leptina (*LEPR*) també segrega en Duroc, amb l'al·lel T afectant positivament IMF i el contingut d'àcids grassos saturats (SFA). La selecció a favor de l'al·lel T del gen *SCD*, particularment en combinació amb la selecció a favor de l'al·lel C de *LEPR*, es confirma com una bona estratègia per millorar la relació MUFA / SFA i per tant la producció de carn més saludable.

I. INTRODUCTION

1. Importance of pig production in Spain

In the past twenty years (1993-2013), pork has been the main meat produced in the globe (FAOSTAT, 2014), with Spain as the current third worldwide producer (EUROSTAT, 2016; Vega, 2016). Within the country, more than 60% of pork is produced across three regions: Cataluña (42.2%), Castilla y León (13.9%) and Aragón (10.4%) (**Figure 1**).

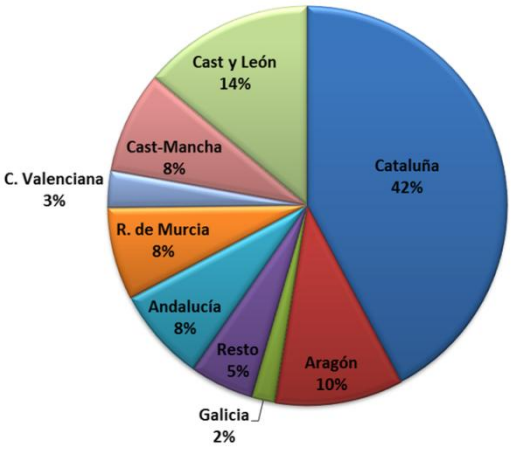

Figure 1. Spanish pork production by autonomous community in 2015
Source: MAGRAMA (2016)

The majority of Spanish farms (79,23%) work under intensive systems (MAGRAMA, 2016), where feed can account for more than 60% of production costs. The decrease in feed prices of the last few years (DARP, 2015) (**Figure 2**) positively impacted the business profitability. Mercolleida is the institution who sets the prices used in 90% of Spanish pork trades; its board is integrated by the main buyers and sellers of hogs in the country (Mercolleida, 2016; Lence, 2005). The market of meat exports is led by pork, whose numbers steadily increased from 603.596 tons in 2006 to 1.076.365 tons in 2014 (ANICE, 2014). Approximately 75% of Spanish pork exports are destined to EU countries, where the main costumers by 2015 were France (27.8%), Portugal (19.7%) and Italy (13.4%), while China remains as the first client outside the EU (MAGRAMA, 2016). The pork industry represented 13% of the Spanish agrarian production in 2015 as well as 36% of final livestock production.

Figure 2. Evolution of Spanish feed wheat prices in the last years (EUR/ton)
Source: Boletín de Mercolleida nº 2.427 - 7 al 11 de septiembre de 2015

The Spanish market offers not only fresh pork but a rich variety of pork products. In fact, since 2009, the consumption of such products surpasses fresh meat and by 2015 was 7.7% higher (MAGRAMA, 2016). The traditional sector of dry-cured hams stand out as both the leading product sold locally as well as exported to foreign markets (OECE, 2015).

2. Dry-cured hams: market relevance and production

Dry meat products are well known worldwide, especially in the Mediterranean markets, due to their particularly sensorial attributes. In Spain, dry-cured hams represent 31% of the total consumption of pork products (Cruz, 2013), which makes them the favorite of consumers. These hams are made from pigs reared intensively or extensively and mainly from the principal commercial pig lines rather than Iberian animals (Chamorro et al., 2008). There are two categories of Spanish dry-cured hams: Serrano (*PDO Teruel* and *PGI Treveléz*) and Iberian, which possesses four European PDOs (protected designations of origin): *Jamón de Guijuelo, Dehesa de Extremadura, Jamón de Huelva* and *Jamón Valle de los Pedroches* (Toldrá, 2015).

The dry-curing process was originally used as a preservation method; however the methodology has been improved to develop a more desirable flavor and a firmer texture (Gonzalez and Ockerman, 2000). The main steps in the processing of dry-cured hams have been summarized by Toldrá (2006) as follows: initially, hams are bledded on a steel belt with pressing rollers and receive the adequate amount of cure salt that is rubbed on the outer surface. During salting, in which hams are kept under refrigeration (below 4 ºC), they are placed in plastic or stainless steel shelves and are given a certain amount of salt. They are left to stand for 10–14 days to allow salt and nitrate penetration into the ham. The main objective of the next stage, named post-salting, is for salt and nitrate diffusion into the full piece. This stage may last between 40 and 60 days, depending on the temperature (usually below 6 ºC) and the size of the ham. Ripening and drying is the following stage, which is conditioned by a large number of variables, such as time, temperature, relative humidity and air velocity. In general, high quality hams are placed in curing chambers under mild conditions (temperatures 14–16 ºC for more than 8 months) and, once they reach the expected moisture loss, are manually smeared with a layer of lard in order to prevent excessive dehydration. Final total weight loss may reach 34–36% in relation to the initial weight.

3. Pork quality

Many factors affect meat quality and its definition, hence several authors have tried to classify them according to different categories: processing and sensory meat quality traits (Ciobanu et al., 2011), product and process quality characteristics (Weissmann, 2014) or experiential and credence quality attributes (Font-i-Furnols and Guerrero, 2014). On the whole they agree that consumers play a key role in the assessment of quality since their personal preferences drive the market demands. On **Table 1** are described the main traits impacting meat quality:

Table 1. Meat quality traits

Processing meat quality traits	
	Water-holding capacity
	Colour
	Firmness
	Cooking loss
	Others: Napole yield, RYR1
Sensory traits: assessed by trained panels or consumers surveys	
	Appearance (colour and marbling)
	Texture (uncooked meat)
	Tenderness (cooked meat)
	Juiciness (cooked meat)
	Flavour (cooked meat)

Modified from Ciobanu et al., 2011.

Studies across different environments using traditional and selected pig breeds have shown that ultimate pH, measured 24 h after slaughter, is correlated with relevant quality aspects such as water-holding capacity, color, firmness and tenderness (Bidner et al., 2004; Boler et al., 2010; Li et al., 2013), there by underpinning an important part of the variation of meat quality traits. All previous characteristics lead to summarize meat quality as a combination of traits that result in a wholesome product that tastes good, has good value and generates repeat sales (Mabry and Bass, 1998).

3.1. Intramuscular fat content

Intramuscular fat (IMF), as described by Hocquette et al. (2010), refers to the amount of fat within muscles which, therefore, cannot be removed before consumption. From a chemistry point of view, it is the chemically extractable fat in a muscle, predominantly from adipocytes and myocytes which lipids can be subdivided in phospholipids, triacylglycerols, mono and diacylglycerols, cholesterol and cholesteryl esters, and free fatty acids (Shi-Zheng and Su-Mei, 2009). The term marbling is used to refer to the fat intermingled with the lean within a muscle, or visible intramuscular fat (Jeremiah, 2001). Different authors have shown the significant and positive correlation between chemically determined IMF and marbling (van der Wal et al., 1992; Faucitano et al., 2004).

Since several years ago, IMF is a relevant trait for the meat industry due to its impact on several sensory and processing characteristics. Fuentes et al. (2013) and Ruiz-Carrascal et al. (2000) evidenced the positive effect of IMF on brightness scores (an appearance trait as color), juiciness and oiliness of Iberian dry-cured hams. Using commercially raised hybrid barrows, Cannata et al. (2010) found that with increased marbling drip loss decreases and sensory tenderness and juiciness scores increase. Consumers within a wide range of ages (18 to 73) ranked with higher acceptability, tenderness and juiciness scores cooked loin chops with increased levels of IMF (Font-i-Furnols et al., 2012). Also with crossbred pork, Fernandez et al. (1999 a,b) showed that IMF was associated with higher juiciness and flavor scores and had positive effects on perception of texture and taste. Studies assessing Warner-Bratzler shear force, an instrumental measure of tenderness, have reported a negative correlation between IMF and this trait (Fortin et al., 2005; Jeleníková et al., 2008), suggesting that selection towards extreme lean content produces "tougher meats". Regarding genetic parameters, different authors have found a moderate-to-high heritability (0.39 - 0.53) for this trait (Cameron, 1990; Suzuki et al., 2005), whereas genetic correlations between IMF and meat quality traits have been shown to be negative with drip loss, cooking loss and pH, and positive with lightness [L] (Suzuki et al., 2005), tenderness, pork flavor intensity and overall acceptability (Lo et al., 1992; Sellier, 1998).

3.2. Fatty acid composition

Muscle lipids are composed of polar lipids (mainly phospholipids) located in the cell membrane and neutral lipids (mostly triacylglycerol) located in the adipocytes along the muscle fibers and in the interfascicular area (De Smet et al., 2004). The neutral lipid fraction represents 60-70% of total intramuscular fatty acids. In the adipose tissue, lipids are stored in triacylglycerol form (Dodson et al., 2010) which main constituents are saturated (SFA) and monounsaturated (MUFA) fatty acids. On the contrary, phospholipids are essential membrane components very rich in polyunsaturated fatty acids (PUFA) (Wood et al., 2004; Zhang et al., 2009). Neutral lipids become predominant in overall fatty acid composition as body fat increases. Conversely, phospholipid content varies little, since it is strictly controlled in order to maintain membrane properties, and therefore the

polyunsaturated-to-saturated fatty acid ratio (P/S) declines throughout the fattening period (De Smet et al., 2004; Wood et al., 2008).

Fatty acids influence technological and organoleptic characteristics of meat quality in several aspects. SFA require higher temperatures to reach their melting point than PUFA, which impacts fat tissue firmness. The oxidation of MUFA leads to rancidity and therefore affects shelf life during retail display. Lipid oxidation also alters color by promoting oxymyoglobin oxidation (Joo et al., 2002; Wood et al., 2003; Li et al., 2015). Cameron and Enser (1991) have shown a general improvement on eating quality traits, such as flavor, tenderness and juiciness, when MUFA concentrations increase and PUFA decrease. The unsaturated C16:1, C18:1n-9 and C18:1n-7 fatty acids have been positive correlated with pork flavor, flavor liking and overall acceptability whereas the opposite is true for the polyunsaturated C18:2n-6, C18:3n-3, C20:3n-6, C20:4n-6, C20:5n-3, C22:5n-3 and C22:6n-3 (Cameron et al., 2000).

The intramuscular lipid content is also important for the dry-cured ham industry because affects quality traits such as the aspect and color of slices, texture and the intensity and persistence of aroma (Gandemer, 2009). For example, during processing the linoleic, arachidonic, oleic, palmitic and stearic fatty acids from phospholipids decrease significantly due to the action of muscle phospholipases. As a consequence, an important amount of free fatty acids are generated of which unsaturated fatty acids are further oxidized to aroma volatile compounds. The oxidation leads to the formation of aliphatic hydrocarbons, alcohols, aldehydes and ketones while some esters could be derived from the interaction of the free fatty acids with alcohols (Toldrá, 2006). Several of those ketones have been correlated with the aromas of dry ham and cured meat, while rancid aroma has been related to aldehydes among others products of lipid oxidation (Buscailhon et al., 1994).

3.3. Impact of dietary fatty acids on human health

The nutritional profile of meat fat has been largely studied because, as an important source of dietary lipids, it can influence health. There is a general agreement that SFA increase LDL cholesterol levels while PUFA have the opposite effect (Grundy, 1997; Decker and Park, 2010). Thus, the increase of the P/S ratio is widely recommended as an effective strategy to reduce the risk of coronary heart disease (Hu and Willett, 2002).

Dietary MUFA, and particularly oleic acid, promote healthy blood lipid profiles, improve insulin sensitivity and regulate glucose levels (Gillingham et al., 2011; Ros et al., 2015; Joris and Mensink, 2016). A high (n-6) : (n-3) PUFA ratio (> 4), which is commonly seen in Western diets, has been associated with an increase in chronic inflammatory diseases such as nonalcoholic fatty liver disease, cardiovascular disease, obesity, inflammatory bowel disease, rheumatoid arthritis and Alzheimer's disease (Patterson et al., 2012).

3.4. Factors influencing fatty acid composition

The age, sex, breed, fat location, muscle type and diet directly influence the fatty acid composition of pork. As growing progresses, pig's fat as well as SFA and MUFA, both in muscle and subcutaneous fat, increase while PUFA decrease (Virgili et al., 2003; Lo Fiego et al., 2010; Bosch et al., 2012). The concentrations of PUFA and SFA on subcutaneous fat decrease and increase, respectively, in the order of male > females > males castrates (Nürnberg and Ender, 1998). Studies comparing different pig lines have found significant differences for SFA, MUFA and PUFA content among breeds and within fat and lean phenotypes (Cameron and Enser, 1991; Wood et al., 2004; Plastow et al., 2005), showing that genetic types with low concentration of total lipid in muscle have higher proportions of PUFA in total lipids. The fatty acid profile also changes between intramuscular fat and backfat: the PUFA/SFA ratio is lower in muscle than in backfat while the PUFA n-6:n-3 ratio has the inverse relationship (Bosch et al., 2012; Raj et al., 2010). Muscles with high concentration of oxidative fibers have greater phospholipid and PUFA content as compared to glycolytic muscles (Hernández et al., 1998; Wood et al., 2004). It is well known that monogastrics absorb dietary fatty acids unchanged before their incorporation into the tissue lipids, thus dietary lipids have a direct and predictable effect on meat fatty acid composition (Nieto and Ros, 2012). As a consequence, diverse nutritional strategies can be used to modify the fatty acid profile of pork, from diets enriched with different oils to changes in dietary protein or specific aminoacids levels (Cameron et al., 2000; Enser et al., 2000; Wood et al., 2008).

4. Pig breeding

4.1. Breeding organization and selection schemes

Commercial pig breeding programs are organized mainly under a three-tier pyramidal structure as follows: nucleus farms → multiplier farms → commercial farms. Genetic improvement is generated in small nucleus populations and disseminated afterwards through multiplier farms aimed to reproduce pigs of outstanding performance at larger scales. The commercial producer acquires gilts from multipliers to produce crossbred piglets using terminal boars, finally this progeny will be destined to fattening and slaughter (Dekkers et al., 2011). Selection is performed considering different objectives between dam and sire lines, thus, for sow production, maternal traits are the main target (total number born, mothering ability, farrowing rate,…) while for boars are production and meat quality (growth rate, water binding capacity, intramuscular fat, meat color,…) (Oldenbroek and van der Waaij, 2015). Nowadays, due to societal concerns, breeding organizations are exploring the inclusion of traits related to welfare and health of pigs, robustness, and the healthiness and sensory quality of pork (Kanis et al., 2005).

European breeding schemes are based mostly in crosses of Large White, Landrace, Duroc and Pietrain pigs (Blasco et al., 1994; Gispert et al., 2007; Leenhouwers & Merks, 2013). The three-way cross of a F1 sow Landrace × Large White with a sire from a well conformed breed is commonly used in Spain for pork production. The choice of the terminal sire depends on the profitability obtained per animal and can vary from heavily muscled boars, like Pietrain, to those with good growth rate and resistance like Duroc (Toldrá, 2002). Spanish premium hams like the *Jamón Ibérico* and *Jamón de Teruel* use Duroc as sire line (B.O Aragón, 2009; BOE, 2014).

The Duroc breed was developed in the US and is one of the main terminal sires used worldwide. The inclusion of Duroc genes in breeding programs has been consistently associated with increases in growth rate, intramuscular fat content and backfat thickness, higher ultimate pH, juiciness, less drip loss, an a redder and more intensive meat color (Candek-Potokar et al., 1998; Edwards et al., 2003; Channon et al., 2004; Choi et al., 2014). Nonetheless, some reports have shown lower flavor liking and acceptability values for Duroc boars as compared to other breeds (Cameron et al., 1990). Regarding maternal lines,

its incorporation improves appetite during lactation, structural soundness and sow longevity (Jones, 1998). The crossbreeding with Duroc also impacts piglet performance, improving birth weight and body max index from birth to weaning, traits positive correlated with survival at weaning (Rootwelt et al., 2012).

4.2. Selection for meat quality

Thanks to the incentive-based marketing system for pork initiated in the 80's, genetic selection for lean pigs became a top objective in breeding programs. As a consequence, marbling fat content fell below 1% of muscle weight (Wood, 1990) and out of the 2%-4% target recommended by the US National Pork Board (Meisinger, 2002) to achieve a balance between eating satisfaction and health concerns. Along the past decades, Barton-Gade (1990), Cameron (1990) and Schwab et al. (2006) have shown that selection for increased leanness reduces meat and eating quality, affecting traits such as tenderness, color, pH and fatty acid content. Nowadays, even when the meat industry does not *paid for* them, several commercial pig lines have breeding goals where pork quality traits represent between 20 to 37% of the total (Knap, 2014).

Fatty acids are genetically determined (Sellier et al., 2010; Gjerlaug-Enger et al., 2010) and therefore their profile can be improved through selection strategies. The heritability of oleic acid, the major fatty acid in pigs, ranges between 0.36 and 0.50 (in muscle) while its correlation with IMF varies between 0.10 and 0.47 (Suzuki et al., 2006; Ros-Freixedes et al., 2012; Ros-Freixedes et al., 2014). This correlation structure is in line with studies showing that selection towards leanness negatively affects oleic acid content (Cameron and Enser, 1991; Ntawubizi et al., 2010). Suzuki et al. (2006) reported negative genetic correlations between oleic acid and drip loss (-0.10), cooking loss (-0.61), lightness (-0.61) and pH (-0.29), while positive with average daily gain (0.25) and tenderness (0.15). A study on genetic parameters for IMF fatty acid composition showed a positive correlation between MUFA (79% was oleic acid) and SFA, as well as negative with PUFA (Ntawubizi et al., 2010). Recently, an experiment to assess the expected genetic response for oleic acid was performed in the Duroc population used in this thesis. The results suggest that selection programs directed at growth rate would have a minor effect on oleic acid whereas those focusing on lean content would decrease it content (Ros-Freixedes et al., 2012). Breeding for fatty acid composition needs to address two main challenges: to define which and how

many fatty acids should be included as criteria in the breeding program, and to find a cost-efficient phenotyping strategy for measuring the trait (or traits) at large-scale (Suzuki et al., 2006).

5. Lipid metabolism

In pigs, liver is the main organ synthesizing long PUFA while the adipose tissue is the primarily site where *de novo* lipogenesis occurs, with the main products being SFA and MUFA (Durant-Montgé et al., 2009). Glucose is the major precursor for oxidation and *de novo* fatty acid synthesis (**Figure 3**) whereas acetate is the preferred substrate in liver (Dunshea et al., 2003). In pigs fed the usual high-carbohydrate diet, *de novo* lipogenesis from glucose accounts for approximately 75% of lipid synthesis.

Glucose is metabolized via glycolysis to pyruvate, which is subsequently converted to citrate via the initial stages of the tricarboxylic acid pathway. Outside the mitochondrion, the citrate is cleaved to acetyl CoA and oxaloacetate by ATP- citrate lyase. Acetyl CoA is carboxylated to malonyl CoA by acetyl CoA carboxylase, which is then polymerised into fatty acids by fatty acid synthetase (Pearce, 1983; Dunshea et al., 2003). The predominant products of *de novo* lipogenesis are palmitic, stearic, oleic and palmitoleic fatty acids, through procedures of elongation and/or desaturation via the interaction of many catalytic enzymes (i.e. reductases, desaturases, elongases). Different factors modulate lipogenesis and, among them, the genes involved in *de novo* synthesis are particularly important. According to the review of Laliotis et al. (2010), the main lipogenic genes isolated in both ruminant and non-ruminant mammalians are Acetyl-CoA carboxylase (*ACC*), Fatty Acid Synthase (*FAS*) and the genes encoding NADPH Generating Dehydrogenases: Glucose 6-Phosphate Dehydrogenase (*G6PD*), 6-Phosphogluconate Dehydrogenase (*6PGD*), Cytosolic NADP Malate Dehydrogenase/Malic Enzyme 1 (*ME1*) and Cytosolic NADP Isocitrate Dehydrogenase (*IDH1*).

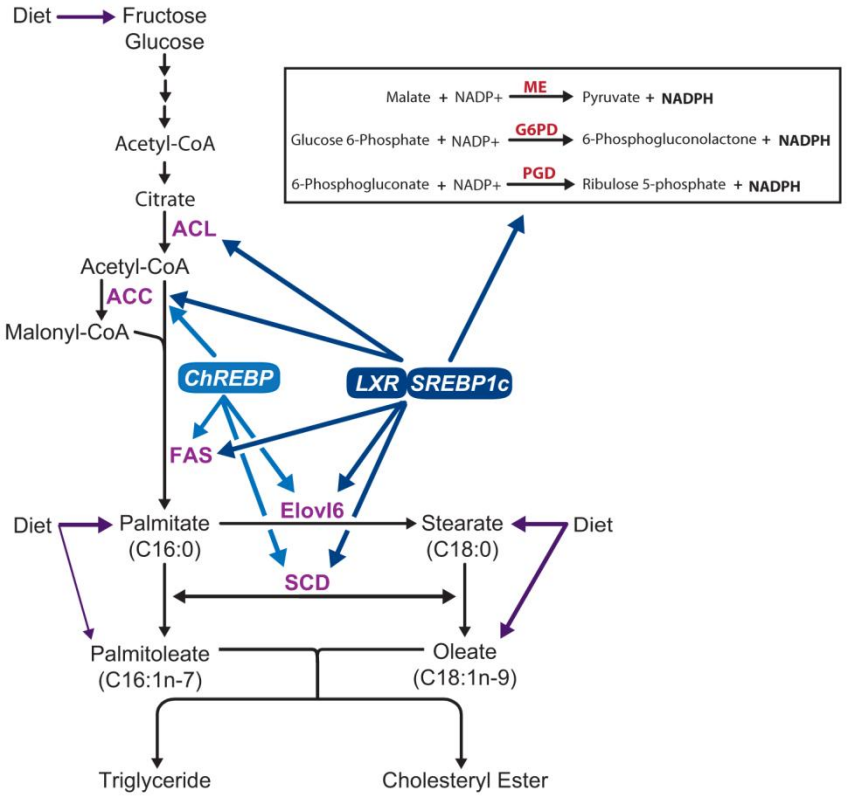

Figure 3. Pathway of de novo synthesis of saturated and monounsaturated fatty acids and regulation of lipogenic genes by transcription factors (LXR, SREBP-1c and ChREBP). NADPH is required for fatty acid synthesis and three chemical reactions yield NADPH (inset). PGC-1β has been shown to coactivate LXR and SREBP-1c. ACL, ATP-citrate lyase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; ELOVL6, elongation of long-chain fatty acids family member 6 (elongase 6); SCD, stearoyl-CoA desaturase; LXR, liver X receptor; SREBP-1c, sterol regulatory element-binding protein 1c; ChREBP, carbohydrate response element binding protein; ME, malic enzyme; G6PD, glucose 6-phosphate dehydrogenase; PGD, Phosphogluconate dehydrogenase (Strable and Ntambi, 2010).

The expression of genes involved in fatty acid metabolism is primarily controlled at the transcription level by several transcription factors, which have the ability to bind on target-sequences of the genes and promote or suppress gene transcription according to the stimulus (i.e. nutrients, hormones, etc.) (Laliotis et al., 2010). The sterol regulatory element binding proteins (SREBP) are present as two isoforms: SREBP-2 is primarily involved in activation of cholesterol synthesis and metabolism while SREBP-1 [a & c] is involved in regulation of fatty acid synthesis and the induction of both cholesterol and fatty acids synthesis. In the liver, SREBP-1c increases expression of *SCD* (stearoyl-CoA desaturase),

ACC, *FAS* and acetyl CoA synthase. The peroxisome proliferator activated receptor (PPAR) is part of the nuclear receptor family. The PPARα form is involved in regulation of β-oxidation and lipolysis in hepatocytes while PPARγ is involved in regulation of fatty acid synthesis in adipocytes. Peroxisome proliferators also stimulate *SCD*-1 transcription levels (White et al., 2013). Other nuclear receptors, such as RAR, RXR, ROR and HNF4, also function as ligand-activated transcription factors modulating and controlling transcription during lipogenesis (Stroup et al., 2000; Tarling et al., 2004).

The lipogenesis process is particularly sensitive to changes in the diet, thus it is stimulated by high carbohydrate diets and, conversely, is inhibited by PUFA and fasting. The hormonal regulation is mediated mainly through insulin, the growth hormone (GH), leptin and the acylation stimulating protein (ASP) (Kersten, 2001).

As showed before, enzymes play a key role in fatty acid metabolism and therefore elucidating the genetic determinism of these biological catalysts can provide a useful tool to manipulate meat fatty acid composition.

5.1. Acetyl-CoA Carboxylase

The Acetyl-CoA carboxylase enzyme (ACC) is a crucial component of fatty acid biosynthesis since catalyzes acetyl-CoA, the principal building block of fatty acids, to derive malonyl-CoA which is the precursor of palmitate (Laliotis et al., 2010) and an inhibitor of the import of fatty acids into the mitochondria for oxidation (Barber et al., 2005). There are two isoenzymes of ACC that are transcribed from different genes: *ACC*-α (ACC-1, ACACA) and *ACC*-β (ACC-2). The *ACC*-α gene is expressed ubiquitously but at higher rates (10-50 μg/g of wet weight) in lipogenic tissues (adipose tissue, liver and lactating mammary gland), and encodes a protein of 265 kDa localized in the cytosol (Travers and Barber, 2001). The *ACC*-β gene is expressed at low levels (1-2 μg/g of wet weight) mainly in heart and skeletal muscle while the liver shows the highest concentrations (20-25% of total hepatic ACC); it encodes a protein of 280 kDa attached to the mitochondrial membrane (Brownsey et al., 2006). The malonyl-CoA catalysed by *ACC*-α is used for the synthesis of long chain fatty acids while, in contrast, the derived from *ACC*-β regulates fatty acid β-oxidation. In liver, where fatty acid synthesis and oxidation are active, both isoforms are highly expressed (Abu-Elheiga et al., 2000; Zu et al., 2013).

5.2. Fatty Acid Synthase

Fatty acid synthase (FAS) catalyzes the synthesis of palmitate in a sequence of seven steps which are NADPH-dependent and utilize one acetyl-CoA and seven malonyl-CoAs as the base molecules to generate the final product (Ronnett et al., 2006). The complete coding sequence of the *FASN* gene in pig has a length of 8044 nucleotides and the corresponding polypeptide includes 2512 aminoacids, with a sequence highly conserved between mammals (goat, horse, cow and human 93% of homology) (Braglia et al., 2014). This multifunctional enzyme is composed of two identical monomers and each one of them contains six functional domains (White et al., 2013). The *FAS* mRNA transcripts have been found in most tissues, including adipose, liver, lung, brain, kidney, and small intestine, and their abundance in a tissue determines the rate of FAS protein synthesis, and ultimately the tissue content of FAS protein (Clarke, 1993).

5.3. Stearoyl-CoA desaturase

The stearoyl-CoA desaturase (SCD) is the rate-limiting enzyme in the synthesis of MUFA, since catalyzes the conversion of SFA, with preference for stearic and palmitic, to their MUFA counterparts, oleic and palmitoleic (Strable and Ntambi, 2010). The porcine *SCD* gene spans a transcription unit of 16,186 bp and encodes a protein of 359 amino acids with a calculated molecular mass of 41.3 kDa (Ren et al., 2004). There are four different isoforms of *SCD* with a tissue-specific distribution. The *SCD*-1 is expressed at high levels in mammalian adipose tissue and liver, *SCD*-2 is abundant in the brain and neuronal tissues, *SCD*-3 is expressed in sebocytes in the skin, as well as in the harderian and preputial glands, and *SCD*-4 is expressed only in the heart (Guillou et al., 2010). Studies in pigs have shown a linear relationship between *SCD* protein expression and total fatty acids in muscle (Doran et al., 2006).

5.4. Elongation of long-chain fatty acids family member 6

Elongation of long-chain fatty acids family member 6 (ELOVL6) is an elongase that catalyzes the addition of two-carbon units primarily to 12, 14, and 16-carbon fatty acid chains (Strable and Ntambi, 2010) and does not possess the capacity to elongate beyond C18 (Jakobsson et al., 2006). The palmitic (C16:0) and palmitoleic (C16:1n-7) fatty acids can be both further elongated by ELOVL6 to yield stearic acid (C18:0) and vaccenic acid

(C18:1n-7) (Guillou et al., 2010). The porcine *ELOVL6* gene spans a transcript of 388 bp and encodes a protein of 74 amino acids (Ensembl gene: ENSSSCG00000025541). The enzyme is ubiquitously expressed especially in tissues with high lipid content such as adipose tissue and liver.

6. Genetic markers as a tool to improve meat quality

Through molecular techniques, gene variants (alleles) that contribute to explain quantitative variations can be identified and subsequently used within genetic improvement programs (**Table 2**). The genotype at each of these loci provides genetic variation information that is incorporated into the genetic models to increase the accuracy of estimated breeding values and the rate of genetic improvement (van der Steen et al., 2005). Molecular information is, then, particularly relevant for the improvement of traits with low heritability such as litter size, disease resistance and longevity, as well as meat quality, since genetic tests have heritability equal to 1 (assuming no genotyping errors), can be done on both sexes and on all animals, at early times in life, and may require the recording of less phenotypic data (Dekkers and Rothschild, 2007). A recent study of Ros-Freixedes et al. (2016) showed that the prediction accuracy for oleic acid content using only the *SCD* single nucleotide polymorphism *AY487830:g.2228T>C* is similar to that obtained with a whole 36k chip. This polymorphism is part of the haplotype explaining 13% of the total additive genetic variance for oleic acid (Estany et al., 2014).

To date, there is still a lot to discover regarding the number of genes controlling meat quality and their interactions. However, different studies aimed to unravel the molecular basis of phenotypic variations have found genetic polymorphisms with important effects on meat quality traits, such as the ones described below.

6.1. Ryanodine receptor 1

A single missense mutation in the Ryanodine receptor 1 (*RYR1*) gene, which modulates the release of Ca^{2+} by the sarcoplasmic reticulum into the skeletal muscle, is responsible for the porcine stress syndrome (PSS), or malignant hyperthermia syndrome (MH). The anesthetic halothane can trigger PSS or MH in pigs (Eikelenboon and Minkema, 1974), being the reason why RYR1 is also known as the Halothane (HAL) gene. In genetically susceptible animals, stress triggers a higher rate of post mortem glycolysis

which generates low pH early post mortem, high temperatures and, as a consequence, high protein denaturation (Rosenvold and Andersen, 2003). Homozygous of the recessive genotype (*nn*) develop the pale, soft, exudative (PSE) meat defect, reducing the production yield of cooked ham and increasing the seasoning loss of dry-cured hams. Interestingly, however, the *RYR1 n* allele has an additive effect for carcass lean percentage, loin muscle area, dressing percentage, 1 h post-mortem pH and meat color score. Therefore, the genotyping of the mutation can be used to take advantage of the leanness and meat quality of the heterozygotes *Nn* while controlling PSS in the *nn* pigs (Ciobanu et al., 2011).

Table 2. Reported candidate genes associated with production and meat quality traits in pigs and its commercial use

Gene name	Gene symbol	Trait(s)	Industry use
Alpha-(1,2)-fucosyltransferase	*FUT1*	Disease resistance	Exclusive use
Calpastatin	*CAST*	Pork quality	Commercially available
Carbonic anhydrase III	*CA3*	Pork quality	
Cholecystokinin type A receptor	*CCKAR*	Feed intake	
Cytochrome b5A	*CYB5A*	Boar taint	
Estrogen receptor 1	*ESR1*	Litter size	Commercially available
Fatty acid binding protein 3	*FABP3*	Carcass composition	
Follicle-stimulating hormone beta	*FSHB*	Litter size	
Growth hormone	*GH*	Carcass composition	
Growth hormone-releasing hormone	*GHRH*	Growth and carcass composition	
Insulin-like growth factor 2	*IGF2*	Growth and carcass composition	Commercially available
Interferon-inducible guanylate-binding protein 1	*GBP1*	Disease resistance	
Leptin	*LEP*	Growth and carcass composition	
Leptin receptor	*LEPR*	Fat content and composition	
Mast/stem cell growth factor	*KIT*	Coat color	Exclusive use
Melanocortin 1 receptor	*MC1R*	Coat color	
Melanocortin 4 receptor	*MC4R*	Growth and carcass composition	Commercially available
Myopalladin	*MYPN*	Carcass composition	
Myostatin	*MSTN*	Growth	
Pituitary-specific transcription factor 1	*POU1F1*	Growth and carcass composition	
Prolactin receptor	*PRLR*	Litter size and boar reproduction	
Protein kinase adenosine monophosphate-activated gamma subunit	*PRKAG3*	Pork quality	Commercially available
Retinal binding protein 4	*RBP4*	Litter size	
Ryanodine receptor 1	*RYR1*	Stress susceptibility, leanness, and pork quality	Commercially available
Stearoyl CoA desaturase	*SCD*	Fatty acid composition	
Titin	*TTN*	Pork quality	
Parentage tests			Exclusive use within some companies, commercially available

Modified from Ernst and Steibel (2013), with additional information from Estany et al. (2014), Óvilo et al. (2005), Rothschild (2010) and Samorè and Fontanesi (2016).

6.2. Rendement Napole gene

A mutation in codon 200 of the protein kinase AMP-activated gamma 3-subunit gene (*PRKAG3*) is responsible for the presence of the dominant allele *RN*$^{-}$ which causes a 70% increase in muscle glycogen content. Consequently, pigs with at least one copy of the *RN*$^{-}$ allele produce meat with lower pH24h, because of post-mortem degradation of glycogen, which is associated with worsened water-holding capacity (WHC) and higher reflectance (Davoli and Braglia, 2008). The mutation is almost completely restricted to the Hampshire breed. A different polymorphism in the same gene, *p.Ile199Val* (II, IV, VV), was also associated with muscle glycogen differences, however its effect is opposite to the RN gene: the favorable homozygote (II) has less drip loss, higher pH24h and darker meat than the other genotypes (Ciobanu et al., 2001; Otto et al., 2007). The favorable additive effect of *p.Ile199Val* is present in all common commercial breeds. These studies suggest that *PRKAG3* plays a key role in the regulation of energy metabolism.

6.3. Calpastatin

Postmortem meat tenderization is associated with myofibrillar protein degradation. Calpain proteinases, like μ- and m-calpain, catalyze protein degradation in post-mortem muscle and improve meat tenderness, hence its inhibitor calpastatin (CAST) affects the rate and extent of tenderization (Ciobanu et al., 2011). Using crossbred pigs, Ciobanu et al. (2004) showed an association between a haplotype in the *CAST* gene and shear force (a measure of tenderness), cooking loss and juiciness. Since then, several studies have confirmed the effect of *CAST* on tenderness by regulating calpain activity (Meyers and Beever, 2008; Lindholm-Perry et al., 2009; Gandolfi et al., 2011; Nonneman et al., 2011) including the work of Ropka-Molik et al. (2014) who showed an association between polymorphisms within the 6th intron of porcine *CAST* and meat colour, pH, water holding-capacity (WHC) and texture parameters such as toughness, firmness, cohesiveness, chewiness, and resilience.

6.4. Stearoyl-CoA desaturase

The stearoyl-CoA desaturase (SCD) is the key enzyme required for the biosynthesis of MUFA (Paton and Ntambi, 2009). For this reason, polymorphisms at the *SCD* gene have been largely studied as possible candidates for fat content and fatty acid

composition. The *SCD* gene co-localizes with some detected quantitative trait loci for fatty acid composition in pigs with Duroc background (Quintanilla et al., 2011; Uemoto et al., 2011a). Findings so far support that there is genetic variation in the *SCD* gene affecting specifically MUFA content in both muscle and adipose tissue (Uemoto et al. 2011b; Maharani et al., 2013). Using Duroc barrows, Estany et al. (2014) showed that an haplotype of three SNP in the promoter of the *SCD* gene explains an important fraction of the total additive genetic variance of total MUFA content, oleic acid content, palmitoleic acid content and MUFA/SFA ratio. These authors also provided substantial evidence that the SNP *AY487830:g.2228T>C*, which localizes in the core sequence of a putative retinoic acid response element, could be the causal mutation.

6.5. Leptin receptor

The leptin receptor (LEPR), localized in the brain and pituitary of the pig, is a mediator of the satiety effect of the leptin hormone (LEP) (Barb et al., 2001). Research studies indicate that both LEP and LEPR play an essential role in food intake and energy balance. Using pig records from F_2 experimental crosses, Óvilo et al. (2005) reported an association between *LEPR* alleles and backfat traits and suggested that differences in appetite could explain the results. Consecutive studies have corroborated the effect of *LEPR* polymorphisms on production traits like voluntary feed intake, body and carcass weight and intramuscular fat content (Rodriguez et al., 2010; Uemoto et al., 2012; Hirose et al., 2014; Gol et al., 2015). Recently, a genome-wide association study confirmed *SCD* and *LEPR* as the two main loci influencing IMF and fatty acid composition in the Spanish Duroc population analyzed in this thesis (Ros-Freixedes et al., 2016).

7. Nutrigenetic regulation of adipogenesis by vitamin A

Vitamin A (retinol) is ingested in the diet as retinyl esters and as provitamin A carotenoids, of which β-carotene is the most active. After ingestion and uptake by intestinal mucosal cells, intestinal enzymes are involved in several processes (**Figure 4**): hydrolysis of retinyl esters; re-esterification of retinol in enterocytes before lymphatic transport; oxidative cleavage of carotenoids to yield retinal; reduction of retinal to retinol; and, oxidation of retinal to retinoic acid. Retinol is then converted back to retinyl esters for storage mainly in the liver (Marill et al., 2003). Retinoic acid (RA) affects adipogenesis by

promoting adipogenic commitment and hampering terminal differentiation of adipocytes, which is expected would promote hyperplasia in preadipocytes and reduce hypertrophy and lipid accumulation in mature adipocytes. RA effect on progenitor cells and mature adipocytes differ due to stage-specific expression of several transcription factors (Wang et al., 2016). RA acts as a ligand for transcriptional factors associated with the RA response elements (RAREs) located in the promoters of RA-responsive genes. The most studied transcriptional factors bound to RAREs are RA receptors (RARs) and retinoid X receptors (RXRs) (Zhang et al., 2015). It is noteworthy that RA seems to regulate the expression of a key gene for endogenous lipogenesis, the stearoyl-CoA desaturase, in part through the interaction with the previously described transcription factors (Mauvoisin and Mounier, 2011).

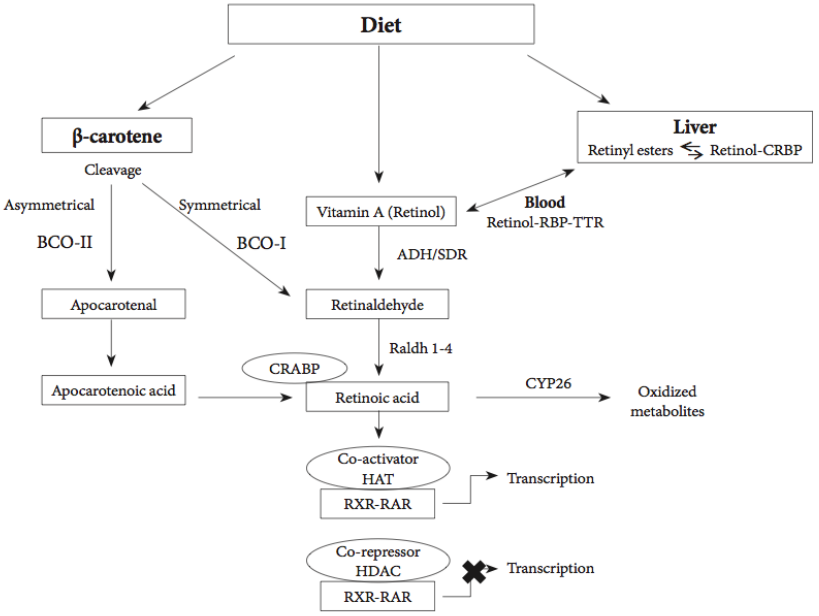

Figure 4. The retinoid metabolic pathway. CRBP, cellular retinol binding protein; RBP, retinol binding protein; TTR, transthyretin; BCO-I, β,β-carotene-15,15′-monooxygenase; BCO-II, β,β-carotene-9′,10′-dioxygenase; CRABP, cellular retinoic acid binding protein; ADH, alcohol dehydrogenase; SDR, short-chain dehydrogenases/reductases; Raldh, retinaldehyde dehydrogenase; CYP26, cytochrome P450 family 26; HAT, histone acetyltransferase; RXR, retinoid X receptor; RAR, retinoic acid receptor; HDAC, histone deacetylase (Rhee and Plutzky, 2012).

The discovery of the important role of retinoic acid on adipogenesis revealed an opportunity to investigate if the manipulation of dietary vitamin A affects meat fat content and composition of livestock species. In Japan, where marbling scores are an important measure of beef quality, Oka et al. (1998) showed a marbling increase in Japanese Black cattle subjected to dietary vitamin A restriction; however, the effect was age-dependent: significant when the treatment started after 15 months but not after 23 months. In Australian Angus, Kruk et al. (2008) found that vitamin A restriction affected favorably IMF without influencing subcutaneous fat and production and meat quality traits. In line with these results, Adachi et al. (1999) found lower serum vitamin A in steers with high marbling as compared to those with low marbling. Researchers in the US found that the effect of vitamin A restriction on IMF seems to differ between beef and dairy cattle. Thus, IMF content of Angus-cross steers was increased by low vitamin A diets after 145 days of withdrawal (Gorocica-Buenfil et al., 2007a), whereas Holstein animals needed almost 100 more days to exhibit a similar effect (Gorocica-Buenfil et al., 2007b). The studies of Siebert et al. (2006) and Gorocica-Buenfil et al. (2008) suggested an effect of vitamin A restriction on the fatty acid profile through an increase in stearoyl-CoA desaturase activity and, consequently, on the MUFA/SFA ratio. Recently, nutrigenetic interactions among dietary vitamin A and genetic polymorphisms have been reported. Accordingly, Ward et al. (2012) found a significant interaction between a SNP in the alcohol dehydrogenase 1C gene (*ADH1C)* and vitamin A supplementation/restriction for IMF.

In the past years several authors have also explored the role of vitamin A on pig's fat deposition. In crossbred animals, Olivares et al. (2009a) and Tous et al. (2014) did not find an effect of different restriction levels of dietary vitamin A on IMF content. A subsequent study of Olivares et al. (2009b) showed an improvement of IMF as a response to vitamin A supplementation in Duroc-sired pigs but not in Landrace × Large White-sired animals, suggesting a role of the pig's genetic background in the treatment response. Contrarily to the previous reports and in agreement with results in beef cattle, Olivares et al. (2011) found an increase of IMF in pigs fed low vitamin A diets. Different experiments with commercial crosses showed modifications of the fatty acid profile of subcutaneous fat and liver due to vitamin A withdrawal but not of muscle (Olivares et al., 2009a,b; Olivares et al., 2011). A wide study on Iberian barrows reported an increase in IMF and MUFA in muscle and subcutaneous fat, as well as intramuscular preadipocyte number, in animals

under restriction of vitamin A (Ayuso et al., 2015ab). However, the effect differed between muscles. Interestingly, changes in the fatty acid profile of subcutaneous fat were coupled with *SCD* gene upregulation. Production and carcass traits were not affected by vitamin A restriction in any of the previously described reports. All in all, research to date shows that further studies are needed in order to elucidate specific strategies for improving fat content and composition through dietary vitamin A manipulation, which should take into account time and duration of the restriction, doses, pig's genetic background, target muscle/tissue, as well as the possible interaction with genetic markers that could be segregating in the population of interest.

II. OBJECTIVES

The objectives of the present thesis are part of a line of research aimed at improving pork quality and, in particular, to find out genetic markers associated to intramuscular fat content and fatty acid composition, notably to oleic acid content. In the framework of this research, Estany et al. (2014) identified a haplotype variant in the stearoyl-CoA desaturase (*SCD*) gene promoter, which localizes in the core sequence of a putative retinoic acid response element, that substantially enhances fatty acid desaturation in Duroc pigs. The impact of this polymorphism needs to be assessed under different production and commercial scenarios including traditional dry-cured hams. Therefore, the following specific objectives were established:

1. To examine whether the effects of the *SCD* and *LEPR* polymorphisms on fat content and composition are maintained at different market carcass weights.

2. To determine whether the effect of the *SCD* polymorphism on fat desaturation, monounsaturated fatty acid, and oleic acid content is still evident in commercial (a) purebred Duroc and (b) Duroc-sired Iberian dry-cured hams.

3. To investigate the impact of carotenoid intake as a source of dietary retinoic acid on fat content and fatty acid composition in pigs from opposite genotypes at the *SCD* gene polymorphism.

III. EXPERIMENTAL SAMPLES AND PROCEDURES

Animals and pork samples

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida (Agreement 2/01, March 2001) and all animal procedures and care performed in accordance with authorization AE2374 issued by the Catalan Ministry of Agriculture, Livestock, and Fisheries, Spain.

Animals, meat and ham samples used in chapters IV, V and VII belong to a purebred Duroc line (Selección Batallé, Riudarenes, Girona, Spain) closed in 1991 and selected for an index including body weight, backfat thickness and intramuscular fat content with the primarily objective of producing premium pork and high quality dry-cured hams. The dry-cured ham slices of '*Jamón Ibérico de Cebo'* analyzed on chapter VI were randomly purchased in different franchised stores of the same supermarket chain in the city of Lleida (Catalonia, Spain). The supplier chosen produces Iberian dry-cured hams using crossbred Duroc × Iberian gilts or barrows from their own nucleus herds, farms, and manufacturing facilities, which facilitates the hams traceability.

Determination of fat content and composition

A representative aliquot from a pulverized freeze-dried sample was used to determine, in duplicate, the individual FA by gas chromatography (Bosch et al., 2009). In brief, fatty acids methyl esters were directly obtained by transesterification using a solution of 20% boron trifluoride in methanol (Rule, 1997). Methyl esters were determined by gas chromatography using a capillary column SP2330 (30 m × 0.25 mm; Supelco, Bellefonte, PA, USA) and a flame ionization detector with helium as carrier gas. Runs were made with a constant column-head pressure of 172 kPa. The oven temperature program increased from 150 to 225ºC at 7ºC/min and injector and detector temperatures were both 250ºC. The quantification was carried out through area normalization with an external mixture of fatty acids methyl esters (Supelco® 37 Component FAME Mix. Sigma, Tres Cantos, Madrid, Spain). The internal standard was 1,2,3-tripentadecanoylglycerol (Tripentadecanoin. Sigma, Tres Cantos, Madrid, Spain). Then, the fatty acid composition was expressed as the percentage of each individual fatty acid relative to total fatty acids. The complete profile for each sample included saturated (SFA: C14:0; C16:0; C18:0; and C20:0); monounsaturated (MUFA: C16:1n-9; C18:1n-7; C18:1n-9; and C20:1n-9); and polyunsaturated (PUFA:

C18:2n-6; C18:3n-3; C20:2n-6; and C20:4n-6) fatty acids. The identification and quantification of the C18:1n-7 isomer was made by using a commercial methyl ester mixture (FAME Column Evaluation Mix. Sigma, Tres Cantos, Madrid, Spain) and was confirmed by mass spectrometry. The fat content was calculated as the sum of the individual fatty acids expressed as triglyceride equivalents (AOAC, 2000) on a dry tissue basis.

Isolation of genomic DNA and genotyping

The isolation of genomic DNA was carried out from muscle samples stored at -80ºC or refrigerated hams. Samples were lysed in the presence of proteinase K and DNA was purified through extraction with phenol:chloroform, followed by ethanol precipitation. Finally, DNA was re-suspended and stored in TE buffer. The quantification and estimation of the quality and purity of genomic DNA was performed using a Nanodrop N-1000 spectrophotometer, considering an A260/A280 ratio of 1.8-2.2 as acceptable. DNA integrity was tested through electrophoresis in a 1% agarose gel.

Pigs were genotyped for the *LEPR NM_001024587:g.1987C>T* and/or the *SCD AY487830:g.2228T>C* SNP, which serve as tag SNP for capturing the variance associated to *LEPR* and *SCD* genes, respectively. The *LEPR NM_001024587:g.1987C>T* SNP at exon 14 (Óvilo et al., 2005) was genotyped by High Resolution Melt analysis (Luminaris Color HRM Master Mix, Thermo Scientific) in a real time thermocycler (CFX-100, Bio-Rad) using 10 ng of genomic DNA and 0.4 µM of each primer **(Table 3)**. The *SCD AY487830:g.2228T>C* SNP was genotyped using an allelic discrimination assay in a reaction mix that contained 1x Universal TaqMan master mix (LifeTechnologies, Grand Island, NY, USA), 0.9 µM Primer mix, 0.2 µM Probe mix and 10 ng of DNA in a final volume of 5 µl.

The sex of the hams was determined using a modified protocol of Sembon et al. (2008) based on the amplification of the amelogenin (*AMEL*) gene. Using published sequences for the porcine amelogenin genes (EMBL/GenBank accession numbers, AB091791 [*AMELX*] and AB091792 [*AMELY*]), a set of PCR primers **(Table 3)** were designed to amplify a portion of intron 2 that was expected to yield PCR products of different sizes between AMELX (450 bp) and AMELY (278 bp). The PCR reaction mix

contained 1x buffer, 2.66 mM of $MgCl_2$, 0.13 mM dNTPs, 0.4 µM of each primer, 0.4 U of Taq DNA polymerase (Biotools, Madrid, Spain) and 40 ng of genomic DNA in a final volume of 15 µl. The amplification was carried out in a Veriti thermocycler (LifeTechnologies) with the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of 96°C for 20 s, 54°C for 30 s and 72°C for 40 s and a final extension step of 72ºC x 5 min. PCR products were run in 1.5% agarose gels and visualized by ethidium bromide staining under UV illumination.

Table 3. Primers used for the amplification of the *AMEL* gene and the genotyping of the single nucleotide polymorphisms in the porcine *SCD* gene promoter (AY487830:*g.2228T>C*) and exon 14 of *LEPR* (NM_001024587:*g.1987C>T*)

Gene/SNP	Primer name	Sequence 5' → 3'
AMEL gene	Primer Forward	TCATGAGGAATCTCTTTGGTA
	Primer Reverse	CCAGAGGTTGTAACCTTACAG
AY487830:*g.2228T>C*	Primer Forward	CCCTTCTTGGCAGCGAATAAAA
	Primer Reverse	CAGGCTGGGTATTTAAAGGCTAGAG
	Probe for C allele	VIC-CGACCGTGTCCTGTATT-NFQ
	Probe for T allele	FAM-CGACCGTATCCTGTATT-NFQ
NM_001024587:*g.1987C>T*	Primer Forward	CAGAGGACCTGAATTTTGGAG
	Primer Reverse	CATAAAAATCAGAAATACCTTCCAG

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CHAPTER IV

The effect of *SCD* and *LEPR* genetic polymorphisms on fat content and composition is maintained throughout fattening in Duroc pigs

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ABSTRACT

The effects of the stearoyl-CoA desaturase (*SCD; AY487830:g.2228T>C*) and leptin receptor (*LEPR; NM_001024587:g.1987C>T*) polymorphisms on fat content and fatty acid (FA) composition were investigated throughout fattening. Samples of *Longissimus thoracis* (LT) and subcutaneous fat (SF) from 214 Duroc barrows were collected from 160 days to slaughter age (220 days) using a longitudinal design. Results indicated that the positive effect of the T allele at the *SCD* gene on monounsaturated FA and of the T allele at the *LEPR* gene on saturated FA are maintained throughout the growing-finishing period, both in LT and SF. In *LEPR*, however, compositional changes, particularly in SF, are a result of increased fatness. There is very limited evidence of genotype by age interaction, and thus it is concluded that the combined selection for the *SCD* T and *LEPR* C alleles is a good strategy to increase the MUFA/SFA ratio regardless of the age at slaughter.

Key words: age; fatty acids; genetic marker; intramuscular fat; meat quality; pork.

1. Introduction

The pig industry mostly relates carcass quality to lean content and conformation. However, there is a constant increase of consumers who attach greater importance to pork quality. Meat quality is not straightforward to define (Wood et al., 2004) and depends on a number of meat attributes. Among them, intramuscular fat (IMF) content has a beneficial impact on tenderness, texture, taste and flavour intensity of pork, particularly for premium fresh pork niches and dry-cured products (Fernandez, Monin, Talmant, Mourot, & Lebret, 1999; Fortin, Robertson, & Tong, 2005; Jeleníková, Pipek, & Miyahara, 2008). Recently, mainly due to health promotion policies, the fatty acid (FA) composition has also entered as a new feature for pork quality. A dietary substitution of saturated fatty acids (SFA) for monounsaturated fatty acids (MUFA) may not only be beneficial against dyslipidemias (Gillingham, Harris-Janz, & Jones, 2011; Roche, 2001) but may also improve organoleptic properties and overall acceptability of pork (Cameron et al., 2000; Cameron & Enser, 1991; Tikk et al., 2007).

Due to the importance of fat content and composition for the meat industry, genes involved in lipid metabolism have been an important target of research in animal breeding. The leptin receptor (*LEPR*) and the stearoyl-CoA desaturase (*SCD*) are two of these genes. *LEPR*, as a mediator of the satiety effect of the leptin hormone, influences overall fatness (Houseknecht, Baile, Matteri, & Spurlock, 1998), while *SCD*, the rate-limiting enzyme

required for the biosynthesis of MUFA from SFA, affects fatty acid composition (Ntambi & Miyazaki, 2004). In pigs, a non-synonymous exonic polymorphism in the *LEPR* gene has been reported to be strongly associated with fatness in an Iberian × Landrace (Óvilo et al., 2005) and in Duroc × Landrace/Large White (Galve et al., 2012) crossbreds. Similarly, a polymorphism has been reported in the promoter region of the *SCD* gene affecting MUFA content in both IMF and subcutaneous fat (SF) of purebred and crossbred Duroc animals (Estany, Ros-Freixedes, Tor, & Pena, 2014; Henriquez-Rodriguez, Tor, Pena, & Estany, 2015). A recent genome-wide association study confirmed *SCD* and *LEPR* as the two main loci influencing IMF and FA composition in Duroc (Ros-Freixedes et al., 2016).

In a previous work, Bosch, Tor, Reixach, & Estany (2012) estimated the evolution of fat content and composition in both IMF and SF throughout the growing–finishing period in pigs from a Duroc line used for high-quality production. These authors showed that the age-related increase of IMF and SF is associated to modifications in the fatty acid profile, with major changes occurring in MUFA and PUFA. Therefore, the objective of this paper was to examine whether the effects of the *SCD* and *LEPR* polymorphisms on fat content and composition affect each other and/or change with age.

2. Material and methods

2.1. Animals and experimental procedures

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida (Agreement 2/01, March 2001) and all animal procedures and care performed in accordance with authorization AE2374 issued by the Catalan Ministry of Agriculture, Livestock, and Fisheries, Spain.

A total of 214 purebred barrows from a Duroc line (Selección Batallé, Riudarenes, Girona, Spain) were used for this research (Bosch et al. 2012). The line was closed in 1991 and since then it has been selected for an index including body weight, backfat thickness and intramuscular fat content with the primarily objective of producing premium pork and high quality dry-cured hams (Solanes et al., 2009). Pigs were produced by 102 sows and 36 boars and raised up to slaughter in three separate batches in a commercial farm. They were allocated in pens of 12 individuals and were given *ad libitum* access to feed. A pelleted growing and finishing diet were given from 110 to 160 days and from 160 to 220 days,

respectively (**Table 1**). Pigs used in the experiment were subjected to repeated sampling for muscle and subcutaneous fat (SF) specimens throughout the finishing period. A biopsy of m. *Longissimus thoracis* (LT) and of SF was taken in 191 pigs at around 185 days (183, SD 4.3). Additionally, samples of both tissues were also taken at 160 days (158.0, SD 6.9; n=81) and at 210 days (207.9, SD 3.0; n=60). Before taking biopsies, the live body weight was measured and backfat thickness (BT) and loin-muscle thickness at 5 cm of the midline between the third and fourth last ribs ultrasonically recorded using the portable equipment Piglog 105 (SFK-Technology, Herlev, Denmark). Biopsies were taken 5 cm deep at the same location where BT was measured and were extracted using 8-mm cannula inserted into spring-loaded biopsy device (PPB-U Biotech, Nitra, Slovakia) as described in Oksbjerg, Henckel, Andersen, Pedersen, & Nielsen (2004). All the necessary measures were taken to prevent animal discomfort during and after the process (Bosch, Tor, Villalba, Puigvert, & Estany, 2003). Muscle and fat samples were trimmed from skin and separately frozen in liquid nitrogen until analysis 1 to 5 months later. Pigs were slaughtered at 220 days (222, SD 3.8) in a commercial slaughterhouse equipped with a carbon dioxide stunning system (Butina ApS, Holbaek, Denmark), where BT and loin-muscle thickness at 6 cm off the midline between the third and fourth last ribs were measured using the Autofom automatic carcass grading (SFK-Technology, Herlev, Denmark). After slaughter, the carcass weight and the carcass length were measured. The carcass length was measured from the anterior edge of the symphysis pubic to the recess of the first rib. The carcass lean percentage was estimated on the basis of 35 measurements of AutoFOM points by using the official approved equation (decision 2001/775/CE, 2001) and the lean weight from carcass weight and lean percentage. After chilling for about 24 h at 2°C, each carcass was divided into primal cuts and the left side ham was weighed. Each ham was trimmed according to customary procedure used for manufacturing traditional dry-cured Spanish ham. Immediately after quartering, a sample of m. *Gluteus medius* from the left side ham was taken. In around 30 pigs per batch a sample of LT and SF at the level of the third and fourth ribs was also collected. These samples were immediately vacuum packaged and stored at –20°C until required for IMF and FA determinations.

2.2. Determination of IMF content and fatty acid composition

Frozen samples were removed from the nitrogen tank or the freezer 12 h prior to laboratory analyses. Biopsy specimens were directly freeze-dried and thereafter thoroughly homogenized by mixing with sand using a glass stirring rod. Due to their small size, dry matter in these samples was calculated as the weight difference before and after freeze-drying, and then the whole sample used for subsequent analyses. Post-mortem samples of LT and m. *Gluteus medius* were completely defrosted, vacuum drip losses were eliminated and muscle and subcutaneous fat were dissected out separately. Once minced, a small quantity of each was used to determine dry matter by drying 24 h at 100 to 102 °C in air oven whereas the rest of the sample was freeze-dried and pulverized using an electric grinder. A representative aliquot from the pulverized freeze-dried specimens was used for chemical analyses.

IMF content was estimated by quantitative determination of the fatty acids by gas chromatography following the methodology described in Bosch, Tor, Reixach, & Estany (2009). Fatty acid methyl esters of both IMF and SF were directly obtained by transesterification using a solution of boron trifluoride 20% in methanol (Rule, 1997). Analysis of fatty acid methyl esters were performed by gas chromatography with a capillary column SP2330 (Supelco, Tres Cantos, Madrid) and a flame ionization detector with helium as the carrier gas at 1 mL/min. The oven temperature program increased from 150 to 225 °C at 7 °C per min, and the injector and detector temperatures were both 250 °C (Tor, Estany, Francesch, & Cubiló, 2005). The quantification was carried out through area normalization by adding into each sample 1, 2, 3-Tripentadecanoylglycerol as internal standard before transesterification. IMF was calculated as the sum of each individual fatty acid expressed as triglyceride equivalents (AOAC, 2000) on a dry tissue basis. IMF and SF fatty acid composition was calculated as the percentage of each individual fatty acid relative to total fatty acids, and expressed as mg/g fatty acid. The proportion of SFA (C14:0; C16:0; C18:0 and C20:0), MUFA (C16:1n−9; C18:1 and C20:1n−9) and PUFA (C18:2n−6; C18:3n−3; C20:2n−6 and C20:4n−6) fatty acid contents were calculated.

Table 1. Composition of the diets (g/kg)

Item	Growing	Finishing
Dry matter	893.2	886.1
Crude lipid	56.3	61
Crude protein	193.6	181.2
Ash	60.4	69.8
Crude fiber	57.1	61.7
Nitrogen free extract	525.8	512.4
ME, MJ/kg	13.4	12.7
Fatty acids, mg/g fatty acid [A]		
C12:0, lauric	4.8	3.2
C14:0, myristic	18.3	16.2
C16:0, palmitic	220.8	229.8
C18:0, stearic	77.4	81.7
SFA	321.3	330.9
C16:1n−9, palmitoleic	22.6	23.6
C18:1n−9, oleic	301.1	294.7
C20:1n−9, eicosenoic	3.7	6.8
MUFA	327.4	325.1
C18:2n−6, linoleic	327.7	311.7
C18:3n−3, linolenic	15.5	19.7
C20:2n−6, eicosadienoic	2.8	3.1
C20:4n−6, arachidonic	1.1	1.3
PUFA	347.1	353.8

[A] SFA, saturated fatty acids (C12:0+C14:0+C16:0+C18:0; MUFA, monounsaturated fatty acids (C16:1n-9+C18:1n-9+C20:1n-9); PUFA, polyunsaturated fatty acids (C18:2n-6+C18:3n-3+C20:2n-6+C20:4n-6).

2.3. Isolation of genomic DNA and genotyping

The isolation of genomic DNA was carried out from muscle samples stored at -80ºC. Samples were lysed in the presence of proteinase K and DNA was purified through extraction with phenol: chloroform, followed by ethanol precipitation. Finally, DNA was re-suspended and stored in TE buffer. The quantification and estimation of the quality and purity of genomic DNA was performed using a Nanodrop N-1000 spectrophotometer; DNA integrity was tested through electrophoresis in a 1% agarose gel.

All pigs were genotyped for the *LEPR NM_001024587:g.1987C>T* and the *SCD AY487830:g.2228T>C* single nucleotide polymorphisms (SNP), which serve as tag SNPs for capturing the variance associated to *LEPR* and *SCD* genes, respectively. The *LEPR NM_001024587:g.1987C>T* SNP at exon 14 (Óvilo et al., 2005) was genotyped by High Resolution Melt analysis (Luminaris Color HRM Master Mix, Thermo Scientific) in a real time thermocycler (CFX-100, Bio-Rad) using 10 ng of genomic DNA and 0.4 µM of each of the following primers: LEPR-F, 5´-CAGAGGACCTGAATTTTGGAG-3´; LEPR-R, 5´-CATAAAAATCAGAAATACCTTCCAG-3´. The *SCD* AY487830:g.2228T>C SNP was

genotyped using an allelic discrimination assay with the primers and probes indicated in Estany et al. (2014). The reaction mix contained 1x Universal TaqMan master mix (LifeTechnologies, Grand Island, NY), 0.2 µM Primer mix, 0.8 µM Probe mix and 10 ng of DNA in a final volume of 5 µl.

2.4. Statistical analyses

The effect of the *SCD* and *LEPR* genotypes by age on body weight, BT, loin-muscle thickness, IMF and FA of LT and SF were estimated on data from biopsies taken at 160, 185, and 210 days of age using a linear mixed model which included the batch (3 levels), the age at measurement (160, 185, and 210 days), the *SCD* genotype (TT, CT and CC), the *LEPR* genotype (CC, CT and TT) and the interaction of genotype by age at measurement as fixed effects and the pig and the residual as random effects. Moreover, data from either biopsies or carcass, were also analyzed independently at each age using a fixed model with the effects of the batch, the *SCD* and *LEPR* genotypes and age, this latter considered here as a deviation from the target age in each time-point (160, 185, 210, and at slaughter at 220 days). As in Bosch et al. (2009), in both approaches the potential bias due to the biopsy size on IMF and FA composition was corrected including in the model for these traits a quadratic polynomial on sample weight. The interaction between genotypes was tested including in the model the corresponding term. The effect of the genotypes was tested following an F-test and multiple pairwise comparisons were done using the Tukey test. All the analyses were performed using the statistical package JMP 8 (SAS Institute Inc., Cary, NC).

3. Results

The average effects of the *SCD* and *LEPR* genotypes on body weight, BT, loin-muscle thickness, IMF and FA composition in both muscle and subcutaneous fat during the finishing period are given in **Tables 2 and 3,** respectively. The effect of both genotypes on LT and SF was consistent across tissues and throughout the finishing period. Thus, pigs carrying the T allele at *SCD* increased MUFA content (452.0 mg /g FA, for TT, and 428.9 mg/g FA, for CC, $P<0.05$, in LT; and 414.3 mg/g FA, for TT, and 400.7 mg/g FA for CC, $P<0.05$, in SF) while the T allele at *LEPR* increased SFA (423.9 mg/g FA, for TT, and 409.8 mg/g FA, for CC, $P<0.05$, in LT; and 420.6 mg/g FA, for TT, and 410.6 mg/g FA, in

SF). In general, the effect of the *SCD* genotypes on the FA profile was greater than for *LEPR* genotypes and in LT than in SF. The T allele at *LEPR* also increased BT (20.9 mm, for TT, and 19.4, for CC, $P<0.05$). The *SCD* genotype did not affect neither BT nor IMF. A significant interaction of *SCD* with age was observed for BT and PUFA, both in LT and SF, and of *LEPR* with age for SFA in LT ($P<0.05$).

Table 2. Least square means (±SE) for production traits, intramuscular fat content (IMF) and fatty acid composition in m. Longissimus thoracis and subcutaneous fat by *SCD* genotype during the finishing period (from 160 to 210 days of age) and interaction of the *SCD* genotype with age [A]

	m. *Longissimus thoracis*				Subcutaneous fat			
	TT	CT	CC	*SCD**age	TT	CT	CC	*SCD**age
No of data	51	164	99		51	159	98	
Body weight, kg	111.2±1.7	113.0±0.9	115.3±1.2					
Backfat thickness, mm	19.2±0.6	20.3±0.3	19.8±0.4	*				
Loin thickness, mm	43.2±0.6	44.1±0.3	44.8±0.4					
IMF, % DM	16.8±0.9	17.4±0.5	16.8±0.7					
C14:0	14.0±0.8	13.8±0.5	13.1±0.6		17.6±0.4	16.6±0.2	17.0±0.3	
C16:0	255.5±2.2	258.1±1.2	257.8±1.6		246.9±2.4^{ab}	246.0±1.3^{b}	251.8±1.8^{a}	
C18:0	133.8±1.8^{c}	141.7±1.0^{b}	148.8±1.3^{a}		139.5±2.4^{b}	143.9±1.3^{b}	153.8±1.7^{a}	
C20:0	1.7±0.1	1.76±0.1	1.79±0.1		1.7±0.2	1.8±0.1	1.8±0.1	
SFA, mg/g FA	404.6±3.7^{b}	415.2±2.0^{a}	421.3±2.7^{a}		406.0±4.2^{b}	408.2±2.3^{b}	424.3±3.2^{a}	
C16:1n-9	34.0±1.1^{a}	32.1±0.6^{a}	28.8±0.8^{b}		22.4±1.1^{a}	20.8±0.6^{ab}	18.4±0.8^{b}	
C18:1	408.7±3.2^{a}	402.5±1.7^{a}	391.6±2.3^{b}		380.3±3.0^{ab}	378.7±1.6^{a}	372.0±2.2^{b}	
C20:1n-9	9.2±0.2^{a}	8.59±0.1^{b}	8.42±0.2^{b}		11.3±0.3	10.8±0.2	10.5±0.2	*
MUFA, mg/g FA	452.0±3.5^{a}	443.2±1.9^{a}	428.9±2.6^{b}		414.3±3.1^{a}	410.4±1.7^{a}	400.7±2.3^{b}	
C18:2n-6	123.2±3.6	122.3±2.0	129.9±2.7	*	158.2±3.0	157.5±1.7	153.7±2.2	*
C18:3n-3	7.7±0.2	7.46±0.1	7.76±0.2		11.9±0.5^{ab}	12.5±0.3^{a}	11.2±0.4^{b}	
C20:2n-6	5.8±0.2	5.66±0.1	5.80±0.1		8.5±0.4	8.5±0.2	7.9±0.3	
C20:4n-6	6.1±0.4	5.9±0.2	6.34±0.3		2.4±0.1	2.5±0.1	2.3±0.1	
PUFA, mg/g FA	142.9±4.1	141.5±2.3	149.9±3.0	*	181.5±3.5	181,1±1.9	175.1±2.6	*
C18:1/C18:0	3.18±0.05^{a}	2.86±0.03b	2.65±0.04^{c}		2.74±0.05^{a}	2.66±0.03^{a}	2.45±0.04^{b}	
C16:1n-9/C16:0	0.13±0.00^{a}	0.12±0.00^{a}	0.11±0.00^{b}		0.09±0.00^{a}	0.09±0.00^{a}	0.07±0.00^{b}	
MUFA/SFA	1.12±0.01^{a}	1.07±0.01^{b}	1.02±0.01^{c}		1.02±0.02^{a}	1.01±0.01^{a}	0.95±0.01^{b}	
MUFA/PUFA	3.29±0.10^{ab}	3.24±0.05^{a}	3.04±0.07^{b}		2.33±0.05	2.31±0.03	2.34±0.04	*
SFA/PUFA	2.95±0.10	3.04±0.05	2.97±0.07		2.28±0.07^{b}	2.29±0.04^{b}	2.48±0.05^{a}	

[A] SFA: C14:0+C16:0+C18:0+C20:0; MUFA: C16:1n-9+C18:1+C20:1n-9; PUFA: C18:2n-6+C18:3n-3+C20:2n-6+C20:4n-6; C18:1:C18:1n-9+C18:1n-7; * Interaction between *SCD* genotype and age significant at p<0.05; * a.b.c Within row and factor, means with different superscripts differ significantly (P<0.05).

Table 3. Least square means (±SE) for production traits, intramuscular fat content (IMF) and fatty acid composition in m. Longissimus thoracis and subcutaneous fat by *LEPR* genotype during the finishing period (from 160 to 210 days of age) and interaction of the *LEPR* genotype with age [A]

	m. *Longissimus thoracis*				Subcutaneous fat			
	CC	CT	TT	LEPR*age	CC	CT	TT	LEPR*age
No of data	82	156	76		80	153	75	
Body weight, kg	110.8±1.4	113.5±1.0	115.1±1.4					
Backfat thickness, mm	19.4±0.5^{b}	19.0±0.3^{b}	20.9±0.5^{a}					
Loin thickness, mm	43.7±0.5	44.8±0.3	43.6±0.5					
IMF, % DM	16.2±0.7	16.5±0.5	18.3±0.7					
C14:0	13.6±0.7^{ab}	12.6±0.5^{b}	14.8±0.7^{a}		17.2±0.4	16.9±0.2	17.0±0,4	
C16:0	256.5±1.8^{ab}	253.0±1.3^{b}	261.8±1.8^{a}	*	246.7±2.0^{ab}	245.4±1.4^{b}	252.6±2.0^{a}	
C18:0	138.32±1.52^{b}	140.1±1.0^{b}	145.9±1.5^{a}		144.4±2.0	143.8±1.3	149.0±2.0	
C20:0	1.6±0.1	1.8±0.1	1.8±0.1		1.9±0.1	1.7±0.1	1.7±0.1	
SFA, mg/g FA	409.8±3.0^{b}	407.4±2.1^{b}	423.9±3.0^{a}	*	410.6±3.5^{ab}	407.4±2.4^{b}	420.6±3.5^{a}	
C16:1n-9	32.2±0.9	31.8±0.6	30.8±0.9		21.0±0.9	21.0±0.6	19.5±0.9	
C18:1	404.4±2.6	402.2±1.8	396.2±2.6		378.5±2.5	379.3±1.7	373.2±2.4	
C20:1n-9	8.6±0.2	8.7±0.1	8.9±0.2		10.7±0.3	10.7±0.2	11.1±0.3	
MUFA, mg/g FA	445.4±2.9^{a}	442.8±2.0^{ab}	435.9±2.9^{b}		410.5±2.6	410.9±1.8	403.9±2.6	
C18:2n-6	124.9±3.0	129.5±2.1	121.1±3.0		156.3±2.5	159.0±1.7	154.2±2.5	
C18:3n-3	7.6±0.2	7.8±0.1	7.4±0.2		11.9±0.4	11.9±0.3	11.8±0.4	
C20:2n-6	5.8±0.1	5.9±0.1	5.6±0.1		8.5±0.4	8.1±0.2	8.3±0.3	
C20:4n-6	6.4±0.4	6.4±0.2	5.5±0.4		2.5±0.1^{ab}	2.6±0.1^{a}	2.2±0.1^{b}	
PUFA, mg/g FA	144.9±3.4^{ab}	149.8±2.3^{a}	139.6±3.4^{b}		179.7±2.9	181.6±2.0	176.3±2.9	
C18:1/C18:0	2.96±0.04^{a}	2.90±0.03^{a}	2.74±0.04^{b}		2.65±0.04^{ab}	2.78±0.03^{a}	2.52±0.04^{b}	
C16:1n-9/C16:0	0.13±0.00	0.13±0.00	0.12±0.00		0.09±0.00	0.09±0.00	0.08±0.00	
MUFA/SFA	1.09±0.01^{a}	1.09±0.01^{a}	1.03±0.01^{b}		1.01±0.01^{ab}	1.02±0.01^{a}	0.96±0.01^{b}	
MUFA/PUFA	3.21±0.08	3.10±0.05	3.27±0.08		2.33±0.04	2.31±0.03	2.34±0.04	
SFA/PUFA	2.94±0.08^{ab}	2.84±0.06^{b}	3.17±0.08^{a}		2.33±0.06	2.29±0.04	2.44±0.06	

[A] See fatty acid abbreviations in Table 2; * Interaction between *LEPR* genotype and age significant at $p<0.05$; * [a.b.c] Within row and factor, means with different superscripts differ significantly ($P<0.05$).

In order to dissect out these interactions, the data were independently analyzed at each age of measurement. The effects of the *SCD* and *LEPR* genotypes on SFA, MUFA and PUFA in LT by age are depicted in **Figures 1 and 2**, respectively. As expected from previous works (Bosch et al., 2012), MUFA increased during the finishing period while PUFA decreased. Similar results were obtained for BT and for FA composition in SF and therefore they are not shown. It can be seen from these figures that the interaction between genotype and age was minor and limited to small changes in magnitude for SFA in *LEPR*. On the whole, the effect of the genotypes on SFA, MUFA, and PUFA showed the same pattern throughout the finishing period, with the T allele at *SCD* increasing MUFA and the T allele at *LEPR* increasing SFA. The joint effect of both genes is accounted for in **Figure 3** using the MUFA/SFA ratio as a target trait. Both in LT and in SF, the proportion of MUFA with respect to SFA was around 15% higher in the TTC- (TT, for *SCD*, and CC or CT, for *LEPR*) as compared to the CCTT (CC, for *SCD*, and TT, for *LEPR*) pigs (1.16 and 1.07, for TTC-, and 1.01 and 0.94, for CCTT, in LT and SF, respectively; $P<0.05$). The difference between this two extreme genotypes for BT, IMF and body weight was not significant ($P>0.05$; data not shown).

The effect of the *SCD* and *LEPR* genotypes on carcass traits, as well as on IMF content and FA composition of the *Gluteus medius* muscle, are presented in **Table 4**. In agreement with results obtained with live measurements, the most striking effects were on FA composition. Thus, pigs carrying the T allele at the *SCD* gene had higher MUFA (471.7 mg/g FA, for TT, and 456.2 mg/g FA, for CC, $P<0.05$) and pigs with the T allele at the *LEPR* gene higher SFA content (417.5 mg/g FA, for TT, and 402.4 mg/g FA, for CC, $P<0.05$). In contrast to the *SCD*-T allele, the *LEPR*-T led to higher levels of IMF (22.5% DM, for TT, and 19.5% DM, for CC, $P<0.05$). Neither of the two genotypes affected BT, loin-muscle thickness, and lean content. Evidence of synergic effects between both genes was limited, with BT and C20:2n-6 being the only traits for which the interaction between *SCD* and *LEPR* was significant ($P<0.05$).

Figure 1. Effect of the *SCD* genotype on SFA, MUFA and PUFA in m. Longissimus thoracis by age. Means with different letters within age differ significantly (P<0.05)

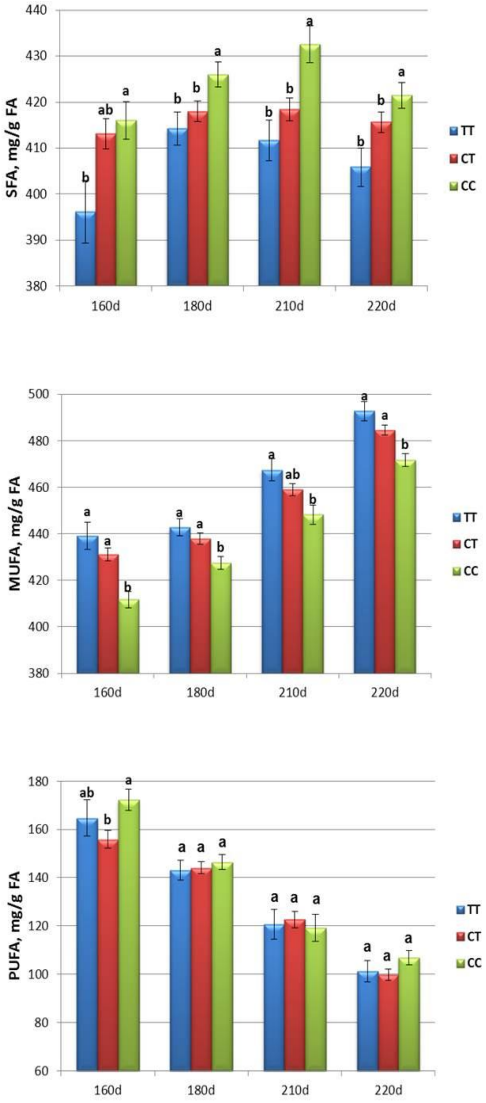


Figure 2. Effect of the *LEPR* genotype on SFA, MUFA and PUFA in m. Longissimus thoracis by age. Means with different letters within age differ significantly (P<0.05)

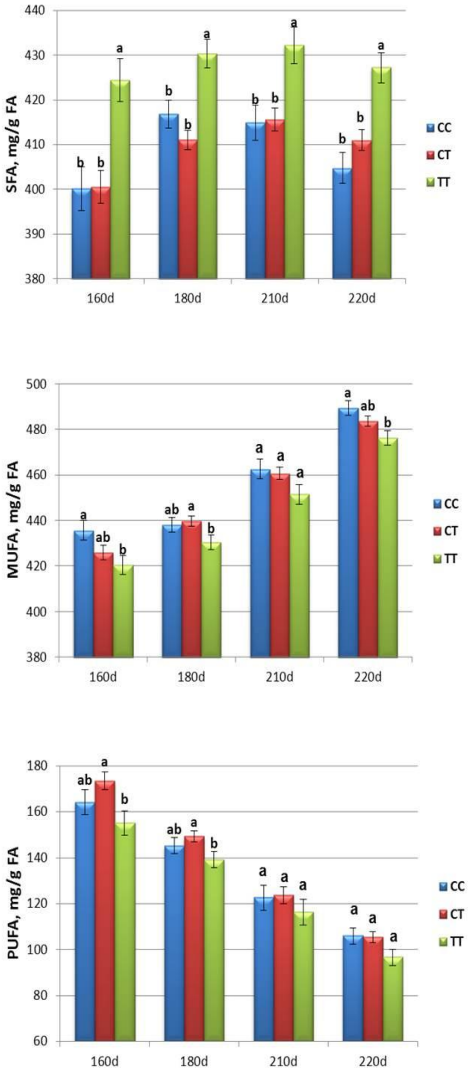
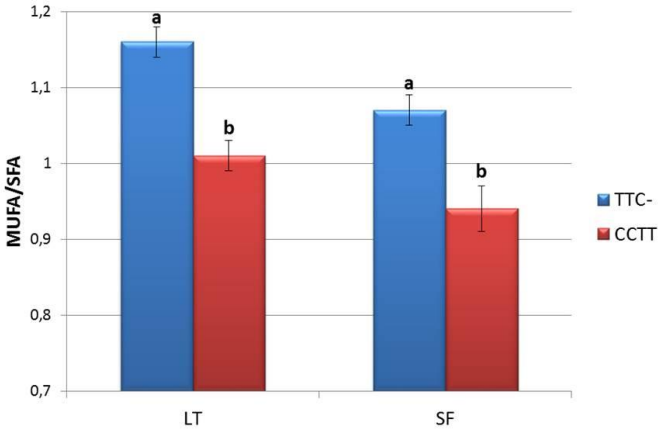


Figure 3. Least square means for the monounsaturated to saturated fatty acid ratio (MUFA/SFA) in m. Longissimus thoracis (LT) and subcutaneous fat (SF) the during finishing period (from 160 to 210 days of age) in the two extreme genotypes at *SCD* (first) and *LEPR* (second) genes. Means with different letters within tissues differ significantly (P<0.05)



4. Discussion

In this study we investigated the effects of two tag polymorphisms, one at the promoter of the *SCD* gene (*AY487830:g.2228T>C*) and another at exon 14 of the *LEPR* gene (*NM_001024587:g.1987C>T*), on fat content and composition during the growing-finishing period. In line with earlier research in Duroc pigs (Estany et al., 2014; Henriquez-Rodriguez et al., 2015), the results obtained confirmed the beneficial effect of the T allele at *SCD* gene on MUFA content and provided new evidence that the T allele at *LEPR*, which is segregating in Duroc, is positively associated with fatness and SFA content, both in muscle and SF. This is in agreement with previous findings with the *LEPR* gene in both Iberian (Muñoz et al., 2009; Óvilo et al., 2010) and Duroc-sired crossbreds (Galve et al., 2012; Muñoz et al., 2011). Also in line with previous reports (Gol et al., 2015), the allelic frequency of the T allele in this study was 0.41, for *SCD*, and 0.48, for *LEPR*, suggesting that both polymorphisms are present at intermediate frequencies in purebred Duroc. Such segregation pattern gives enough scope for using both SNPs to reduce the heterogeneity of Duroc-sired pig products.

Table 4. Least square means (±SE) for carcass traits, intramuscular fat content (IMF) and fatty acid composition in m. Gluteus medius at slaughter (220 days of age) by *SCD* and *LEPR* genotypes [A]

	SCD genotype			*LEPR* genotype			*SCD*LEPR*
	TT	CT	CC	CC	CT	TT	
No of pigs	33	110	71	58	104	50	
Carcass weight, kg	102.2±2.6	104.18±1.1	104.33±1.5	101.1±2.3	105.5±1.2	104.1±1.9	
Carcass backfat thickness, mm	24.2±0.9	24.5±0.4	23.2±0.5	24.0±0.8	23.7±0.4	24.1±0.6	*
Carcass loin thickness, mm	42.9±2.3	43.1±1.0	44.6±1.3	43.8±2.0	44.2±1.0	42.7±1.6	
Carcass length, cm	86.9±0.9	87.2±0.4	87.4±0.7	86.9±0.9	87.6±0.4	87.0±0.8	
Lean, %	42.0±1.2	41.7±0.5	43.0±0.7	42.0±1.1	42.8±0.5	42.0±0.8	
Lean weight, kg	42.8±1.4	43.3±0.6	44.5±0.8	42.3±1.2	44.9±0.6	43.4±1.0	
Ham weight, kg	12.6±0.3	13.0±0.1	13.3±0.2	12.9±0.3	13.3±0.2	12.7±0.3	
IMF, % DM	20.3±1.3	20.8±0.6	20.5±0.7	19.5±1.2^{ab}	19.6±0.6^{b}	22.5±0.9^{a}	
C14:0	16.3±1.0	15.4±0.4	14.6±0.6	15.7±0.9^{ab}	14.1±0.4^{b}	16.6±0.7^{a}	
C16:0	256.7±2.3	253.6±1.0	254.3±1.3	253.3±2.1^{b}	251.2±1.1^{b}	260.0±1.6^{a}	
C18:0	127.0±2.3^{c}	135.0±1.0^{b}	142.2±1.3^{a}	131.6±2.1^{b}	133.4±1.0^{b}	139.2±1.6^{a}	
C20:0	1.54±0.2	1.52±0.1	1.71±0.1	1.6±0.1	1.5±0.1	1.6±0.1	
SFA, mg/g FA	401.7±4.4^{ab}	405.5±1.9^{b}	412.8±2.5^{a}	402.2±4.0^{b}	400.3±2.0^{b}	417.5±3.0^{a}	
C16:1n-9	42.2±1.4^{a}	40.0±0.6^{a}	37.7±0.8^{b}	40.1±1.2	40.4±0.6	39.4±0.9	
C18:1	420.6±4.4^{ab}	419.9±1.9^{a}	410.2±2.5^{b}	418.2±4.0	417.8±2.0	414.6±3.1	
C20:1n-9	8.9±0.3	8.6±0.1	8.4±0.1	8.4±0.2^{ab}	8.4±0.1^{b}	9.0±0.2^{a}	
MUFA, mg/g FA	471.7±4.4^{a}	468.5±1.9^{a}	456.2±2.5^{b}	466.7±4.0	466.7±2.0	463.1±3.0	
C18:2n-6	108.1±3.8	107.0±1.7	111.4±2.2	111.5±3.5^{ab}	113.1±1.7^{a}	101.8±2.7^{b}	
C18:3n-3	6.2±0.3	5.9±0.1	6.1±0.1	6.3±0.2	6.1±0.1	5.8±0.2	
C20:2n-6	5.0±0.2	5.1±0.1	5.2±0.1	5.0±0.2	5.3±0.1	5.0±0.1	*
C20:4n-6	7.3±0.7	8.0±0.3	8.3±0.4	8.2±0.6^{ab}	8.5±0.3^{a}	6.8±0.5^{b}	
PUFA, mg/g FA	126.6±4.6	126.0±2.0	131.0±2.6	131.1±4.2^{ab}	133.1±2.1^{a}	119.5±3.2^{b}	
C18:1/C18:0	3.34±0.08^{a}	3.14±0.04^{b}	2.90±0.05^{c}	3.22±0.07^{a}	3.26±0.04^{a}	3.00±0.06^{b}	
C16:1n-9/C16:0	0.16±0.01^{a}	0.16±0.00^{a}	0.15±0.00^{b}	0.16±0.00	0.16±0.00	0.15±0.00	
MUFA/SFA	1.18±0.02^{a}	1.16±0.01^{a}	1.11±0.01^{b}	1.16±0.02^{a}	1.17±0.01^{a}	1.11±0.01^{b}	
MUFA/PUFA	3.81±0.13	3.8±0.06	3.61±0.08	3.65±0.12^{ab}	3.61±0.06^{b}	3.95±0.09^{a}	
SFA/PUFA	3.26±0.12	3.3±0.05	3.26±0.07	3.15±0.11^{b}	3.11±0.06^{b}	3.56±0.09^{a}	

[A] See fatty acid abbreviations in Table 2; * Interaction between *SCD* ad *LEPR* genotypes significant at $p<0.05$; [a.b.c] Within row and factor, means with different superscripts differ significantly ($P<0.05$)

The polymorphism at the *SCD* gene did not show relevant undesirable effects, particularly on carcass traits and composition. Contrarily, the *LEPR* polymorphism, although had a positive impact on IMF, it also affected overall fatness. It has been suggested that the effects of *LEPR* can be an indirect consequence of increased feed intake (Óvilo et al., 2005), since the leptin receptor mediates the satiety effect of leptin (Barb, Hausman, & Houseknecht, 2001; Houseknecht et al., 1998). This hypothesis was corroborated by the results reported by Rodríguez et al. (2010), who found a positive effect of the T allele on body weight and voluntary feed intake. In the present study we found an effect of *LEPR* on BT and IMF, but not on body weight. However, dealing with a larger dataset on production and carcass traits from the same line used here, Gol et al. (2015) were able to detect that *LEPR* not only affect BT and IMF but also body and carcass weight. These results would confirm that, although subjected to variations due to sampling location, muscle or equipment of measurement, the T allele at *LEPR*, likely through increased feed intake, results in heavier and fatter pigs. Although BT is easy to modify with conventional breeding, it is always interesting to have available for use in genetic evaluations a genetic marker explaining a significant percentage of the genetic variation of IMF content and composition (Ros-Freixedes et al., 2016) and of the unfavorable correlation of BT with these traits (Ros-Freixedes et al, 2014).

Several authors have shown that the fatty acid profile of muscle and SF changes during fattening. Bosch et al. (2012), using the same Duroc as here, reported increased SFA and MUFA content while decreased PUFA content from 5.5 to 7.5 months of age. The same trend was observed in commercial crossbreds by Lo Fiego, Macchioni, Minelli, & Santoro (2010), from 6 to 9.5 months, and by Virgili et al. (2003), from 8 to 10 months. The results obtained here reflect the same evolution as in these experiments regardless of the markers. Interestingly, however, the effect of the markers may offset the effect of age in terms of fatty acid composition. Thus, for example, the CC pigs at the *SCD* gene had more SFA in LT at 160 days (415.9±4.1 mg/g FA) than the TT at 220 days (405.8±4.2 mg/g FA), or similarly, the TT pigs at the *LEPR* gene had more SFA at 160 days (424.4 ±4.8 mg/g FA) than the other two genotypes at 220 days (CC: 404.8±3.4 mg/g FA; CT: 411.0±2.3 mg/g FA). The combined effect of the *SCD* and *LEPR* markers was analyzed for the MUFA/SFA ratio, a trait commonly used to assess the impact of dietary fat on health (Pacheco et al., 2006; Voisin et al., 2015). Both in muscle and SF, the MUFA/SFA ratio

was on average 15% higher in pigs jointly displaying the beneficial *SCD* TT and *LEPR* C- genotypes as compared to pigs with the CCTT genotype. This result shows that the combined use of both markers could be useful to produce healthier meat. However, the use of the *LEPR* C- genotype, which is associated to lower IMF and higher PUFA, may affect negatively the technological and sensory attributes of dry-cured hams production (Ruiz-Carrascal, Ventanas, Cava, Andrés, & García, 2000; Gandemer,2009).

The effects of the *SCD* and *LEPR* SNPs have proved to be consistent throughout the whole finishing period and in both LT and SF. Rodríguez et al. (2010) observed that the magnitude of the effect of *LEPR* on feed intake and average daily gain increased with age. In this study, however, we did not observe an interaction pattern between genotype and age. It should be noted, however, that we have only investigated the age interval covering the late fattening period, from 95 to 130 kg, where the effect of *LEPR* genotypes on body composition are already manifested. In fact, Rodríguez et al. (2010) did not find any effect of *LEPR* on body weight and feed intake until 65 kg. The effect of both polymorphisms on fat composition was in general more relevant in muscle than in SF, which is in accordance with the fact that the composition of neutral lipids in IMF is more aligned to endogenous fatty acid synthesis and remodeling rather than to dietary fat (Wood et al., 2008). Not only age but fat content determine fatty composition. For SFA in particular, Bosch et al. (2012) showed that fat content is what most influences SFA. To test whether the effect of *LEPR* was mainly a matter of scale, the difference between genotypes for SFA was adjusted for IMF (in *Gluteus medius* and LT) and BT (in SF). The effect of *LEPR* on SFA at constant fat content was lower, still significant ($P<0.05$) in *Gluteus medius* and LT but not in SF (410.5±3.4 mg/g FA, for CC; 408.1±2.3 mg/g FA, for CT; and 417.5±3.4 mg/g FA, for TT). This suggests that with regards to *LEPR*, compositional changes, particularly in SF, are due to overall increased fatness.

In a previous research we showed that the T allele at *SCD* behaved additively (Estany et al., 2014), but results are less clear and more controversial for *LEPR*, in part because only some experiments included the three genotypes. Thus, while *LEPR* effects were found to be mainly additive (Rodríguez et al., 2010; Galve et al., 2012), complete dominance is not discarded (Pérez-Montarelo et al., 2012; Uemoto et al., 2012). Even though we have not tested specifically for dominance, the results obtained (see, for

instance, **Figure 1**) would support the existence of a dominant effect with allele T acting as recessive, in line with other results in purebred Duroc (Uemoto et al., 2012; Gol et al., 2015). The statistical gene-gene interactions can lead to changes in magnitude or direction of the effects observed phenotypically (Mackay, 2014). Evidence of epistatic interaction between *LEPR* and *SCD* are minor and constrained to small-magnitude changes in BT, in line with the interaction of *LEPR* with other genes related to fat metabolism, such as the leptin (Perez-Montarelo et al., 2012) or the *MC4R* (Galve et al., 2012) genes. However, there are recent reports providing clues for possible dominant by additive interactions of *LEPR* with the *SCD* (Gol et al., 2015) and *PRKAG3* (López-Buesa, Burgos, Galve, & Varona, 2013) genes. More powerful designs are needed to detect and confirm these potential dominant and epistatic effects.

5. Conclusions

The present research confirms the positive effect of the T allele at the *SCD* gene (*AY487830:g.2228T>C*) on MUFA and provides new evidence on the positive effect of the T allele at the *LEPR* gene (*NM_001024587:g.1987C>T*) on SFA, both in LT and SF in Duroc pigs. However, contrarily to *SCD*, our findings show that the effect of *LEPR*, particularly in SF, is due to increased overall fatness. There is limited evidence of synergic effects between *SCD* and *LEPR* genes and of the interaction between them and age. Accordingly, their join effects are mostly additive and remain stable throughout all the finishing period. It is concluded that the combined selection for the *SCD* T and the *LEPR* C alleles is a good strategy to increase the MUFA/SFA ratio regardless of the age at slaughter.

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CHAPTER V

A polymorphism in the Stearoyl-CoA Desaturase gene promoter increases monounsaturated fatty acid content in dry cured ham

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ABSTRACT

Data on 125 dry-cured hams from purebred Duroc pigs were used to examine whether the favorable effect of the T allele in the promoter region of the stearoyl-CoA desaturase gene (*AY487830:g.2228T>C*) on monounsaturated fatty acid (MUFA) content in green ham is maintained after the curing process. It is shown that pigs carrying the T allele produced dry-cured hams with increased C16:1, C18:1n-9, C18:1n-7, and MUFA and decreased C18:0 and saturated fatty acid (SFA) content. The TT pigs had 1.39% more MUFA and 1.62% less SFA than the CC animals, while gilts had 0.74% more MUFA and 0.34% less SFA than barrows. The correlation between MUFA in green and dry cured hams (n=53) was high (r=0.88), with TT pigs being more effective in retaining increased MUFA in green hams until the end of the curing period. It is concluded that increasing the presence of the T allele could have more impact than gender to produce hams with a high level of MUFA.

Key words: dry-cured ham; fatty acid composition; genetic marker; intramuscular fat; meat quality; pigs.

1. Introduction

The dietary replacement of carbohydrates and saturated fatty acids (SFA) by monounsaturated fatty acids (MUFA) benefits human health. It has been shown that dietary MUFA increase HDL cholesterol, decrease LDL cholesterol and may even improve insulin sensitivity (FAO/WHO, 2008; Gillingham, Harris-Janz, & Jones, 2011). With regard to pork, MUFA content and, most particularly, oleic acid (C18:1n-9) content has been positively correlated with organoleptic properties such as flavor, tenderness, juiciness, pork flavor, flavor liking and overall acceptability (Cameron & Enser, 1991; Cameron et al., 2000; Tikk et al., 2007).

The stearoyl-CoA desaturase (*SCD*) is the rate-limiting enzyme required for the biosynthesis of the most abundant MUFA in pork, the oleic (C18:1n-9) and the palmitoleic (C16:1n-9) acids, which are produced by desaturating at the Δ9 position their respective saturated fatty acid precursors, the stearic acid (C18:0) and the palmitic acid (C16:0) (Guillou et al., 2010). Recently, Estany et al. (2014) reported in Duroc pigs a haplotype in the promoter region of the *SCD* gene affecting fat desaturation in pork. The single nucleotide polymorphism *AY487830:g.2228T>C* is part of this haplotype. Pigs carrying the T allele show higher (C16:1n-9)/C16:0 and C18:1/C18:0 ratios and higher levels of C16:1n-9, C18:1 (C18:1n-9+C18:1n-7) and MUFA in raw muscle while the intramuscular fat (IMF) content remains unchanged.

Dry-cured ham is a traditional product with enormous economic importance for the meat industry in the Mediterranean area, being the most preferred pork product by consumers in Spain (Enge et al., 1998). The IMF and MUFA content, particularly C18:1, is a quality criterion 58 of green ham suitability for dry-curing, since they affect the color and aspect of ham slices, the texture of hams and the intensity and persistence of aroma (Ruiz-Carrascal et al., 2000; Wood et al., 2008; Gandemer, 2009). Therefore, the objective of this paper was to examine whether the favorable effect of the T allele at the *AY487830:g.2228T>C* polymorphism on fat desaturation and MUFA content is maintained in commercial dry-cured hams. The relationship between fatty acid composition in green and dry-cured samples is discussed in relation to the effects of this polymorphism.

2. Material and methods

2.1. Animals and samples

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida.

Data on 125 dry-cured hams from a purebred Duroc line were used for the analyses. The line was completely closed in 1991 and since then it has been selected for an index including body weight, backfat thickness, and intramuscular fat (IMF) (Solanes et al., 2009; Ros-Freixedes et al., 2012a). At about 75 d of age piglets were moved to the fattening units, where they were penned by sex (8 to 12 pigs per pen) until they were slaughtered at 210 d in a commercial abattoir. During the fattening period pigs had ad libitum access to commercial diets. At slaughter, a sample of *Gluteus medius* (GM) muscle was collected from the ham of 53 barrows reared in the same batch (B1). After chilling for about 24 h at 2ºC, all GM samples were vacuum packed and stored at -18ºC for 28 days until analysis (Bosch et al., 83 2009). These storage conditions have been shown not to affect the fatty acid composition of the pig muscle (Hernández, Navarro, & Toldrá, 1999). The hams of these barrows were traced throughout the dry-curing process. Once dry-cured, a sample of the dry cured *Biceps femoris* (BF) muscle was dissected out from a slice of the mid-ham for fatty acid composition analysis (**Figure 1**). Additionally, 72 BF samples were obtained by the same procedure by randomly sampling two batches (B2; B3) of untraced dry-cured Duroc hams from the same line. The gender of the pigs producing these hams (barrows or gilts) was determined as indicated below.

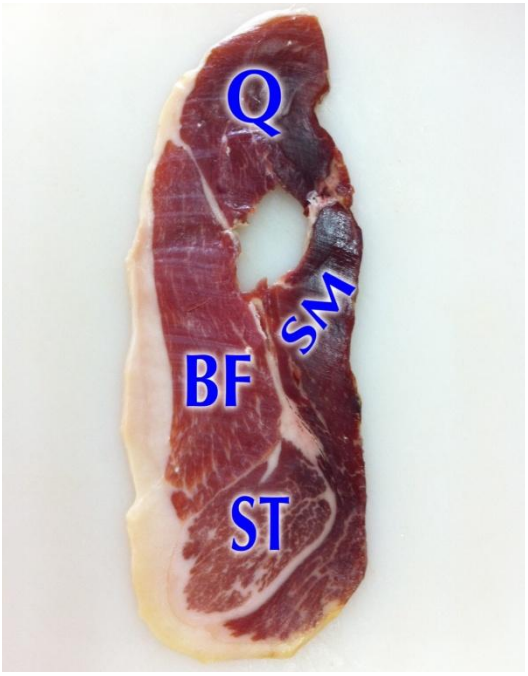


Figure. 1. Sample of dry-cured mid-hamsection before dissection of the muscle Biceps femoris (BF) used for this study and the muscles Quadriceps (Q), Semitendinosus (ST), and Semimembranosus (SM)

2.2. Dry-curing process

After trimming, the green hams go through four processing steps. First, in the salting step, hams are covered with salt and kept for 10 to 14 d at 2°C. In the resting step, hams are allowed to dry slowly for 3 months within a slow ramp of increasing temperatures (from 3 to 10°C). Then a first ripening step takes place by exposing the ham for 6 months at 9 to 10°C. In the last ripening step (aging cellar) the ham is kept at room temperature for at least 16 months, until one third of the initial weight is lost (Pena et al., 2013). The traced hams in B1 were divided into two aging groups according the green weight: the hams from the first group (C27) were aged for 18 months, for a total processing time of 27 months, while the second group (C30) aged three extra months up to 30 months of a total processing time.

2.3. Determination of IMF content and composition

Defrosted GM and dry-cured BF samples 108 were freeze-dried and pulverized previous to fat analysis. The IMF content and fatty acid (FA) composition were determined in duplicate by quantitative determination of the individual FA by gas chromatography (Bosch et al., 2009). In brief, FA methyl esters were directly obtained by transesterification using a solution of 20% boron trifluoride in methanol (Rule et al., 1997). Methyl esters were determined by gas chromatography using a capillary column SP2330 (30 m × 0.25 mm; Supelco, Bellefonte, PA) and a flame ionization detector with helium as carrier gas. Runs were made with a constant column-head pressure of 172 kPa. The oven temperature program increased from 150 to 225ºC at 7ºC/min and injector and detector temperatures were both 250ºC. The quantification was carried out through area normalization with an external mixture of FA methyl esters (Supelco® 37 Component FAME Mix. Sigma, Tres Cantos, Madrid). The internal standard was 1,2,3-tripentadecanoylglycerol. Then, the FA composition was expressed as the percentage of each individual FA relative to total FA. The complete profile for each sample included saturated (SFA: C14:0, C16:0, C18:0, and C20:0); monounsaturated (MUFA: C16:1n-9, C18:1, and C20:1n-9); and polyunsaturated (PUFA; 18:2n-6, C18:3n-3, C20:2n-6, and C20:4n-6) FA. In the dry-cured ham samples the C18:1n-9 and the C18:1n-7 isomers of C18:1 were separated. The identification and quantification of C18:1n-7 was made by using a commercial methyl ester mixture (FAME Column Evaluation Mix. Sigma, Tres Cantos, Madrid) and was confirmed by mass spectrometry. The IMF content in each muscle was calculated as the sum of the individual FA expressed as triglyceride equivalents (AOAC, 2000) on a dry tissue basis.

2.4. Isolation of genomic DNA and genotyping

The isolation of genomic DNA was carried out from muscle samples stored at -80ºC. Samples were lysed in the presence of proteinase K and DNA was purified through extraction with phenol:chloroform, followed by ethanol precipitation. Finally, DNA was re-suspended and stored in TE buffer. The quantification and estimation of the quality and purity of genomic DNA was performed using a Nanodrop N-1000 spectrophotometer; DNA integrity was tested through electrophoresis in a 1% agarose gel.

The *AY487830:g.2228T>C* polymorphism was genotyped using an allelic discrimination assay with the primers and probes indicated in Estany et al. (2014). The reaction mix contained 1x Universal TaqMan master mix (LifeTechnologies, Grand Island, NY), 0.2 μM Primer mix, 0.8 uM Probe mix and 10 ng of DNA in a final volume of 5 μl.

The sex of the 72 untraced hams was determined using a modified protocol of Sembon et al. (2008) based on the amplification of the amelogenin (*AMEL*) gene. Using published sequences for the porcine amelogenin genes (EMBL/GenBank accession numbers, AB091791 [*AMELX*] and AB091792 [*AMELY*]), a set of PCR primers were designed (AMEL-F, 5´-TCATGAGGAATCTCTTTGGTA-3´; AMEL-R, 5´-CCAGAGGTTGTAACCTTACAG-3´) to amplify a portion of intron 2 that was expected to yield PCR products of different sizes between *AMELX* (450 bp) and *AMELY* (278 bp). The PCR reaction mix contained 1x buffer, 2.66 mM of MgCl2, 0.13 mM dNTPs, 0.4 μM of each primer, 0.4 U of Taq DNA polymerase (Biotools, Madrid, Spain) and 40 ng of genomic DNA in a final volume of 15 μl. The amplification was carried out in a Veriti thermocycler (LifeTechnologies) with the following conditions: initial denaturation at 158 95°C for 5 min, 35 cycles of 96°C for 20 s, 54°C for 30 s and 72°C for 40 s and a final extension step of 72ºC x 5 min. PCR products were run in 1.5% agarose gels and visualized by ethidium bromide staining under UV illumination.

2.5. Statistical analyses

The effect of the *SCD* genotype on fatty acid content in the dry-cured hams was estimated on data from traced (C27 and C30 from B1) and untraced (B2 and B3) hams using a fixed effects model, which included the genotype (TT, CT and CC), the gender (barrow and gilt), and the batch (C27; C30; B2; and B3). The effect of the genotype was also independently estimated for each ham status (green or dry-cured) using only B1 traced hams. Moreover, two additional models were fitted based on these data to further investigate the effect of the genotype by ham status. The first model, which was used to estimate the relationship between MUFA in dry-cured and green muscle by *SCD* genotype, described the fatty acid content in the dry-cured ham as a function of the genotype, the aging time (C27 and C30), the fatty acid content in green ham, and the genotype by green ham's fatty acid content interaction. The second model was used to estimate the partial correlation between green and dry-cured muscles for fatty acid composition traits adjusted

for ham status and genotype. In this model the fatty acid content in green and dry-cured hams were jointly analyzed using a mixed model including the genotype, the ham status (green; C27, and C30), and the interaction between them as fixed effects and the ham as a random effect. The random variance and the residual variance were estimated by restricted maximum likelihood. As a result, the intraclass correlation between fresh and dry-cured fatty 183 acid composition was calculated as the random variance divided by the sum of the random variance and residual variance. Fixed effects were tested following an F-test. Multiple pairwise comparisons between genotypes and ham status were tested by a Tukey test. All the analyses were performed using the statistical package JMP 8 (SAS Institute Inc, Cary, NC).

3. Results and discussion

3.1. Effect of the SCD genotype on dry-cured ham fatty acid composition

The fatty acid composition in the dry-cured BF muscle differed by *SCD* genotype and gender (**Table 1**). The genotype influenced all the desaturation indexes which are commonly used as indirect measures of *SCD* activity (C16:1n-9/C16:0; C18:1/C18:0; and MUFA/SFA; Smith et al., 2002). In line with the results Estany et al. (2014) obtained in fresh pork, dry-cured hams of TT genotype had the greatest *SCD* activity and the CC the lowest, with CT hams exhibiting an intermediate phenotype. These findings were confirmed in the traced subset of hams, when green and dry-cured samples were analyzed separately (**Figure 2**), and also for C18:1n-9 and C18:1n-7, when the two isomers of C18:1 in the dry-cured hams were treated independently (**Table 2**). As a result of the greater desaturation activity, hams with the TT genotype showed increased C16:1n-9, C18:1n-9, C18:1n-7, and MUFA contents ($P<0.05$) and decreased C18:0 and SFA contents ($P<0.05$). Thus, pigs with the TT genotype had 1.39% more MUFA than the CC and 1.62% less SFA, while maintaining similar levels of PUFA. The difference between TT and CC hams for C18:1 was 0.95% (0.65%, for C18:1n-9, and 0.34%, 208 for C18:1n-7). As in green hams, the genotype did not affect the IMF content, thereby suggesting that the primary effect of the polymorphism is to replace SFA with MUFA. Several quantitative trait loci (Pena et al., 2013) and candidate gene markers (Ramos et al., 2008; Renaville et al., 2010) have been described to affect production and quality of dry-cured hams. In particular, a polymorphism at the *SCD* gene has been associated to fat lightness, which might be interpreted as a result

of a modification of the MUFA/SFA ratio (Renaville et al., 2010). However, none of reported markers have been investigated in relation to dry-cured ham fatty acid composition.

The gender affected fatty acid composition, with barrows displaying a higher amount of MUFA and lower PUFA levels than gilts, a result which is in agreement with previous studies indicating that barrows show greater concentrations of MUFA but lower PUFA as compared to gilts (Garitano et al., 2013; Zhang et al., 2007). The difference between barrows and gilts for MUFA and C18:1 (0.74% and 0.47%, respectively) was almost half of the differences due to the genotype. Therefore, in terms of fatty acid composition, increasing the frequency of the T allele had more impact than gender differences for producing a meat product with a higher level of MUFA. However, in contrast to what we have seen for the *SCD* genotype, the effect of castration of male pigs on fatty acid composition was not independent of the IMF content, which was 2.44% higher in barrows than in gilts. Thus, the difference between barrows and gilts for MUFA (51.17% vs. 50.43%) and C18:1 (46.78% vs. 46.31%) vanished after adjusting for IMF content (50.95% vs. 50.60% and 46.60% vs. 46.46%, respectively). This suggests that the primary effect of castration is to trigger endogenous fat biosynthesis, with the concomitant 233 effect of increasing MUFA. In fact, Yao et al. (2011) concluded that higher body fat deposition in castrated male pigs resulted mainly from increased transcription of lipogenic genes. In terms of fatty acid composition, this means that the main effect of castration is on enhancing the SFA/PUFA and MUFA/PUFA ratios and not the MUFA/SFA ratio as seen for the *SCD* genotype.

Table 1. Least square means (±SE) for intramuscular fat content and fatty acid composition in dry-cured *Biceps femoris* muscle by *SCD* genotype and gender

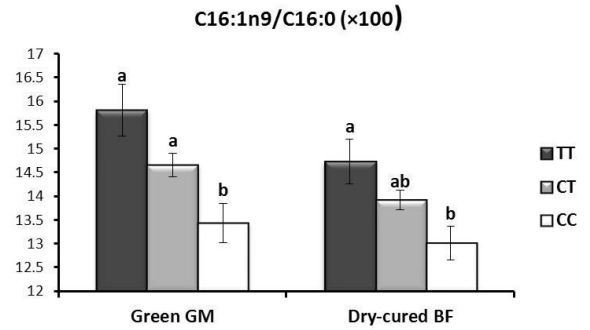
	SCD genotype			Gender	
Fatty acid, % FA [A]	TT	CT	CC	Barrows	Gilts
n	29	59	37	84	41
% DM	52.59±0.74	54.37±0.52	53±0.62	53.79±0.42	52.86±0.82
IMF, % DM	13.65±0.62	13.91±0.44	14.47±0.52	15.23±0.36[a]	12.79±0.69[b]
C14:0	1.49±0.02	1.5±0.01	1.52±0.02	1.52±0.01	1.48±0.02
C16:0	24.38±0.15	24.59±0.11	24.73±0.13	24.68±0.09	24.44±0.17
C18:0	10.78±0.14[c]	11.34±0.10[b]	12.02±0.12[a]	11.41±0.08	11.35±0.15
C20:0	0.17±0.00	0.17±0.00	0.17±0.00	0.17±0.00	0.17±0.00
SFA	36.82±0.26[c]	37.59±0.18[b]	38.44±0.22[a]	37.79±0.15	37.45±0.29
C16:1n-9	3.63±0.08[a]	3.51±0.05[a]	3.26±0.06[b]	3.58±0.04[a]	3.36±0.08[b]
C18:1	46.92±0.23[a]	46.79±0.16[a]	45.93±0.19[b]	46.78±0.13	46.31±0.25
C20:1n-9	0.79±0.01	0.79±0.01	0.77±0.01	0.8±0.01[a]	0.76±0.01[b]
MUFA	51.35±0.26[a]	51.08±0.18[a]	49.96±0.22[b]	51.17±0.15[a]	50.43±0.29[b]
C18:2n-6	9.22±0.19	8.86±0.13	9.03±0.16	8.75±0.11[b]	9.33±0.21[a]
C18:3n-3	0.5±0.01	0.49±0.01	0.48±0.01	0.49±0.01	0.49±0.01
C20:2n-6	0.44±0.01	0.43±0.01	0.43±0.01	0.42±0.01	0.44±0.01
C20:4n-6	1.67±0.07	1.54±0.05	1.67±0.06	1.38±0.04[b]	1.87±0.08[a]
PUFA	11.83±0.26	11.32±0.18	11.6±0.22	11.04±0.15[b]	12.13±0.29[a]
C16:1n-9/C16:0 (x100)	14.93±0.30[a]	14.29±0.21[a]	13.20±0.26[b]	14.54±0.18	13.74±0.34
C18:1/C18:0	4.38±0.06[a]	4.15±0.04[b]	3.83±0.05[c]	4.13±0.04	4.11±0.07
MUFA/SFA	1.40±0.01[a]	1.36±0.01[b]	1.30±0.01[c]	1.36±0.01	1.35±0.02
MUFA/PUFA	4.42±0.12	4.59±0.08	4.41±0.1	4.72±0.07[a]	4.23±0.13[b]
SFA/PUFA	3.19±0.09	3.40±0.06	3.40±0.08	3.49±0.05[a]	3.17±0.10[b]

[A] IMF: intramuscular fat content; SFA=C14:0+C16:0+C18:0+C20:0; MUFA=C16:1n-9+C18:1+C20:1n-9; PUFA=C18:2n-6+C18:3n-3+C20:2n-6+C20:4n-6; C18:1=C18:1n-9+C18:1n-7.

[a,b,c] Within row and factor, means with different superscripts differ significantly (P<0.05)

Figure 2. Least square means for the fatty acid desaturation ratios (A) C16:1n-9/C16:0, (B) C18:1/C18:0 (C18:1n-9+C18:1n-7) and (C) monounsaturated to saturated (MUFA/SFA) in green *Gluteus medius* (GM) and dry-cured *Biceps femoris* (BF) muscles by *SCD* genotype (n TT=7; CT=34; CC=12). Columns with different superscripts within group differ significantly (P<0.05)

(A)



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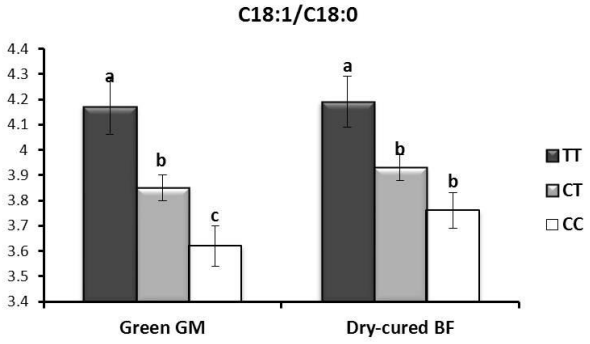

(C)

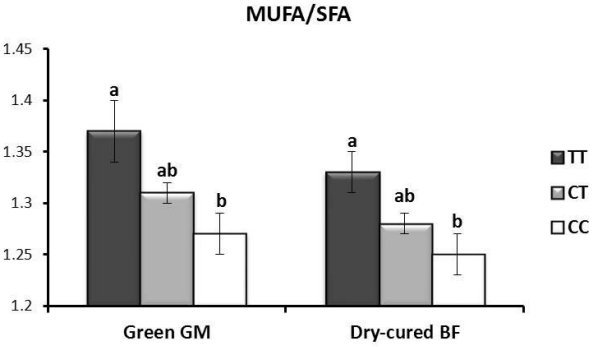

Table 2. Least square means (±SE) for oleic (C18:1n-9) and vaccenic (C18:1n-7) C18:1 isomers and associated desaturation ratios in dry-cured *Biceps femoris* muscle by *SCD* genotype and gender

	SCD genotype			Gender	
Fatty acid, % FA	TT	CT	CC	Barrows	Gilts
n	29	59	37	84	41
C18:1n-9	42.50±0.21[a]	42.49±0.15[a]	41.85±0.18[b]	42.47±0.12	42.09±0.23
C18:1n-7	4.42±0.05[a]	4.30±0.04[a]	4.08±0.04[b]	4.32±0.03	4.21±0.06
C181n-9/C18:1n-7	9.65±0.12[b]	9.92±0.09[b]	10.30±0.10[a]	9.88±0.07	10.03±0.13
C18:1n-7/C16:0 (x100)	18.23±0.25[a]	17.57±0.18[a]	16.53±0.21[b]	17.58±0.14	17.32±0.28
C18:1n-9/C18:0	3.97±0.06[a]	3.77±0.04[b]	3.49±0.05[c]	3.75±0.03	3.74±0.06

[a,b,c] Within row and factor, means with different superscripts differ significantly (P<0.05)

3.2. Relationship between green and dry-cured hams composition

The phenotypic correlations between raw fatty acid composition in green Gluteus medius and dry-cured Biceps femoris are presented in **Table 3**. Despite representing different muscles, the correlations between green and dry-cured samples were high, with values ranging from 0.59, for C18:3, to 0.92, for C16:1n-9. In particular, all the correlations for the ratios C16:1n-9/C16:0, C18:1/C18:0 and MUFA/SFA, as well as for MUFA and C18:1, were in the range of 0.84 to 0.89. However, the relationship of fatty acid composition between green and dry-cured hams differed according to the *SCD* genotype (**Figure 3**). Thus, for MUFA content, one of the target traits of this polymorphism, an interaction (P<0.05) was found between the MUFA content in green ham and the genotype, which contributed to explaining the MUFA content in dry-cured ham. The regression coefficients of dry-cured ham MUFA on green ham MUFA were 1.16±0.14, 0.71±0.07, and 0.65±0.14, for TT, CT, and CC genotypes, respectively. This result indicates that TT pigs, as compared to CC, are more effective in retaining increased MUFA in green hams until the end of the curing period. The variability on fatty acid content, both in green and dry cured samples, was low, in line with previously reported estimates (Ros-Freixedes et al., 2014b). In contrast, the correlations between green and cured muscles were higher than those calculated in this same population among fresh muscles (Ros-Freixedes et al., 2014c). Thus, although it needs to be confirmed in larger data sets, the correlation structure between fatty acid composition in green and dry-cured hams suggest that fatty acid composition in green hams is a good predictor of fatty acid composition in dry-cured hams.

The interaction between genotype and ham status for fatty acid composition was analyzed under a repeatability model, given that the variability displayed by individual fatty acids in the dry-cured samples, although somewhat lower, was very similar to that of green samples (**Table 3**). Results from this model indicated that there were no significant ($P>0.05$) interactions between the genotype and the ham status (data not shown) and that the correlations between green and dry-cured samples did not substantially change after adjusting for genotype. However, they highlighted that dry-curing cause a shift towards increased SFA/PUFA and MUFA/PUFA ratios, particularly in hams subjected to long aging periods (**Table 4**). The percentages of C18:2n-6, C20:2n-6, C20:4n-6 decreased after dry-curing, in agreement with what has been reported by Martín et al. (1999) for Iberian dry-cured ham. During processing, lipids undergo lipolysis and oxidation, which form free fatty acids and volatile compounds (Gandemer, 2009; Bermúdez et al., 2014). Because PUFA are the preferred substrates of such reactions, their proportion with respect to SFA and MUFA decrease during curing (Narváez-Rivas et al., 2008). In relative terms, the major fatty acid compositional change while dry-curing was the replacement of PUFA with SFA, with MUFA, as well as C18:1, remaining rather stable throughout all the processing period. The observed differences between the two processing times (C27 and C30) in 283 MUFA and C18:1 may be explained by the different initial green weight of the hams allocated to each group (12.51 kg, for C27, and 12.96 kg, for C30, $P<0.05$), which resulted in different levels of ultimate IMF. Thus, the C30 dry-cured hams, which were subjected to a total processing time of 30 months, showed a higher level of IMF than C27 hams, with a total processing time three months shorter (17.43% DM, for C30, and 13.84 % DM, for C27, $P<0.05$) and it is known that MUFA and C18:1 increased with IMF (Bosch et al., 2012). Overall, these results showed that the benefits of the T allele of the *SCD* genotype on MUFA and C18:1, particularly when they expressed with respect to SFA and C18:0, do not drop off during processing.

Figure 3. Relationship between monounsaturated (MUFA) content in green *Gluteus medius* and dry-cured *Biceps femoris* muscles by *SCD* genotype

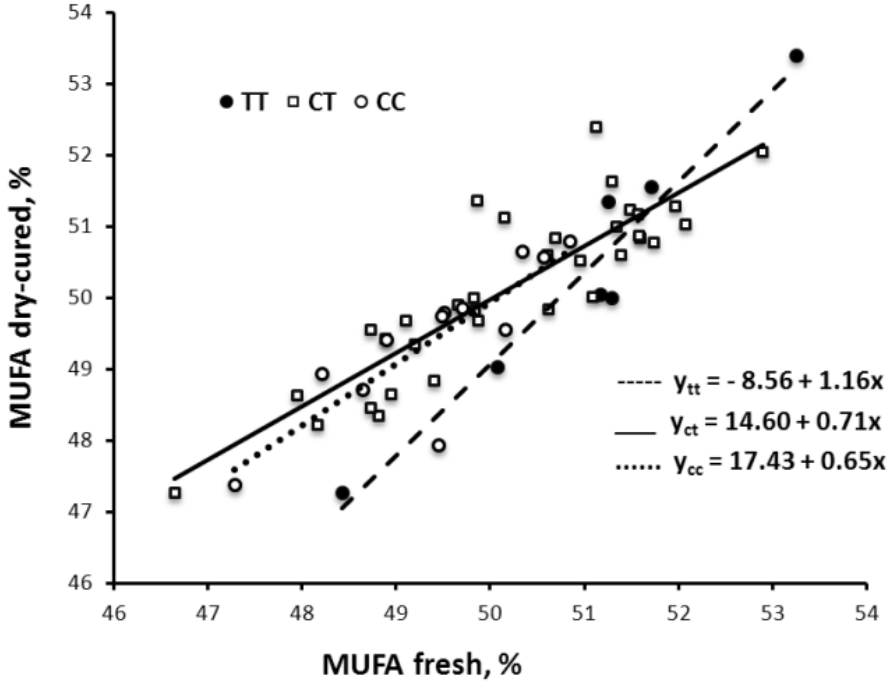

Table 3. Phenotypic correlations (r) between green (G) *Gluteus medius* and dry-cured (C) *Biceps femoris* muscles and standard deviation (SD) for fatty acid composition traits

	Total [A]			Partial [A]	
Fatty acid, % FA [B]	r	SD_G	SD_C	r	SD
C14:0	0.86	0.1	0.09	0.86	0.1
C16:0	0.88	0.83	0.7	0.85	0.78
C18:0	0.85	0.73	0.64	0.8	0.6
C20:0	0.72	0.01	0.02	0.7	0.01
SFA	0.85	1.35	1.16	0.82	1.21
C16:1n-9	0.92	0.4	0.33	0.89	0.34
C18:1	0.84	1.17	0.99	0.89	1.1
C20:1n-9	0.77	0.08	0.06	0.88	0.07
MUFA	0.86	1.38	1.14	0.88	1.24
C18:2n-6	0.79	1.07	0.78	0.76	0.95
C18:3n-3	0.59	0.05	0.04	0.57	0.05
C20:2n-6	0.66	0.06	0.04	0.63	0.05
C20:4n-6	0.64	0.35	0.22	0.65	0.3
PUFA	0.77	1.4	0.99	0.75	1.24
C16:1n-9/C16:0 (x100)	0.89	1.51	1.22	0.85	1.25
C18:1/C18:0	0.89	0.31	0.27	0.88	0.26
MUFA/SFA	0.88	0.07	0.06	0.87	0.06
MUFA/PUFA	0.79	0.66	0.53	0.81	0.61
SFA/PUFA	0.79	0.52	0.44	0.8	0.49

[A] Correlation and standard deviations between raw values (Total) and adjusted for ham status (G; C at 27 months, and C at 30 months) and genotype (TT; CT; and CC) using a repeatability model. All correlations are significant ($P<0.05$)

[B] SFA=C14:0+C16:0+C18:0+C20:0; MUFA=C16:1n-9+C18:1+C20:1n-9; PUFA=C18:2n-6+C18:3n-3+C20:2n-6+C20:4n-6; C18:1=C18:1n-9+C181n-7

Table 4. Least square means (±SE) for fatty acid composition in green *Gluteus medius* and dry-cured *Biceps femoris* aged for 27 and 30 months

		Dry-cured	
Fatty acid, % FA [A]	Green	27 m	30 m
n	52	36	16
C14:0	1.53±0.02[b]	1.60±0.02[a]	1.58±0.02[a]
C16:0	24.63±0.13[b]	25.71±0.14[a]	25.69±0.19[a]
C18:0	11.87±0.10	11.75±0.11	11.63±0.16
C20:0	0.16±0.00[b]	0.18±0.00[a]	0.17±0.00[b]
SFA	38.19±0.20[b]	39.24±0.21[a]	39.06±0.32[a]
C16:1n-9	3.61±0.06[a]	3.52±0.06[b]	3.66±0.08[ab]
C18:1	45.88±0.19[a]	45.54±0.19[b]	46.14±0.26[a]
C20:1n-9	0.76±0.01	0.77±0.01	0.76±0.02
MUFA	50.25±0.21[a]	49.83±0.22[b]	50.58±0.29[a]
C18:2n-6	9.27±0.16[a]	8.87±0.17[b]	8.26±0.27[b]
C18:3n-3	0.45±0.01[b]	0.47±0.01[a]	0.44±0.02[ab]
C20:2n-6	0.42±0.01[a]	0.38±0.01[b]	0.35±0.02[b]
C20:4n-6	1.42±0.05[a]	1.25±0.06[b]	1.19±0.10[b]
PUFA	11.56±0.21[a]	10.96±0.22[b]	10.26±0.36[b]
C16:1n-9/C16:0 (x100)	14.66±0.21[a]	13.70±0.22[b]	14.23±0.31[ab]
C18:1/C18:0	3.88±0.04	3.89±0.05	3.98±0.06
MUFA/SFA	1.32±0.01[a]	1.27±0.01[b]	1.29±0.01[ab]
MUFA/PUFA	4.42±0.10[b]	4.59±0.11[b]	5.02±0.16[a]
SFA/PUFA	3.36±0.08[b]	3.62±0.09[a]	3.86±0.13[a]

[A] SFA=C14:0+C16:0+C18:0+C20:0; MUFA=C16:1n-9+C18:1+C20:1n-9; PUFA=C18:2n-6+C18:3n-3+C20:2n-6+C20:4n-6; C18:1=C18:1n-9+C18:1n-7

[a,b,c] Within row, means with different superscripts differ significantly P<0.05

4. Conclusions

The present work provides evidence that the favorable effect of the T allele at the *AY487830:g.2228T>C* polymorphism on fatty acid composition of green hams is maintained in dry-cured hams. The phenotypic correlations between fatty acids show a strong and positive relationship between green and dry-cured samples, with TT pigs being more effective in retaining increased MUFA in green hams until the end of the curing period. It is concluded that selection for the T allele is a good alternative to increase MUFA

and C18:1 content of pork intended to produce high quality dry-cured hams without affecting the total fat content of the product.

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CHAPTER VI

A polymorphism in the Stearoyl-CoA Desaturase gene promoter influences monounsaturated fatty acid content of Duroc ×Iberian hams

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ABSTRACT

Data on 74 dry-cured hams from Duroc × Iberian pigs were used to examine whether the tag polymorphism *AY487830:g.2228T>C* in the promoter region of the stearoyl-CoA desaturase [*SCD*] gene affect fat desaturation and monounsaturated fatty acid (MUFA) as previously described in purebred Duroc hams. Samples were taken from sliced trays of dry-cured hams marketed as Jamón Ibérico de cebo, which were randomly purchased from the same supplier in different stores of the same supermarket chain. Genomic DNA was isolated from each sample to genotype for *SCD* and gender. Also, a freeze-dried sample of two slices was used to determine fat content and fatty acid (FA) composition by gas chromatography. The effect of the genotype (TT and CT) and the gender (barrows and gilts) was estimated under a Bayesian setting. Results showed that the *SCD* polymorphism was associated to fat composition but not to fat content, with TT hams showing increased C18:1n-7, C18:1n-9, C20:1n-9 and MUFA (probability between 0.92-0.98) and decreased C18:2n-6, C20:4n-6 and polyunsaturated FA (PUFA) (probability between 0.91-0.99) as compared to the CT. As a result, the TT hams had more MUFA (0.95%) and a higher MUFA/PUFA ratio (0.43) than the CT. Barrows had more saturated FA (SFA) and less PUFA than gilts. No differences in MUFA content were found between genders. The *SCD* polymorphism had a greater impact on MUFA than using hams from barrows instead of gilts. It is concluded that the *SCD* polymorphism is a good tool to increase MUFA and MUFA/PUFA ratio in Duroc crossbred dry-cured hams.

Additional key words: dry-cured ham; fatty acid composition; genetic marker; meat quality; pigs.

Abbreviations used: B (barrows); CT (heterozygous genotype for the polymorphism *AY487830:g.2228T>C*); DM (dry matter); FA (fatty acids); G (gilts); IMF (intramuscular fat); MCMC (Markov chain Monte Carlo); MUFA (monounsaturated fatty acids); PUFA (polyunsaturated fatty acids); *SCD* (stearoyl-CoA desaturase); SE (standard error); SNP (single nucleotide polymorphism); SFA (saturated fatty acids); TT (alternative homozygous genotype for the polymorphism *AY487830:g.2228T>C*).

1. Introduction

The dry-cured ham industry is a traditional sector with an enormous economic importance in the Mediterranean area, particularly in Spain, where it is the pork product most preferred by consumers (Enge et al., 1998; ANICE, 2014). The intramuscular fat content (IMF) and fatty acid (FA) composition, especially the monounsaturated fatty acid (MUFA) content, influence the final quality of the dry-cured products, since they affect color, aspect and texture of ham slices, as well as the intensity and persistence of aroma (Ruiz-Carrascal et al., 2000; Gandemer, 2009; Lorido et al., 2015). Moreover, the

consumption of dietary MUFA promotes healthy blood lipid profiles, mediates blood pressure, improves insulin sensitivity and regulates glucose levels (Gillingham et al., 2011).

MUFA represent the most abundant fatty acids in pork and oleic acid (C18:1n-9) is its major component, accounting for approximately 35-50% of total fatty acids (López-Bote, 1998; Wood et al., 2008; Tejerina et al., 2012). It has been shown that the oleic acid content is positively correlated with pork flavor, flavor liking and overall acceptability (Cameron et al., 2000; Tikk et al., 2007). A relevant part of the variation in MUFA and oleic acid has a genetic origin and thus can be attributed to differences between breeds (Wood et al., 2004; Reixach et al., 2008) and individuals within breed (Ros-Freixedes et al., 2012). A recent research of Estany et al. (2014) has shown that a haplotype in the promoter region of the stearoyl-CoA desaturase (*SCD*) gene, which enhances fat desaturation and, as a result, MUFA and oleic acid in muscle and subcutaneous fat, segregates in Duroc. This beneficial effect is maintained in purebred dry-cured Duroc hams (Henriquez-Rodriguez et al., 2015), despite the fatty acid compositional changes occurring through lipolysis and oxidation in the curing process (Narváez-Rivas et al., 2008; Gandemer, 2009).

The Iberian hams represent about 20% of total turnover of Spain´s dry-cured ham industry (Belmonte, 2006; Chamorro et al., 2008; Cruz, 2013). The Spanish Ministry of Agriculture guidelines (BOE, 2014) establishes two types of labelling for Iberian hams according to their genetic background: 100% Iberian (produced from purebred Iberian pigs) and Iberian (produced using Iberian crossbred pigs with a maximum 50% of Duroc). Nowadays, however, most of the Iberian marketed hams, around 94%, are based on crosses of Iberian sows with Duroc boars (Cruz, 2013). The objective of this study was to assess whether the haplotype segregating at the *SCD* gene in Duroc also affects the MUFA content of marketed Duroc × Iberian dry-cured hams.

2. Material and methods

2.1. Sample collection

Seventy four dry-cured ham sliced packs of '*Jamón Ibérico de Cebo'* (fodder fed Iberian ham) from the same supplier were randomly purchased in 15 different franchised stores of the same supermarket chain in the city of Lleida (Catalonia, Spain) and surroundings between July 2013 and January 2014. The supplier is known to produce the

Iberian dry-cured hams using crossbred Duroc × Iberian females or barrows from their own nucleus herds, farms, and manufacturing facilities. To randomize unidentified effects, each pack was taken from a different production batch. All packs were vacuum-packaged and kept in refrigerated shelves. Two standard entire slices per pack were freeze-dried and pulverized previous to fat analysis.

2.2. Determination of fat content and composition

A representative aliquot from the pulverized freeze-dried sample was used to determine, in duplicate, the individual FA by gas chromatography (Bosch et al., 2009). In brief, FA methyl esters were directly obtained by transesterification using a solution of 20% boron trifluoride in methanol (Rule, 1997). Methyl esters were determined by gas chromatography using a capillary column SP2330 (30 m × 0.25 mm; Supelco, Bellefonte, PA, USA) and a flame ionization detector with helium as carrier gas. Runs were made with a constant column-head pressure of 172 kPa. The oven temperature program increased from 150 to 225ºC at 7ºC/min and injector and detector temperatures were both 250ºC. The quantification was carried out through area normalization with an external mixture of FA methyl esters (Supelco® 37 Component FAME Mix, Sigma, Tres Cantos, Madrid). The internal standard was 1,2,3-tripentadecanoylglycerol (Tripentadecanoin, Sigma, Tres Cantos, Madrid). Then, the FA composition was expressed as the percentage of each individual FA relative to total FA. The complete profile for each sample included saturated (SFA: C14:0; C16:0; C18:0; and C20:0); monounsaturated (MUFA: C16:1n-9; C18:1n-7; C18:1n-9; and C20:1n-9); and polyunsaturated (PUFA: C18:2n-6; C18:3n-3; C20:2n-6; and C20:4n-6) FA. The identification and quantification of the C18:1n-7 isomer was made by using a commercial methyl ester mixture (FAME Column Evaluation Mix, Sigma, Tres Cantos, Madrid) and was confirmed by mass spectrometry. The fat content was calculated as the sum of the individual FA expressed as triglyceride equivalents (AOAC, 2000) on a dry tissue basis.

2.3. Isolation of genomic DNA and genotyping

The isolation of genomic DNA was carried out from refrigerated Iberian hams. Samples were lysed in the presence of proteinase K and DNA was purified through extraction with phenol:chloroform, followed by ethanol precipitation. Finally, DNA was re-

suspended and stored in TE buffer. The quantification and estimation of the quality and purity of genomic DNA was performed using a Nanodrop N-1000 spectrophotometer; DNA integrity was tested through electrophoresis in a 1% agarose gel.

The single nucleotide polymorphism *AY487830:g.2228T>C* , which was selected as a tag SNP to evaluate the effect of alternate haplotypes, was genotyped using an allelic discrimination assay with the primers and probes indicated in Estany et al. (2014). The reaction mix contained 1x Universal TaqMan master mix (LifeTechnologies, Grand Island, NY), 0.2 µM primer mix, 0.8 µM probe mix and 10 ng of DNA in a final volume of 5 µL.

The sex of the hams was determined using a modified protocol of Sembon et al. (2008) based on the amplification of the amelogenin (*AMEL*) gene. Using published sequences for the porcine amelogenin genes (EMBL/GenBank accession numbers, AB091791 [*AMELX*] and AB091792 [*AMELY*]), a set of PCR primers were designed (AMEL-F, 5´-TCATGAGGAATCTCTTTGGTA-3´; AMEL-R, 5´-CCAGAGGTTGTAACCTTACAG-3´) to amplify a portion of intron 2 that was expected to yield PCR products of different sizes between AMELX (450 bp) and AMELY (278 bp). The PCR reaction mix contained 1x buffer, 2.66 mM of MgCl2, 0.13 mM dNTPs, 0.4 µM of each primer, 0.4 U of Taq DNA polymerase (Biotools, Madrid) and 40 ng of genomic DNA in a final volume of 15 µL. The amplification was carried out in a Veriti thermocycler (LifeTechnologies) with the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of 96°C for 20 s, 54°C for 30 s and 72°C for 40 s and a final extension step of 72ºC × 5 min. PCR products were run in 1.5% agarose gels and visualized by ethidium bromide staining under UV illumination.

2.4. Statistical analyses

The effect of the *SCD* genotype on fatty acid content and composition was estimated under a Bayesian approach by fitting a model which included the genotype (TT and CT) and the gender (barrows and gilts). Each trait was assumed to be conditionally distributed as follows:

$$\mathrm{y} \quad \mathrm{b}, \sigma_e^2 \sim \mathrm{N} \quad \mathrm{Xb}, \mathrm{I}\sigma_e^2 \ ,$$

where **b** is the vector including the effects of genotype and gender; **X** is the known incidence matrix relating the observations with the systematic effects; **I** is the identity matrix; and σ^2_e is the residual variance. Bounded uniform priors were used to represent vague previous knowledge of **b** and for the residual variance. Marginal posterior distributions for all unknowns were estimated using Gibbs sampling. Chains of 60,000 samples with a burn-in period of 10,000 were used. One sample of each 10 was saved to avoid high correlations between consecutive samples. The Monte Carlo SE was estimated using effective sample size to take into account the autocorrelation in the MCMC samples. Convergence was tested using the Z-criterion of Geweke. Inferences were done from the marginal posterior distributions of the differences between two genotypes or genders. In particular, the following parameters were calculated: the mean of the marginal posterior distribution of the difference (D); the highest posterior density interval for D at 95% (HPD95%); the probability (p) of D being greater (if D>0) or lower (if D<0) than zero; and the limit k of the interval [k, +∞), if D>0, or (–∞, k], if D<0, at 80% of probability. All the analyses were performed using the statistical package Rabbit developed by Institute for Animal Science and Technology (Valencia, Spain; http://www.dcam.upv.es/dcia/ablasco/Publi.htm).

3. Results

For all the analyzed traits, the Monte Carlo SE was very small and the Geweke test did not detected lack of convergence.

3.1. Effect of SCD genotype

Allelic frequencies of the *SCD* gene by gender are presented in **Table 1**. The allele frequencies were 0.79 and 0.21, for allele T and C, respectively. Because only one sample with genotype CC was found, it was discarded for further analyses. Features of estimated marginal posterior distributions of the difference between TT and CT genotypes for fat content and fatty acid composition in Duroc × Iberian hams are shown in **Table 2**.

Results provided no evidence for an association between the *SCD* genotype and fat content. However, they clearly support the hypothesis that the investigated polymorphism affects fatty acid composition. Thus, the hams produced by TT pigs showed greater values for C18:1n-9, C18:1n-7, C20:1n-9 and MUFA (with p from 0.92 to 0.98) and lower values

for C18:2n-6, C20:4n-6 and PUFA (with p ranging 0.91 to 0.99) as compared to the CT. Differences between genotypes were much lower for SFA, the most evident being for C18:0 (with p of 0.83). The effect of the polymorphism was higher than one-third of the standard deviation of the trait for C18:1n-9, C18:1n-7, C20:1n-9, MUFA, C18:2n-6, C20:4n-6 and PUFA. The TT hams had a probability of 80% of having at least 0.37% more C18:1n-9 and 0.56% more MUFA than the CT. As a result, the 18:1n-9/C18:0, MUFA/SFA, MUFA/PUFA, and SFA/PUFA ratios were higher in the TT hams. In particular, the MUFA/PUFA ratio was 0.43 higher in the TT than in the CT hams (with p of 1.00).

Table 1. Number of samples by gender and genotype and allelic frequencies at the *SCD* gene in sampled Duroc × Iberian hams

Gender	Number of samples		Allelic frequency	
	TT	CT	T	C
Barrows	20	18	0.76	0.24
Gilts	23	13	0.82	0.18
Total	43	31	0.79	0.21

3.2. Effect of the gender

Features of estimated marginal posterior distribution of the difference between barrows and gilts for fat content and fatty acid composition in Duroc × Iberian hams are given in **Table 3**. The hams were evenly distributed by gender, with 51.1% from barrows and 48.6% from gilts, suggesting, because packs were randomly taken, that males and gilts are indistinctly used for producing dry-cured hams.

The barrows did not differ from gilts for fat content (the probability of barrows showing higher fat content than gilts was 0.53), but they showed greater values for SFA (C14:0, and C16:0, with p of 0.99 and 0.94 respectively), and lower for PUFA (C18:2n-6 and PUFA with p of 0.99). Results concerning MUFA were more disparate. Thus, while barrows exhibited higher levels of C16:1n-9, C18:1n-7 and C20:1n-9 (with p ranging from of 0.84 to 1.00), they showed lower values of C18:1n-9 (although only with p of 0.73). The effect of the gender was higher than one-third of the standard deviation of the trait for C14:0, C16:0, C18:1n-7, C20:1n-9, C18:2n-6, and PUFA. As a result, the ratios

SFA/PUFA and MUFA/PUFA were, respectively, 0.24 and 0.28 higher in barrows than in gilts (with p of 0.98 and 0.97, respectively).

Table 2. Features of the estimated marginal posterior distribution of the difference between *SCD* genotypes for fat content (IMF) and fatty acid composition in Duroc × Iberian dry-cured hams[1]

Trait[2],%	$Mean_{TT}$	$Mean_{CT}$	Mean D_{TT-CT}	$HPD_{95\%TT-CT}$	*p*	$k_{80\%}$
n	43	31				
Fat, % DM	27.77	27.20	0.57	-2.55; 3.79	0.64	
DM	62.11	62.11	0.00	-2.34; 2.40	0.51	
C14:0	1.41	1.41	0.00	-0.06; 0.05	0.48	
C16:0	24.80	24.93	-0.13	-0.63; 0.39	0.70	
C18:0	10.35	10.6	-0.24	-0.72; 0.24	0.83	-0.03
C20:0	0.18	0.21	-0.03	-0.09; 0.04	0.78	
SFA	36.76	37.10	-0.35	-1.27; 0.61	0.76	
C16:1n-9	3.16	3.05	0.11	-0.08; 0.31	0.87	0.03
C18:1n-9	45.62	44.93	0.69	-0.05; 1.46	**0.96**	0.37
C18:1n-7	3.82	3.68	0.13	-0.03; 0.31	**0.93**	0.06
C20:1n-9	0.90	0.88	0.02	-0.01; 0.05	**0.92**	0.01
MUFA	53.50	52.54	0.95	0.05; 1.91	**0.98**	0.56
C18:2n-6	8.33	8.81	-0.48	-0.88; -0.06	**0.99**	-0.31
C18:3n-3	0.47	0.48	-0.01	-0.08; 0.06	0.62	
C20:2n-6	0.36	0.37	-0.01	-0.03; 0.01	0.79	
C20:4n-6	0.59	0.68	-0.09	-0.20; 0.04	**0.91**	-0.03
PUFA	9.75	10.34	-0.59	-1.06; -0.08	**0.99**	-0.38
C18:1n-9/C18:0	4.46	4.27	0.20	-0.05; 0.45	**0.94**	0.09
C16:1n-9/C16:0	0.13	0.12	0.01	0.00; 0.01	0.89	0.00
MUFA/SFA	1.46	1.42	0.04	-0.02; 0.10	**0.92**	0.02
MUFA/PUFA	5.56	5.12	0.43	0.14; 0.74	**1.00**	0.31
SFA/PUFA	3.82	3.63	0.19	-0.03; 0.44	**0.95**	0.09

[1]Mean D_{TT-CT}: mean of the marginal posterior distribution of the difference between genotype TT and CT; $HPD_{95\%TT-CT}$: highest posterior density interval for D_{TT-CT} at 95%; *p*: posterior probability of D_{TT-CT} being greater (if $D_{TT-CT} > 0$) or lower (if $D_{TT-CT} < 0$) than zero; and $k_{80\%}$: limit of the interval $[k, +\infty)$ (if $D_{TT-CT} > 0$) or $(-\infty, k]$ (if $D_{TT-CT} < 0$) at 80% of probability (k is only displayed when D_{TT-CT} and $k_{80\%}$ are of the same sign). $p > 0.9$ are written in bold.

[2] DM, dry matter; SFA, saturated fatty acids (C14:0+C16:0+C18:0+C20:0); MUFA, monounsaturated fatty acids (C16:1n-9+C18:1n-9 + C18:1n-7+ C20:1n-9); PUFA, polyunsaturated fatty acids (C18:2n-6+C18:3n-3+C20:2n-6+C20:4n-6).

Table 3. Features of estimated marginal posterior distribution of the difference between barrows (B) and gilts (G) for fat content (IMF) content and fatty acid composition in Duroc × Iberian dry-cured hams[1]

Trait[2], %	$Mean_B$	$Mean_G$	Mean D_{B-G}	$HPD_{95\%B-G}$	*p*	$k_{80\%}$
n	38	36				
Fat, % DM	27.56	27.42	0.14	-2.87; 3.31	0.53	
DM	62.27	61.95	0.32	-1.93; 2.69	0.60	
C14:0	1.44	1.39	0.06	0.01; 0.10	**0.99**	0.04
C16:0	25.06	24.67	0.40	-0.09; 0.88	**0.94**	0.18
C18:0	10.49	10.46	0.02	-0.47; 0.50	0.54	
C20:0	0.19	0.20	-0.01	-0.08; 0.06	0.59	
SFA	37.20	36.67	0.53	-0.36; 1.47	0.87	0.12
C16:1n-9	3.15	3.06	0.09	-0.09; 0.28	0.84	0.01
C18:1n-9	45.16	45.39	-0.24	-0.95; 0.52	0.73	
C18:1n-7	3.83	3.68	0.15	-0.01; 0.31	**0.96**	0.08
C20:1n-9	0.91	0.87	0.04	0.01; 0.07	**1.00**	0.03
MUFA	53.04	53.00	0.03	-0.86; 0.92	0.53	
C18:2n-6	8.32	8.82	-0.50	-0.90; -0.11	**0.99**	-0.33
C18:3n-3	0.46	0.49	-0.03	-0.10; 0.04	0.83	0.00
C20:2n-6	0.36	0.38	-0.01	-0.04; 0.01	**0.92**	-0.01
C20:4n-6	0.63	0.64	-0.01	-0.14; 0.11	0.57	
PUFA	9.77	10.33	-0.56	-1.03; -0.08	**0.99**	-0.36
C18:1n-9/C18:0	4.34	4.39	-0.05	-0.31; 0.19	0.66	
C16:1n-9/C16:0	0.13	0.12	0.00	-0.01; 0.01	0.65	
MUFA/SFA	1.43	1.45	-0.02	-0.08; 0.04	0.75	
MUFA/PUFA	5.48	5.20	0.28	0.00; 0.57	**0.97**	0.16
SFA/PUFA	3.84	3.60	0.24	0.00; 0.47	**0.98**	0.14

[1]Mean D_{B-G}: mean of the marginal posterior distribution of the difference between barrows and gilts; $HPD_{95\%B-G}$: highest posterior density interval for D_{B-G} at 95%; *p*: posterior probability of D_{B-G} being greater (if $D_{B-G} > 0$) or lower (if $D_{B-G} < 0$) than zero; and $k_{80\%}$: limit of the interval $[k, +\infty)$ (if $D_{B-G} > 0$) or $(-\infty, k]$ (if $D_{B-G} < 0$) at 80% of probability (k is only displayed when D_{B-G} and $k_{80\%}$ are of the same sign). *p* >0.9 are written in bold.

[2]See Table 2 for abbreviations.

4. Discussion

Iberian hams, including Duroc × Iberian crossbreds, are characterized by a high level of fat infiltration, which is very appreciated by consumers and provides a high degree of marbling, a firm texture and an intense, delicate and very special flavor (Fernández et al., 2007). The official regulation concerning the required genetic types that can be used under the designation Iberian (BOE, 2014) are not always perfectly fulfilled by some producers and therefore it might be possible to find some Duroc × Iberian hams with a proportion of Duroc alleles greater than 50%. However, this is certainly not the case here because, as indicated above, all sampled packs were from pigs of identified origin. Moreover, the observed genotypic frequencies (0.58, for TT, and 0.42, for CT genotypes, with only 1 CC) is in line with the hypothesis that allele T is almost fixed in Iberian and segregating at an intermediate frequency in Duroc (Estany et al., 2014).

The IMF content has a positive influence on texture and appearance traits of hams, such as oiliness, brightness and juiciness (Ruiz-Carrascal et al., 2000). In this study the *SCD* genotype did not affect fat content, in agreement with what has been reported in Estany et al. (2014) for IMF in three distinct raw muscles. Similarly, the fat content was not affected by the gender, which is in accordance with what has been observed by some authors using Duroc × Iberian crossbred barrows and castrated and intact females (Serrano et al., 2008; Cordero et al., 2010), but not all. In particular, Muñoz et al. (2011) found that barrows had more IMF than intact females. However, these works were carried out with raw rather than dry-cured samples. Using samples of dry-cured Serrano hams, Soriano et al. (2005) did not find an effect of sex on IMF. However, because we analyzed entire slices, samples also included intermuscular fat and the untrimmed subcutaneous fat. Therefore, as determined, fat content here should be considered as total edible fat rather than IMF. Nevertheless, taking together, these results suggest that both the *SCD* polymorphism and the use of barrows instead of gilts do not exert a relevant influence on the edible fat content.

The main purpose of this study was to investigate the effect of the *SCD* polymorphism on the fatty acid profile of the edible fat in an entire slice. This approach was used for its simplicity but also because, from previous studies, we know that the correlation of the fatty acid profile between fat tissues is relatively high (Ros-Freixedes et al., 2014) and the effect of the polymorphism is consistent across muscles and fat tissues (Estany et

al., 2014). Fatty acid composition affects eating and meat quality traits of pork, particularly flavor, tenderness and juiciness (Antequera et al., 1992; Ruiz et al., 1999; Ruiz-Carrascal et al., 2000). In contrast to fat content, we found that both the *SCD* polymorphism and the gender influenced the fatty acid profile of fat. In line with previous results in purebred Duroc, either with raw meat (Estany et al., 2014) or with dry-cured hams (Henriquez-Rodriguez et al., 2015), the T allele increased MUFA (+0.95%). However, here, contrarily to these previous works, the increase in MUFA was at expense of PUFA (-0.59%), especially C18:2n-6 (-0.48%), rather than SFA (-0.35%). The side-effect of the marker on PUFA content is not obvious to explain, although it might be influenced by the sampling scheme used, which includes fat sources other than IMF, or by differing ripening conditions. It is known that the subcutaneous fat contains more PUFA than IMF (Ros-Freixedes & Estany, 2013) and that PUFA decrease across ripening due to oxidative reactions (Martín et al., 1999; Narváez-Rivas et al., 2008; Henriquez-Rodriguez et al., 2015). Therefore, changes in PUFA are expected to be more marked in samples from subcutaneous fat subjected to long dry-curing periods. The substitution of MUFA for PUFA is a side effect that indirectly may benefit the quality of the ham, since it is known that PUFA increase susceptibility to oxidative processes and off-flavors (Cava et al., 1999).

The substitution effect of the T allele for C18:1n-9 and MUFA (0.69% and 0.95%) was very close to the values obtained by Estany et al. (2014) for C18:1 (C18:1n-9+C18:1n-7, 0.70%) and MUFA (1.02%), thereby confirming that the favorable effect of the T allele on fat desaturation observed in purebred Duroc raw muscles is maintained in Duroc × Iberian dry-cured hams. The favorable effect of allele T on MUFA in Duroc × Iberian had already been reported in raw muscle (Estany et al., 2014). Although quality attributes of dry-cured ham are subjected to complex polygenic inheritance patterns (Pena et al., 2013), a number of associated genetic markers have been reported to use in pig breeding programs (Stalder et al., 2005; Renaville et al., 2010; Gou et al., 2012). However, only one of them, and likely as a result of a correlated change in overall fatness, affected the fatty acid composition of dry-cured hams (Reina et al., 2012). In practical terms, the results obtained indicate that the *SCD* polymorphism can be a good tool to discriminate among Duroc boars by their transmittance ability for increased MUFA content in ham. In fact, we have proved that its effects can even be detected in the total edible fat of dry-cured ham slices from commercial packs randomly sampled.

Barrows, as compared to gilts, increased SFA and decreased PUFA. It is a generalized result that castration decreases PUFA (Garitano et al., 2013; Henriquez-Rodriguez et al., 2015), but it is not clear whether this is mainly caused as a result of increased SFA, MUFA, or both. While in our experiment barrows had a greater proportion of SFA (but not MUFA) than gilts, in that of Garitano et al. (2013), based on Duroc × (Landrace × Large White) crossbreds, the barrows had more MUFA (but not SFA). On the other hand, although using raw muscle samples, Ramirez & Cava (2007) did not find differences for SFA, MUFA and PUFA between barrows and gilts in different crossbreds of Duroc × Iberian. However, interestingly, our findings proved that, in commercial Duroc × Iberian dry-cured hams, the investigated *SCD* polymorphism can have a greater impact on MUFA than using hams from barrows instead of gilts.

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CHAPTER VII

Carotenoid intake and *SCD* genotype exert complementary effects over fat content and fatty acid composition in Duroc pigs

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ABSTRACT

Nutritional and genetic strategies are needed to enhance intramuscular fat (IMF) and monounsaturated fatty acid (MUFA) content without altering carcass leanness. Dietary vitamin A restriction has been suggested to specifically promote IMF while a polymorphism of the stearoyl-CoA desaturase (*SCD*) gene has shown to specifically increase MUFA. The purpose of this study was to investigate the combined effects of pro-vitamin A (PVA) carotenoid intake and *SCD* genotype (*AY487830:g.2228T>C*) on hepatic retinoid content and on liver, muscle (LM and gluteus medius, GM), and subcutaneous fat (SF) content and fatty acid composition. Following a split-plot design, 32 castrated Duroc pigs, half of each of the two homozygous *SCD* genotypes (CC and TT) were subjected from 165 to 195 d of age to two finishing diets differing in the PVA carotenoid content (C+ and C). Both diets were identical except for the corn line used in the feed. The C+ diet was formulated with 20% of a carotenoid-fortified corn (M37W-Ph3) while the C diet used instead 20% of its near isogenic M37W line, which did not contain PVA carotenoids. No vitamin A was added to the diets. The C diet was estimated to provide at most 1300 IU of vitamin A/kg and the C+ diet to supply an extra amount of at least 800 IU vitamin A/kg. As compared to the C diet, pigs fed with C+ diet had threefold more retinoic acid ($P < 0.01$) and fourfold more SCD gene expression in liver ($P = 0.06$). The diet did not affect performance traits and backfat thickness, but pigs in the C+ diet had less fat (4.0% vs 5.0%, $P = 0.07$) and MUFA (18.3% vs 22.5%, $P = 0.01$) in liver, less IMF (5.4 % vs 8.3%, $P = 0.04$) in GM, and more fat content (90.4% vs 87.9, $P = 0.09$) and MUFA (48.0% vs 46.6%, $P = 0.04$) in SF. The TT genotype at the *SCD* gene increased MUFA ($P < 0.05$) in all tissues (liver: 21.4% vs 19.5%, LM: 55.0% vs 53.1%; GM: 53.9% vs 51.7%, and SF: 48.0% vs 46.7%, for TT and CC genotypes, respectively). Liver fat and MUFA content declined non-linearly with liver all-trans retinoic acid, indicating a saturation point at relatively low all-trans retinoic acid content. The results obtained provide evidence for a complementary role between dietary PVA and *SCD* genotype, in the sense that the TT pigs fed with a low-PVA diet are expected to show higher and more monounsaturated IMF without increasing total fat content.

Key words: growth, intramuscular fat, liver, oleic acid, pork, vitamin A.

1. Introduction

Intramuscular fat (**IMF**) and monounsaturated fatty acid (**MUFA**) content play major roles in meat quality and healthy diets profile. The continued selection for lean content practiced for decades in commercial pig lines has led to IMF below recommended levels for consumer satisfaction (Wood et al., 2008). Several genetic (Ros-Freixedes et al., 2012) and nutritional (Doran et al., 2006) strategies have been investigated to increase IMF and without increasing subcutaneous fat (**SF**). Regarding genetics, molecular markers with a specific effect over MUFA could be very useful for selection. This is the case of a mutation in the promoter of the stearoyl-coA desaturase (***SCD***) gene, which increases MUFA but not overall fatness (Estany et al., 2014). As for nutritional strategies, adjusting dietary vitamin A at critical stages for IMF adipocyte formation can

specifically promote IMF without increasing SF (Wang et al., 2016). Previous experiments in cattle and pigs provided evidence that restricting vitamin A intake in finishing diets improves IMF (D'Souza et al., 2003; Pickworth et al., 2012) and MUFA (Ayuso et al., 2015a,b) with minimal impact on carcass composition. These effects can be partly explained because vitamin A, after being absorbed, is converted into retinoic acid, which coregulates key adipogenic genes, including *SCD* (Wang et al., 2016). Interestingly, the mutation described in Estany et al. (2014) is positioned in the core sequence of several putative retinoic acid response elements.

The β-carotene and other pro-vitamin A (**PVA**) carotenoids are a source of dietary vitamin A. Different staple crops biofortified with vitamins are available (Farré et al., 2011), including high carotenoid corn producing high levels of β-carotene (Zhu et al., 2008). Therefore, the objective of this study was to investigate the combined effect of PVA carotenoid intake and *SCD* genotype on IMF and MUFA. The relationship of hepatic retinoids with liver *SCD* expression and fat content and composition is also discussed.

2. Materials and methods

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida (Agreement CEEA 02-04/14) and performed in accordance with authorization 7704 issued by the Catalan Ministry of Agriculture, Livestock, Fisheries and Food, Spain.

2.1. Pigs and experimental design

Thirty-two castrated Duroc pigs, half of which carried the CC genotype and half the TT genotype for the *AY487830:g.2228T>C* polymorphism at the *SCD* gene (Estany et al., 2014), were chosen for the experiment. All of them were from the same Duroc genetic line (Ros-Freixedes et al., 2012), which was completely closed in 1991 and since then selected for an index including BW, backfat thickness (**BT**), and IMF (Solanes et al., 2009). The pigs were sampled from litters produced by 23 dams and 7 sires which were born within the range of 4 d. The piglets were castrated within the first week of age and, at 70 days (SD 1.3), moved to the CEP Pig Research Centre, Torrelameu, Lleida, where they were kept until slaughter in 8 pens of 4 pigs for a 2 x 2 split-plot arrangement. The two treatments consisted of two finishing diets differing in the PVA carotenoid content and the two *SCD* homozygotes. Thus, each diet was given to four alternate pens, each one housing

two CC and two TT piglets of similar weight. Sires were equally represented by diet, with at least 4 sires having offspring of each genotype.

2.2. Diets

The pigs were subjected to a four-phase feeding program during the experiment. In all phases the pigs had ad libitum access to cereal-based commercial diets but only in the fourth, which was given during the finishing period from 165 to 195 days of age, the diets were formulated to specifically contain different amount of PVA carotenoids. In the first three phases (from 70 to 110 d; from 110 to 140 d; and from 140 to 165 d), four pens were fed a regular-protein diet (17%, 16%, and 15% CP, respectively) and four with a low-protein diet (15%, 14% and 13% CP, respectively). Diets in the first phase were supplemented with 8000 IU vitamin A per kg of feed and diets in the second and third phase with 6500 IU per kg of feed. A full description of the diets used in these three first phases is given in supplementary **Table S1**. At the start of the fourth and last phase, four pens (two from the regular and two from the low-protein treatment) were fed an enriched–carotene diet (C+) and four pens (two from the regular and two from the low-protein treatment) a control diet (C). The two diets in the fourth phase were identical in terms of both ingredients and chemical composition (**Table 1**) except for the corn line used in the feed formulation. Thus, the C+ diet was prepared using the M37W-Ph3 carotenoid-fortified corn (Zhu et al., 2008) and the C diet using its near isogenic M37W line, which only contains traces of carotenoids (primarily lutein and zeaxanthin). The nutrient composition of these two corn genetic types is reported in supplementary **Table S2**. No vitamin-mineral premix, including vitamin A, was added in the formulation of the feed for C+ and C diets. The C diet was estimated to provide at most 1300 IU of vitamin A/kg and the C+ diet to supply an extra amount of at least 800 IU vitamin A/kg. These feeds were manufactured in one batch at the beginning of the fourth phase. Nutrient and fatty acid (**FA**) analyses were performed in duplicate as described in Cánovas et al. (2009). Feed protein was hydrolyzed by incubating a sample of 500 mg in 5 mL 6N HCl for 12 h at 110ºC. An aliquot of 25 µL of hydrolysate was evaporated under nitrogen steam and re-diluted in 500 µL of water/acetonitrile (20/80 vol/vol). Quantitation of lysine was performed by using an UPLC coupled to a triple quadrupole mass spectrometer system. Carotenoids in whole corn were extracted by using the method of Folch et al. (1957) and analyzed as indicated below for liver retinoids.

Table 1. Ingredients and nutrient content of the diet fed during the finishing period (as-fed basis)

Item	g/kg
Ingredients	
Barley	571.5
Canola	90.3
Wheat shorts	52.6
Corn [1]	206.6
Blended animal: vegetal fat (3:5)	39.6
Monocalcium phosphate	9.4
Salt	8.9
Calcium carbonate	7.2
L-Lysine (50%)	5.4
DL-Methionine (99%)	0.7
L-Threonine	0.7
Nutrient content	
Dry matter	875.1
Crude protein[2]	121.5
Total Lysine, %	0.7
GE, MJ/kg	16.0
Crude fat	61.2
Crude fiber	43.1
Ash	47.1
Fatty acids, % FA	
C16:0	20.4
Total SFA [3]	26.9
C18:1,cis-9	34.7
Total MUFA [4]	38.2
C18:2,cis-9,12	31.3
Total PUFA [5]	34.9

[1]The C+ diet used the fortified corn line M37W-Ph3 (7.0 μg/g DM of β-carotene) while the C diet used its near isogenic corn line M37W (non-detected β-carotene content). The C+ diet is estimated to provide 800 IU of vitamin A/ kg more than the C diet.
[2] Amino acid content (%) calculated according to FEDNA (2010): Lys= 0.68; Ileu =0.38; Met =0.26; Thr =0.44; Val= 0.52; Met + Cys= 0.49; Trp =0.12.
[3]Total SFA, saturated fatty acids: C10:0 + C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0 + C24:0
[4]Total MUFA, monounsaturated fatty acid: C14:1 cis-9 + C16:1, cis-9 + C17:1, cis-10

+ C18:1, trans-9 + C18:1, cis-9 + C20:1, cis-11 + C22:1, cis-13
[5]Total PUFA, polyunsaturated fatty acids: C18:2,trans-9,12 + C18:2,cis-9,12 + C18:3,cis-6,9,12 + C18:3,cis-9,12,15 + C20:2,cis-11,14 + C20:3,cis-11,14,17 + C20:4,cis-5,8,11,14

2.3. Performance traits and sample collection

Weekly records of BW were taken throughout the experiment. The average feed intake per pen was also calculated on a weekly basis. At the end of the finishing period the pigs were humanely euthanized and carcass BT at 6 cm off the midline between the third and fourth last ribs was ultrasonically estimated (Renco Corp., Minneapolis, MN, US). Then, a representative sample of liver, SF and muscles gluteus medius (**GM**) and LM were taken and stored at -80ºC until required.

2.4. Analysis of vitamin A metabolites in liver

Liver samples and standards were stored in the dark and handled under red light. Liver tissue samples where lyophilized and ground to powder in liquid nitrogen to avoid thermal degradation. An aliquot of 100 mg of freeze-dried tissue was accurately weighed in a 15 mL Falcon tube. Then, 200 ng of all-trans-retinoic acid-D5 (Toronto Research Chemicals, Toronto, Canada) was spiked in 50 µl of acetonitrile/methanol/acetone (7/3/5; vol/vol/vol) containing 0.01% of butylated hydroxytoluene. At this point, 2 mL of acetonitrile/water (1/1; vol/vol) and 60 µL 4N HCl were added to each sample, which were then vortexed for 30 min. Retinoids were extracted twice with 5 mL of hexane by vortexing for 5 min and centrifuging at 1811 *g* for 5 min at room temperature. Extracts were evaporated under a stream of nitrogen at 32ºC and reconstituted in 1 mL of acetonitrile/methanol/acetone (7/3/5; vol/vol/vol) containing 0.01% of butylated hydroxytoluene. Finally, extracts were filtered through a 0.20 µm PTFE filter and transferred to an amber autosampler vial.

Quantitation of retinoids was performed using a UPLC Acquity system (Waters, Milford, MA, USA), equipped with an Acquity UPLC BEH C18 column (2.1 x 150 mm; 1.7 µm). Solvent A was water/methanol 98/2 with 0.1% formic acid; solvent B was acetonitrile/methanol 7/3 with 0.1% formic acid. The gradient included four steps. Initial conditions were 30% A and 70% B maintained for 1 min at 0.4 mL/min. Then, from 30% to 100% B in 13 min; from minute 13 to 23 flow was switched to 0.6 mL/min. From minute

23 to 25 initial conditions were regained. Weak and strong washing solvents were 80/20 acetonitrile/water and isopropanol, respectively. The injection volume was 2.5 µL. Quantitation was performed by using a Multiple Reaction Monitoring method in a Waters XEVO TQD mass spectrometer (Micromass MS Technologies, Manchester, UK). The system was equipped with an APCI source operated in positive ion mode. The parameters in the source were set as follows: source temperature, 150ºC; probe temperature 450ºC; cone gas flow 150 L/h; collision gas flow 0.25 mL/min. Retinoid transitions (supplementary **Table S3**) were determined by using commercial standards (all-trans retinol, all-trans retinal, keto-retinoic acid, all-trans-4-hidroxy retinoic acid, all-trans-retinoic acid, 9-cis-retinoic acid, 9,13-cis-retinoic acid, 13-cis-retinoic acid, all-trans retinoic-d5 acid, and all-trans-5-6-epoxy retinoic acid were purchased from Toronto Research Chemicals, Toronto, Canada; retinyl-palmitate was purchased from Sigma-Aldrich Quimica SL, Spain). Cone voltage and collision energy were optimized individually. Calibration curves were constructed from the commercial compounds and diluted to a series of appropriate concentrations with acetonitrile/methanol/acetone (7/3/5; vol/vol/vol) containing 0.01% of butylated hydroxytoluene. Data were processed using QuanLynx software. For retinoid quantification all-trans retinoic acid-d5 was used as internal standard.

2.5. *Fatty acid analysis*

A representative aliquot from each pulverized freeze-dried sample was used for fat analysis. The IMF content and FA composition of liver, GM, LM and SF was determined in duplicate by gas chromatography (Bosch et al., 2009). Fatty acid methyl esters were directly obtained by transesterification using a solution of 20% boron trifluoride in methanol (Rule, 1997). Methyl esters were determined by gas chromatography using a capillary column SP2330 (30 m × 0.25 mm, Supelco, Bellefonte, PA, USA) and a flame ionization detector with helium as carrier gas. Runs were made with a constant column-head pressure of 172 kPa. The oven temperature program increased from 150 to 225ºC at 7ºC/min and injector and detector temperatures were both 250ºC. The quantification was carried out through area normalization after adding into each sample 1,2,3-tripentadecanoylglycerol as internal standard. Intramuscular fat content was predicted as the sum of each individual fatty acids expressed as triglyceride equivalents (AOAC, 1997).

2.6. Analysis of gene expression

Total RNA was isolated from the liver of all pigs with TRI reagent (Sigma-Aldrich, St Louis, MO, USA) using the manufacturer's instructions. Two µg of RNA was digested with Turbo DNA-free DNase (Fisher Scientific, Madrid, Spain) and retrotranscribed into cDNA using RevertAid Premium Reverse Transcriptase (Fisher Scientific) using standard conditions with a mix of oligo(dT) and random primers at 1.25 µM each. Reactions were incubated for 10 min at 25ºC and 20 min at 37ºC. Upon completion, cDNA were diluted 1:30 in H_2O and stored at -40ºC. Quantitative PCR reactions for the *SCD*, and two reference genes (*RPL32* and *B2M*) were set up in triplicate for each sample with primers and conditions as described in Estany et al., (2014).

2.7. Statistical analysis

The effects of diet and *SCD* genotype were analyzed with a mixed model, which included the diet (C+ and C), the genotype (TT and CC) and the diet by genotype interaction as fixed effects, and the pen nested within diet as a random effect. Because feed intake was recorded on a pen basis, the model for feed intake and feed conversion ratio only included the diet. The analyses were performed either with or without adding in the model the covariates age at slaughter (for BW, BT, fat content in liver and IMF) or fat content (for FA composition traits). The potential effect of the diet given in earlier feeding phases was analyzed using the above model. No differences were observed between the regular and the low-protein diet on target traits and therefore the previous diet was not included in the model. The relationship amongst retinoids was assessed using a multivariate restricted maximum likelihood correlation analysis and that of retinoids with *SCD* gene expression and liver fat content and composition with a log-log regression analysis. Results were considered statistically significant at $P \leq 0.05$ whereas trending effects were discussed at $P \leq 0.10$. All the analyses were performed using the statistical package JMP 12 (SAS Institute Inc., Cary, NC, USA).

3. Results

Upon absorption, PVA carotenoids are partially converted into vitamin A (retinol, retinal, retinoic acid and intermediate compounds) mainly in gut and liver. Liver, in addition, is the main site of vitamin A storage and metabolism. Thus, we first assessed the

impact of differential dietary carotenoid intake on the hepatic retinoid profile and then the combined effect of carotenoid intake and *SCD* genotype on fat content and composition. Results were used thereafter to investigate the relationship amongst liver retinoid content, *SCD* gene expression and fat content and composition. Because the interaction between diet and the *SCD* genotype were detected ($P < 0.10$) for a very low number of traits, means are separately presented for each factor and interactions only discussed where relevant. In line with previously reported studies, means are presented adjusted for covariates, although results did not change when excluding them from the analyses.

3.1. Effect of diet and genotype on liver retinoid content

The diet, but not the genotype, influenced the retinoid content of liver (**Table 2**). However, there was evidence of interaction between the diet and the *SCD* genotype for all-trans retinol ($P = 0.02$) and all-trans-5-6-epoxy retinoic acid ($P = 0.06$), for which the effect of the diet was only evident in the CC pigs. Thus, for all-trans retinol, there was a favorable effect of the C+ diet in the CC pigs (12.0 μg/g DM vs 4.6 μg/g DM, $P < 0.01$) but not in the TT pigs (5.6 μg/g DM vs 6.3 μg/g DM, $P = 0.77$). Feeding pigs with the C+ diet increased all-trans retinoic acid (2.8-fold), 13-cis retinoic acid (1.8-fold) and all-trans-5-6-epoxy retinoic acid (2.2-fold) in liver with respect to the C diet ($P < 0.01$). This trend was also observed for all-trans retinol ($P = 0.10$), but not for all-trans retinal ($P = 0.16$) and retinyl palmitate content ($P = 0.50$). Other analyzed retinoids (all-trans-4-hidroxy retinoic acid; 9-cis-retinoic acid; 9,13-cis-retinoic acid; and all-trans-5-6-epoxy retinoic acid) were not detected in liver. The correlations between liver retinoids were moderate to strong, ranging from 0.56 to 0.87, except for retinyl palmitate, the main storage form, which was not correlated with the rest of retinoids (**Table 3**).

Table 2. Concentration of retinoids in liver by diet and *SCD* genotype

	Diet[1]		*SCD* genotype[2]		Diet*_SCD_
Retinoid[3], µg/g DM	C	C+	CC	TT	P-value
All-trans retinol	5.5±1.2	8.7±1.1	8.2±1.2	6.0±1.0	0.02
All-trans retinal	3.2±0.4	4.2±0.4	3.6±0.5	3.7±0.5	0.14
All-trans retinoic acid	0.2±0.0[b]	0.5±0.0[a]	0.3±0.1	0.3±0.0	0.26
13-cis retinoic acid	0.1±0.0[b]	0.1±0.0[a]	0.1±0.0	0.1±0.0	0.27
Epoxy retinoic acid (x10)	0.1±0.0[b]	0.3±0.0[a]	0.2±0.0	0.2±0.0	0.06
Retinol/Retinal	2.1±0.3	2.0±0.3	2.3±0.2	1.8±0.2	0.08
Retinoic/Retinal (x10)	0.3±0.1[b]	0.6±0.1[a]	0.4±0.1	0.5±0.1	0.41
Retinyl palmitate	782.8±126.3	655.4±122.1	680.7±120.8	757.6±108.9	0.16

[1]Diet C+: diet with 20% carotenoid-fortified corn M37W-Ph3; Diet C: diet with 20% of the near isogenic M37W line, which does not contain pro-vitamin A carotenoids.
[2]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*
[3]Epoxy retinoic acid: all-trans-5-6-epoxy retinoic acid. All-trans-4-hidroxy retinoic acid; 9-cis-retinoic acid; 9,13-cis-retinoic acid; and keto-retinoic acid were analyzed but not detected.
[a,b] Within row and factor, means with different superscripts differ significantly ($P \leq 0.05$).

Table 3. Correlation among retinoids in liver

Retinoid[1]	R	RL	RA	RC	RE	RP
All-trans retinol (R)	-	**0.77**	**0.69**	**0.82**	**0.86**	0.04
All-trans retinal (RL)		-	**0.69**	**0.61**	**0.72**	-0.10
All-trans retinoic acid (RA)			-	**0.59**	**0.56**	-0.11
13-cis retinoic acid (RC)				-	**0.87**	-0.22
All-trans-5-6-epoxy retinoic acid (RE)						-0.24
Retinyl palmitate (RP)						-

[1]Correlations in bold are significant at $P \leq 0.05$

3.2. Effect of diet and genotype on fat content and composition

The diet and the *SCD* genotype had no effect on weight gain, feed intake or feed conversion ratio while pigs were raised on the experimental diets (supplementary **Table S4**). The difference observed between *SCD* genotypes in BW (P = 0.03) reflected the heavier BW of TT pigs at the start of the experimental feeding phase (P = 0.05). In general,

the C+ diet decreased fat content in liver and muscle (**Table 4**), but not in SF. Thus, pigs fed on diet C+ had less fat in liver (4.0% vs 5.0%, P = 0.07), in LM (4.2% vs 4.8%, P = 0.26), and in GM (5.4% vs 8.3%, P = 0.04) than diet C-fed pigs. In contrast, the C+ diet not only did not affect BT (P = 0.53) but showed a trend to increase fat content in SF (90.4% vs 87.9%, P = 0.09). The diet also had an impact on FA composition, although with opposite effects in liver (where MUFA decreased from 22.5%, in diet C, to 18.3%, in diet C+, P = 0.01) and SF (where MUFA increased from 46.6%, in diet C, to 48.0%, in diet C+, P = 0.04). Accordingly, the pigs fed on diet C+ had a lower MUFA/SFA ratio in liver (0.53 vs 0.65, P = 0.01). As expected, the *SCD* genotype did not alter fat content in any of the analyzed tissues but affected the MUFA/SFA ratio in all of them. As compared to CC pigs, the TT pigs showed a greater MUFA/SFA ratio in liver, GM, LM and SF due to an increment in MUFA at the cost of SFA (**Table 4**). The effects of the diet, the *SCD* genotype on each individual fatty acid, together with the significance of their interactions, are shown in supplementary **Tables S5**, **S6**, **S7** and **S8**, for liver, LM, GM, and SF, respectively.

Table 4. Least square means (±SE) for backfat thickness, intramuscular fat (IMF) content and fatty acid composition in liver, muscle and subcutaneous fat by diet and *SCD* genotype

	Diet[1]		*SCD* genotype[2]		Diet**SCD**
Trait[3]	C	C+	CC	TT	P-value
n	14	15	13	16	
Liver					
Fat, %	5.0±0.3	4.0±0.3	4.3±0.3	4.6±0.3	0.50
SFA, %	34.6±0.2	34.9±0.2	35.0±0.2	34.5±0.2	0.39
MUFA, %	22.5±0.7^{a}	18.3±0.7^{b}	19.5±0.7^{b}	21.4±0.6^{a}	0.94
PUFA, %	42.9±0.7^{b}	46.8±0.7^{a}	45.5±0.6	44.1±0.6	0.86
MUFA/SFA	0.65±0.02^{a}	0.53±0.02^{b}	0.56±0.02^{b}	0.62±0.02^{a}	0.77
m. longissimus					
IMF, %	4.8±0.4	4.2±0.3	4.5±0.3	4.5±0.3	0.67
SFA, %	36.4±0.4	35.2±0.4	36.7±0.4^{a}	34.9±0.4^{b}	0.71
MUFA, %	53.5±0.6	54.6±0.5	53.1±0.5^{b}	55.0±0.5^{a}	0.27
PUFA, %	10.0±0.4	10.3±0.4	10.2±0.3	10.1±0.3	0.11
MUFA/SFA	1.48±0.03	1.56±0.03	1.45±0.03^{b}	1.58±0.03^{a}	0.59
m. gluteus medius					
IMF, %	8.3±0.8^{a}	5.4±0.8^{b}	6.8±0.8	6.9±0.7	0.55
SFA, %	34.5±0.3	34.4±0.3	35.3±0.4^{a}	33.6±0.4^{b}	0.65
MUFA, %	52.4±0.5	53.2±0.5	51.7±0.5^{b}	53.9±0.5^{a}	0.51
PUFA, %	13.0±0.7	12.4±0.6	13.0±0.6	12.4±0.5	0.34
MUFA/SFA	1.52±0.02	1.56±0.02	1.47±0.02^{b}	1.61±0.02^{a}	0.80
Subcutaneous fat					
Backfat thickness, mm	24.7±1.1	25.8±1.1	24.5±1.0	26.0±0.9	0.37
Total fat, %	87.9±0.9	90.4±0.9	88.7±0.7	89.5±0.7	0.78
SFA, %	36.1±0.4	36.1±0.3	37.4±0.6^{a}	34.9±0.5^{b}	0.61
MUFA, %	46.6±0.4^{b}	48.0±0.4^{a}	46.7±0.4^{b}	48.0±0.4^{a}	0.96
PUFA, %	17.4±0.5	15.8±0.4	16.1±0.5	17.0±0.5	0.62
MUFA/SFA	1.31±0.02	1.33±0.02	1.26±0.03^{b}	1.38±0.03^{a}	0.74

[1]Diet C+: diet with 20% carotenoid-fortified corn M37W-Ph3; Diet C: diet with 20% of the near isogenic M37W line, which does not contain pro-vitamin A carotenoids.

[2]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*
[3]In muscles and subcutaneous fat: SFA, saturated fatty acids: C10:0+C12:0+C14:0+C16:0+C17:0 +C18:0 +C20:0; MUFA, monounsaturated fatty acids: C16:1n-9+C17:1n-7+C18:1n-7+C18:1n-9+C20:1n-9; and PUFA, polyunsaturated fatty acids:C18:2n-6+C18:3n-3+C20:2n-6+C20:4n-6. In liver, SFA also included C13:0+C15:0+C22:0; MUFA, C15:1n-5+C20:1n-9+C24:1n-9; and PUFA: C20:3n-6+C20:5n-3+C22:6n-3.
[a,b]Within row and factor, means with different superscripts differ significantly ($P \leq 0.05$)

3.3. Relationship of fat content and composition with liver retinoids

Liver fat content showed a negative correlation with liver retinoids, except for retinyl palmitate, where no association was detected. This general trend was most evident for liver all-trans retinoic acid (P = 0.03; **Figure 1**). The plot of liver fat content against liver all-trans retinoic acid exhibited a nonlinear relationship, with greater expected responses at low levels of retinoids, indicating the existence of a saturation point at higher contents. Liver FA composition also changed with liver retinoid content. In particular, MUFA in liver was negatively correlated to all measured retinoids (see **Figure 2** for all-trans retinoic acid, P < 0.01) whereas the opposite was observed for PUFA. The only exception was for retinyl palmitate, which only showed a negative association with SFA (P < 0.01). These relationships were maintained after adjusting for fat content, thereby denoting that these compositional changes were due to a direct effect of retinoids on liver FA composition rather than to an indirect effect resulting from correlated changes in fat content. The pattern of relationships of liver retinoids with IMF in GM and LM was consistent, although less strong, with that observed with liver fat content. In contrast, liver retinoids were not correlated with MUFA and MUFA/SFA in GM, LM and SF. However, a negative effect of the three retinoic acids on PUFA content in SF was found (P < 0.05).

Figure 1. Relationship between fat content and all-trans retinoic acid [log (fat content) =1.28 - 0.14* log (all-trans-retinoic acid); R^2 = 0.16] in the liver of pigs with CC (●) and TT (●) genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*

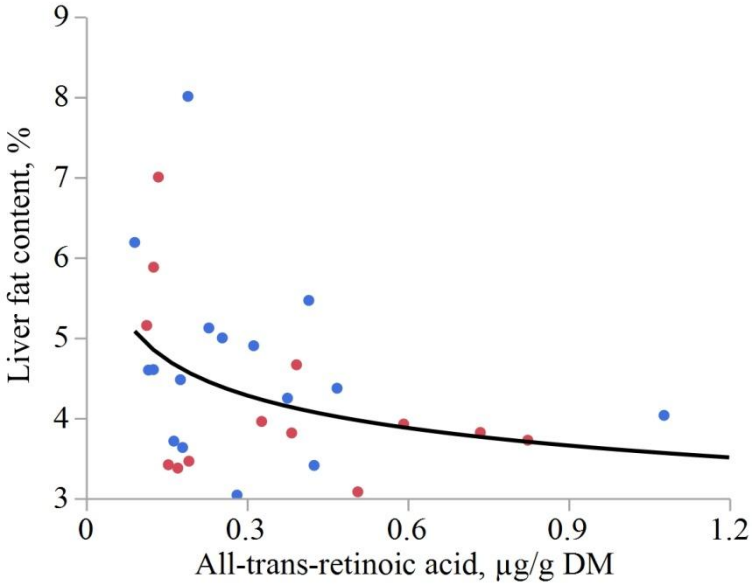
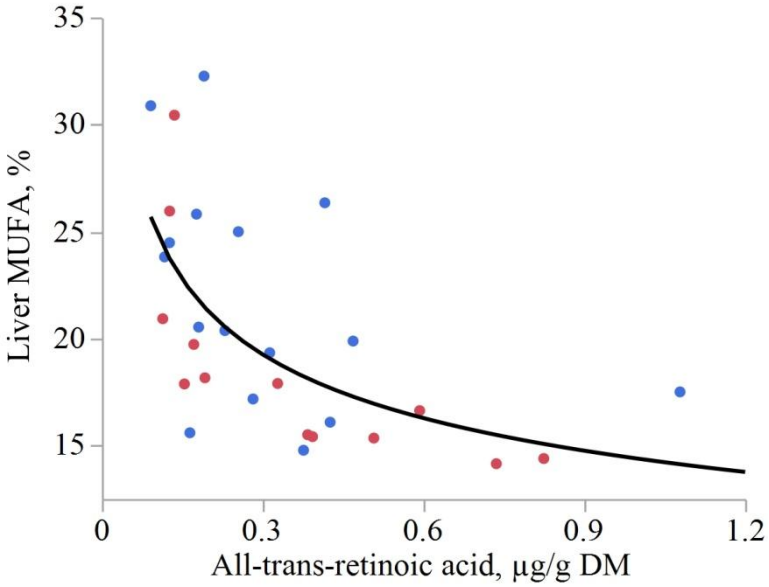
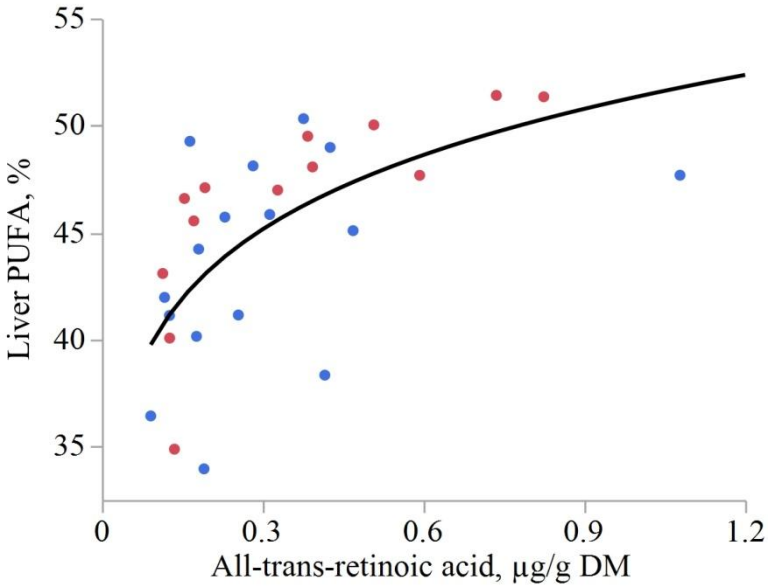


Figure 2. Relationship of (**a**) monounsaturated [log (MUFA) = 2.67- 0.24 * log (all-trans-retinoic acid); R^2 = 0.42] and (**b**) polyunsaturated [log (PUFA) = 3.94 + 0.11* log (all-trans-retinoic acid); R^2 = 0.37] fatty acids with all-trans retinoic acid in the liver of pigs with CC (🔴) and TT (🔵) genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*

a)



b)



3.4. Effect of diet and genotype on SCD gene expression

In liver, the C+ diet induced a 4.5-fold increase of *SCD* gene expression compared to the C diet, regardless of the *SCD* genotype (**Figure 3**, P = 0.06). In line with this result, liver *SCD* gene expression increased with the three retinoic acids (**Figure 4** for all-trans retinoic acid; P < 0.01), with a similar trend for all-trans retinol (P = 0.10). No relationship between *SCD* gene expression and all-trans retinal (P = 0.18) and retinyl palmitate (P = 0.17) was detected. The higher expression of liver *SCD* was associated with lower (P = 0.01) MUFA and higher (P = 0.01) PUFA (**Figure 5**), but not to changes in total liver fat and SFA. Regarding the other tissues, liver *SCD* expression was positively correlated to IMF in GM (P = 0.01).

Figure 3. Least square means for *SCD* gene expression in liver by diet (C: diet with the near isogenic M37W line; C+: diet with the carotenoid-fortified corn M37W-Ph3) and *SCD* genotype (CC and TT genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*). Means with different letters within effect differ at P ≤ 0.10

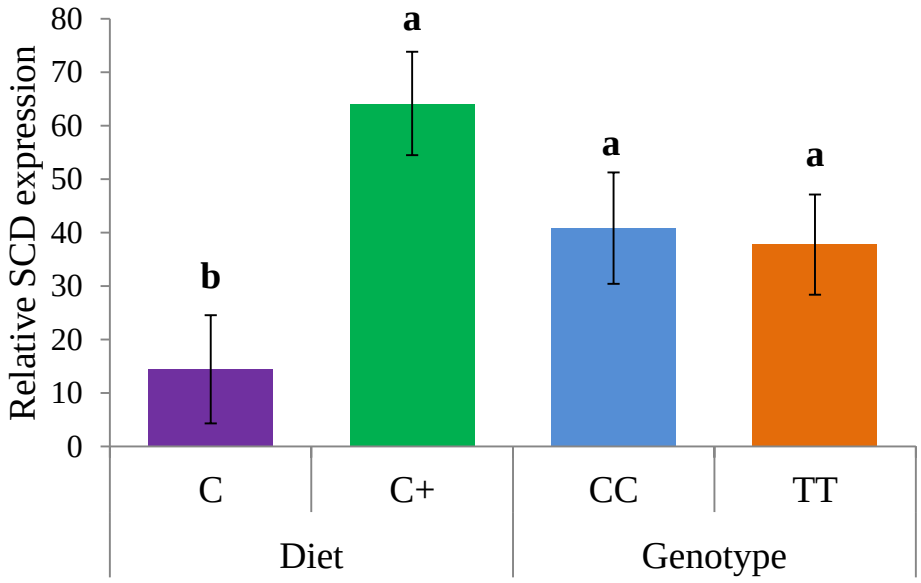


Figure 4. Relationship between *SCD* expression and all-trans retinoic acid [log (relative SCD expression) = 4.32 + 0.94 * log (all-trans-retinoic acid); R^2 = 0.26] in the liver of pigs with CC (●) and TT (●) genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*

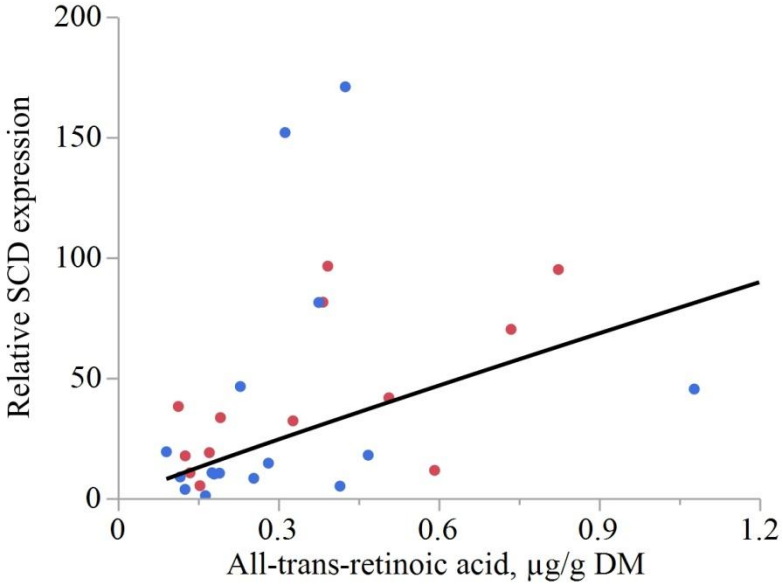
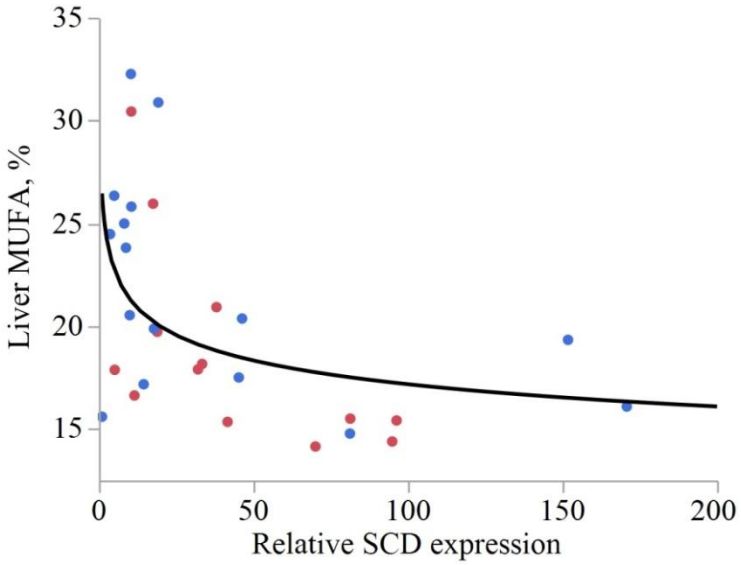
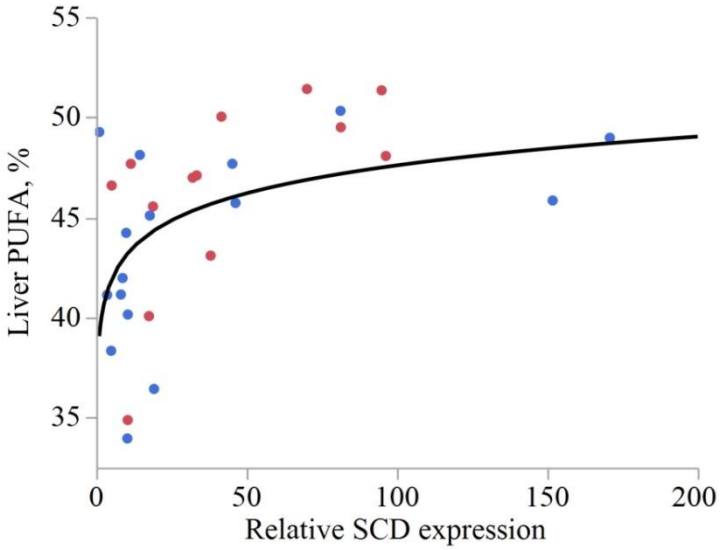


Figure 5. Relationship of (**a**) monounsaturated [log (MUFA) = 3.28 - 0.09 * log (relative SCD expression); R^2 = 0.22] and (**b**) polyunsaturated [log (PUFA) = 3.67 + 0.04 * log (relative *SCD* expression); R^2 = 0.21] fatty acids with *SCD* gene expression in the liver of pigs with CC (🔴) and TT (🔵) genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*

a)



b)



4. Discussion

Vitamin A is crucial for many physiological functions, daily BW gain, and immune status. As pigs are not very efficient converting β-carotene to vitamin A, with only around one third of the conversion efficiency observed in rats or poultry (Mc Dowell, 2000), commercial diets are regularly supplemented with PVA compounds. The two most common types of feed supplementation are PVA carotenoids (mostly β-carotene) and preformed vitamin A (mainly retinyl esters). Carotenes are absorbed and converted into all-trans retinoic acid mainly in the gut and the liver, where it is further processed into other active compounds. Excess of vitamin A is stored in the liver as retinyl palmitate, which represents around 50 to 80% of total retinol (Blomhoff and Blomhoff, 2006).

The carotenoid-rich (C+) diet increased the content of retinoids in liver, in line with reported trials supplying different amounts of commercial vitamin A (Olivares et al., 2009a; Olivares et al., 2011; Tous et al., 2014). In the body, vitamin A is found in three main circulating vitamers differing in their oxidation state (Bonet et al., 2003): the hydroxyl form (retinol), the aldehyde form (retinal) and the carboxylic acid form (retinoic acid). Our results indicate that all three are well correlated and retinol serves as a good predictor of the other two. Reported values of liver retinol, the most abundant of the three and, as such, the most referenced retinoid, vary across experiments and species. The amount of liver retinol observed in our trial should be considered in the low range of reported values. However, the main retinoid molecule in the liver was retinyl palmitate (the storage form), which did not differ significantly between C and C+-fed pigs. Then, considering all retinoids, the observed total amount of vitamin A in terms of retinol equivalents was within the expected range (EFSA, 2008). Different reports suggest that PVA carotenoids and retinol have the same effect on liver retinol levels. Condron et al. (2014), in feedlot cattle, did not find any difference in liver retinol, retinyl palmitate and retinoic acid between diets equivalently supplemented with synthetic β-carotene or retinyl palmitate. In laying hens, no differences in liver retinol content were found between diets supplemented either with high carotenoid corn or retinol (Moreno et al., 2016).

Our experiment indicates that restricted PVA diets (like diet C) promote IMF deposition with no change in production traits, in line with previous findings in cattle (Siebert et al., 2006; Gorocica-Buenfil et al., 2007; Kruk et al., 2008; Pickworth et al.,

2012) and pigs (D'Souza et al., 2003; Olivares et al., 2011; Ayuso et al., 2015a,b). Nonetheless, in pigs, the favorable effect of restricted vitamin A intake on IMF is less consistent across genetic types (Olivares et al., 2009c) and more variable with respect to the intensity and duration of the restriction period (Olivares et al., 2009a; Olivares et al., 2011) and the target muscle (Ayuso et al., 2015a). Moreover, this effect was not replicated in all experiments (Olivares et al., 2009b; Tous et al, 2014). Several factors can explain such heterogeneous responses. To start with, pre-experimental conditions determine the amount of vitamin A stored in the liver, which can become high (O'Byrne and Blaner, 2013) and can provide active retinol compounds to the body for a certain amount of time. Also, differences in the timing of fat development among genetic types, tissues and muscles are additional contributing factors. For instance, with respect to C+ diet, the effect of C diet on fat accumulation was greater in GM (1.5-fold) than in LM (1.14-fold), intermediate in liver (1.25-fold) and negligible in SF. Thus, IMF and BT are distinctly affected by dietary restriction of carotenoids, probably due to the differential effect of retinoic acid on lipid accumulation with age. The development of adipocytes is essential for fat accretion and active vitamin A vitamers modulate adipogenesis through acting as a ligand for retinoic acid receptors. Studies in pigs have shown that preadipocyte hyperplasia is an early process, with a fast decline after 40 days of age, at a time when hypertrophy begins, increasing markedly with age (Dunshea and D'Souza, 2003). However, differences in fat development across breeds can modulate this timing. Thus, in Iberian pigs, Ayuso et al. (2015b) showed that the preadipocyte number in LM increased when vitamin A supplements were not added to the feed from two months of age. Subcutaneous fat develops and matures prior to IMF and thus preadipocyte formation and hyperplasia happen earlier in SF than in IMF. This means that during growth hypertrophy is the key driver for SF deposition (Nakajima et al., 2011) and hyperplasia for IMF (Damon et al., 2006; Hausman et al., 2014). Because vitamin A is expected to promote hyperplasia in preadipocytes and reduce hypertrophy in mature adipocytes, its intake at critical periods for IMF formation and accretion can lead to enhance IMF content without increasing BT. It has been postulated that the differential effect of retinoic acid on progenitor cells and mature adipocytes is due to the stage-specific expression of related transcription factors (Wang et al., 2016). Altogether, our results are indicative that a carotenoid-restricted finishing diet, even for a short period, enables pigs to enhance IMF without substantial variation in BT. In accordance with the expected effects of vitamin A over fat deposition, it can be hypothesized that a greater response could have

been obtained if restriction at finishing had been accompanied of enhanced-vitamin A starter or growing diets.

The pigs fed with diet C+ had higher levels of the three vitamers (retinol, retinal and retinoic acid) in liver than the control pigs and accumulated less fat in this tissue, confirming the inhibitory role of retinoids in fat deposition. The liver fat content decreases with all-trans retinoic acid in liver, but the response was higher at low concentrations, suggesting a saturation point at relatively low values. Thus, the relative high response of the diet on liver fat could be due to the low content of all-trans retinoic acid found in our trial as compared to other reported trials. Pigs under the C diet were provided with a feed containing the equivalent to 1300 IU of vitamin A/kg, which roughly corresponds to the National Research Council recommended intake of vitamin A for growing pigs (NRC, 2012), and pigs fed C+ diet had an extra daily intake of around 1000 IU of vitamin A. These values of vitamin A intake allows us to postulate that a relatively small but continuous amount of carotenoid intake over the recommended level would suffice to induce changes in fat metabolism and beneficial effects in the prevention and treatment of fat liver-related diseases.

The effect of the diet showed an opposite pattern on the FA composition of liver and SF, with diet C+ decreasing MUFA in liver but increasing it in SF. Carotenoids also increased liver *SCD* expression by 4.5-fold, in line with expectations (Ntambi and Miyazaki, 2004). This enzyme is directly involved in the biosynthesis of MUFA from SFA. However, in liver there is a clear inverse relationship between *SCD* expression levels and MUFA content. In liver, the endogenously synthesized MUFA are the main substrate for the synthesis of hepatic triglycerides and cholesterol esters, which are released to the bloodstream in the form of very-low-density lipoprotein (VLDL) (Hodson and Fielding, 2013). Thus, in agreement with previous reports, enhanced *SCD* expression results in lower fat and MUFA content through the release of de novo fatty acids to circulating VLDL (Peter et al., 2011; Silbernagel et al., 2012) which indirectly affects PUFA liver content. Taken together, these results confirm the relationship between dietary PVA, liver *SCD* expression and total fat and MUFA content in liver reported in lean humans and mice but not in obese individuals (Peter et al., 2011). Circulating MUFA are delivered in VLDL target tissues (muscle, heart and adipose), increasing MUFA content in SF, the main tissue

of fatty acid uptake. Previous studies in pigs have not found consistent results of dietary vitamin A on liver, SF and muscle FA profile. In contrast with our results, Olivares et al. (2009b) found that dietary vitamin A increased SFA and decreased PUFA in liver. The trends observed by Ayuso et al. (2015a), although not significant, were more in line with ours. Results in SF were also contradictory, with studies finding a negative effect of vitamin A on MUFA (Olivares et al., 2009b; Ayuso et al., 2015a) while others did not. In general, as here, little effects of dietary vitamin A were observed for muscle FA profile, although some works described an increase in SFA (Ayuso et al., 2015a,b). In part, this could be likely because results in other experiments were not adjusted for fat content. Further research is needed to elucidate the role of vitamin A and its metabolites on FA metabolism. However, in agreement with the diet effects, MUFA in liver decreased (and PUFA increased) with all-trans retinoic content.

Consistently with earlier reports (Estany et al., 2014; Henriquez-Rodriguez et al., 2016), the *SCD* genotype affected FA composition, with the TT genotype increasing MUFA and MUFA/SFA ratio in all tissues, but not fat content. However, in contrast to diet, the major effect of the *SCD* genotype on fatty acid composition was in muscle and SF and not in liver. In fact, the *SCD* genotype had no significant impact on hepatic *SCD* gene expression, in agreement with Estany et al. (2014), who found that the *SCD* genotype affected *SCD* gene expression in muscle and SF but not in liver. This will confirm that the footprint of the *SCD* genotype is more important in IMF and SF, the sites where de novo fatty acid synthesis mostly occurs in pigs. Thus, the positive effect of the TT genotype on liver MUFA is difficult to interpret. But it is a fact that the effect of C+ diet on liver retinoids (all-trans retinol and all-trans-5-6-epoxy retinoic acid) was greater in the CC pigs and some evidence indicating that the TT pigs could be less sensitive to the action of liver retinoids for *SCD* gene expression and MUFA ($P < 0.01$, for MUFA over all-trans retinal by *SCD* genotype). A more powerful experimental design is needed to confirm this result and to investigate the interaction between diet and *SCD* genotype including retinoic acid and gene expression distribution throughout tissues. Duration, timing and intensity of the restriction period, as well as nutrient composition of the whole diets are also factors for further research. Results so far, however, provide evidence for a complementary role between dietary PVA and *SCD* genotype, in the sense that the TT pigs fed with a low-PVA

diet are expected to show higher (because of the diet) and more monounsaturated (because of the *SCD* genotype) IMF without increasing total fat content.

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SUPPLEMENTARY TABLES

Table S1. Nutrient content of the diets fed from 70 to 165 days of age (as-fed basis)

	Diet[1]					
	From 70 to 110 d		From 110 to 140 d		From 140 to 165 d	
	LP	RP	LP	RP	LP	RP
Dry matter, g/kg	889.5	892.5	875.8	877.4	879.3	881.5
Crude protein, g/kg	153.0	173.0	140.0	155.0	126.0	147.0
ME, MJ/kg	13.3	13.5	13.0	13.2	13.0	13.2
Crude fat, g/kg	49.0	57.0	52.0	59.0	46.0	66.0
Crude fiber, g/kg	35.0	36.0	36.0	39.0	46.0	54.0
Ash, g/kg	71.0	99.0	71.7	69.4	60.7	59.6

[1]LP: low-protein diet; RP: regular-protein diet.
Vitamins A, D3 and E added per kg of feed from 70 to 110 d: 8000 IU, 1500 IU and 25 mg respectively.
Vitamins A, D3 and E added per kg of feed from 110 to 165 d: 6500 IU, 2000 IU and 15 mg respectively.

Table S2. Nutrient content of M37W-Ph3 and M37W corn lines used in the enriched-carotene (C+) and control (C) diets

	Corn line	
	M37W-Ph3	M37W
Dry matter, g/kg	841.2	849.1
Crude protein, g/kg	111.4	115.7
ME, MJ/kg	16.0	15.9
Crude fat, g/kg	37.9	47.6
Crude fiber, g/kg	20.4	27.8
Ash, g/kg	17.2	23.2
β-carotene, µg/g DM	7.0	nd[1]
β-cryptoxanthin, µg/g DM	2.2	nd[1]
Fatty acids, % FA		
C16:0	15.4	15.2
Total SFA [2]	18.2	17.6
C18:1,cis-9	32.9	33.7
Total MUFA [3]	33.5	34.2
C18:2,cis-9,12	46.5	46.6
Total PUFA [4]	48.3	48.2

[1]nd: non-detected.
[2]Total SFA, saturated fatty acids: C10:0 + C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0 + C24:0
[3]Total MUFA, monounsaturated fatty acid: C14:1 cis-9 + C16:1, cis-9 + C17:1, cis-10 + C18:1, trans-9 + C18:1, cis-9 + C20:1, cis-11 + C22:1, cis-13
[4]Total PUFA, polyunsaturated fatty acids: C18:2,trans-9,12 + C18:2,cis-9,12 + C18:3,cis6,9,12 + C18:3,cis-9,12,15 + C20:2,cis-11,14 + C20:3,cis-11,14,17 + C20:4,cis-5,8,11,14

Table S3. Multiple Reaction Monitoring (MRM) parameters for retinoid quantification

Analyte	Retention time (min)[1]	MRM Transitions Q1 > Q3 *m/z*	Cone voltage (V)	Collision energy (eV)
All-trans retinol	7.59	269.19 > 93.05	30	20
		269.19 > 213.10		15
All-trans retinal	8.16	285.19 > 161.04	30	10
		185.19 > 175.10		15
Retinyl-palmitate	19.28	269.19 >93.05	30	20
		269.19 > 213.1		15
Keto-retinoic acid	2.73	297.23 >159.03	30	20
		297.23 >241.09		15
All-trans-4-hidroxy retinoic acid	2.25	299.25 > 95.09	30	10
		299.25 >199.11		15
All-trans-retinoic acid	7.9	301.15 >123.03	30	20
		301.15 > 159.05		
9-cis-retinoic acid	7.74	301.15 >123.03	30	20
		301.15 > 159.05		
9,13-cis-retinoic acid	7.46	301.15 >123.03	30	20
		301.15 > 159.05		
13-cis-retinoic acid	7.27	301.15 >123.03	30	20
		301.15 > 159.05		
All-trans retinoic acid-d5	7.84	306.27 >127.02	30	15
		306.27 > 162.11		20
All-trans-5-6-epoxy retinoic acid	4.5	317.29 > 107.05	30	25
		317.29 >135.04		
		403.37 > 137.09		25

[1]Q1: first mass analyzer; Q3: second mass analyzer; m/z: mass-to charge ratio; V: volt; eV: electron volts

Table S4. Least square means (±SE) for production traits by diet and *SCD* genotype

	Diet[1]		*SCD* genotype[2]		P-value
Trait	C	C+	CC	TT	Diet*_SCD_
Number of pigs	14	15	13	16	
Weight at 165 d, kg	98.9±1.2	98.2±0.9	94.0±2.4	103.2±2.2	0.56
Weight at 195 d, kg	119.3±2.0	121.1±1.8	114.9±2.8[b]	125.5±2.4[a]	0.89
Total weight gain, kg	21.3±1.5	23.7±1.5	22.6±1.3	22.4±1.2	0.25
Total feed intake, kg %DM	74.7±3.4	84.6±3.4	-	-	
Feed conversion ratio, %DM	3.5±0.2	3.6±0.2	-	-	

[1]Diet C+: diet with 20% carotenoid-fortified corn M37W-Ph3; Diet C: diet with 20% of the near isogenic M37W line, which does not contain pro-vitamin A carotenoids.
[2]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*
[a,b]Within row and factor, means with different superscripts differ significantly ($P \leq 0.05$)

Table S5. Least square means (±SE) for fatty acid composition traits in liver by diet and *SCD* genotype

	Diet[1]		*SCD* genotype[2]		P-value
Trait, %	C	C+	CC	TT	Diet**SCD*
n	14	15	13	16	
C10:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.06
C12:0	0.1±0.0[a]	0.1±0.0[b]	0.1±0.0	0.1±0.0	0.55
C13:0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.88
C14:0	0.6±0.0[a]	0.4±0.0[b]	0.5±0.0	0.5±0.0	0.71
C15:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.32
C16:0	16.0±0.4[a]	13.5±0.4[b]	14.4±0.4[b]	15.1±0.3[a]	0.26
C17:0	0.5±0.0[b]	0.7±0.0[a]	0.6±0.0	0.5±0.0	0.44
C18:0	16.8±0.5[b]	19.6±0.4[a]	18.8±0.4[a]	17.6±0.4[b]	0.57
C20:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.30
C22:0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.06
C15:1n-5	0.3±0.0[b]	0.3±0.0[a]	0.3±0.0	0.3±0.0	0.56
C16:1n-9	1.1±0.1[a]	0.8±0.1[b]	0.8±0.1[b]	1.1±0.1[a]	0.31
C17:1n-7	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.12
C18:1n-7	1.8±0.1[a]	1.6±0.0[b]	1.7±0.0[b]	1.8±0.0[a]	0.19
C18:1n-9	18.4±0.7[a]	14.7±0.6[b]	15.8±0.6[b]	17.4±0.5[a]	0.77
C20:1n-9	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.60
C24:1n-9	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.07
C18:2n-6	16.5±0.3[a]	15.2±0.3[b]	15.8±0.3	15.9±0.3	0.26
C18:3n-3	0.8±0.1[a]	0.5±0.1[b]	0.6±0.0	0.7±0.0	0.31
C20:2n-6	0.6±0.0	0.7±0.0	0.7±0.0	0.7±0.0	0.73
C20:3n-6	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.27
C20:4n-6	22.3±0.9[b]	27.6±0.9[a]	25.7±0.8	24.2±0.7	0.42
C20:5n-3	0.4±0.0	0.5±0.0	0.5±0.0	0.4±0.0	0.40
C22:6n-3	1.6±0.1	1.7±0.1	1.6±0.1	1.6±0.1	0.88

[1]Diet C+: diet with 20% carotenoid-fortified corn M37W-Ph3; Diet C: diet with 20% of the near isogenic M37W line, which does not contain pro-vitamin A carotenoids.
[2]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*
[a,b] Within row and factor, means with different superscripts differ significantly ($P \leq 0.05$).

Table S6. Least square means (±SE) for fatty acid composition traits in Longissimus muscle by diet and *SCD* genotype

	Diet[1]		*SCD* genotype[2]		P-value
Trait, %	C	C+	CC	TT	Diet*SCD
n	14	15	13	16	
C10:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.90
C12:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.46
C14:0	1.6±0.0	1.6±0.0	1.6±0.0	1.6±0.0	0.44
C16:0	23.4±0.2	22.8±0.2	23.2±0.2	22.9±0.2	0.89
C17:0	0.2±0.0	0.2±0.0	0.2±0.0[a]	0.2±0.0[b]	0.28
C18:0	10.7±0.3	10.1±0.3	11.2±0.3[a]	9.7±0.2[b]	0.52
C20:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.77
C16:1n-9	3.7±0.2	3.9±0.2	3.5±0.2[b]	4.1±0.2[a]	0.54
C17:1n-7	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.34
C18:1n-7	4.0±0.1	4.3±0.1	3.9±0.1[b]	4.4±0.1[a]	0.32
C18:1n-9	44.8±0.3	45.4±0.3	44.6±0.4	45.6±0.3	0.38
C20:1n-9	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.75
C18:2n-6	7.5±0.4	7.7±0.4	7.6±0.3	7.6±0.3	0.10
C18:3n-3	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.02
C20:2n-6	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.10
C20:4n-6	1.7±0.1	1.9±0.1	1.8±0.1	1.8±0.1	0.95

[1]Diet C+: diet with 20% carotenoid-fortified corn M37W-Ph3; Diet C: diet with 20% of the near isogenic M37W line, which does not contain pro-vitamin A carotenoids.
[2]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*
[a,b] Within row and factor, means with different superscripts differ significantly ($P \leq 0.05$).

Table S7. Least square means (±SE) for fatty acid composition traits in Gluteus medius by diet and *SCD* genotype

	Diet[1]		*SCD* genotype[2]		P-value
Trait, %	C	C+	CC	TT	Diet*SCD
n	14	15	13	16	
C10:0	0.9±0.2	0.8±0.2	1.0±0.2	0.7±0.2	0.97
C12:0	0.2±0.0	0.2±0.0	0.2±0.1	0.2±0.0	0.42
C14:0	1.5±0.1	1.6±0.1	1.5±0.1	1.7±0.1	0.58
C16:0	21.7±0.3	21.8±0.3	21.8±0.3	21.8±0.3	0.57
C17:0	0.2±0.0	0.2±0.0	0.3±0.0[a]	0.2±0.0[b]	0.33
C18:0	9.9±0.2	9.7±0.2	10.4±0.2[a]	9.1±0.2[b]	0.80
C20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.79
C16:1n-9	3.4±0.2	3.6±0.2	3.2±0.2[b]	3.8±0.2[a]	0.81
C17:1n-7	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.29
C18:1n-7	3.8±0.1	4.0±0.1	3.6±0.1[b]	4.2±0.1[a]	0.95
C18:1n-9	44.0±0.3	44.7±0.3	43.7±0.4	45.0±0.4	0.47
C20:1n-9	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.60
C18:2n-6	10.2±0.6	9.5±0.6	10.2±0.5	9.6±0.5	0.26
C18:3n-3	0.6±0.0	0.5±0.1	0.6±0.0	0.5±0.0	0.25
C20:2n-6	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.32
C20:4n-6	1.7±0.2	1.9±0.2	1.7±0.1	1.8±0.1	0.73

[1]Diet C+: diet with 20% carotenoid-fortified corn M37W-Ph3; Diet C: diet with 20% of the near isogenic M37W line, which does not contain pro-vitamin A carotenoids.
[2]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*
[a,b] Within row and factor, means with different superscripts differ significantly ($P \leq 0.05$).

Table S8. Least square means (±SE) for fatty acid composition traits in subcutaneous fat by diet and *SCD* genotype

	Diet[1]		*SCD* genotype[2]		P-value
Trait, %	C	C+	CC	TT	Diet*SCD
n	14	15	13	16	
C12:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.59
C14:0	1.4±0.0[a]	1.3±0.0[b]	1.3±0.0[b]	1.4±0.0[a]	0.31
C16:0	22.8±0.3	22.7±0.3	22.9±0.3	22.6±0.3	0.66
C17:0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.83
C18:0	11.4±0.2	11.6±0.2	12.5±0.4[a]	10.4±0.3[b]	0.67
C20:0	0.2±0.0	0.2±0.0	0.2±0.0[a]	0.2±0.0[b]	0.60
C16:1n-9	2.1±0.1	2.0±0.1	1.9±0.1[b]	2.2±0.1[a]	0.84
C17:1n-7	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.95
C18:1n-7	2.7±0.0	2.7±0.0	2.5±0.1[b]	2.8±0.1[a]	0.76
C18:1n-9	40.6±0.4[b]	42.1±0.4[a]	41.0±0.4	41.7±0.3	0.93
C20:1n-9	1.0±0.0	1.1±0.0	1.1±0.0	1.0±0.0	0.79
C18:2n-6	15.2±0.4	13.8±0.4	14.1±0.5	14.9±0.4	0.64
C18:3n-3	1.0±0.0	1.0±0.0	1.0±0.0	1.1±0.0	0.51
C20:2n-6	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.0	0.59
C20:4n-6	0.4±0.0	0.3±0.0	0.3±0.0	0.4±0.0	0.46

[1]Diet C+: diet with 20% carotenoid-fortified corn M37W-Ph3; Diet C: diet with 20% of the near isogenic M37W line, which does not contain pro-vitamin A carotenoids.
[2]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*
[a,b] Within row and factor, means with different superscripts differ significantly ($P \leq 0.05$).

VIII. GENERAL DISCUSSION

The selection for lean content developed in the last decades decreased pork fat content negatively affecting traits such as tenderness, color, pH, IMF content and the fatty acid profile (Barton-Gade, 1990; Cameron, 1990; Cameron et al., 2000; Schwab et al., 2006). Today's consumers demand an improvement of those meat and eating quality characteristics in the frame of a healthy diet. These demands are being addressed (in part) by the industry by developing commercial pig lines where pork quality traits represent 20 to 37% of the total breading goal (Knap, 2014). Among traits influencing consumer's satisfaction, fatty acid composition has received special attention because of the role it plays as a source of dietary lipids and as components of technological aspects of meat quality. Increases in SFA consumption increase the risk of coronary heart disease whereas MUFA and PUFA have the opposite effect (Decker and Park, 2010; Joris and Mensink, 2016), while the n-6:n-3 PUFA ratio has been related to cancer risk (Williams et al., 2011). In meat, the profile of fatty acids affects fat color and firmness, shelf life and flavor development (Wood et al., 2003) as well as different quality traits of dry-cured products (Ruiz-Carrascal et al., 2000; Gandemer, 2009). The monounsaturated oleic acid, which accounts for approximately 35-50% of pork total fatty acids (López-Bote, 1998; Tejerina et al., 2012; Wood et al., 2008), not only promotes a healthy blood lipid profile but it is also positively correlated with pork flavor, flavor liking and overall acceptability (Cameron et al., 2000).

Due to the nutritional and technological importance of fatty acids for pork production, genes involved in fatty acid metabolism have been an important target of research in animal breeding. After reports showed that a relevant part of the variation in oleic acid has a genetic origin (Gjerlaug-Enger et al., 2010; Ros-Freixedes et al., 2012), Estany et al. (2014) found a haplotype in the *SCD* (stearoyl-CoA desaturase) gene promoter affecting this and others MUFA in Duroc pigs. The *SCD* is the rate-limiting enzyme required for the biosynthesis of oleic and palmitoleic acids, which are produced by desaturating at the Δ9 position their respective precursors: the stearic and palmitic acids (Guillou et al., 2010). Pigs carrying the favorable alleles of the haplotype reported by Estany et al. (2014) have higher C16:1n-9/C16:0 and C18:1/C18:0 ratios and higher levels of C16:1n-9, C18:1 and MUFA in raw muscle while IMF content remains unchanged.

The *SCD* polymorphism can be a useful tool to improve MUFA and oleic acid content without increasing total fatness in pigs. Therefore, it becomes important to validate its effects in different production and commercial scenarios. In **chapter IV** of this thesis we examined whether the effect of the *SCD* marker on fat content and composition changes at different market weights, a relevant aspect since different studies have shown modifications in the fatty acid profile of pigs as they grow and fat content increases (Virgili et al., 2003; Lo Fiego et al., 2010; Bosch et al., 2012). The interaction between the *SCD* and a *LEPR* polymorphism was also tested, given that *SCD* and *LEPR* are the two main loci influencing IMF and fatty acid composition in this Duroc population (Ros-Freixedes et al., 2016).

The results confirmed the association of the T allele at the *SCD* gene with increased MUFA. Conversely, and in line with previous reports in Iberian and Duroc crossbreds (Muñoz et al., 2009; Galve et al., 2012), the T allele at *LEPR* increased SFA. However, while the effect of *SCD* on MUFA was independent of fat content, the effect of *LEPR* on SFA was not, being the higher SFA values of the allele a consequence of increased fatness. Additionally to these traits, previous studies with larger data sets of the same population (Gol et al., 2015) observed that the *LEPR* polymorphism also affected body and carcass weight and feed intake. Thus, in Duroc × Iberian pigs, Rodriguez et al. (2010) found a positive effect of the *LEPR* T allele on voluntary feed intake. Some authors (Óvilo et al., 2005) have suggested that the *LEPR* T allele increases weight and fat content through and indirect effect on feed intake, since the leptin receptor mediates the satiety effect of leptin (Houseknecht et al., 1998; Barb et al., 2001). The results of the present study add further evidence to support such hypothesis.

Altogether, the effect of both genotypes was consistent across tissues and throughout the finishing period. Interactions of genotype with age were only detected for backfat thickness and PUFA, for *SCD*, and for SFA in m. longissimus thoracis, for *LEPR*. However, they only showed minor effects which were limited to small changes in magnitude. The modifications of the fatty acid profile throughout fattening in both tissues followed the same trend reported in several works (Virgili et al., 2003; Lo Fiego et al., 2010; Bosch et al., 2012), with MUFA increasing and PUFA decreasing regardless of the markers. Interestingly, the IMF of CCTT pigs (CC at *SCD*, TT at *LEPR*) had more SFA at 160 days than the TTCT or TTCC genotypes at 220 days, two months later. When analyzed

together, the MUFA/SFA ratio was on average 15% higher in pigs with the TTCT or TTCC genotypes than the CCTT in both muscle and subcutaneous fat. Thus, the combined use of both markers can be a useful tool for breeders to produce healthier pork. However, further work needs to assess the impact of these two polymorphisms at younger ages, since reducing weight at slaughter could be a future strategy to deal with boar taint if surgical castration is banned (Xue and Dial, 1997; Thomsen et al., 2015). Moreover, because selection for the *LEPR* C allele is expected to decrease IMF and to increase PUFA, caution must be taken on the negative impact that this action could have on the technological and sensory characteristics of dry-cured hams.

The Duroc line used in this thesis is a commercial line that was closed in 1991 and since then it has been selected for an index including body weight, backfat thickness and IMF content, with the primary objective of producing premium pork and high quality dry-cured hams. For dry-cured ham production, IMF content and fatty acid composition are important attributes, since both exert strong influence on quality traits such as color, aspect and texture of ham slices and intensity and persistence of aroma (Ruiz-Carrascal et al., 2000; Gandemer, 2009). During curing, fatty acids undergo lipolysis and oxidation which modifies the fatty acid profile of the cured ham as compared to raw meat (Motilva et al., 1993; Cava et al., 2003; Pateiro et al., 2015). The gender of the hams influences the final quality of the dry-cured products due to differences in IMF and fatty acid composition among gilts, males, and barrows (Garitano et al., 2013; Zhang et al., 2007). Therefore, in **Chapter V** it was assessed whether the effect of the *SCD* polymorphism on fat desaturation and monounsaturated fatty acid content is maintained in traditional purebred Duroc dry-cured hams from gilts and barrows.

In agreement with results obtained in different muscles (Estany et al., 2014), the T allele at the *SCD* gene also had greater C16:1n-9, C18:1n-9, C18:1n-7 and MUFA content in dry-cured ham. A batch of traced samples from green hams to the end of curation confirmed that the beneficial effect of the T allele on MUFA remains after curing and that the polymorphism did not affect IMF content. Phenotypic correlations between green and cured samples showed a positive and strong relationship between individual fatty acids, suggesting that fatty acid composition of green hams could be a good predictor of fatty acids in dry-cured hams even when measured in different muscles. These results are

supported by an earlier study of Ros-Freixedes et al. (2014), whom using the same population found similar correlations among fatty acids between m. gluteus medius and m. longissimus thoracis. Interestingly, further analysis showed that these relationships differ by *SCD* genotype for MUFA content. Thus, a regression analysis of MUFA in dry-cured ham on MUFA in green ham indicated that the green hams carrying the TT *SCD* genotype retain initial MUFA more effectively throughout the curing process. The results obtained in this thesis show that the beneficial effect of the T allele on desaturation ratios, oleic acid and MUFA content does not vanish after curing.

The effect of gender on fatty acid composition of dry-cured hams was similar to reports on raw meat (Garitano et al., 2013; Zhang et al., 2007), with barrows displaying higher MUFA and lower PUFA than gilts. The difference among barrows and gilts for MUFA and oleic acid was almost half of the difference due to the *SCD* genotype. However, the differences between barrows and gilts vanished after adjusting for fat content, showing that the effect is basically explained by IMF increase. All in all, regarding fatty acid composition, the T allele is more decisive than the difference between barrows and gilts as a tool to increase MUFA and oleic acid content of dry-cured hams.

In the Spanish market, dry-cured hams occupy the first place of sales among pork products (Enge et al., 1998; ANICE, 2014). The Iberian ham (*Jamón Ibérico*) is a label carefully overseen by government guidelines which represents approximately 20% of total turnover of Spain´s dry-cured ham industry. Official guidelines (BOE, 2014) establish two types of labelling for these hams according to their genetic background: 100% Iberian (produced from purebred Iberian pigs) and Iberian (produced using Iberian crossbred pigs with a maximum 50% of Duroc). Nowadays, most of the Iberian marketed hams, around 94%, are based on Duroc-sired Iberian crossbreds (Cruz, 2013). It has been reported that crossbreeding can affect sensory characteristics and the fatty acid profile of Iberian hams (Carrapiso et al., 2003; Petrón et al., 2004). Therefore, the **chapter VI** examined whether the effect of the *SCD* polymorphism is evident in commercial Duroc-sired Iberian dry-cured hams from gilts and barrows.

In this experiment, entire slices of vacuum packed "*Jamón Ibérico de Cebo*" (fodder fed Iberian ham) were used to investigate the effect of the *SCD* marker on the fatty acid profile of the edible fat that consumers get when buying dry-cured ham at a local

grocery store. This simple approach takes advantage of previous reports indicating that the correlation of fatty acids between tissues is relatively high (Ros-Freixedes et al, 2014) and that the effect of the polymorphism is consistent across fat tissues (Estany et al., 2014). The genotypic frequencies found (0.58, for TT, and 0.42, for CT; only 1 CC ham) support previous findings suggesting that the T allele is fixed in Iberian pigs (Estany et al., 2014). The *SCD* genotype as well as the gender affected the fatty acid profile. The T allele at the *SCD* gene increased MUFA in line with previous reports in raw meat (Estany et al., 2014) and dry-cured hams (chapter V). The increase in MUFA at expenses of PUFA instead of SFA might have been influenced by the presence of different type of fats (IMF, intermuscular fat, and untrimmed subcutaneous fat) in the analyzed sample, which are known to differ in fatty acid composition (Bosch et al., 2012). In addition, it should be taken into account that differences in ripening times and in conditions of refrigerated storage affect the fatty acid profile and the rate of lipid oxidation of dry-cured hams (Cava et al., 2009; Salazar et al., 2015). The previously described factors could have also influenced the results obtained for gender differences, since contrary to earlier results (Garitano et al., 2013: chapter V) barrows increased SFA and decreased PUFA as compared to gilts.

Taken together, results in **chapters V** and **VI** allow confirming that the *SCD* genotype is a useful tool to increase MUFA content in both purebred and crossbred Duroc dry-cured hams. The genotypic frequencies obtained in these experiments indicate that is possible to select in favor of the T allele to improve the MUFA/SFA ratio. On a next stage, it would be worth investigating if differences in MUFA/SFA due to the *SCD* marker affect hams organoleptic properties and hence consumers acceptance using, i.e., the willingness to pay approach (Biermann et al., 2016).

The fat content and composition of pork can also be modified through nutritional strategies. In this regard, adjusting dietary vitamin A at critical stages for IMF adipocyte formation can specifically promote IMF without increasing subcutaneous fat (Wang et al., 2016). Previous experiments in cattle and pigs provided evidence that restricting vitamin A intake in finishing diets improves IMF (D'Souza et al., 2003; Pickworth et al., 2012) and MUFA (Ayuso et al., 2015a,b) with minimal impact on carcass composition. These effects can be partly explained because vitamin A, after being absorbed, is converted into retinoic

acid, which coregulates key adipogenic genes, including *SCD* (Wang et al., 2016). The target mutation of this thesis is positioned at the core sequence of several putative retinoic acid response elements. Hence, the **chapter VII** assessed the combined effects of pro-vitamin A carotenoid intake and the *SCD* genotype on liver, muscle and subcutaneous fat content and composition.

The 2 × 2 split-plot design consisted in two diets differing in pro-vitamin A carotenoid content (carotenoid-rich and carotenoid-restricted) and the two *SCD* homozygotes (TT and CC). The carotenoid-rich diet increased the content of retinoids in liver and it was found that three main circulating forms of vitamin A, retinol, retinal and retinoic acid, are well correlated and therefore retinol serves as a good predictor of the other two. Besides having higher levels of the main vitamers, pigs fed with the carotenoid-rich diet also accumulated 20% less fat in the liver than the carotenoid-restricted diet, confirming the inhibitory role of retinoids in fat deposition. The liver fat content decreased with all-trans retinoic acid in liver, but the response was higher at low concentrations, suggesting a saturation point at relatively low values. Taking into account that pigs fed the carotenoid-rich diet had an extra daily intake of around 1000 IU of vitamin A as compare to the carotenoid-restricted group, results suggest that a small but continuous amount of carotenoid intake over the recommended level would suffice to induce relevant changes in fat metabolism and beneficial effects in the prevention of fat liver-related diseases.

The carotenoid-restricted diet increased IMF deposition with no change in production traits, in line with previous findings in cattle (Siebert et al., 2006; Gorocica-Buenfil et al., 2007; Kruk et al., 2008; Pickworth et al., 2012) and pigs (D'Souza et al., 2003; Olivares et al., 2011; Ayuso et al., 2015a,b). The effect of the restriction was greater in muscle than in liver and absent in backfat thickness. Studies in pigs have shown that preadipocyte hyperplasia is an early process, with a fast decline after 40 days of age, at a time when hypertrophy kicks off, increasing markedly with age (Dunshea and D'Souza, 2003). Subcutaneous fat develops and matures prior to IMF and thus preadipocyte formation and hyperplasia happen earlier in subcutaneous fat than in IMF. Therefore, during growth hypertrophy is the key driver for subcutaneous fat deposition (Nakajima et al., 2011) and hyperplasia for IMF (Damon et al., 2006; Hausman et al., 2014). Because vitamin A is expected to promote hyperplasia in preadipocytes and reduce hypertrophy in

mature adipocytes, its intake at critical periods for IMF formation and accretion can lead to enhance IMF content without increasing backfat thickness. In this regard, our results showed that a carotenoid-restricted finishing diet, even for a short period, enables pigs to enhance IMF without substantial variation in backfat thickness.

The diets showed an opposite effect on the fatty acid composition of liver and subcutaneous fat, with the carotenoid-rich diet decreasing MUFA in liver but increasing it in subcutaneous fat. Carotenoids also increased liver *SCD* expression in line with expectations (Ntambi and Miyazaki, 2004). This enzyme is directly involved in the biosynthesis of MUFA from SFA. However, in liver there is a clear inverse relationship between *SCD* expression levels and MUFA content. In liver, the endogenously synthesized MUFA are the main substrate for the synthesis of hepatic triglycerides and cholesterol esters, which are released to the bloodstream in the form of very-low-density lipoprotein (VLDL) (Hodson and Fielding, 2013). Thus, in agreement with previous reports, enhanced *SCD* expression results in lower fat and MUFA content through the release of *de novo* fatty acids to circulating VLDL (Peter et al., 2011; Silbernagel et al., 2012) which indirectly affects PUFA liver content. Taken together, these results confirm the relationship between dietary pro-vitamin A carotenoids, liver *SCD* expression and total fat and MUFA content in liver reported in lean humans and mice but not in obese individuals (Peter et al., 2011). Circulating MUFA are delivered in VLDL target tissues (muscle, heart and adipose), increasing MUFA content in SF, the main tissue of fatty acid uptake. Previous studies in pigs have not found consistent results of dietary vitamin A on liver, subcutaneous fat and muscle fatty acid profile (Olivares et al., 2009; Ayuso et al., 2015a,b). However, in agreement with the diet effects, MUFA decreased (and PUFA increased) with all-trans retinoic content in liver.

Consistently with earlier reports (Estany et al., 2014; Chapter IV), the *SCD* genotype affected fatty acid composition, with the TT genotype increasing MUFA and MUFA/SFA ratio in all tissues, but not fat content. In contrast to diet, the major effect of the *SCD* genotype on fatty acid composition was in muscle and subcutaneous fat and not in liver. Considering that the *SCD* genotype had no significant impact on hepatic *SCD* gene expression, results suggest that the footprint of the *SCD* genotype is more important in IMF and subcutaneous fat, the sites where *de novo* fatty acid synthesis mostly occurs in pigs.

Thus, results so far support the hypothesis that a pro-vitamin A carotenoid restricted diet at finishing and the TT genotype at the *SCD* gene complement well each other to simultaneously increase IMF and MUFA without increasing total fat content.

Opportunities for further research include exploring potential interactions between diet and *SCD* genotype, retinoic acid and gene expression distribution throughout tissues, duration, timing and intensity of the restriction period, as well as nutrient composition of the whole diets. Moreover, it would be interesting to determine the relationship between *SCD* gene and protein expression in order to get the whole sequence from DNA to phenotype, since it is known that correlations between RNA and protein levels can vary widely among tissues (Gry et al., 2009) due to different factors (Maier et al., 2009). Given that nutrients can induce epigenetic modifications which can alter gene expression patterns (Feil and Fraga, 2012), it could be investigated if the effect of the carotenoid-fortified corn M37W-Ph3 on the epigenome varies with respect to its near isogenic M37W line and commercial supplements of vitamin A.

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IX. CONCLUSIONS

1. The positive effect of the T allele at the *AY487830:g.2228T>C* polymorphism at the *SCD* gene on oleic acid and MUFA is confirmed throughout the growing-finishing period. The *LEPR NM_001024587:g.1987C>T* marker is also segregating in Duroc, with the T allele affecting positively IMF and SFA but also total fat content. The effect of this *LEPR* SNP is also maintained during the growing-finishing period. Therefore, the combined selection for the *SCD* T and *LEPR* C alleles is a good strategy to increase the MUFA/SFA ratio regardless of the age at slaughter.

2. The effect of the T allele at the *SCD* gene on oleic acid and MUFA still remains after dry-curing. In both purebred Duroc and crossbred Duroc × Iberian, the effect of the *SCD* SNP had a greater impact on MUFA than using hams from barrows instead of gilts. There is a strong positive relationship between MUFA in green and dry-cured hams, with TT pigs being more effective in retaining increased MUFA in green hams until the end of the curing period.

3. A pro-vitamin A carotenoid restricted diet at finishing and the TT genotype at the *SCD* gene complement well each other to simultaneously increase IMF and MUFA without increasing total fat content. Pro-vitamin A carotenoid intake increases hepatic retinoic acid content and *SCD* gene expression and decreases liver fat and MUFA content. Liver fat and MUFA content declined non-linearly with liver all-trans retinoic acid, suggesting a saturation point at relatively low all-trans retinoic acid content.

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