EFFECT OF DIFFERENT DIETARY FACTORS ON INTRAMUSCULAR FAT CONTENT IN PIGS

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DOCTORAL THESIS

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FAIG CONSTAR que aquest treball, titulat “Effect of different dietary factors on intramuscular fat content in pigs”, que presenta Núria Tous Closa per a l’obtenció de títol de Doctor, ha estat finalitzat sota la meva direcció al subprograma de Nutrició de monogàstrics de IRTA i que acompleix els requeriments per poder optar a la Menció Europea.

Constantí, 20 de juliol de 2012

El director de la tesi doctoral

Dr. Enric Esteve Garcia

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ABBREVIATIONS

AA	Arachidonic acid
ACACA	Acetyl-CoA carboxylase gene
ACC	Acetyl-CoA carboxylase
ALA	α-linoleic acid
AMP	Adenosine monophosphate
AMPK	Protein kinase activated by AMP
Ap2	Adipocyte Protein 2
ATGL	Adipose triglyceride lipase
ATP	Adenosine Triphosphate
BCCAA	Branched chain amino acid
C/EBPß	CCAAT/enhancer binding protein ß
cAMP	Cyclic AMP
CREB	cAMP response element-binding
d	Day
DFAT	Dedifferentiated fat cells
DFD	Dark firm and dry
DHA	Docosahexaenoic acid
ECM	Extracellular matrix
EPA	Eicosapentanoic acid
FA	Fatty acids
FAS	Fatty acid synthase
FASN	Fatty acid synthase gene
FG	Fast-twitch glycolytic
FOG	Fast-twitch oxidative glycolytic
GLUT-4	Glucose transporter type 4
GW	Growth hormone
HSL	Hormone sensitive lipase
IGF-I	Insulin-like growth factor-1
IMF	Intramuscular fat
LA	Linoleic acid
LD	*Longissimus dorsi*
LT	*Longissimus thoracis*
LPL	Lipoprotein lipase
LW	Live weight
miRNA	Micro ribonucleic acid

mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
p.m.	Post mortem
PKA	Protein kinase A
PPARα	Peroxisome proliferator alpha
PPARγ	Peroxisome proliferator gamma
PPARδ	Peroxisome proliferator delta
Pref-1	Preadipocyte factor-one
PSE	Pale soft and exudative
PUFA	Polyunsaturated fatty acid
RA	Retinoic acid
RAREs	Retinoic acid response elements
RARs	Retinoic acid receptors
RBP	Retinol binding protein
RXRs	Retinoid X receptors
SCD	Steaoryl-CoA desaturase
SDH	Succinate dehydrogenase
SFA	Saturated fatty acids
SO	Slow-twitch oxidative
SREBP	Sterol regulatory element-binding protein 1
SREBPF1	Sterol regulatory element-binding protein factor 1
STRA6	Stimulated by retinoic acid gene 6 homolog
TAG	Triacylglycerols
TGF	Transforming growth factor
TTR	Termed transthyretin

RESUM

A partir dels anys 80 es van començar a seleccionar genèticament porcs amb una menor deposició de greix i millor de l'eficiència de conversió de l'aliment, el que va resultar en un augment del percentatge de magre, augmentant així el valor econòmic de les canals. Per altra banda, la reducció de greix de les canals també va ser d'interès pels consumidors, ja que el consum d'àcids grassos saturats provinent dels animals és associat a un major risc de patir malalties cardiovasculars. Aquesta selecció es va dur a terme amb èxit i les noves línees genètiques resultants van reduir el seu contingut en greix. Tanmateix, entre aquests es va veure inclòs la quantitat de greix dipositada a l'interior del múscul, coneguda com la grassa intramuscular (GIM) la qual es va reduir de 2-4 % fins al 1 % i en alguns casos per sota aquest valor. La reducció de la GIM es va veure negativament associada a alguns paràmetres sensorials, com per exemple la tendresa i la suculència de la carn de porc. Per tal d'avaluar si el consumidor espanyol percebia aquestes diferències, es va fer un estudi de consumidors. Aquest, consistia en primer terme en una prova organolèptica, on el consumidor havia d'avaluar quatre porcions de llom cuits al forn i embolicats amb paper d'alumini pertanyents a quatre grups diferents segons el seu contingut en GIM (0.96 ± 0.30 %; 2.11 ± 0.07 %; 3.72 ± 0.26 % and 5.78 ± 0.19 %). En segon terme es va procedir a realitzar una avaluació visual de quatre llonzes de porc que diferien en el seu contingut de GIM (o vetejat de grassa) com en el primer cas. Els resultats d'aquest estudi van mostrar que des del punt de vista gustatiu tots els consumidors preferien la carn amb un contingut de GIM més alt. En contra, des del punt de vista visual es van obtenir dos segments de població, un que preferia la carn sense greix i l'altre que preferia la carn amb més greix. Així doncs, es va concloure que la quantitat de GIM té un efecte positiu en la percepció gustativa. Diferents estratègies nutricionals s'han proposat per tal d'augmentar aquests valors de GIM. Entre aquestes, en aquesta tesis s'han avaluat: l'àcid linoleic conjugat (ALC), la vitamina A, la proteïna, la lisina, l'arginina, i la

leucina. L'avaluació d'aquestes estratègies es va distribuir en quatre proves experimentals diferents.

L'efecte del ALC en la deposició de greix en porc és un tema molt discutit a la literatura, tot i això, s'han observat discrepàncies entre els resultats obtinguts per diferents grups i el seu mode d'acció sembla no estar clar. En la primera prova, Per tal de determinar si l'addició de ALC en dietes d'acabat augmenta la GIM es va utilitzar una dosis més alta (4%) en relació a d'altres estudis. La deposició de grassa de animal es va veure reduïda com molts altres autors havien reportat prèviament, però el seu efecte sobre la GIM no es va observar tot i l'addició d'una dosi elevada. A més, la incorporació de ALC a la dieta va augmentar-ne el seu contingut en el múscul, obrint vies per tal d'enriquir amb ALC la carn. En aquest estudi també es va avaluar l'efecte del ALC en el perfil d'àcids grassos i l'expressió de gens relacionats amb el metabolisme lipídic en diferents teixits i els resultats suggereixen que el mode d'acció del ALC és específic per cada teixit i que és més actiu en teixits amb un caràcter més oxidatiu (i.e. fetge i múscul *semimembranosus*). A més, tots el canvis observats a nivell transcripcional no varen ser reflectits en la deposició de greix de l'animal, suggerint que hi ha mecanismes postranscripcionals que afecten a la deposició de greix.

En el segon experiment, es varen avaluar tres dietes que diferien en el seu contingut en vitamina A: una dieta on no es va addicionar (la font varen ser els carotens dels ingredients), una on es va administrar un nivell similar al requeriment segons el NRC (1,250 IU vitamina A/kg) i una tercera on se li va afegir el corrector habitual per les dietes comercials (5,000 IU vitamina A/kg). El nivell de vitamina A de la dieta no va afectar els paràmetres de creixement. Tanmateix, la grassa perirenal, la subcutània i la GIM varen ser reduïdes quan els animals es varen alimentar amb les dietes sense l'addició de vitamina A, tot i que en els dos últims casos la diferència no va ser significativa. Aquests resultats suggereixen una reducció de l'engreixament de l'animal quan la vitamina A no es addicionada a la dieta, contrari a la hipòtesis inicial. Així, la dieta sense vitamina A va resultar amb

una major proporció de múscul. El contingut de retinol del fetge va augmentar quan els animals es varen alimentar amb nivells més alts de vitamina A, però els animals alimentats sense, també varen produir retinol en quantiats petites a partir del ß-carotè de la dieta. Per tant, el fet de que els paràmetres de creixement no haguessin estat afectats per les dietes sense vitamina A es podría deure a dues raons: que la quantitat de retinol present al fetge es la necessaria per el desenvolupament i/o que la disminució del greix pot pot ser balançada per un augment del magre.

En la tercera prova experimental es varen dissenyar quatre tractaments per tal d'avaluar l'efecte de la proteïna, lisina i la interacció entre ambdós, ja que a la majoria d'estudis quan un dels paràmetres és reduït l'altre n'és reduït com a conseqüència. Aquests tractaments es varen regir per: alta proteïna alta lisina; alta proteïna baixa lisina; baixa proteïna alta lisina i baixa proteïna baixa lisina. Els nivells de proteïna, fins i tot en els descrits com a 'alta proteïna' van ser més baixos que els utilitzats en d'altres estudis, per tal de reduir l'excreció de nitrogen al medi. Pel que fa a la GIM es va observar una interacció entre el nivell de proteïna i el de lisina, essent els animals que varen rebre les dietes altes en proteïna baixes en lisina o les dietes baixes en proteïna altes en lisina els que varen dipositar més GIM. Per contra, la reducció de proteïna va produir un engreixament general de l'animal, mentre que la reducció en lisina en dietes altes en proteïna en va penalitzar el creixement. La dieta baixa amb proteïna i alta amb lisina podria ser considerada per producció porcina ja que la disminució de proteïna no va afectar els paràmetres de creixement, va augmentar la GIM i també produiria una reducció de l'excreció de nitrogen a l'ambient.

En les anteriors proves experimentals s'havien utilitzat animals amb el creuament (Duroc x Landrace), ja que la línia Duroc és coneguda pel seu alt contingut en GIM. En l'última prova, però, es va canviar a una genètica més magra, en aquest cas (Duroc x Landrace) x Pietrain. Es van avaluar sis tractaments diferents: (1) proteïna normal, (2) proteïna normal + arginina, (3) proteïna normal + leucina, (4) proteïna

normal + arginina + leucina, (5) baixa proteïna, (6) baixa proteïna + arginina + leucina. La quantitat de lisina digestible va ser la mateixa per totes les dietes, inclús en les baixes en proteïna. L'àcid glutàmic es va addicionar per reemplaçar les addicions d'arginina i leucina i mantenir el nivell de proteïna constant. També en aquesta prova, els nivells de proteïna van ser baixos en relació a d'altres estudis. Els animals alimentats amb la dieta baixa en proteïna + arginina + leucina se'ls va reduir el consum, guany mig diari i incrementar l'índex de conversió del aliment, suggerint un antagonisme entre la leucina i els altres aminoàcids de cadena ramificada. Els animals alimentats amb la dieta proteïna normal + arginina i proteïna normal + leucina varen tendir a guanyar més pes comparats amb la dieta proteïna normal, suggerint que aquests aminoàcids eren limitants en les dietes de proteïna normal. El greix subcutani i la ventresca es varen reduir per l'addició d'arginina o la reducció de proteïna, mentre que el percentatge de magre i el filet (només en dietes baixes en proteïna) varen augmentar. El vetejat de grassa al múscul (GIM visible) i la GIM es varen reduir per l'addició d'arginina, contrari a la hipòtesis inicial. Els altres tractaments no varen modificar el contingut de GIM, ni tant sols al reduir el nivell de proteïna, contrari al que s'havia observat al experiment prèvi. Els resultats suggereixen que la reducció de proteïna només augmenta la GIM en genotips més grassos que el d'aquest estudi, i l'arginina redueix la deposició de greix incloent la GIM, augmentant la deposició de magre.

Finalment i per concloure, aquesta tesi demostra que els consumidors espanyols aprecien la carn amb més GIM, si bé a l'hora de la decisió de compra hi ha dos grups amb criteris totalment oposats, indicant que per un segment de la població la qualitat gustativa esperada no és la mateixa que la percepció a l'hora de la compra. És difícil augmentar la GIM a través de la dieta ja que la resposta als factors dietaris semblen dependre de manera important del component genètic, ja que el mateix tractament va respondre de manera diferent en dos creuaments diferents. De tots els tractaments dietaris estudiats, la reducció de la proteïna mantenint el nivell de lisina va ser el més eficaç en augmentar el nivell de GIM sense afectar els

paràmetres productius, però només en el creuament Duroc x Landrace, i no en el (Duroc x Landrace) x Pietrain. L'absència de resposta en el GIM en els experiments presentats en aquesta tesis contrasta amb d'altres estudis els quals havien assajat els mateixos tractaments, fins i tot en alguns casos la tendència va ser contrària. De la informació disponible, els factors que poden modificar la resposta semblen dependre del genotip dels animals utilitzats.

RESUMEN

A partir de los años 80 se empezaron a seleccionar genéticamente los cerdos con una mejor eficiencia de conversión del alimento y una menor deposición de grasa, lo que resultó en un aumento del porcentaje de magro, aumentando así el valor económico de las canales. Además, la reducción de grasa de las canales también fue de interés para los consumidores, ya que el consumo de ácidos grasos saturados provenientes de animales se relacionó con un mayor riesgo a padecer enfermedades cardiovasculares. Esta selección se realizó con éxito y las líneas genéticas resultantes redujeron su contenido en grasa. Asimismo, entre estos se vio también reducida la cantidad de grasa depositada en el interior del músculo, conocida como grasa intramuscular (GIM), la cual pasó de un 2-4 % a valores de 1 % o en algunos casos inferiores. La reducción de GIM se vio negativamente asociada a algunos parámetros sensoriales como por ejemplo la terneza y la jugosidad de la carne de cerdo. A fin de evaluar si el consumidor español percibía estas diferencias, se realizó un estudio de consumidores. Este consistía en primer lugar en un prueba organoléptica, dónde el consumidor tenía que evaluar cuatro porciones de lomo cocidas al horno y envueltas de papel de aluminio pertenecientes a cuatro grupos diferentes según su contenido de GIM (0.96 ± 0.30 %; 2.11 ± 0.07 %; 3.72 ± 0.26 % and 5.78 ± 0.19 %). En segundo lugar, se realizó una evaluación visual de cuatro chuletas de cerdo que diferían en su contenido de GIM (o veteado de la grasa) como en el primer caso. Los resultados de este estudio mostraron que des del punto de vista gustativo todos los consumidores preferían la carne con un contenido de GIM más alto. Sin embargo, des del punto de vista visual se obtuvieron dos segmentos de población, uno que prefería la carne sin grasa y otro que la prefería con grasa. Así pues, se observó que la GIM tiene un efecto positivo en la percepción gustativa de la carne. Diferentes estrategias nutricionales han sido propuestas a fin de incrementar el contenido de GIM. Entre las mismas, en esta tesis se han evaluado: el ácido linoleico conjugado (ALC), la vitamina A, la proteína,

la lisina, la arginina y la leucina. La evaluación de estas estrategias fue distribuida en cuatro pruebas experimentales diferentes.

El efecto del ALC en la deposición de grasa de cerdo es un tema ampliamente discutido en la literatura, sin embargo, se han observado discrepancias entre los resultados de diferentes grupos y su modo de acción no parece ser claro. En la primera prueba, para determinar si la adición de ALC en dietas de acabado aumenta la GIM se utilizó una dosis más alta (4%) en relación a otros estudios. La deposición de grasa del animal se vio reducida como otros autores habían reportado previamente, pero su efecto sobre la GIM no se observó a pesar de haber utilizado una dosis alta. Además la incorporación de ALC en la dieta produjo un aumento del mismo en el músculo, lo que hace posible enriquecer con ALC la carne. En este estudio también se evaluó el efecto del ALC en el perfil de ácidos grasos y la expresión de genes relacionados con el metabolismo lipídico en diferentes tejidos y los resultados sugieren que el modo de acción del ALC es especifico para cada tejido y que es más activo en tejidos con un carácter más oxidativo (i.e. hígado y músculo *semimembranosus*). Además, los cambios observados en la transcripción de genes no son siempre reflejados en la deposición de grasa, sugeriendo que hay mecanismos post-transcripcionales que afectan la deposición de grasa.

En el segundo experimento, se evaluaron tres dietas que diferían de su contenido en vitamina A: una dieta donde no se adicionó (la fuente fueron los carotenos presentes en los ingredientes), una donde se administró un nivel similar al requerimiento según el NRC (1,250 IU vitamina A/kg) y una tercera donde se añadió el corrector habitual de dietas comerciales (5,000 IU vitamina A/kg). El nivel de vitamina A de la dieta no afectó a los parámetros de crecimiento. Sin embargo, una reducción de la grasa perirenal i una reducción, a pesar de no ser significativa, de la grasa subcutánea i GIM se observó en los animales que recibieron las dietas sin vitamina A, sugiriendo que cuando no se adiciona vitamina A a la dieta se produce una reducción del engrasamiento general del animal, contrario a la

hipótesis inicial. Consequentemente, el tratamiento sin vitamina A resultó con una mayor proporción de músculo. El contenido de retinol en el hígado aumentó al aumentar el nivel de vitamina A de la dieta, pero los animales que fueron alimentados sin vitamina A, a pesar que en pequeñas cantidades, también produjeron retinol a partir del β-caroteno presente en la dieta. Por lo tanto el hecho que los parámetros de crecimiento no hubiesen estado afectados por las dietas sin vitamina A puede ser debido a dos razones: que la cantidad de retinol presente en el hígado es la necesaria para el desarrollo i/o que la disminución de grasa sea balanceada por el incremento de magro.

En la tercera prueba experimental se diseñaron cuatro tratamientos experimentales a fin de evaluar el efecto de la proteína, lisina e interacción entre ambos, ya que en la literatura la mayoría de estudios cuando un parámetro es reducido el otro se reduce como consecuencia. Estos tratamientos se rigieron por: alta proteína alta lisina, alta proteína baja lisina, baja proteína alta lisina y baja proteína baja lisina. Los niveles de proteína, incluso los descritos como 'alta proteína' fueron más bajos que los utilizados en otros estudios, para reducir la excreción de nitrógeno al medio. En cuanto a la GIM se observó una interacción entre el nivel de proteína y el de lisina, siendo los animales que recibieron las dietas altas en proteína bajas en lisina o las dietas bajas en proteína altas en lisina los que depositaron más GIM. Por lo contrario, la reducción de proteína produjo un engorde general del animal, mientras que la reducción de lisina y niveles altos de proteína penalizó el crecimiento. La dieta baja en proteína y alta lisina podría utilizarse en producción porcina ya que la disminución de proteína no afectó a los parámetros de crecimiento, aumentó la grasa intramuscular y también produciría una reducción de nitrógeno al ambiente.

En las anteriores pruebas experimentales se utilizaron animales del cruce (Duroc x Landrace), ya que la línea Duroc es conocida por su alto contenido en GIM. En la última prueba, pero, se decidió cambiar a una línea genética más magra para comprobar si se obtenían los mismos resultados al reducir el nivel de proteína de la

dieta, en este caso (Duroc x Landrace) x Pietrain. Se evaluaron seis tratamientos diferentes: proteína normal, proteína normal + arginina, proteína normal + leucina, proteína normal + arginina + leucina, baja proteína, baja proteína + arginina + leucina. La cantidad de lisina digestible fue la misma para todos los trataminentos, incluso en las dietas bajas en proteína. El ácido glutamico se adicionó para reemplazar las adiciones de arginina y leucina, manteniendo así el nivel de proteína constante. También en esta prueba, los niveles de proteína fueron más bajos en relación a otros estudios. Los animales alimentados con las dietas bajas en proteína + arginina + leucina vieron reducido su consumo, ganancia de peso diaria e incrementado el índice de conversión del alimento, sugiriendo un antagonismo de la leucina con los otros aminoácidos de cadena ramificada. Los animales alimentados con las dietas proteína normal + arginina o proteína normal + leucina tendieron a ganar más peso, sugiriendo que estos aminoácidos eran limitantes en la dieta de proteína normal. La grasa subcutánea y la panceta fueron reducidas por la adición de arginina a la dieta o la reducción de proteína, mientras que el porcentaje de magro y filete (este solo en dietas bajas en proteína) fueron aumentados. El veteado de la grasa del músculo (GIM visible) y la GIM fueron reducidos por la adición de arginina, contrario a la hipótesis inicial. Los demás tratamientos no modificaron el contenido de GIM, tampoco la reducción de proteína, contrario a lo que se había observado en el anterior experimento. Los resultados sugieren que la reducción de proteína sólo aumenta la GIM en genotipos más grasos que los de este estudio, y que la arginina reduce la deposición de grasa incluida la GIM, aumentando la deposición de magro. Finalmente y para concluir, esta tesis demuestra que los consumidores españoles aprecian la carne con más GIM, si bien a la hora de la decisión de compra hay dos grupos con criterios totalmente opuestos, indicando que para un segmento de población la calidad gustativa esperada no es la misma que la percepción en el momento de la compra. Aumentar la GIM a través de la dieta es difícil, ya que la respuesta a los factores dietarios parece depender de manera importante del

componente genético, el mismo tratamiento respondió de manera diferente en dos cruzamientos diferentes. De todos los tratamientos dietarios estudiados, la reducción de la proteína manteniendo el nivel de lisina fue el más eficaz en aumentar el nivel de GIM sin afectar a los parámetros productivos, pero sólo en el cruzamiento Duroc x Landrace, y no en el (Duroc x Landrace) x Pietrain. La ausencia de respuesta en la GIM en los experimentos presentados en esta tesis contrasta con otros estudios los cuales habían evaluado los mismos tratamientos, incluso en algunos casos la tendencia fue contraria. De la información disponible, los factores que pueden modificar la respuesta, dependen del genotipo de los animales utilizados.

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EFFECT OF DIFFERENT DIETARY FATORS ON INTRAMUSCULAR FAT CONTENT IN PIGS
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ABSTRACT

Since the eighties, pigs were genetically selected to improve feed efficiency and to reduce body fatness, which resulted in an increase of carcass lean percentage and a consequent increase of the economic value of the carcasses. Moreover, the reduction of fatness was also of interest to consumers, because a high consumption of saturated fat from animals was associated to a higher risk of cardiovascular diseases. The genetic selection succeeded, resulting in new genetic lines with less body fat. Furthermore, the fat content within the muscle, known as intramuscular fat (IMF) was also reduced from 2-4 % to 1 % or even lower values. The reduction of IMF was negatively associated to some sensorial parameters of pork such as tenderness or juiciness. In order to evaluate if the Spanish consumers could detect these differences, a consumer test was realized. First, it consisted of an organoleptic section, where consumer had to evaluate four loin portions cooked in the oven and wraped with aluminum foil from four groups differing in IMF content (0.96 ± 0.30 %; 2.11 ± 0.07 %; 3.72 ± 0.26 % and 5.78 ± 0.19 %). Secondly, consumers were asked to determine their purchase decision of four loin chops depending on its IMF content (or marbling), the same as in the first evaluation. The results from this study showed that from the point of view of taste, all consumers preferred the pork with the highest IMF while from the point of view of purchase decision, two segments of population were observed, one preferring the meat with the highest IMF and one preferring the meat with the lowest IMF content. Thus, it could be concluded that IMF has a positive effect in taste perception of pork. Different nutritional strategies have been proposed to increase the IMF content. Among those evaluated in this thesis are: conjugated linoleic acid (CLA), vitamin A, protein, lysine, arginine and leucine. The evaluation of these strategies was performed with four different experimental trials:

The effect of CLA in pig fat deposition is widely discussed in the literature, however, discrepancies in the results of different groups are found and its mode of action is

not clear. In the first trial, in order to determine whether CLA addition in pig finishing diets increases IMF content, a high dose (4%) was used compared with other studies. Animal fatness was reduced as was reported previously by other authors, however, IMF was not modified despite the high dose used. Additionally, dietary supplementation with CLA, led to an increase of this product in muscle, which suggests that it is possible to enrich pork with CLA. In this study the effect of CLA in fatty acid profile and expression of genes related with lipid metabolism in different tissues was also evaluated. Results suggest that CLA is acting in a tissue-specific way and that it is more active in the tissues with a more oxidative capacity (i.e. liver and *semimembranosus* muscle). Changes in transcription were not always reflected in changes in fat deposition, suggesting that post-transcriptional mechanisms may influence fat deposition.

In the second experiment, three diets differing on their vitamin A content were evaluated: one diet without vitamin A (the retinol source was the carotenes present in the ingredients), one diet with the similar requirements to NRC (1,250 IU vitamin A/kg) and a third diet with current typical vitamin A in commercial diets (5,000 IU vitamin A/kg). Vitamin A did not affect the growth performance parameters. However, a reduction of perirenal fat and, although not significant, subcutaneous fat and IMF were observed in animals fed diets without vitamin A, suggesting a decrease of overall fatness when vitamin A is omitted in the diet, contrary to the initial hypothesis. Thus, the diet without vitamin A resulted in a higher proportion of muscle. Retinol content in the liver increased as the level of vitamin A in the diet increased, the smaller amounts found in animals fed without vitamin A might originate from the β-carotene present in the diet. Thus, omission of dietary vitamin A may not affect the growth of animals for two reaons: animals fed non vitamin A diets had some retinol content in the liver that might have been enough for the animal development and the decrease of fat might have balanced the increase of lean meat.

In the third trial, four experimental treatments were designed in order to evaluate the effect of protein, lysine and their interactions because in most of the studies, when one parameter is reduced the other one is reduced as well. The treatments were: high protein and high lysine, high protein and low lysine, low protein high lysine and low protein low lysine. The levels of protein, even the levels in the high protein diets were lower than those reported in other studies in order to reduce the amount of nitrogen excreted to the environment. An interaction between protein and lysine was observed for IMF, and animals which received high protein low lysine or low protein high lysine diets showed the highest values of IMF. A reduction of dietary protein produced an increase of whole animal fatness and a reduction of lysine in high protein diets depressed performance. Thus, the low protein high lysine diet could be used because the growth performance parameters were not affected, IMF was increased and nitrogen excretion to the environment would be reduced.

In the above mentioned studies was used pigs with the genotype (Duroc x Landrace), as Duroc is recognized to have higher IMF content. On the other hand, in the last trial the genotype was changed to a leaner one, (Duroc x Landrace) x Pietrain. Six experimental treatments were evaluated: (1) normal protein, (2) normal protein + arginine, (3) normal protein + leucine, (4) normal protein + arginine + leucine, (5) low protein, (6) low protein + arginine + leucine. The amount of digestible lysine was the same for all treatments even in the low protein diets. Glutamic acid was added to the diets replacing arginine and leucine, in order to maintain the level of protein. In this study the levels of dietary protein were also lower compared with other studies. Animals fed low protein + arginine + leucine diets had a reduced feed intake and weight gain and an increased feed to gain ratio, suggesting an antagonism between leucine and the other branched chain amino acids. Animals fed normal protein + arginine or normal protein + leucine tended to increase weight gain with respect to the normal protein diet, suggesting that these amino acids were limiting in normal protein diets. Subcutaneous fat and

belly were reduced by arginine addition or protein reduction, while lean carcass meat percentage and tenderloin (only in low protein) were increased. Marbling (visible IMF) and IMF were reduced by the addition of arginine in the diet, contrary to the initial hypothesis. The other treatments did not modify the IMF content, even for the low protein diet, in contrast to the previous experiment. Results suggest that reduction of dietary protein only increases IMF content in fatter genotypes than the one used in the present study, and arginine reduces overall animal fat deposition including IMF, increasing lean deposition.

Finally and as a conclusion, this thesis shows that Spanish consumers appreciate pork with higher IMF content, although two different groups with opposite criteria were found at the point of purchase, indicating that for a segment of population the quality of pork expected is not the same as the perception at the purchase, because even they appreciate high IMF, they will not buy it if marbling is high. To increase IMF content through dietary strategies is not easy, because response to dietary factors seems to depend more on genetic origin. From all the treatments studied, reduction of dietary protein in high lysine diet was the more effective treatment increasing the content of IMF without affecting growth performance parameters, but this only occurred in the crossbreed Duroc x Landrace, and not in (Duroc x Landrace) x Pietrain. Absence of response of experimental treatments on IMF contrasts with other studies evaluating the same treatments, with in some cases the tendency observed that was the opposite. From the available information, the factors that may affect the response seem to depend on the genotype of the animals.

INTRODUCTION

INTRODUCTION

Pigs are basically exploited for food production, either for raw meat or for processed products (i.e. bacon, ham, sausages or pâtés). China was the country that produced and consumed more pork (49,500 and 49,810 metric tons of carcass weight equivalent[1], respectively) in 2011 followed by Europe 27[2] (22,530 and 20,545 metric tons of carcass weight equivalent, respectively) according to a report by United States Department of Agriculture (**USDA**), Agriculture Foreign Countries. In Europe, pig production is concentrated in a few countries, with Denmark, Germany, Spain, France, the Netherlands and Poland having more than two thirds of the breeding pigs between them. Spain is also one of the European countries that consume a lot of pork, with 51 kg/year per capita in 2009 (European commission Eurostat). For this reason pork quality is a field in research of great interest, especially for pork industry.

1. Role of fat in pork quality

1.1. Meat composition

Skeletal muscle is quantitatively the most important tissue of the pig body. At commercial slaughtering weight of 100 kg of live weight (**LW**), tissue muscular represents around 70% and adipose tissue the 20-25 % of the LW (Lebret and Mourot, 1998). Meat is composed of muscular fibers, connective, adipose, and vascular tissues and nervous. Muscle type composition, fiber areas and the capillary density of specific muscles are important factors influencing many peri- and post-mortal processes and thereby meat quality.

[1] The weight of meat cuts and meat products converted to an equivalent weight of a dressed carcass. Includes bone, fat, tendons, ligaments, and inedible trimmings (whereas product weight may or may not)

[2] The European Union is composed of 27 sovereign member states: Austria, Belgium, Bulgaria, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, the Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, and the United Kingdom.

Muscle fibers can be classified according to their metabolic, contractile and color properties (Klont et al., 1998). Gauthier (1969) used a method of fiber typing based on histochemical reactions of aerobic oxidative capacity, using the reference enzyme succinate dehydrogenase (**SDH**). Three major fiber types were distinguished: red, intermediate and white. Basically this method reflects differences in mitochondrial content. Another frequently used and reliable method for histochemical classification of muscle fibers is based in sensitivity of ATPase activity after exposure to either high or low pH. Muscular cell fibers contain the same isoforms of myosin, which is composed of six amino acid chains, two heavy and four light chains (Fig. 1). The myosin heavy chain is both a structural protein and an enzyme which hydrolyses ATP. ATPase stains were developed by labeling the inorganic phosphate precipitate when myosin hydrolyses ATP in presence of Ca^{+2} and they allow distinguishing between slow type I fibers and fast type IIA or IIB fibers (Brooke and Kaiser, 1970).

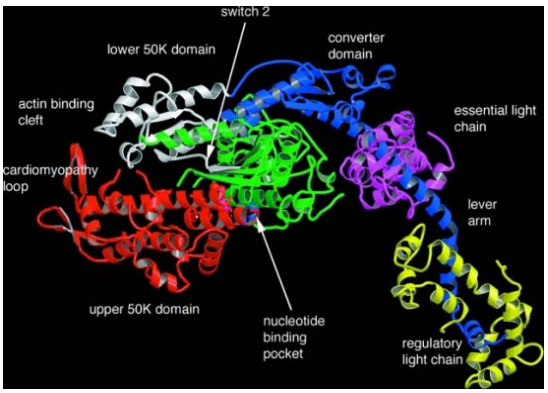

Fig. 1: The structure of the head region of myosin.

Structure determined by (Rayment et al., 1993). The catalytic domain is shown on the left. The upper 50K, lower 50K, nucleotide binding, and converter domains are colored in red, white, green, and blue respectively. The relay helix is colored in blue with its distal end connected to the actin binding side in the lower 50K domain by a blue strand, shown at the top of the molecule.

Peter et al. (1972) combined the histochemical staining for the oxidative enzyme NADH tetrazolium reductase and ATPase which resulted in three major fiber types: slow-twitch oxidative (**SO**), fast-twitch oxidative glycolytic (**FOG**) and fast-twitch glycolytic (**FG**). The SO fibers corresponded to type I, but FOG and FG did not fully match with the fiber types IIA or IIB which makes the classification systems based on stains for enzymes involved in oxidative metabolism and ATPase activity incompatible. Nowadays, immunohistochemical methods are available, which use poly and monoclonal antibodies and divide fast fiber subtypes in: IIA, IIX and IIB which could explain the incompatibility between the classification systems mentioned above (Klont et al., 1998).

Muscle **connective tissue** provides a structure to the muscle and is composed of ground substance, fibers and connective tissue cells. A portion of these elements of the connective tissue comprise collagen I and adipocytes. Collagen is an important parameter to the meat industry as an increased amount of this component may impact on the meat toughness and meat quality (Karunaratne et al., 2005). However, collagen is not as important for quality in pigs because they are young when they are slaughtered. Morphologically, there are three discrete collagen depots in the muscle: the epi-, peri-, and endomysiums. The connective tissue sheath surrounding individual muscles and continuous with the tendon joining other muscle or bones is the epimysium. The epimysium is often thick and tough and resistant to both shear and solubilization. However, it is easily (and usually) separated from cuts of meat and is generally not considered to be a factor in meat quality. The three-dimensional collagen network that surrounds large and small bundles of muscle fiber and contains intramuscular lipid deposits and vasculature is perimysium and the layer of connective tissue encircling each fiber and overlying the basement membrane is the endomysium (McCormick, 1999). These two cannot be separated from cuts of meat and thus, may influence meat quality.

The muscular **adipose tissue** should be distinguished between intermuscular fat and intramuscular fat (IMF). The intermuscular fat is deposited between muscles

and the intramuscular fat is the infiltration of fat in the muscle. The structural lipids are found in the cellular membranes and they are mainly composed by phosphoglycerides and cholesterol and minor amount of sphingolipids. On the other hand, the storage lipids contain almost exclusively triacylglycerols and they are found in the differentiated adipocytes, in the muscle periphery for intermuscular fat and associated to the membrane of the muscular bundles (intercellular) or as lipid droplets in the muscular fibers (intracellular) for intramuscular fat. The IMF may vary from 1% to 6% of the muscle but only represents 1 to 2 % of the lipids in the carcass (Bout and Girard, 1988). The amount and the quality of intramuscular fat are elements related with flavor, aroma and tenderness of meat (Coma and Piquer, 1999).

Vascular and **nervous systems**, also present in the muscle are less related with meat quality but they are important for the role they develop in the muscle function. Skeletal muscles have an abundant supply of blood vessels and nerves. This is directly related to the primary function of skeletal muscle, namely, contraction. Before a skeletal muscle fiber can contract, it has to receive an impulse from a nerve cell. The vascular system permits the regulation of the body temperature and the rapid transport of blood and its elements between the distinct parts of the body depending on the functional need.

1.2. Meat quality parameters

Consumer ideally desire attractive, economical priced products with desirable color, which are nutritious and healthy, tender, juicy, and flavorful, with no fat or additives. At the point of purchase, the consumer visually assesses meat products for size, shape, color, fat to lean and lean to bone ratio, texture and cost for serving (McGill, 1981). Consumers buy a product based on the balance of these factors which are determined by past personal experience in order to assure the maximum eating satisfaction. Once a product has been purchased consumers respond to aromatic taste and mouthful sensations during consumption, which result in

hedonic or value judgments, based upon past personal experience. In general, meat quality can be defined as a set of sensorial, technological, nutritional and hygienic characteristics (Lebret and Mourot, 1998). Some attributes, mainly the organoleptic characteristics have an important geographical and cultural component (i.e. in Japan is appreciated dark meat with a high content of intramuscular fat which would be rejected in our society).

In the group of **technological properties**, the water holding capacity is a primordial characteristic for the elaboration of processed meat products. The water holding capacity mainly depends on the pH changes during the *post mortem* transformation of the muscle to meat. When the decline of pH approaches the isoelectric point (5. 0 -5.1) muscular proteins reduce the capacity to hold water (Briskey and Wismer-Pedersen, 1961). The changes of pH after slaughter are mainly due to the degradation of glycogen to acid lactic through glycogenolysis and glycolysis in anaerobic conditions. The role of glycogen in the liver is mainly to keep the level of blood glucose constant, however, the glycogen in the muscle acts as an energy source for quick mobilization especially in the cases of anaerobic metabolism through glycogenolysis. The rapid breakdown of muscle glycogen caused by severe, short-term stress just prior to slaughter, for example during off-loading, handling, holding in pens and stunning results in a meat which becomes very pale soft and exudative (**PSE**) with pronounced acidity (pH values of 5.4-5.6 immediately after slaughter). Allowing pigs to rest for one hour prior to slaughter and quiet handling will considerably reduce the risk of PSE. On the other hand, when the muscle glycogen has been used up during the period of handling, transport and pre-slaughter and as a result, after slaughter, there is little lactic acid production, which results in dark firm and dry (**DFD**) meat with a high pH (often > 6.5). PSE are unsuitable for processed meats or products which would swim in an extra fluid and DFD meats highly promote the growth of microorganisms neutrophils (Gregory and Grandin, 1998).

Nutritional quality takes into account the human nutritionist recommendations such as reduction of fat ingestion, of saturated fatty acids (**SFA**) and increase of PUFA, particularly the n-3 family fatty acids (**FA**) such as linolenic acid (C18:3 n-3), eicosapentanoic acid (**EPA**; 20:5 n-3) and docosahexanoic acid (**DHA**; C22:6 n-3). The SFA are associated with an increase of blood cholesterol levels, which is a risk for cardiovascular diseases. However, there is an emerging evidence that palmitic (C16:0) and stearic (C18:0) acids do not contribute to coronary heart disease indicating that the perception that all SFA are unhealthy is incorrect (Moloney and Dunsany, 2002). Humans cannot synthesize all FAs, so it is important to include them in the diet. Among essential C_{18}, α-linolenic acid (ALA, C 18:3 n-3) and linolenic acid (LA, C 18:2 n-6) are the precursors of two families of PUFA. The n-3 and n-6 PUFA have two primary functions: they are structural components of the membranes and serve as substrate for eicosanoids synthesis (Guil-Guerrero, 2007). Some clinical trials support a positive effect of n-3 FA on decreasing platelet aggregation, blood pressure, circulating TAG and producing modest changes in serum cholesterol and lipoproteins (Wijendran and Hayes, 2004). Competition exists between n-3 and n-6 families at the level of desaturation and chain elongation because both pathways are regulated by the same rate-limiting enzymes. However, these enzymes appear to give preference to the n-3 over the n-6 pathway (Fig. 2, Vance and Vance, 1991). Additionally, excessive intakes of n-6, relative to n-3 produce larger amounts of eicosanoid metabolic products from arachidonic acid (AA), specifically prostaglandins, thromboxanes, leukotrienes, hydroxyl fatty acids, and lipoxins than those formed from n-3 FA. The eicosanoids from AA are biologically active in very small qualtities and, if they are formed in large amounts, they contribute to the formation of thrombus and atheromas; to allergic and inflammatory disorders, particularly in susceptible people; and to proliferation of cells. Thus, a diet rich in n-6 FA shifts the physiological stat to one that is prothromotic and proaggregatory, with increases in blood viscosity, vasoplasm, and vasoconstriction and decreases in bleeding time (Simopoulos,

2008). Often meat, and particularly pork is judged by its content of saturated fat (Mattson and Grundy, 1985) but should be reconsidered because a portion of 100 g of loin only has about 2g of fat.

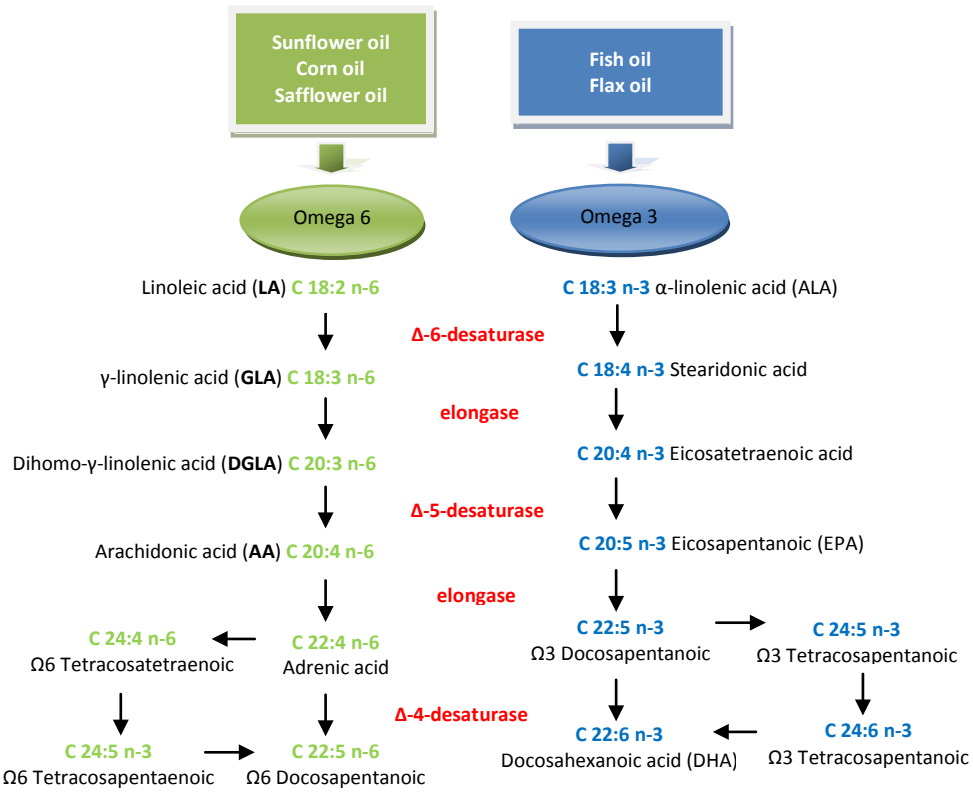

Fig. 2: Metabolic pathway of long-chain PUFAs.

The competition between FA of the n-3 and n-6 families occurs at the level of desaturation and chain elongation. With Δ6 desaturase enzyme, C 18:3 n-3 is a better substrate than C 18:2 n-6. Accordingly the abundance of C 18:3 n-3 can effectively decrease formation of C 20:4 n-6 from C 18:2 n-6. (Adapted from Vance and Vance, 1991)

Hygienic quality of meat is a basic requirement for the consumer. It can alter the proliferation of harmful microorganisms or toxic substances. For this reason, regulatory authorities usually provide the compulsory national framework for food/meat hygiene programs through laws and regulations and monitor the implementation of such laws. At the meat industry level, it is the primary responsibility of individual enterprises to develop and apply efficient meat hygiene

programs specifically adapted to their relevant range of production (Heinz and Hautzinger, 2007).

The **organoleptic properties** are perceived by the human senses (vision, smell, taste, touch and hearing) and are related to three major attributes: appearance (color, size, shape) flavor (odor, taste) and texture (mouth feeling, viscosity and hearing; Mason and Nottingham, 2002). Some of these organoleptic properties of meat are described below:

Meat color can be included as a visual aspect. Color depends on the amount of myoglobin which is related with the percentage of red fibers and its oxidation state. Myoglobin is a compact globular protein consisting of globin (apo protein) and an iron containing heme group, Fe-protoporphyrin, which is the chromophore of myoglobin (Stryer, 1981). The color of myoglobin is determined by its redox state and by the type of ligand bound: deoxymyoblobin (Fe^{+2}) without ligand give a purple color, oxymyoglobin (Fe^{+2}) ligated with O_2 give a bright cherry red color and metmyoblobin (Fe^{+3}) ligated to H_2O (pH<8) give a brown coloration (Fig. 3; Lindahl, 2005). The amount of visible intramuscular fat may also turn the color of meat a bit whitish or yellowish. The final color of the meat will be given by the combination of those two parameters, influencing the consumer decision. Normally, the consumer is looking for a meat with homogenous color (Melton et al., 1996), neither pale nor dark.

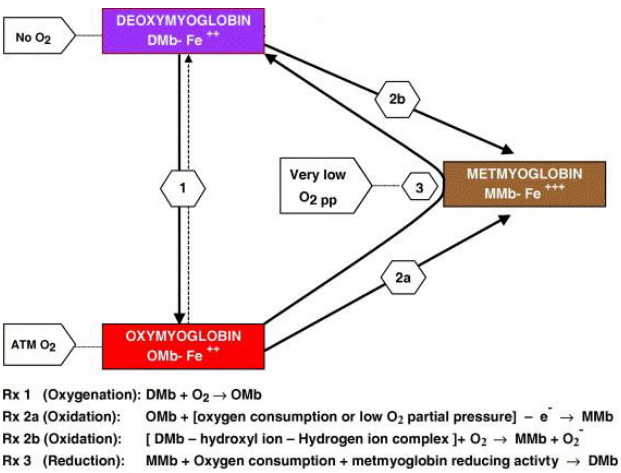

Fig. 3. Visible myoglobin redox interconversion on the surface of meat (Adapted from Mancini and Hunt, 2005)

Tenderness is the easiness of meat cutting or chewing and it mainly depends on: *post mortem* factors such as sarcomer length, temperature, pH and proteolysis and the intrinsic properties of the muscle, i.e. fibre type composition, collagen and IMF content together with factors related to breed, genotype, growth rate and nutrition (Maltin et al, 2003). Tough meat cannot be recognized by the eye at the moment of purchase. However, because IMF has an influence on the tenderness, the most 'marbled' pork cuts may be an indication of tenderness although it is not the only parameter affecting it.

Juiciness is the feeling of the liberation of juice during chewing and is related with the amount of free water in the meat. The juiciness of meat depends on the raw meat quality (water holding capacity, IMF content, concentration of glycogen or rearing conditions; Eikelenboom et al., 1996a/1996b, Jonsäll et al., 2001) and on the cooking procedure (the center temperature and the cooking procedure -heating time/ temperature/ heating method-; Bejerholm and Aaslyng, 2003). Aaslyng et al. (2003) suggested that juiciness experienced initially in the chewing process depends only on the water content of the meat, whereas juiciness experienced later in the chewing process is determined by water and intramuscular fat content and the saliva production during chewing. Girard et al. (1988) also showed that IMF influences favorably meat juiciness.

Flavor corresponds to the taste and olfactory perception during the tasting. Basically, it depends on the lipid composition but may be also caused by the sexual odor. Meat from about 5-10 % boars may produce an unpleasant odor when the meat is heated or cooked due to the content in andostenone and skatole (Bonneau et al., 1992; Udesen, 1998). In order to prevent these defects, and consequently avoid the depreciation of the carcasses, in some European countries (exception: United Kingdom, Ireland, Cyprus, Portugal and Spain) castration is performed systematically on young males, excluding the ones destined for reproduction. Thus, fresh pig meat consumed is almost exclusively from females or precociously castrated males (Girard et al., 1988). On the other hand, the nature of lipids rich in

polyunsaturated fatty acids (PUFA) undergoes peroxidation when they are stored for a long time, giving rancidity and off-flavors to the product. Lipid oxidation is initated when a labile hydrogen atom (next to a double bond) is abstracted from a site on the fatty acyl chain by the action of sunlight or the presence of a metal catalyst (iron, copper, cobalt…), with the production of a free lipid radical which reacts rapidly with oxygen to form a peroxyradical (primary products of oxidation). The peroxyradical abstracts a hydrogen from another hydrocarbon chain yielding a hydroperoxide and new free radical which can perpetuate the chain reaction and giving aldehydes and carboxylic acids leaing the development of oxidative rancidity (Ladikos and Lougovois, 1990). Feeding strategies as the incorporation of high vitamin E levels in the diet decreases the susceptibility to lipid oxidation in pig *longissimus dorsi* muscle (Gonzalez and Tejeda, 2007). Furthermore, Mattes (2009) proposed a “fatty” taste as a primary taste and Khan and Besnard (2009) supported this affirmation suggesting that the lingual receptor CD36 is implicated in the perception of dietary fat as a gustatory lipid sensor. However, confirmation of “fatty” taste will require additional studies that verify that these observations are taste specific. Cameron et al. (2000) found that for neutral intramuscular lipids, the C18:2 n-6, C18:3 n-3, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:5 n-3, and C22:6 n-3 were negatively correlated with pork flavor and overall acceptability. In contrast C16:1, C18:1 n-9 and C18:1 n-11 were positively correlated with pork flavor and overall acceptability. In the phospholipid fraction C18:2 n-6, C20:4 n-6 and C22:4 n-6 were positively correlated with pork flavor and overall acceptability. Tikk et al. (2007) associated a high level of PUFA in fried pork chops with sweet odor (correlation = 0.53). In oven roasts, PUFA were negatively correlated with sourish odor (-0.49), piggy odor (-0.45) and piggy flavor (-0.53).

It can be concluded that fat in meat supplies essential fatty acids and fat soluble vitamins and that IMF plays an essential role in sensory perception. The perception of healthiness and sensory expectation are important criteria that influence the decision to purchase a particular food product. Due largely to consumer preference

for low-fat products and with the aim of improving financial returns, the meat industry began in the early 1980s to modify production systems to produce animals with faster growth rates and with less fat (i.e. the fat content of the carcasses has decreased in Britain by over 30 % for pork; Moloney and Dunsany, 2002). Enhancing organoleptic properties has not been a primary objective, consumer complaints of the lack of taste in pork from modern lean breeds and the frequent reference to more strongly favored meat of former times implies that meat quality has been deteriorated and presents a challenge for the pork industry. Pork industry is faced with the dilemma of producing meat with a minimal amount of visible fat in order to alleviate the health concern of the consumer, but at the same time producing sufficient IMF which is known to contribute substantially to meat quality factors such as juiciness or tenderness, and hence satisfy the eating experience of the consumer. Note that marbling (visible IMF) is a subjective measurement and IMF an objective measurement and that not all of the IMF is visible. However, because IMF is not the only parameter affecting meat quality characteristics, the association of high IMF with an improvement of some of the meat quality parameters is not clear (Ngapo and Gariepy, 2008; Table 1).

Table 1: Studies relating IMF and meat quality

References	Relation between IMF and meat quality traits
Positive effect	
Davis et al. (1975)	Significant correlation with juiciness, tenderness and overall acceptability of meat (0.42, 0.41, and 0.41, respectively)
Gandemer et al. (1990)	↑ IMF: most appreciated by panel test
Hodgson et al. (1991)	Significant correlation with juiciness (0.65) and overall acceptability of the meat (0.51)
Fernandez et al. (1999a)	↑ IMF: favorable effects on sensory attributes
Brewer et al. (2001)	↑ marbling: lighter colored chops, less acceptable appearance but ↑ juiciness, tenderness and flavor
Heyer and Lebret (2007)	Correlated with juiciness ($r = 0.62$; $P = 0.001$)
Moeller et al. (2010)	IMF fat levels of 5-6% would improve pork flavor, small incremental of juiciness or tenderness
Weak positive effect	
Henry et al. (1963)	Correlated with tenderness (0.37) and flavor (0.23) but not with juiciness
DeVol et al. (1988)	Correlated with tenderness, juiciness and flavor (0.32, 0.21, and 0.23, respectively)
Casteels et al. (1995)	Correlated with tenderness (0.39), juiciness (0.43) and taste intensity (0.28) but no correlation when was corrected for the genetic background
Channon et al. (2004)	Only correlated with flavor (0.31)
Rincker et al. (2008)	Limited effects on juiciness, tenderness, pork flavor and oiliness
Negative effect	
Judge et al. (1960)	Correlated with firmness (0.35) poor correlated with tenderness, juiciness and flavor were (-0.3, 0.13 and 0.1, respectively).
Candek-Potokar et al. (1988)	Pigs increased in age and weight: lower sensory quality in spite of the ↑ IMF; only a weak correlation with flavor (0.29)
Huff-Lonergan et al. (2002)	No correlated with juiciness, but correlated with firmness (0.31) or flavor (0.23)
No influence	
Hovenier et al. (1993)	No correlation with tenderness
Blanchard et al. (2000)	No correlated with any eating quality parameters
van Laack et al. (2001)	Not results in improved tenderness
Lonergan et al. (2007)	Small variation in texture and tenderness of pork loin with pH between 5.80 and 5.50, but not at greater or lesser pH.

Some reports proposed a threshold level of IMF that will ensure a pleasing eating experience but it differs between different studies (Table 2). The selective breeding designed to increase the lean to fat ratio in the carcasses led to many modern genotypes with an IMF percentage of only 1 % (e.g. in Large White pigs) compared with 2-4 % in the 1960s, becoming a problem in terms of meat quality parameters, such as juiciness and tenderness of fresh meat products (Wood et al., 2008).

Table 2: Suggested IMF thresholds to achieve a pleasing eating experience

IMF (%)	Sensory notes	Meat and sensory methods	Reference
1.0 minimum	Based primarily on tenderness	LD chops, frozen (ageing not given), grilled, 80ºC core	Wood (1990)
1.5 minimum	Function of attributes describing tenderness	LL, frozen (ageing not given), roasted, 72ºC core	Fortin et al. (2005)
2.0-3.0	Based on tenderness and flavour	LD chops, frozen 5-7 d *p.m*, grilled, 65ºC core	Bejerholm and Barton-Gade (1986)
2.0-4.0	US National Pork Boards quality targets	Not specified	Meisinger (2002)
2.5-3.0	Based on tenderness	LD chops, frozen 2 d *p.m.*, roasted, 75ºC core	DeVol et al. (1988)
2.5-3.5	Texture and acceptability	LL chops, frozen 1 month *p.m*, grilled at 180ºC	Fernandez et al. (1999b)
>3.0	Palatability, juiciness and tenderness	Not specified	Daszkiewicz et al. (2005)
>4.0	Tenderness, juiciness and flavour	LD, frozen 24 h *p.m.*, roasted, 70-75ºC core, cooled to ambient	Gandemer et al. (1990)

Adapted from (Ngapo and Gariepy, 2008); LD: *longissimus dorsi*; LL: *longissimus thorcacis*; d: day; *p.m.*: *post mortem*

2. Importance of lipid biochemistry in pigs

This thesis is mainly focused on the storage of fat because of the relation of IMF and meat quality parameters described above. Nonetheless, the other roles of fat in the organism are as well important for a correct development of the animal. So, a brief summary is presented before to go deeply into lipid metabolism.

Lipid is the term used in a biochemical context instead of fat which is mainly used to refer to solid part of all lipids. Lipid classification is divided between simple and complex lipids. The simplest lipids are the fatty acids (**FA**), which are the constituents of many more complex lipids and which substantially contribute to the physical appearance of fats. Fats rich in unsaturated fatty acids are liquid at room temperature, whereas those with a higher content of saturated fatty acids (SFA) are more solid. The introduction of a double bound in *cis* geometric configuration results in a bending of the chain with a change of approximately 30^{o} from the

linearity of the saturated chain. Indeed, firmness increases as the hydrocarbon chains are longer. The reason is simple: long saturated fatty acid can pack closely together, to form regular, semicrystalline structures and *cis* double bound makes molecular packing more difficult. In agreement, Davenel et al. (1999) reported that the presence of two saturated fatty acids in a same triacylglycerol gives them a high melting point, increasing the solid phase of the lipids in pig adipose tissue.

The biological functions of lipids include energy storage (i.e. triacylglycerols), structural components of cell membranes (i.e. glycerophospholipids, glycosohingolipids and cholesterol), signaling molecules (i.e. steroid hormones or eicosanoids), lipid soluble vitamins and constituents of bile acids (Mathews and Van Holde, 1966).

The long hydrocarbon chains of fatty acids (**FA**) are extraordinarily efficient for **energy storage**, because they contain carbon in a fully reduced form and will therefore yield a maximum amount of energy on oxidation. They are much more efficient energy stores than carbohydrates and for this reason are used as energy storage for live organisms. FAs are largely stored in the form of triacylglycerols (**TAG**) in fat cells (adipocytes) found in the adipose tissue. Almost the entire volume of each cell is filled by a fat droplet.

The **structural lipids**, glycerophospholipids, glycosohingolipids and cholesterol form the integral part of the cell membranes and act as cell limits, mediating the cellular transport, acting as barriers between cell compartments and as production sites of many important biochemical substances for the metabolism.

Lipids may also act as **metabolic signals**:

- *Steroid hormones* are synthesized from cholesterol and control metabolism at gene level. They react with intracellular protein receptors, and the hormone-receptor complexes bind to specific sites on the genome and regulating the transcription of neighboring genes.
- *Prostaglandins, thromboxanes* and l*eukotrienes*, collectively are called eicosanoids because of their common origin from C 20, particularly arachidonic

acid. Eicosanoids act like hormones on target cells. However, because they act locally or near their sites of synthesis, they are considered as local hormones. Moreover, the actions of a given prostaglandin may vary in different tissues.

The four lipid-soluble **vitamins** (A, E and K) isoprenoids and (D) steroid are diverse in their function. Vitamin A plays a key role in vision, vitamin D regulates calcium and phosphorus metabolism, vitamin E appears to play an antioxidant role and vitamin K is involved in blood coagulation. Still, other functions have been assigned to some of these vitamins.

Bile acids are steroid derivates with detergent properties which emulsify dietary lipids in the intestine and thereby promote fat digestion and absorption.

2.1. Pig lipid metabolism

The formation or degradation of TAG depends on the energetic status of the animal. When pig is in a positive energy balance, the 'excess' of energy is stored in adipose tissue increasing whole animal fat deposition. This increase is caused by a high storage of fatty acids (mainly SFA and MUFA) arising from *de novo* fatty acid synthesis of carbonated chains from the carbohydrate and amino acid catabolism (Gandemer, 2002). Additionally, the composition of FA in the pig adipose tissue is also dependent on the fatty acid origin and quantity included in the diet. The contribution of each of these 2 pathways (exogenous or *de novo*) depends on the amount of fat in the diet.

2.1.1. Exogenous fat source, digestion and absorption

In practical diets for pigs, fat is added at less than 10 % and typically less than 5 % of the diet. In the case of the pigs, dietary fat inhibits *de novo* FA synthesis and alters FA profile of adipose tissue and other tissues (Hausman et al., 2009). Hence, FA may be deposited without modification or modified in less or more intensity (elongation or desaturation) in the different tissues. Digestion and absorption of

dietary fat depends on the pancreatic secretions and the bile acids. Kloareg et al. (2005) found that approximately 70% of the digested n-6 FA and 50% of n-3 were retained by the animal, while only 30% was oxidized. Duran-Montgé et al. (2008) demonstrated that when unsaturated FA are provided in lower amounts they are highly incorporated within the tissues (80-90 %), while when larger amounts are provided they are not incorporated at the same rate (around 60 %) suggesting that some are oxidized.

The TAG, the most abundant class of lipids in feed, arrive to the small intestine where they undergo an emulsification process, favoring the action of lipase and colipase which act selectively in the position 1 and 3 of triglycerides (Fig. 4). The short or medium (< C 14:0) chain FA may be hydrolyzed by a pregastric esterase and gastric lipase before their arrival in the duodenum. Pancreatic juice contains the phospholipase A_2 which once activated by trypsin is able to hydrolyze glycerophospholipids producing lysolecithins. The esters of exogenous (from the diet, if it is the case) or endogenous (from the bile acids) cholesterol can be hydrolyzed in the duodenum by cholesterol-ester-hydrolase or cholesterol esterase. The free short or medium chain FA by themselves and the 2-monoglycerides, the free long chain fatty acids, lysolecithins and cholesterol solubilized with the micelles may be incorporated into the enterocyte. In the enterocyte, short or medium chain FA cross the epithelial cell without esterification and are transferred to the liver through portal vein as free FA transported by albumin, while long chain FA are activated in presence of coenzyme A to acyl-coenzyme A which bound with glycerol to synthesized again TAGs. Cholesterol is also sterified again in the enterocyte. The products from the fat digestion will form part of the lipoproteins called chylomicrons which transport the lipid molecules from the intestine to the peripheral tissues. The chylomicrons are lipoproteins rich in TAG (85%), additionally the other components are: phospholipids (7%), cholesterol esters (4%), cholesterol (2%), and proteins (2%). Finally, chylomicrons are transferred to the intercellular spaces and then to the lymph joining the

bloodstream (Perez et al., 1986). The TAGs are hydrolyzed to glycerol and FAs on the capillary endothelium of the peripheral tissues by lipoprotein lipase. The free fatty acids obtained are absorbed for the tissues and can be used as energy source or stored as TAG in the adipocytes. Hence, differences in the lipoprotein lipase activity between different tissues control the distribution of the plasmatic TAGs. The remnant chylomicrons are captured by liver through specific receptors and depredated in the hepatic lysosomes (Mathews and Van Holde, 1966).

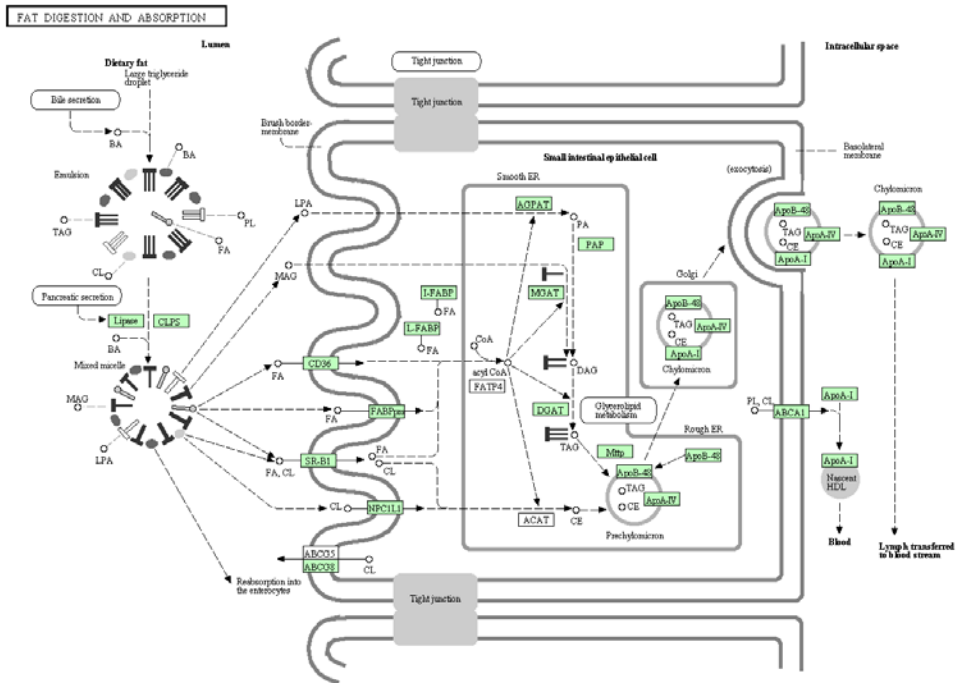

Fig. 4. Fat digestion and absortion – *Sus scrofa* (pig)

From: Kyoto Encyclopedia of Genes and Genomes (KEGG)

2.1.2. Endogenous synthesis

The majority of fuel stored in most of the animal cells is in the form of fat. However, difference in *de novo* fatty acid synthesis rate or site of lipogenesis exists between different species. Human, rodent and avian species has liver as primary site while for pigs, dogs, cats, cattle and sheep or goats adipose tissue is the major site (Bergen and Mersmann, 2005). With the exception of newborn piglets that

hepatic lipogenesis is high (Mersmann and Phinney, 1973), the low capacity of liver for fatty acid synthesis from glucose indicates that adipose tissue plays a major role in pigs, if not nearly exclusive in fatty acid synthesis as previously reported by O'Hea and Leveille (1969). The difference in the site of fatty acid synthesis and the pattern of consequent lipid trafficking, influences overall animal lipid metabolism including the role of regulatory hormones and transcription factors.

In monogastric animals fatty acid synthesis uses carbohydrates or proteins, but in a positive energy balance they mostly use carbohydrates in order to keep the plasma glucose constant (5mM). Nowadays, the tendency is to use diets low in fat content (2-4% of the diet) and the consequence of feeding low fat diets is that the organism must synthesize a considerable fat amount *de novo*.

Biosynthesis of palmitate

Glucose once metabolized via glycolysis to pyruvate, enters in the mitochondrion, traverses the initial steps of the tricarboxylic acid cycle to citrate, which in excess exits the mitochondrion, is cleaved to acetyl-CoA by ATP-citrate lyase, is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (**ACC**), and then is polymerized to long chain fatty acid by fatty acid synthase (Fig. 5; Stanton and Mersmann, 1986). The cellular cytoplasm is where the FA chain grows in sequences of two carbons by the incorporation of one acetyl-CoA to obtain the final product, palmitic acid (C16:0).

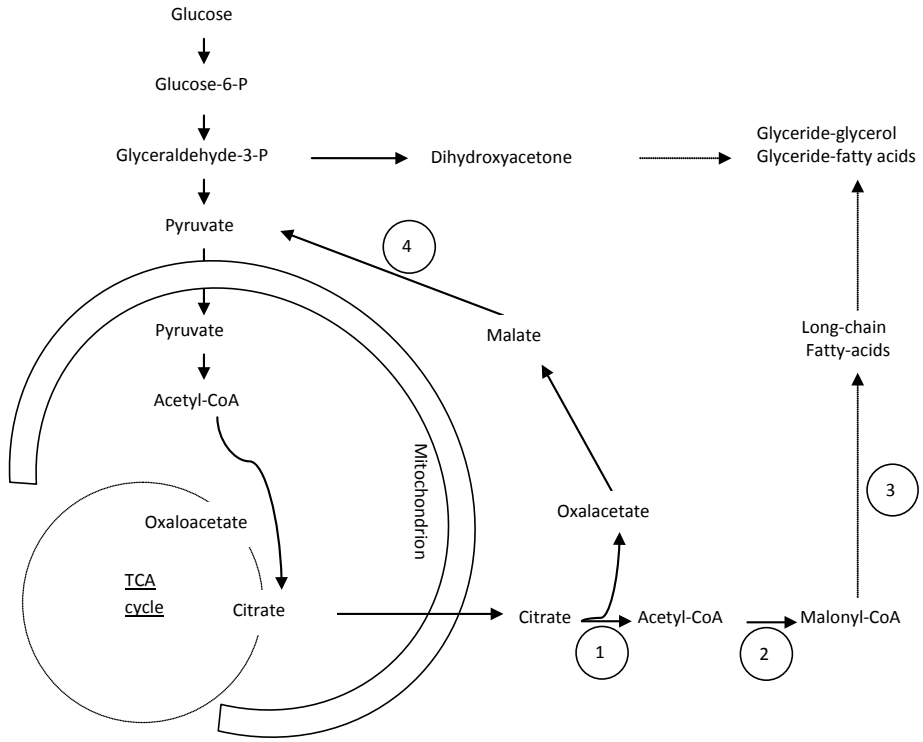

Fig. 5: Fatty acid biosynthesis

1: ATP citrate lyase; 2: Acetyl CoA carboxylase; 3: Fatty acid synthase; 4: malic enzyme dehydrogenase (Adapted from Stanton and Mersmann, 1986).

Chain elongation from palmitate

Many eukaryotic cells have the capacity for 2-carbon chain elongation, both from endogenously synthesized fatty acids and from exogenous dietary fatty acids. There are two primary systems for elongation in liver, brain and other tissues, one in the endoplasmatic reticulum, and the other one in mitochondria. The most active fatty acyl chain elongation system is the one associated with the endoplasmic reticulum and the difference between the two locations is the 2-carbon donor, while in endoplasmic reticulum it is malonyl-CoA, in the mitochondria it is acetyl-CoA. Recent evidence indicates that liver peroxisomes also contain acetyl-CoA dependent elongation system. The four component reactions occuring in the 2-

carbon elongation process are: 1) condensation of fatty acyl-CoA and malonyl-CoA or acetyl-CoA (depending if the reaction is carried out in the endoplasmatic reticulum or the mitochondria, respectively); 2) elongation; 3) dehydration; 4) reduction (Fig. 6; Vance and Vance, 1991). In animals all of the enzymatic activities are associated in a complex named fatty acid synthase (**FAS**).

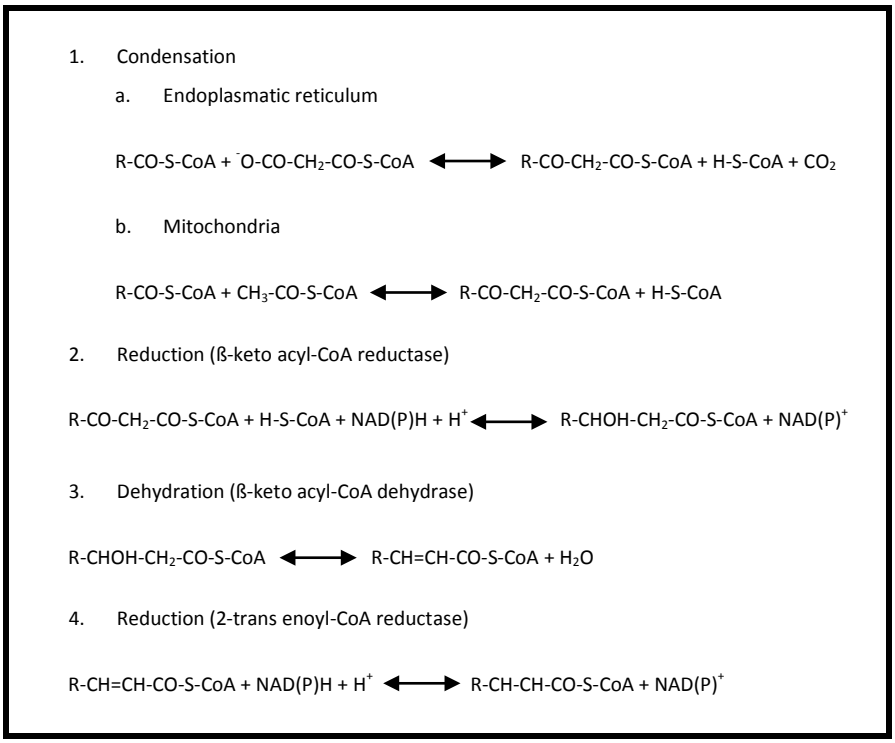

Fig. 6: Reactions in 2-carbon chain elongation of long chain fatty acids

Adapted from: Vance and Vance (1991).

Desaturation

The MUFA, which are needed to maintain the fluidity of the membrane, are formed in mammalian systems by direct oxidative desaturation (a removal of two hydrogen molecules) of a preformed long chain SFA. The Δ9 desaturase is the predominant, if not the exclusive desaturation enzyme in the endoplasmatic reticulum of liver, mammary gland, brain, testes and adipose tissue. The desaturase component is

largely within the microsomal membrane, with the active center exposed to the cytosol. For many tissues 14 to 18 carbon saturated acyl chains are good substrates, with stearoyl-CoA being the most active. One example is the synthesis of oleic acid (18:1 cis-9) the major fatty acid in meat, which is mainly formed from stearic acid (18:0) by the enzyme stearoyl Co-A desaturase (Kouba et al., 2003). A higher preference to desaturation for dietary C 18:0 than for C 16:0 was reported by Raju and Reiser, (1970) and Rhee et al. (1997) while Nakagawa and Uchiyama (1968) reported the contrary. The Δ9 desaturation system consists of three major components: 1) NADH-cytochrome b_5 reductase; 2) cytochrome b_5; and 3) a terminal desaturase component or cyadine-sensitive protein (Fig. 7; Vance and Vance, 1991).

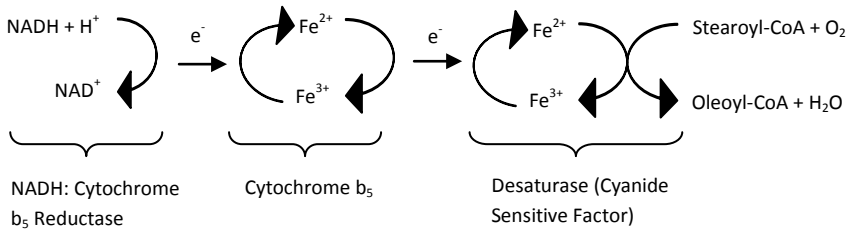

Fig. 7: Diagrammatic representation of the Δ9 desaturase complex, including the electron transport proteins

Adapted from: Vance and Vance (1991).

All eukaryotic organisms contain PUFA in the complex lipids of their membranes, and most mammalian tissues can modify acyl chain composition by introducing more than one double bound. The first double bond introduced into a saturated acyl chain is generally in the Δ9 position so that substrates for further desaturation contain either a Δ9 double bond or one derived from the Δ9 position by chain elongation. Further desaturation is an oxidative process requiring molecular oxygen, reduced pyridine nucleotide and an electron transfer system consisting of a cytochrome and related reductase enzyme. Animal systems cannot introduce double bonds beyond Δ9 position. Thus, second and subsequent double bonds are always between an existing bond and the carboxyl end of the acyl chain. Plants, on

the other hand, generally introduce second and third double bonds between the existing double bond and the terminal methyl group. Consequently, in animals double bonds are inserted at the Δ9, Δ6, Δ5 and Δ4 positions. Conjugated double bonds are extremely rare in mammals. Accordingly, given the limitations of mammalian desaturases, chain elongation usually alternates with desaturation to maintain methylene interruption in PUFA chain. Additionally, al bonds introduced by oxidative desaturation are in the *cis* geometric configuration. *Trans* are introduced to animal systems through diet or intestinal bacteria (Fig. 8; Vance and Vance, 1991).

Biosynthesis of TAG

Most of acyl-CoA synthesized and glycerol-3-phosphate enter to the production of TAG synthesis. Glucose is the substrate that provides most of the glyceride-glycerol for TAG synthesis as a branch form the glycolytic pathway.

2.1.3. Fat oxidation

The release of fat from fat depots in the adipose tissue is controlled hormonally to satisfy the needs of the body in energy generation. Fat catabolism is started with the hydrolysis of TAG to produce free FA and glycerol. Approximately, a 95 % of the energy from the fat oxidation comes from the FA and only a 5% from glycerol. The carbons of the FA chain are metabolized to 2-carbon segments in form as Acetyl-CoA, except a small proportion which contain an odd number of carbons.

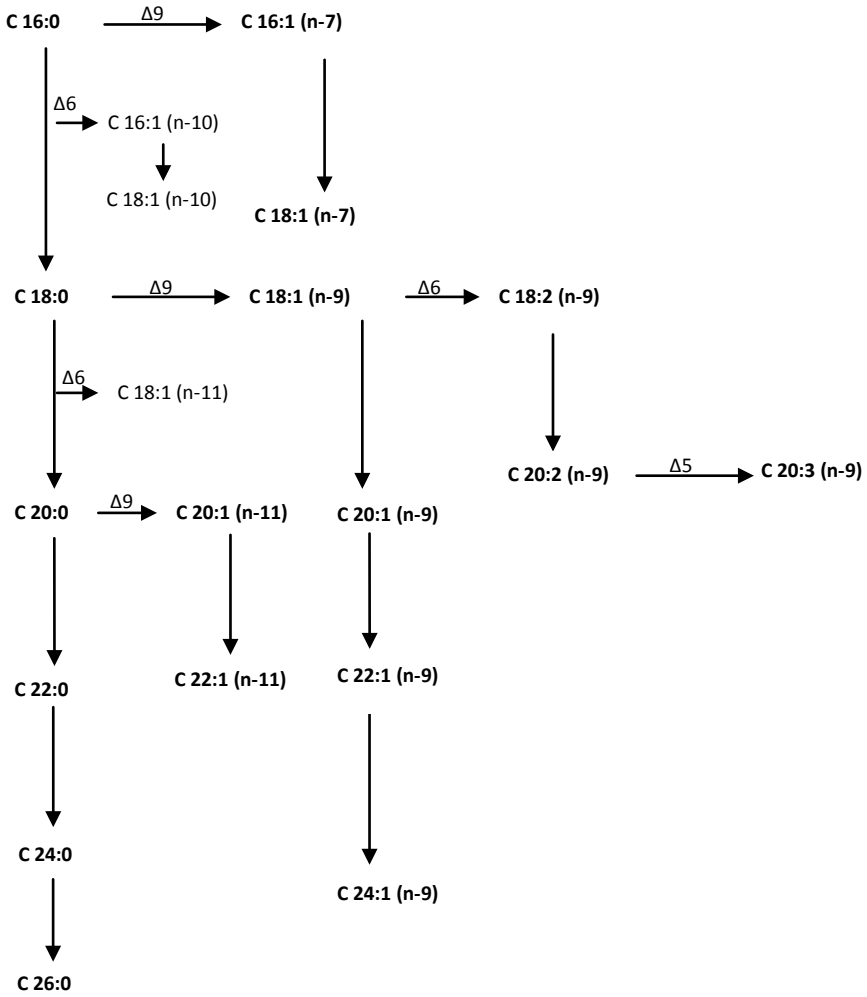

Fig. 8: Major pathways of *de novo* fatty acid biosynthesis by desaturation and chain elongation in animal tissues.

Note that alternating sequence of desaturation in the horizontal direction and chain elongation in the vertical direction. Boldness individual fatty acids reflect, in general way, relative accumulation in tissues. (Adapted from: Vance and Vance, 1991).

Under stress or fasting the triacylglycerol lipase is activated and hydrolyzes the FA in the 1st or 3rd position in the glycerol. Consecutively, the diacylglycerol lipase and monoacylglycerol lipase hydrolyze the other two remaining FA. These FA are linked with albumin to the bloodstream and enter into the tissues by passive diffusion. The glycerol also released into the bloodstream is captured by hepatic cells where

it is used as substrate to the synthesis of glucose. The FAs present in the cytosol must be incorporated into the mitochondrial matrix to undergo the oxidation procedure. Because of the impermeability of the inner membrane, FAs have to be activated with acyl-CoA and ATP degradation through acyl-CoA ligase in the external membrane. The acyl is transferred to carnitine by carnitine acyltransferase I resulting in an acyl-carnitine derivate, which can be transported thorough carnitine acyltransferase II into the mitochondrial matrix. An acyl-CoA is synthesized again inside the matrix and the remaining carnitine is transported to the intermembrane space. In the mitochondrial matrix the oxidation is started in the carbon ß clipping each time a fragment of 2 carbons in form of acetyl-CoA. Each step is composed by 4 cyclic reactions: 1) dehydrogenation to produce an enoyl derivate; 2) hydration of the double bond resulting; 3) dehydrogenation of the hydroxyl group; and 4) thiolysis to produce acetyl-CoA and a two carbons shorter acyl-CoA. The acetyl-CoA produced can enter to the citric cycle where can be oxidized to CO_2 and the NADH and $FADH_2$ produced during the process, are subsequently used in the electron transport chain to produce ATP, the energy currency of the cell (Fig. 9; Mathews and Van Holde, 1966).

In the case of the MUFA and PUFA, two additional enzymes are needed: enoyl-CoA isomerase and 2,4-dienoil-CoA reductase, respectively. The procedure is the same as for the SFA but before the release of the 2 carbons containing the double bound *cis*, the conformation is changed to *trans* by the two enzymes mentioned before. In the oxidation of odd chain FA the substrate obtained in the last cycle contains 5 carbons and the thiolytic fragmentation produces acetyl-CoA and propionyl-CoA. Propionyl-CoA has to be metabolized before being able to enter into the citric acid cycle (Mathews and Van Holde, 1966)

Cytosol

Mitochondrial matrix

Fig. 9: An Overview of the route of fatty acid oxidation

Adapted form Mathews and Van Holde (1966)

2.1.4. Adipogenesis

Adipocytes are the main cells from adipose tissue; they contain almost exclusively TAGs representing the chemical form in which the fatty acids are stored in adipose tissues. The fat cells are isolated or in clusters and they have an ovoid shape with only one lipidic inclusion which is found in the periphery of the cytoplasm (Girard et al., 1988). Preadipocytes and adipocytes are important in establishing the overall

fatness of the carcass, as well as being the main components of marbling which is valued by the consumer of meat products. Adipogenesis is an inclusive term describing the proliferation, differentiation, and conversion of the cells into lipid-assimilating cells found within fat tissue (Dodson and Fernyhough, 2008). Increases in fat cell number originating from adipocyte precursor cells are referred as "proliferation" and "differentiation" is the transition from undifferentiated fibroblast-like preadipocytes into mature round lipid-filled fat cells and is characterized by a change in morphology from fibroblastic to unilocular appearance of the mature fat cell (Hausman et al., 2001). The cellular development associated with adipose tissue growth involves both cellular hypertrophy (increase size) and hyperplasia (increase in number). Hypertrophy is the result of excess TAG accumulation in existing preadipocytes and hyperplasia results from the recruitment of new adipocytes from precursor cells in adipose tissue and involves proliferation and differentiation of preadipocytes. Both hypertrophy and hyperplasia are associated with a positive energy balance (Hausman et al., 2001). Traditionally, cells undergoing adipogenesis are thought as being terminally differentiated when they express numerous cellular or molecular markers reflective of lipid assimilation into storage triacylglycerols. The cellularity of an adipose tissue depot may be function of 3 different cell populations. Firstly, non-differentiated stem cells may experience a trans-differentiation event to become preadipocytes, which possess the ability to accumulate lipid. Secondly, proliferative-competent preadipocytes from embryonic development that are found in adipose depots may add conversion-competent cells to the enlarging adipose tissue depot. Finally, mature cells may reinitiate proliferation and add new cells to the growing adipose tissue. The fate of adipocyte precursor cells depends on the convergence of multiple factors including adhesion of cells to the surrounding extracellular matrix (ECM) or to neighboring heterologous and homologous cells, the mix of growth factors and endocrine environment, neutral inputs and the availability of macro- and micro-nutrients (reviewed: Hausman et al., 2009).

2.1.5. Regulation of lipid metabolism and adipogenesis

As a global regulator of lipid metabolism, leptin has to be mentioned. It is a protein hormone synthesized primarily by adipose tissue and secreted into the bloodstream. It is well correlated with adiposity, reflecting adipose tissue mass and plays a key role regulating energy intake and energy expendure, including appetite and mebabolism. Leptin binds to neuropeptide Y in the brain that signals that the body has enough to eat, producing the feeling of satiety. In a long term positive balance (but not in acute feeding events) leptin is increased in order to limit fat deposition while in a negative energy balance leptin concentrations in plasma and adipose tissue decrease rapidly and profoundly as a result of food deprivation. (Barb et al., 2001).

Biosynthesis

Metabolic energy needs to be stored, i.e. feeding energetic diets.

Hormones: *Insulin* acts increasing the levels of glucose inside the cell, hence increasing glycolysis and the reaction of pyruvate dehydrogenase increasing acetyl-CoA used for the synthesis of FA (Mathews and Van Holde, 1966). Insulin increases the activities of (Δ5, Δ6 and Δ9) desaturases in fasted animals in short term, but repress activity with prolonged re-feeding.

Enzymatic regulation: One important enzyme related to lipogenesis is the malic enzyme (Wise and Ball, 1964). It is present in high concentrations in adipose tissue and increases upon refeeding in proportion to the length of the previous period of fasting. Elevated carboxykinase levels divert oxalacetate to carbohydrate formation, while elevated malic enzyme diverts this 4-carbon acid to pyruvate (Young et al., 1964). A positive correlation higher than 0.70 was found between intramuscular fat and malic enzyme activity, hence, malic enzyme could be an important factor affecting the level of intramuscular fat contents (Mourot and

Kouba, 1998). Furthermore, the concentration of acyl-CoA is reduced by insulin stimulating the synthesis of FA.

Fasting reduces Δ9 desaturase activity and re-feeding increases its activity in liver (Vance and Vance, 1991). In a review, Flowers and Ntambi (2009), suggested that dietary conditions that promote increased insulin secretion will result in high SCD1 activity, which is associated with an accumulation of adipose triglyceride stores. In addition to the evidence that diets low in fat increase liver Δ9 desaturase activity, there is evidence that dietary polyunsaturated acids particularly linoleic acid, selectively inhibit monoene formation (Vance and Vance, 1991). Kouba and Mourot (1998) and Kouba et al., (2003) showed this inhibition in the pig.

Although the response of Δ5 and Δ6 desaturases to dietary alterations are less influenced than Δ9, the enhancement of desaturase activity observed upon refeeding after fasting can be suppressed by glucagon or cAMP in the case of Δ6 desaturase activity but not for Δ5 desaturase (Vance and Vance, 1991).

Transcription factors: SREBPs are synthesized as large precursor proteins that remain in the endoplasmatic reticulum membrane. After proteolytic cleavage, the amino terminal domain migrates to the nucleus and activates target genes (Nakamura and Nara, 2004). SREBP-1c preferentially enhances transcription of genes involved in fatty acid, triglyceride and phospholipid synthesis whereas SREBP-1a and SREBP-2 activate genes involved in cholesterol synthesis. The regulation of gene transcription by fatty acids could be due to changes in the activity or the abundance of transcription factors but fatty acids or cholesterol do not bind to SREBP proteins; instead, they induce changes in nuclear abundance of this transcription factor. Thus, PUFA may decrease the expression of SREBP-1c (Pégorier et al., 2004). Furthermore, the induced hypertrophy of porcine adipocytes would be in part due to the increase of TAG content, FAS activity and SREBP-1c mRNA level (Li and Yang, 2008). Because insulin treatment of primary hepatocytes increased SREBP-1c mRNA but activation of FAS gene expression required both insulin and high glucose, SREBP-1c is probably activated at both the

transcriptional and post-transcriptional levels by insulin (Osborne, 2000).

Beta oxidation

Metabolic energy is needed, i.e. during fasting or stress situations.

Hormones: The hormones *adrenaline* and *glucagon* are secreted as a response to low blood glucose levels. These hormones activate the enzyme adenylyl cyclase in the plasmatic membrane of the adipocytes which produces a second intracellular messenger, cyclic AMP (cAMP). Protein kinase A (PKA), dependent of cAMP is activated and phosphorylates the perilipin activating the hormone sensitive lipase (HSL) which starts the hydrolysis of the TAG in free FA and glycerol (Nelson and Cox, 2009). Perilipin is a protein found in adipocytes that clearly restrains the action of TAG lipases under basal conditions. Caveolin-1 may facilitate phosphorylation of perilipin, and therefore increase the surface area of neutral lipid droplets increasing the accessibility for lipases (Duncan et al., 2007). Granneman et al. (2007) reported that perilipin is a lipid droplet scaffold protein that regulates the trafficking of lipolytic effectors in response to PKA activation.

Glucagon blocks the response of Δ6 desaturase but has little effect on Δ9 desaturase activity. Adrenaline also suppresses Δ5 and Δ6 desaturase activities but enhances Δ9 desaturase. Δ6 and Δ5 desaturase activities are similarly inhibited by glucocorticoids, other steroids and adrenocorticotropic hormone (ACTH; Vance and Vance, 1991).

Enzymatic regulation: In the liver, the acyl-CoA formed in the cytosol can follow two different pathways: ß-oxidation or synthesis of TAG and phospholipids. Carnitine acyltransferase I is inhibited by malonyl-CoA, the first compound in the synthesis of FAs, thus avoiding the oxidation pathway when the synthesis is activated. Additionally, when the relation between [NADH]/[NAD^+] is high, ß-hydroxyacyl-CoA (3^{rd} step of ß-oxidation) is inhibited and high concentrations of acetyl-coA inhibit thiolase (4^{th} step of ß-oxidation; Nelson and Cox, 2009). During fasting the reduction of the levels of ATP and the increase of AMP activates the

AMPK (protein kinase activated by AMP) which phosphorylates the acetyl-CoA carboxylase reducing the synthesis of malonyl-CoA and activating the transport of the acyl-FA into the mitochondrial matrix through the carnitine complex. Then, ß-oxidation is activated and ATP synthesized (Nelson and Cox, 2009).

Transcription factors: PPARα acts in the muscle, adipose tissue and liver on a set of essential genes for the FA oxidation among which there are: carnitine acyltransferase I and II and acyl-CoA dehydrogenase. In a state of energy demand as fasting, the release of glucagon can act through cAMP and the transcription factor CREB to activate certain genes related with lipid catabolism (Nelson and Cox, 2009). An overexpression of a recently identified lipase, the desnutrin/ATGL reveals an increase in TAG breakdown and release of FA (Jaworski et al., 2007).

Adipogenesis

Hormones: Adipocyte differentiation is under the regulation of multiple hormones and growth factors. These initiate intracellular signal transduction through cell surface or intracellular receptors expressed on preadipocytes. Hormonal stimulation of preadipocytes leads to an increase in the level of intracellular cAMP, which is considered as a prerequisite for initiating differentiation. Adipocyte differentiation may be induced by both IGF-I (insulin-like growth factor-1) and insulin. Growth hormone (GH) stimulates the IGF-1 in undifferentiated cells promoting cell proliferation (Wabitsch et al., 1996). Glucocorticoids promote adipogenesis down-regulating the expression of preadipocyte factor-one (Pref-1), a plasma membrane protein that inhibits differentiation (Smas et al., 1999; Sul et al., 2000). Growth factors such as epidermal growth factor and transforming growth factor (**TGF**) inhibit adipose tissue development (Kokta et al., 2004). Other regulators of adipocyte metabolism include prostaglandins, which may promote or inhibit adipogenic conversion depending on the type of prostaglandin (reviewed: Hausman et al., 2009).

Transcription factors: C/EBPs are a family of transcription factors, composed of six members called C/EBPα to C/EBPζ. They promote the expression of certain genes through interaction with their promoter. The different members of C/EBP family can form homodimers, heterodimers with another form of the C/EBPs and with other transcription factors. The dimerization is required for the activity of C/EBPs to bind specifically to DNA through a palindromic sequence in the major groove of the DNA. The C/EBP proteins also contain activation domains at the N-terminus and regulatory domains. These proteins are found in hepatocytes, adipocytes, hematopoietic cells, spleen, kidney, brain and many other organs. C/EBPs proteins are involved in different cellular responses like in the control of cellular proliferation, growth and differentiation, metabolism, immunology and many others (Nerlov, 2007). When C/EBPß is phosphorylated its phosphorylation leads to acquisition of DNA-binding activity by C/EBPß. C/EBPß can now transcriptionally activate the two key adipogenic transcription factors, C/EBPα and PPARγ (peroxisome proliferator-activated receptors gamma). These factors along with ADD1/SREBP-1c, coordinately activate the genes responsible for maintaining the adipocyte phenotype. ADD1/SREBP-1c can activate expression of PPARγ, but its significance during differentiation is unclear (Otto and Lane, 2005). PPARγ form part of a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes. PPARγ is mainly expressed in adipose tissue and SFA and PUFA are potent ligands. The modulation of gene transcription is due to binding of the heterodimer PPAR/RXR (retinoic x receptor) to a specific DNA sequence, the peroxisome proliferator responsive element (PPRE; Pégorier et al., 2004). Overexpression of PPARγ reflects transformation/transdifferentiation of hepatocytes towards adipocytes (Yu et al., 2003) and it will likely implicate the regulation of other adipocyte-specific genes in addition to aP2 (Tontonoz et al., 1994). Furthermore, PPARδ alone with its ligand modulates adipogenic genes via expression of endogenous PPARγ (Yu et al., 2008b). While differentiation involves PPARγ, lipid-filling of the adipocyte relies on a late subset of genes and depending

on depot specificity involves GLUT-4 or any number of metabolic markers (Fernyhough et al., 2007). SREBPF1 mRNA level and IMF were positively correlated, SREBPF1 mRNA level increased as IMF content did. Hence, SREBPF1 gene is a promising marker for IMF-based selection of pigs (Chen et al., 2008). The expression of genes encoding elongases and desaturases increased simultaneously with those involved in fatty acid and triacylglycerol synthesis during porcine adipogenesis (Samulin et al., 2009).

2.2. Pig fat depots

The pig has the highest degree of adiposity among animals. This is primarily a result of its highly developed subcutaneous fat depots; nevertheless, edible meat contains a relatively small amount of fat. The adipose tissue in the new born piglet only represents about 1-2 % of the total carcass weight (Mersmann, 1974) while anatomically separable fat in pigs of 100kg body weight represents between 20 and 35 kg (Lebret and Mourot, 1998). Seventy percent of this is localized in subcutaneous fat, 20-25 % in intermuscular fat (between muscles), 5 % in perirenal fat and intramuscular (within muscle) fat only represents 1-2%, a very small proportion of the total body lipids (Henry, 1977). The increase of carcass adipose tissue of young pigs (between 1 and 2 months) is due primarily to increases in the number of adipose cells (adipocytes). Between 2 and 5 months as the animal grows, hyperplasia diminishes and hypertrophy of existing cells assumes primary responsibility for the increase in adipose tissue mass at the same time that the rate of lipogenesis decreases. After 5 months there are no significant increases in adipose cell number; consequently, adipose mass increases solely by the process of cell enlargement (Anderson and Kauffman, 1973) except in genotypes that had not been selected against fatness, so they are still propense to deposit fat by increasing the adipose cell number in this last stage (Hood and Allen, 1977). Because fat is deposited at a lower rate than muscle during the first periods of postnatal life and at a greater rate than lean tissues when animals get older, the concentration of fat

in the muscle will inevitably increase later in an animal's life (Hocquette et al., 2010). Intramuscular adipose tissue develops later and behaves differently from subcutaneous adipose tissue with regard to development of cellularity and metabolic capacity. The average cell volume of adipocytes begins to increase slowly form 8 weeks of age and then increases very rapidly after 16 weeks. The total number of adipose cells per muscle increases continuously from birth to 24 weeks of age. The rate of increase is somewhat greater in the later stage of animal growth (Lee and Kauffman, 1974). As fat is deposited fat cells with higher enzyme activities may increase in size more rapidly and consequently result in fewer cells per unit area of adipose tissue (Anderson et al., 1972).

Subcutaneous fat is composed by 82-84 % of lipids, 10-11 % of water and 6-7 % of protein (Girard et al., 1988), however, subcutaneous adipose tissue has much higher fatty acid content than muscle (Wood et al., 2008). In pigs slaughtered at around 90kg LW, backfat thickness can be used to predict carcasses fatness because of the high correlation observed between it and: percentage of fat shoulder (0.68), loin (0.68), belly (0.65) and ham (0.61). However, backfat thickness cannot be used to predict the amaunt of IMF in the *longissimus dorsi* muscle because the correlation observed was very low (0.08; Henry et al., 1963). This difference is because although intramuscular adipose tissue seems to possess adequate levels of lipogenic enzymes for *in situ* lipid synthesis (Mourot and Kouba, 1998), proteins involved in both anabolic and energy-yielding catabolic pathways are down-regulated in intramuscular adipocytes compared with subcutaneous, visceral, or intermuscular adipocytes, suggesting that the metabolic activity of intramuscular adipocytes is low (Gondret et al., 2008). Therefore, the lower lipogenic enzyme activities are accompanied by a smaller cell sizes and lower lipid contents (Lee and Kauffman, 1974).

The percentage of IMF differs between muscle types; Morcuende et al. (2007) found that IMF was higher in glycolytic than in oxidative muscles (higher in *biceps femoris* and *longissimus dorsi* than *psoas maior* muscle). In agreement with

Gondret and Hoquette (2006), they suggested that not all the lipids are synthesized *in situ* but are deposited by the transport of fatty acids from other fat depots, which could produce differences among muscles in the ability of depositing IMF beyond their muscular metabolic type.

The most common SFA in meat products are mysristic acid (C 14:0), palmitic acid (C 16:0) and stearic acid (C 18:0). Major MUFA include palmitoleic acid (C 16:1 n-7) and oleic acid (C 18:1 n-9), and the major PUFA include linoleic acid (C 18:2 n-6), linolenic acid (C 18:3 n-3) and arachidonic acid (C 20:4 n-6; Hausman et al., 2009). The PUFA incorporated in the different fat depots come from the oils or seeds used to formulate the diets (Henry, 1972). Additionally, a gradient of decreasing unsaturation from the outer layer to the inner layer of subcutaneous adipose tissue, to intermuscular adipose tissue, and to flare fat, which may reflect the adaptation to the environmental temperature is observed (Sink et al., 1964; Monziols et al., 2007). The same gradient of decreasing unsaturation was observed in the different backfat layers of sows. The component acids of the three "inner" layers were found to be practically identical, but the outermost layer contained slightly less palmitic acid and about 4 % less stearic acid, these being compensated by the presence of correspondingly more oleic acid (Dean and Hilditch, 1933). Apple et al. (2009a) reported that the inner backfat layer had the greatest proportions of all SFA and the least portions of all PUFA, whereas the outer layers had the least percentage of all SFA but the greatest percentages of all MUFA. Even though the middle and outer subcutaneous fat layers had similar PUFA percentages. Difference in the saturation between the different fat depots could be explained because intra-abdominal tissues depend more on lipogenesis, whereas subcutaneous tissues relies more upon capture of free fatty acids (Cousin et al., 1993). IMF content was positively related to the total SFA proportion (r = 0.374) and the total MUFA percentage (0.579) and inversely correlated with PUFA (-0.637; Ntawubizi et al., 2009) and it has more MUFA of C16:1 and C18:1 and less PUFA of C18:2 than other fat depots (Suzuki et al., 2006). Additionally, marbling scores and

MUFA increase in parallel suggesting that steaoryl-CoA desaturase (SCD) gene expression is closely associated and/or necessary for marbling adipocyte differentiation (Smith et al., 2009).

3. Factors that affect fat deposition

The quantity of fat deposition including IMF depends on gender, the age, nutritional and environmental factors and on the breed.

3.1. Genetics

Henry (1977) found that genetic factors influence morphological and metabolic modifications of adipose tissue including the percentage of IMF. Genetic variation in meat quality is affected by three factors: differences between breeds, effects due to major genes and heritability (William et al., 2005).

Different pig breeds can be easily classified accordingly the amount of fat they are able to deposit. Most reports place the meat from Duroc or Duroc-sires in a favorable light. Explanations for differences in meat quality among breeds have been sought in differences in meat characteristics, particularly the high IMF content of Duroc relative to white European breeds (Gandemer et al., 1990; Ngapo and Gariepy, 2008). Channon et al. (2004) found that meat from 100% Duroc pigs was juicer and had a higher IMF content than pork from 0 and 50 % Duroc pigs. Large White and French Landrace are known as breeds with normal muscle development, Pietrain and Belgian Landrace are known as heavily musculated breeds with a reduced amount of IMF. The percentage of fatness is around 24% for the first two, 16.7 % for Pietrain and 20.4 % for Belgian Landrace (Girard et al., 1988). Hence, Chinese (Meishan) and American (Duroc) breeds with a higher content of IMF compared with the Europeans breeds (Large White, Landrace or Pietrain) (Gondret and Hoquette, 2006) could improve the sensory perception of the meat (Fjelkner-Modig and Persson, 1986). Introducing Duroc as finisher male in

lean genetic lines, pigs with similar growth parameters (in some cases a slight reduction) as leaner breeds may be obtained, but containing less lean and more fat (including IMF and hence, improving organoleptic properties of meat) (Edwards et al., 1992).

In fat breeds, the high fat content can be attributed to an increased endogenous fatty acid synthesis or a diminished fat degradation (Miao et al., 2008) and hypertrophy (Henry, 1977). In agreement with this, Scott et al. (1981) found that obese animals had larger adipocytes and, consequently, fewer cells per gram of subcutaneous adipose tissue than did lean animals and the rates of lipogenesis were highly stimulated in obese animals compared with lean pigs, although unstimulated lipolytic rates were not different among breed types. In addition, metabolic regulation differences between breeds may also be due to the expression of different genes. Liu et al. (2009) identified 40 genes differently expressed in *longissimus dorsi* muscles of lean or fatty pigs at slaughter weight. They are involved in metabolic processes, cell communication, binding and responses to stimulus. The group with a high intramuscular fat content was also characterized by the down-expression of genes playing a negative role in adipogenesis. A gene that impacts on IMF content without affecting carcass fat deposition in cross population of European and Chinese pigs was identified; however, it remains to be isolated in other pig genotypes (Janss et al., 1997).

Difference in the amount of carcass fat also leads to differences in FA profile. In genetic types with low concentration of total lipid in muscle, the phospholipid fraction is a high proportion of the total and has higher proportions of PUFA. This occurs because the increase of IMF is correlated with neutral lipid while the phospholipids remain constant (Wood et al., 2008). On the other hand, breeds with a greater fat deposition will have a higher content of SFA (Morcuende et al., 2007).

Nonetheless, it is difficult to select the animals with a high IMF content keeping constant the fat content of the other depots (Gondret and Hoquette, 2006). Nowadays pigs may be slaughtered at very young age, while IMF develops late. As a

result, IMF is not very developed when pigs are slaughtered and the selection against body fatness resulted in a reduction of IMF percentage because, although moderate, there is a positive correlation between IMF and carcass fatness (0.30; Sellier, 1998). However, Suzuki et al. (2009), found a lower correlation between backfat thickness and IMF (0.19) in pigs slaughtered at 105 kg LW, suggesting that genetic variation of fat accumulation differs depending on anatomical location. Heritability of IMF is relatively high (h^2 = 0.26 to 0.86, mean of 0.50) in pigs (Sellier, 1998) and although various genetic markers associated with IMF deposition or marbling have been reported, the results are often inconsistent (Hocquette et al., 2010).

3.2. Slaughtering Age

Feed efficiency decreases with age, and more energy from feed is converted to fat. In new breeds selected against fatness, the maximum of lean deposition is close to the slaughtering body weight. Increasing slaughtering age impacts meat quality traits, as bonds between collagen fibers increase, its solubility is reduced and consequently meat firmness increases, affecting negatively meat tenderness (Hill, 1966). In addition, increasing live weight (above 100kg) the lean meat proportion decreases and subcutaneous fat increases with a moderate growth of IMF which may compensate or even increase meat tenderness (Stupka et al., 2008). By increasing slaughtering age, FA are also modified increasing SFA from 28.1 kg to 68.1 kg LW (similar values between 68.1 and 113.6 kg LW) and reducing PUFA in *longissimus* muscle (Apple et al., 2009c). Furthermore, birth weight of piglets also impacts the final animal fat deposition, when animals are slaughtered at the same body weight, animals with a lower birth weight (lower than 850 g) have a higher backfat and IMF deposition (Lebret and Mourot, 1998).

3.3. Gender

In general, growth of castrated males is similar to that of entire males and higher than females but feed efficiency from castrated males is worse than females and entire males, which reflects a lower lean percentage and higher backfat thickness compared with entire males, while females are intermediate (Fjelkner-Modig and Persson, 1986). A 3.6 mm thicker backfat was observed in barrows than in gilts (Lo Fiego et al., 2005b), while Stupka et al. (2008) and Ntawubizi et al. (2009) found that backfat and IMF levels were similar for female and male castrated. Channon et al. (2004) reported that female pigs had 0.4% more IMF percentage than entire males (1.50 % vs. 1.08 %, respectively). An earlier development of adipose tissue in barrows (Bout and Girard, 1988) and higher lipogenic activities may be the responsible for the increase of body fatness in castrated males (Lebret and Mourot, 1998).

Neutral lipids of females IMF are more unsaturated and less saturated compared with barrows or entire males (Bout and Girard, 1988; Suzuki et al., 2006). The sum of all PUFA tends to be higher in subcutaneous adipose tissue from entire males compared with females mainly due to their thinner backfat (Wood, 1990). A higher index for Δ5 and Δ6 desaturase and elongase activity for PUFA metabolism, indicating a greater potential for the synthesis of long chain PUFA was observed in females compared with castrate males without sex effect on enzyme activities involved in MUFA metabolism (Ntawubizi et al., 2009).

Sensory attributes were not affected by sex (Fjelkner-Modig and Persson, 1986). However, Martel et al. (1988) reported that due to the higher content of IMF, meat from castrated males had better organoleptic properties than female or entire males, although other parameters such as genetic type and slaughtering age also affect meat sensorial properties.

3.4. Environment

One of the environmental factors that highly affect pig fat desposition and FA composition is temperature at which animals are reared. Temperature may affect pig growth performance parameters, feed efficiency and the carcass fatness. Pigs grown at low temperatures (below 16ºC) are fatter than pigs grown at higher temperatures. The optimum growth performance parameters are achieved at temperatures between 16 and 21 ºC. Furthermore, temperature may influence the degree of FA unsaturation. Generally, fat of pigs grown at high temperarture is more consistent than that of pigs grown at low temperature, although FA composition also depends on enery intake and the fat source provided in the diet. Other environmental parameters that may influence pig adiposity are air humidity, the rate of illumination and the rearing conditions, but the one that seems to have higher influence in the fat tissue is temperature (Henry, 1972).

3.5. Diet

There is considerable evidence indicating that manipulating the nutrient composition of swine diets may offset the negative effects of genetic predisposition and/or handling on pork quality, and may actually enhance pork quality traits (Apple, 2002). Sundrum et al. (2011) observed that feeding regime was the main source of variation for IMF content in *longissimus* muscle when compared with sex and birth weight.

3.5.1. Feeding level

As energy intake increases, protein accretion increases linearly until it reaches an upper limit, when this limit is reached energy goes into fat accretion. It has been proposed to use a high energy density during early stages of growth and progressively lower energy density as the pig grows towards market weight (Pettigrew and Esnaola, 2001). Feed restriction consists in providing feed in a lower

amount than animals would eat in *ad libitum* conditions. It may be light (5-10 %) or severe (10-20 %) during the growing or finishing phases. The aim is to reduce fat depots while improving growth efficiency. Daily feed intake should be adjusted in function of animal age or LW, gender and genetic type. In fat genetic lines, restriction should be established earlier than in leaner types and castrated males may be more affected than females (Henry, 1977). Restriction from 30-70 kg LW increases lean meat content due to reduced carcass fatness. After re-alimentation from 70-110 kg LW fatness and lean meat is similar to animals fed *ad libitum*, suggesting that compensatory growth modifies the composition of weight gain with more lipid than protein deposition at carcass level. Feed restriction reduced IMF in *bicep femoris* but significance was not reached in *longissiumus muscle*. After re-alimentation animals fed *ad libitum* for the whole period had higher IMF content, indicating that feed restriction influenced more adipose than muscular tissue deposition (Heyer and Lebret, 2007). Consequently to the reduction of fatness, these animals had more PUFA than pigs fed *ad libitum* (Lebret et al., 1999) and increased the elongation rate (Kloareg et al., 2005).

3.5.2. Balance between the main nutrients (protein, carbohydrates and lipids)

A high level of protein during fattening allows animals to express their potential for muscular development (Lebret et al., 1999). An increase of lipid content in isoenergetic diets results in a higher backfat thickness and an increase of potential for lipid synthesis. Reduction of dietary carbohydrates, with a consequent reduction of lipogenic substrates reduces the lipogenic activity (Lebret and Mourot, 1998).

Dietary protein and lysine levels

Proteins are a sequence of amino acids arranged in a certain order. In most swine diets, a portion of each amino acid that is present is not biologically available to the

animal. This is because most proteins are not fully digested and amino acids are not fully absorbed, and also because not all absorbed amino acids are metabolically available. Therefore, the bioavailable amino acid content of the ingredients being used to formulate pig diets must be known. The amino acids that cannot be synthesized or synthesized at insufficient rate to permit optimal growth or reproduction are termed essential and they are dependent on the species and stage of development (*e.g.* Methionine). Although there are 20 primary amino acids, not all of them are essential dietary components. The non essential may be synthesized through intermediaries of glycolysis, citric acid cycle or pentose phosphate pathway and the nitrogen is provided from glutamate and glutamine (Fig. 10; Nelson and Cox, 2009).

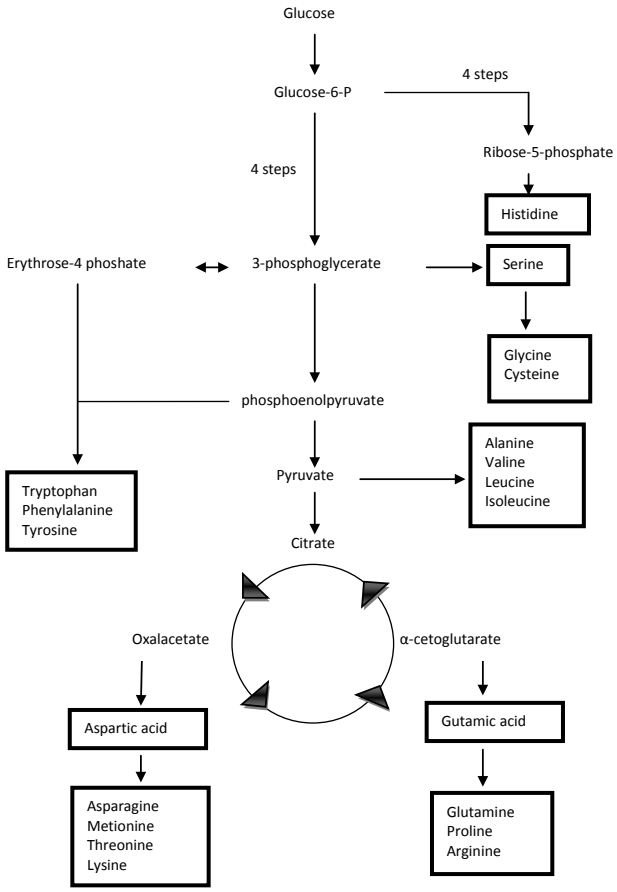

Fig. 10: General diagram of the biosynthesis of amino acids
Adapted from Nelson and Cox (2009)

In diets rich in protein and amino acids or situations of protein turnover, amino acids in animals present oxidative degradation. The amino group can be reused for the synthesis of new amino acids or other nitrogenous products or eliminated through the urea cycle (Fig. 11; Nelson and Cox, 2009). The remaining carbon skeletons may be totally or partially degraded to acetoacetyl-CoA or acetyl-CoA known as ketogenic amino acids (leucine and lysine) or degraded to pyruvate, α-ketoglutarate, succinyl-CoA, fumarate or oxalacetate known as glycogenic amino acids (alanine, cysteine, serine, glycine, proline, glutamine, glutamic acid, arginine, histidine, methionine, valine, asparagine and aspartic acid) or some may be ketogenic or glycogenic (tryptophan, phenylalanine, tyrosine, threonine and isoleucine; Nelson and Cox, 2009 Chapter 18)

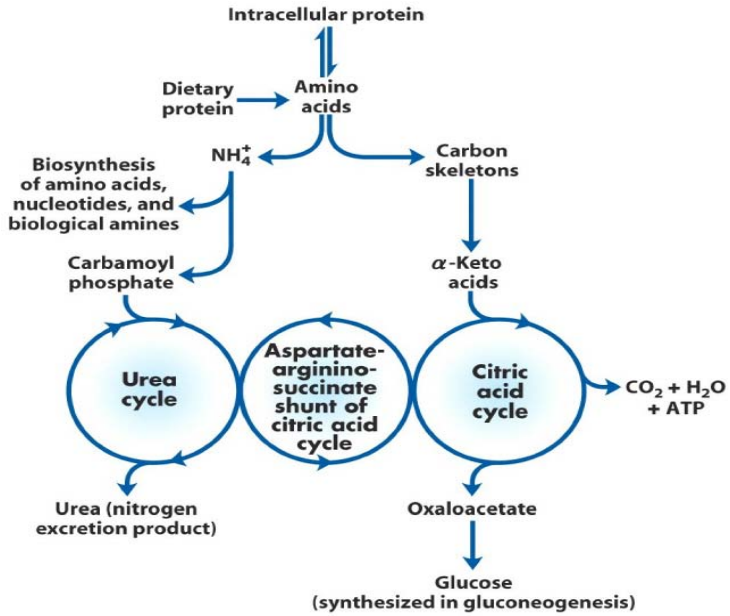

Fig. 11: Overview of the amino acid catabolism

Source: Nelson and Cox (2009)

About protein and amino acids some concepts have to be mentioned:

Deficiency is the lack of an essential amino acid in the diet. Limiting dietary concentration of amino acids causes reduced concentrations of the limiting amino

acids in plasma and tissues. Low concentrations of amino acids at the intracellular sites of protein synthesis reduce the rate of protein synthesis, thereby limiting physiologic process such as growth, lactation or egg production that depend upon a maximal rate of protein synthesis (Austic, 1977).

Imbalance refers to circumstances where the particular balance of amino acids in the diet results in poorer performance. One of the consistent physiological consequences of imbalance is a depression in the concentration of the first-limiting amino acid in blood and a marked increase in the levels of amino acids added to the diet. Result is a reduction of feed intake (Austic, 1977) without change in feed efficiency.

Antagonisms are specific interactions where an excessive level of one amino acid causes an increase in the requirement for a structurally or chemically related amino acid. There are two major interactions: one involving lysine and arginine, and the other one involving the three branched chain amino acids (**BCAAs** excess dietary lysine increases the requirement for arginine and an excess of one of three BCAAs increases the requirement for the other two; Austic, 1977). In contrast to the imbalance, feed efficiency is impaired, as the limiting amino acid is catabolized.

Toxicity refers to effect caused by excessive levels of individual amino acids (Austic, 1977).

Ideal protein consists of a combination of amino acids in exactly the right proportions for maintenance and lean tissue growth. Hence, the ideal protein may be variable depending on the physiological state of the animal. Feedstuffs with high quality protein (amino acid pattern relatively similar to the pig's needs) and the addition of crystalline amino acids to the diet containing low quality protein result in diets meeting the essential amino acid requirements at lower levels of dietary protein (NRC, 1998).

It appears that reducing the levels of dietary crude protein or lysine affect the proportions of protein and fat in the pig carcasses. Apparently, dietary protein reduction may reduce pig development and increase the fatness of whole animal

and lysine as the major limiting amino acid in pig diets plays a pivotal role in energy metabolism in porcine muscle determining growth rate. Leaner and less marbled meat have been observed form pigs fed a high protein/lysine diet, and higher IMF content is found in meat from pigs fed low protein/lysine diet. Da Costa et al. (2004) explain a high IMF content by the fact that low protein content restricts muscle growth, resulting in surplus energy being converted into intramuscular fat. The increase of IMF may also be related with high glycolytic and oxidative potential or larger adipocytes in muscles of pigs fed low protein diets (Gondret and Lebret, 2002; Ruusunen et al., 2007). The increase of IMF in low lysine level diets is caused by higher adipogenesis, *de novo* fatty acid synthesis, reduced rate of β-oxidation of fatty acids (Katsumata et al., 2005) and higher abundance of PPARγ in muscles (Katsumata, 2011). However, in most of the studies both parameters (protein and lysine) were reduced at the same time, which makes difficult to attribute the observed effect to lysine or to protein (Reviewed in Table 3). On the other hand, reducing the intake of dietary protein would reduce the nitrogen excreted by pigs, which is interesting from the point of view of the environment.

During the last decade, some studies supplementing diets with aminoacids such as leucine or arginine have shown a modification of the distribution of fat in the carcass resulting in an increase of IMF (Table 3). Leucine is a ketogenic amino acid, the carbon skeleton of which is converted to acetyl-CoA and acetoacetate in muscle tissue which can be used to synthesize FA. As BCAAs it is an essential amino acid and therefore must be continuously available for protein synthesis. Nevertheless, it is clear that leucine is special among the BCAAs. Leucine promotes global protein synthesis by signaling an increase in translation, promotes insulin release, and inhibits autophagic protein degradation. However, leucine's effects are self-limiting because leucine promotes its own disposal by an oxidative pathway, thereby terminating its positive effects on body protein accretion. A strong case can therefore be made that the proper leucine concentration in the various compartments of the body is critically important for maintaining body protein

levels beyond simply the need of this essential amino acid for protein synthesis (Harris et al., 2004).

In relation with arginine, there is growing evidence that arginine regulates the metabolism of energy substrates (FA, glucose and amino acids) partly through the production of nitric oxide (NO) and NO is synthesized from L-arginine by NO synthase in virtually all cell types. As a signaling molecule, physiological levels of NO stimulate glucose uptake, as well as glucose and FA oxidation in skeletal muscle, heart, liver and adipose tissue, inhibit synthesis of glucose, glycogen and lipid in target tissues (i.e. liver and adipose tissue), and enhance lipolysis in subcutaneous adipocytes. Thus, modulation of the arginine-NO pathway through dietary supplementation with L-arginine aid in reducing unfavorable fat mass in animals of agricultural importance (Jobgen et al., 2006).

Although, quantitatively the most important function of amino acids is the synthesis of proteins, they also have a role as precursors of non-protein substances (such as hormones, neurotransmitters and others) and as energy source (Gutiérrez-Preciado, 2010).

Table 3: Summary of published reports evaluating the effects of dietary protein/lysine reduction or arginina/leucine supplementation in pig carcass composition.

Reference	Gender	Genetic	Wt range	Energy/Protein/Lysine Leucine/Arginine	Difference P and lys	Dietary ingredients	Response
Reduction of protein							
Le Bellego et al. (2002)	Barrows n= 66	PI x (LD x LW)	27 – 100 kg	Growing: DE: 14.34; 14.15MJ/kg P: 20.1; 15.6 % Lys: 1.02; 0.97 % Finishing: DE: 14.30; 14.09 MJ/kg P: 17.5; 13.3 % Lys: 0.84; 0.79 %	Growing: P: 4.5 % Lys: 0.05 % Finishing: P: 4.2 % Lys: 0.05 %	Wheat, corn, SBM	No effect protein level in backfat deposition or % of fat carcass
Doran et al. (2006)	Intact male pigs n=26	DU x (LW x LD)	40-100 kg	DE: all 14 MJ/kg Lys: (-) Prot: PKO 21; PKO18; SBO 21; SBO 18; PO 21; PO 18	P: 2 % Lys: (-)	Barley, wheat, SBM	↓ P: ↑ FA content in muscle (86 %) and adipose tissue (8 %) and ↑ SCD activity, ACC and FAS expression in the muscle but not in adipose tissue
Reduction of lysine							
Witte et al. (2000)	Gilts n=72	PIC 326 sires x C15 dams	90 - 126 kg	ME: both 14.2 MJ/kg P: 10.3; 10.4 % Lys: 0.48; 0.64 %	P: 0.1 % Lys: 0.16 %	Corn, SBM	↓ Lys: ↓ growth efficiency, ↑ backfat thickness, ↓ carcass lean percentage, ↑ IMF (19 %) in LM
Bidner et al. (2004)	Gilts n=64	Hybrid line base on ($RN^{-}/^{-}$ or $rn^{+}/^{+}$)	75 – 100 kg	DE: both 14.2 MJ/kg P: 10.35; 10.43 % Lys: 0.57; 0.69 %	P: 0.08 % Lys: 0.12 %	Corn, SBM, SBO	↓ Lys: ↑ backfat, ↓ carcass lean and ↑ IMF in LM

Reference	Gender	Genetic	Wt range	Energy/Protein/Lysine Leucine/Arginine	Difference P and lys	Dietary ingredients	Response
Katsumata et al. (2005)	Gilts n=11	(LD x LW) x DU	62 – 110 kg	DE: 14.5 MJ/kg (all) Growing: P: 11.1 % (all) Lys: 0.65; 0.43 % Finishing: P: 10.7 % (all) Lys: 0.68; 0.40 %	Growing: P: 0 % Lys: 0.22 % Finishing: P: 0 % 0 Lys: 0.28 %	Corn, SBM	↓ Lys: no effect in SCF, ↑ IMF in LM (91 %), ↓ PUFA in LM
Zhang et al. (2008)	Barrows n=54	PIC	20-50-80-90 kg	DE: both 14.22 MJ/kg (all periods) Early growing: P: 14.70, 14.54 % Lys/DE: 0.66; 0.49 g/MJ Later growing: P: 12.21;, 11.92 % Lys/DE: 0.53; 0.39 g/MJ Finishing: P: 10.50; 10.21 % Lys/DE: 0.43; 0.31 g/MJ	Early growing: P: 0.16 % Lys/DE: 0.17 g/MJ Later growing: P: 0.29 % Lys/DE: 0.14 g/MJ Finishing: P: 0.29 % Lys/DE: 0.13 g/MJ	Corn, wheat, SBM, SBO	↓Lys: ↓ feed efficiency and ↑ marbling (15 %) but not IMF
Boler et al. (2011)	Inmunocastrated males n=96	PIC 337 x PIC 1050	23-45-68-91-129	DE: (-) ; Prot: (-) Lys: Growing: 1.2; 1.3; 1.4; 1.5 % Developing: 1.0; 1.1; 1.2; 1.3 % Finishing 1: 0.8; 0.9; 1.0; 1.1 % Finishing 2: 0.7; 0.8; 0.9; 1.0 %	Lys: 0.3 % Prot: (-)	(-)	↓ Lys: ↓ final BW; no effect on backfat deposition or loin depth; ↑marbling and IMF in LM

Reference	Gender	Genetic	Wt range	Energy/Protein/Lysine Leucine/Arginine	Difference P and lys	Dietary ingredients	Response
Reduction of both, protein and lysine							
Karlsson et al. (1993)	Intact males and gilts n=82	Swedish YR	25-90 kg	ME: both 11.9 MJ/kg P: 18.5; 13.1 % Lys: 0.96; 0.64 %	P: 5.4 % Lys: 0.32 %	Standard feeding regimen	↓P: ↓ADG, lean %, ↑ IMF in LM (60 %) and BF (67 %), ↓ glycolytic and ↑ oxidative capacity in muscles
Goerl et al. (1995)	Gilts n=72	GP, H	26 – 104 kg	ME: 13.8; 13.8; 13.8; 13.7; 13.6; 13.6 % P: 10; 13; 16; 19; 22; 25% Lys: 0.36; 0.55; 0.75; 0.95; 1.16; 1.35 %	P: 15 % Lys: 0.99 %	Corn, SBM	↓ P: ↑backfat and IMF up to 174 % in LM or 54 % in ham muscle (comparing the extrems)
Loughmiller et al. (1998)	Gilts n=64	PIC	54 (64 d)	DE: (-) P: 13.4; 10.5 % Lys: 0.72; 0.51 %	P: 2.9 % Lys: 0.72, 0.21 %	Sorghum, Cornstarch, SBM, SBO	↓ Lys: ↓ ADG and feed efficiency and ↑ backfat thickness
Szabo et al. (2001)	Barrows and gilts n=96	Dutch LD	30-60-105 kg	DE: 14.6 MJ/kg Growing: P: 12.0; 8.7 % Lys/DE: 0.5; 0.36 g/MJ Finishing: P: 10.7; 7.8 % Lys/DE: 0.42; 0.30 g/MJ	Growing: P: 3.3 % Lys/DE: 0.15 g/MJ Finishing: P: 2.9 % Lys/DE: 0.12 g/MJ	Barley, wheat, tapioca	↓ Lys/DE : ↓ADG, ↓ feed efficiency, ↓ muscle volume , ↓ fat carcass content but had no effect in IMF
Gondret and Lebret (2002)	Pigs n=30	DU x (LD x LW)	30 – 110 kg	DE: 13.6; 13.0 MJ/kg P: 18.3; 13.3 % Lys: 0.95; 0.50 %	P: 5 % Lys: 0.45 %	Barley, wheat, corn, wheat bran, SBM, animal fat, molasses	↓ P: ↓ ADG and ADFI, no effect backfat thickness, ↑ IMF (40 %), ↑ adipocyte size, ↓ activity of malic enzyme, G6PDH and LDH suggesting a decreased lipogenic capacity

Reference	Gender	Genetic	Wt range	Energy/Protein/Lysine Leucine/Arginine	Difference P and lys	Dietary ingredients	Response
D'Souza et al. (2003)	Females n=50	LW x LD x DU	24 – 104 kg	DE: all 13.3 MJ/kg P: 18.7; 17.7; 16.9 % Available lys: 0.60; 0.51; 0.42 g/MJ DE	P: 1.8 % Available lys: 0.18 g/MJ DE	Barley, wheat, lupins, SBM, Blood meal, fish meal, canola oil	↓P: ↑ IMF up to 108 % (1.3, 1.9, 2.7 %) without affecting SCF
Da Costa et al. (2004)	Entire male n=48	DU based	9-12 wk	DE: 14; 13 MJ/kg P: 20; 16 % Lys: 1.14; 0.68 %	P: 4 % Lys: 0.46 %	Barley, wheat, SBM, corn gluten	↓P: ↓ ADG, no effect on SCF, ↑ IMF in LM and PM, ↑ expression of genes involved in breakdown of glycogen, fatty acids and proteins, glycolysis, oxidative phosphorilation and ATP synthesis
Wood et al. (2004)	Entire male n=192	Du, LW, BE, TW	9-12 wk	DE: 14; 13 MJ/kg P: 20; 16 % Lys: 1.14; 0.68 %	P: 4 % Lys: 0.46 %	Barley, wheat, wheat-feed, SBM, corn gluten	↓P: ↓ ADG (except BE), no effect on SCF thickness, ↓ muscle percentage, ↑ SCF, ↑ marbling in LM and PM
Pérez et al. (2006)	Gilts and barrows n=60	Pic	93 kg (27 d)	EM: all 13.8 MJ/kg P: 17.18; 16.95; 17.66 % Lys: 0.95; 1.05; 1.15 %	P: 0.71 % Lys: 0. 20 %	Corn, SBM, tallow	↓ Lys: ↑ backfat thickness,↓ loin depth, ↑IMF in LM
Teye et al. (2006a)	Male and female n=60	DU x (LWxLD)	40 -100 kg	EM: all 14 MJ/kg P: 20.9; 18.1 % Lys: 1.0; 0.7 %	P: 2.8 % Lys: 0.3%	Barley, wheat, SBM	↓ P: ↓ ADG, ↓ feed efficiency, no effect backfat thickness, ↑ IMF, SFA and MUFA and ↓ PUFA in LM (70 %)
Teye et al. (2006b)	Male and female n=60	DU x (LWxLD)	40 -100 kg	EM: all 14 MJ/kg P: 20.9; 18.1 % Lys: 1.0; 0.7 %	P: 2.8 % Lys: 0.3%	Barley, wheat, SBM	↓ P: ↑ total lipid, SFA and ↓ PUFA in SCF

Reference	Gender	Genetic	Wt range	Energy/Protein/Lysine Leucine/Arginine	Difference P and lys	Dietary ingredients	Response
Ruusunen et al. (2007)	Gilts and barrows n=40	Finnish LD Finnish YR Finnish LD x Finnish YR	24 – 107 kg	NE: 8.9; 8.8 MJ/kg P: 16.0; 18.7 % Dig Lys: 0.57; 0.90 %	P: 2.7 % Dig Lys: 0.33%	Barley, SBM	↓ P: ↓ ADG and lean meat carcass, ↑ fat carcass and glycolytic potential of LM and SM
D'Souza et al. (2008)	Gilts n= 63	LW x LD x DU	73 d – 163 d	Grower: DE: all 14.3 MJ/kg P: 18.7; 17.7 % Available Lys: 0.6; 0.51 g/MJ Finisher: DE: 13 MJ/kg P: 18.2 % Available Lys: 0.5 g/MJ	Grower: P: 1 % Available Lys: 0.9 g/MJ Finisher: P: 0 % Available Lys: 0 g/MJ	Barley, wheat, lupins, soybean, blood, meat and bone meal, fish meal, canola oil	↓ Lys/DE: No effect subcutaneous fat, ↑ IMF (29 %)
Kamalakar et al. (2009)	Gilts and barrows n=60	YR	25 – 50 – 79 – 111 kg	Grower: DE: all 14.3 MJ/kg P: 16.9; 14.9; 12.1 % Lys: 0.95; 0.76; 0.57 % Finisher 1: DE: all 14.4 MJ/kg P: 14.8; 13.0; 10.7 % Lys: 0.75; 0.60; 0.45 % Finisher 2: DE: all 14.4 MJ/kg P: all 12.5 % Lys: all 0.6 %	Grower: P: 4.8 % Lys: 0.38 % Finisher 1: P: 4.1 % Lys: 0.3 % Finisher 2: P: 0 % Lys: 0 %	Corn, SBM	↓ Lys: ↓ ADG and feed efficiency, ↓ LM area, ↑ marbling

Reference	Gender	Genetic	Wt range	Energy/Protein/Lysine Leucine/Arginine	Difference P and lys	Dietary ingredients	Response
Conde-Aguilera et al. (2011)	Barrows n=58	Iberian	10 -25 kg	DE: 14.99; 14.65; 15.11; 14.88 MJ/kg DM P: 20.1; 17.6; 14.9; 12.3 % Lys/ME: 0.99; 0.88; 0.73; 0.62 g/MJ	P: 7.8 % Lys/ME: 0.37 g/MJ	Barley; SBM; fish meal	↓P: ↓ADG and feed efficiency, ↓ carcass protein and ↑ carcass fat
Guo et al. (2011)	Pigs n=20	DU x LW x LD	74 – 133 kg	DE: 13.9; 14 MJ/kg Phase I P: 11.24; 22.66 % Dig Lys: 0.57; 1.12 % Phase II P: 10.07; 22.66 % Dig Lys: 0.46; 1.12 %	Phase I P: 11.36 % Dig Lys: 0.55 % Phase II P: 12.53 % Dig Lys: 0.66 %	Corn, SBM	↓ Prot: no effect on SCF; ↑ IMF (89 %) and ↑PPARγ and H-FABP expression in LM
Rodríguez-Sánchez et al. (2011)	Gilts and barrows n=120	Du x (LD x LW)	100 – 130 kg	NE: all 9.54 MJ/kg P: 14.5; 14.3; 14.0 % Lys: 0.71; 0.66; 0.59 %	P: 0.5 % Lys: 0.12 %	Barley, wheat, corn, canola meal, SBM, blended animal-vegetable fat	↓ Lys: ↓ ADG and ADFI, ↑ backfat thickness, no effect in LM IMF and SFA, MUFA or PUFA
Supplementation with leucine							
Hyun et al. (2003)	Barrows and gilts n=40	DU x YR	78 – 115 kg	ME: 14.3; 14.2 MJ/kg P: 14.1; 15.4 % 0 vs. 2 % Leu		Corn, corn starch, SBM	+ Leu: ↓ ADG, tended ↑ backfat thickness, ↑ marbling and IMF in LM (42 %)
Hyun et al. (2007)	Barrows n=36	PIC (L329 x C22)	73 – 127 kg	ME: all 13.8 MJ/kg P: 12.1; 12.7; 13.5; 12.8; 12.8; 12.8; 13.9 % Lys: 0.5; 0.7 % Leu: 1; 2; 3 %		Corn, corn starch, SBM, SBO	+ Leu: no effect performance or backfat thickness, 2 % and 3 % Leu ↑IMF (86 and 53 %, respectively) in pigs fed ↓ Lys but not ↑ Lys

Reference	Gender	Genetic	Wt range	Energy/Protein/Lysine Leucine/arginine	Difference P and Lys	Dietary ingredients	Response
Supplementation with arginine							
Tan et al. (2009)	Barrows n=24	DU x LD x LW	41 – 90 kg	DE: 14.3 MJ/kg P: 16.5 1 % Arg vs. 1 % Ala		Corn; SBM, Wheat; SBO	+ Arg: ↑ADG, ↑ muscle content, ↓ carcass fat content, ↑ IMF in LM (70 %) but not in SM.
Tan et al. (2011)	Barrows n=16	DU x LD x LW	41 – 90 kg	DE: 14.3 MJ/kg P: 16.5 1 % Arg vs. 1 % Ala		Corn, SBM, Wheat; SBO	+ Arg: ↑ LPL activity in muscle but ↓ in backfat, resulting in a ↑ or ↓regulation of lipogenic genes in skeletal muscle or SCF, respectively and ↑ regulation of lipolytic genes SCF

ADFI: average daily feed intake; ADG: average daily gain; Ala: alanine; Arg: arginine; BE: Berkshire; BF: *Biceps femoris*; BW: body weight; d: days; Dig: digestible; DU: Duroc; DE: Digestible energy; DM: dry matter; FA: fatty acid; GP: gene pool; H: Hampshire; IMF: intramuscular fat; G6PDH: gluycose-6-phosphate dehydrogenase; LD: Landrace; LDH: lactate dehydrogenase; lys: lysine; LM: *longissumus* muscle; LW: Large White; ME: metabolizable energy; MJ: mega joules; NE: net energy; PI: Pietrain; PKO: palm kernel oil; PM: *Psoas* muslce PO: Palm oil; P: Protein; SBM: Soybean meal; SBO: soybean oil; SCF: subcutaneous fat; SM: *semimembranosus* muscle; TW: Tamworth; wk: weeks; YR: Yorkshire; (-) no information found

3.5.3. Dietary fat content and its composition in fatty acids

Monogastric animals like pigs have the capacity to deposit FA from diet without modification, when animals are fed isoenergetic, isoproteic and isolipidic diets, making possible to modify the carcass characteristics and meat lean through dietary source of FA (Lebret et al., 1999). Thus, modification of animal diets can easily increase the proportion of unsaturated FA and reduce the SFA in meat, milk and eggs. Consuming a greater proportion of these beneficial fatty acids as pork of an everyday diet will appeal to the public, as opposed to taking dietary supplements (Woods and Fearon, 2009). The influence of genotypes and sexes of pig on body FA composition is much lower than that of fat supplements and this effect is more pronounced in backfat than in IMF (Flachowsky et al., 2008).

Addition of 5% of fat in pig diets increased backfat depths with little to no impact on live pig performance or carcass composition, but FA composition differed depending on dietary fat source, soybean oil resulted in a higher polyunsaturation and beef tallow in a higher saturation and monounsaturation of pork (Apple et al., 2009b). In a study, where different fat sources were studied, it was found that pigs fed a non fat diet had the highest values for SFA. Animals fed high-oleic sunflower oil had high values of MUFA and low levels in SFA. MUFA were low in sunflower oil, linseed oil and fish oil, while PUFA were high in sunflower oil and linseed oil and low in high-oleic sunflower oil, tallow and non fat fed animals (Duran-Montgé et al., 2008). Similar results were reported by Skiba et al. (2011), feeding pigs with tallow increases the amount of SFA and MUFA, feeding with rapeseed oil increases the amount of MUFA and feeding with linseed oil increases the content of all PUFA (mainly C 18:3 n-3) in subcutaneous fat. The replacement of beef tallow by sunflower oil produced an increase of PUFA in adipose tissue, liver and loin at expenses of SFA and MUFA, without affecting other characteristics of meat quality (Mitchaothai et al., 2007). Reducing the linoleic content of diets for swine during 6 to 8 weeks prior to slaughter will result in a reduction in linoleic and an increase of

MUFA in the carcass (Gatlin et al., 2002). These studies show that direction of the changes occurred in fat FA content is in the line of the FA content in particular fat sources. Thus, using diets with high levels of C 18:2 n-6, the proportion of this FA in tissues increases linearly as the dietary intake increases (Wood et al., 2008).

Beneficial effects of n-3 long chain PUFA are well documented. Whilst some may be synthesized from α-linolenic acid, recent data indicate this source to be very limited. In many parts of Europe daily intake of EPA and DHA by adults and especially young adults is less than 100 mg/day, which is low compared with the UK recommendation, 450 mg EPA + DHA/day. Hence, enrichment of animal products may help to provide the required amount of these two FA (Givens and Gibbs, 2008). Because cereal-based diets commonly offered to pigs supplies mainly n-6 FA and a small amount of n-3, linseed and fish oil are incorporated in pig diets to transfer these FA to meat products (Woods and Fearon, 2009). Feeding a diet of 100 g of linseed/kg to pigs increased, the n-3 content in muscle and in adipose tissue and promoted the hypertrophy of *longissimus dorsi* muscle, *quadriceps femoris* muscle mass and *semitendinosus* muscle (Huang et al., 2008). Increasing the daily consumption of C 18:2 n-6 and C 18:3 n-3 enhanced their deposition in the body but the efficiency of utilization of C 18:2 n-6 is lower compared with C 18:3 n-3 (coefficient 0.67 vs. 0.79, respectively) and depends not only on the amount consumed, but also on their origin (Raj et al., 2010). Increasing flaxseed meal in pig diets also increased the α-linolenic acid content from 11 to 47 mg/g of backfat and from 5 to 10 mg/g of loin tissue (Eastwood et al., 2009). DHA and EPA from fish oil diets were more efficiently incorporated into tissues than the α-linolenic acid from the linseed diet. A level of 2.31 % EPA and 3.53 % DHA in fish diets resulted in 1.37 and 1.02 % of EPA and DHA, respectively in muscle lipid fraction, whereas 16 % of α-linolenic acid in linseed diet only resulted in 1.24 % of α-linolenic acid in muscle lipid fraction. Nonetheless the α-linolenic acid incorporation was similar when feeding linseed during 17 weeks prior slaughter or only during the 9 weeks prior slaughter while the greatest EPA or DHA proportions

were obtained when fish oil was fed during 17 weeks. The slightly lower dietary linoleic acid supply in fish diets resulted in a significant lower n-6 proportion in meat. However, an increased n-3 supply in linseed diets was not accompanied by a decreased deposition of n-6 FA, except for arachidonic acid. DHA proportion was only achieved when fish oil was included in pig diet, suggesting than DHA formation is strictly regulated and cannot be influenced by dietary supply of the precursors (Haak et al., 2008). Duran-Montgé et al. (2008) also reported that adding a 10 % of linseed oil resulted in an increase of EPA in different tissues, however, an increase of DHA only was achieved when animals received diets supplemented with 10 % of fish oil. Jaturasitha et al. (2007), enriching pig diets with tuna oil from 30 to 90, 100 and 110 kg of slaughter weight found a reduction of n-3 FA in proportion with increasing slaughter weight, thus to achieve the maximum n-3 deposition they proposed to slaughter animals early stages. The same group reported that addition of tuna oil during a short period at the end or during the whole fattening period produced the similar increase of n-3 in adipose tissue and muscle, however, the deposition of n-3 was slightly lower when tuna oil was added at the early stage of fattening period. In compensation to the shifts in n-3 FA proportion, a decline in proportions of n-6 FA occurred (Jaturasitha et al., 2009). The increase of long chain n-3 PUFA in adipose tissue and particularly in the muscle of pigs fed fish oils, produces fishy odors and flavors when critical tissue levels are exceeded (Wood et al., 2008). In order to avoid fishy odors and flavors, other strategies such as the incorporation of chia (*Salvia hispanica L.*), a relative new crop recognized as good source of n-3 FA in finishing pig diets have been proposed. Addition of chia produced a reduction of SFA and an increase of n-3 FA in meat without negatively affect meat taste (Coates and Ayerza, 2009).

Control of gene expression by fatty acids

The amount and type of fat in the diet impacts many other aspects of metabolism including lipoprotein pathways, lipid synthesis and oxidation, adipocyte differentiation, and cholesterol metabolism. It has become increasingly apparent that many of these effects may be due to direct modulation of expression of key genes through the interaction of fatty acids with certain transcription factors. Peroxisome proliferator-activated receptors (PPARs), the liver X receptors (LXRs), hepatic nuclear factor 4 (HNF-4) and sterol regulatory binding proteins (SREBPs) represent four of such factors (Salter and Tarling, 2007; Fig. 12).

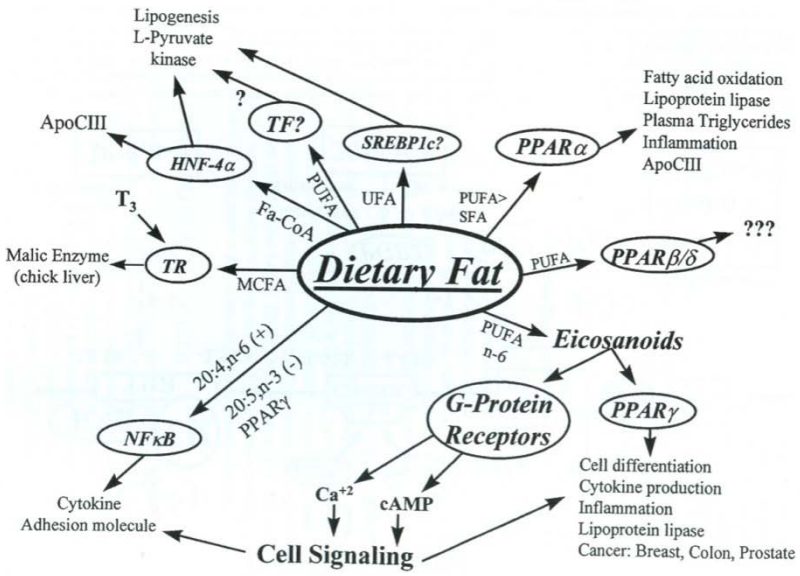

Fig. 12: Fatty acids or their metabolites can potentially activate multiple pathways affecting gene expression.

The diagram illustrates the fatty acids or their metabolites that regulate specific transcription factors. These transcription factors are linked to specific cellular processes involving metabolism, eicosanoids/growth factors production cell growth, and differentiation. G-proteins; G-proteins-linked receptors; TF?: unknown transcription factors; TR: thyroid hormone receptors; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MCFA: medium chain fatty acids; Fa-CoA: fatty acyl coenzyme A (Adapted from: Jump and Clarke, 1999).

These factors are regulated by (a) direct binding of FA, fatty acyl-coenzyme A, or oxidized FA; (b) oxidized FA (eicosanoids) regulation of G-protein-linked cell surface receptors and activation of signaling cascades targeting the nucleus; or (3) oxidized FA regulation of intracellular calcium levels, which affect cell signaling cascades targeting nucleus (Fig. 13).

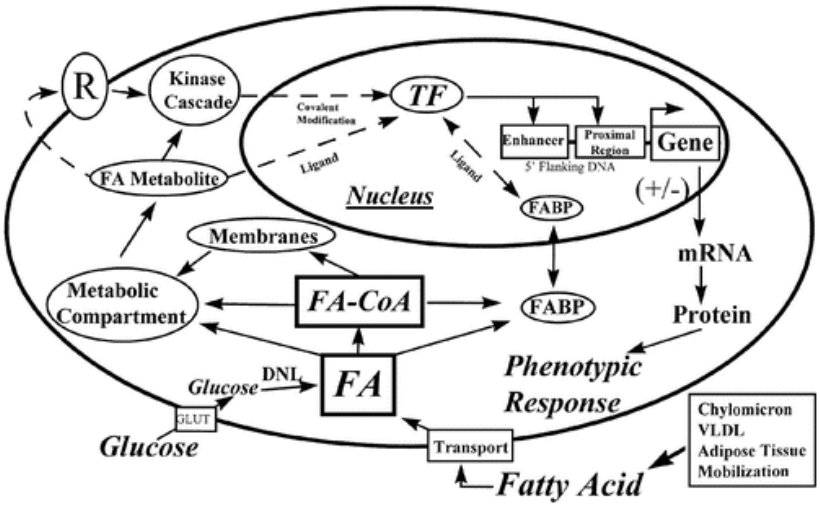

Fig. 13: Fatty acid regulation of gene transcription.

Fatty acids enter cells through membrane-associate transporters, undergo metabolic conversions and affect many cellular processes. Some of these processes involve change in gene transcription leading to changes in cell growth and differentiation, metabolism and production of various molecules such as triglycerides, apoproteins, cytokines, eicosanoids and adhesion molecules. R: receptor; TR: transcription factor; FABP: fatty acid binding protein; GLUT: glucose transporter; FA: fatty acid; Fa-CoA: fatty acyl coenzyme A; VLDL: very-low-density lipoprotein; DNL: *de novo* lipogenesis. The 5'-flanking DNA contains *cis*-regulatory elements involved in basal promotor activity (proximal region) and stimulated activity (enhancer). Both regions can be targeted by fatty acid-regulated transcription factors (Jump and Clarke, 1999).

At the cellular level, the physiological response to FA depends on (a) the quantity, chemistry, and duration of the fat ingested; (b) cell-specific FA metabolism (oxidative pathways, kinetics, and competing reactions); (c) cellular abundance of specific nuclear and membrane receptors; and (d) involvement of specific transcription factors (Jump and Clarke, 1999). In addition, long chain FAs may induce message accumulation by two different mechanisms: transcriptional or

post-transcriptional, most likely message stabilization (Distel et al., 1992). No fat-added diet showed the highest mRNA abundance in adipose tissue of genes involved in the synthesis of palmitic acid (ACACA and FASN) and desaturation (SCD). SFA are equivalent (or more potent) inhibitors of lipogenesis than unsaturated FA. Thus, animals fed diets with high SFA content decrease the abundance of mRNA FASN, ACACA and SDC (Duran-Montge et al., 2009). PUFA are less potent than SFA in increasing adipocyte numbers, but are more effective in inducing preadipocyte differentiation (Fernyhough et al., 2007). Luo et al. (2009) reported that increasing the n-3 PUFA enrichment in the muscle of growing-finishing pigs by feeding diets with 10 % of linseed oil could activate PPARδ, which may upregulate the expression of PPARγ and consequently aP2 and LPL to promote adipogenesis, thus increasing IMF. A metabolite of DHA (not identified) is also proposed as the ligand binding to and activating porcine PPARγ and increasing the expression of fatty acid binding protein (aP2; Yu et al., 2008a).

Conjugated linoleic acid (CLA) as fat source

The term conjugated linoleic acid refers to a mixture of positional and geometric isomers of linoleic acid (c9, c12 C18:2; Fig. 14), which means that a single carbon separates the two double bounds.

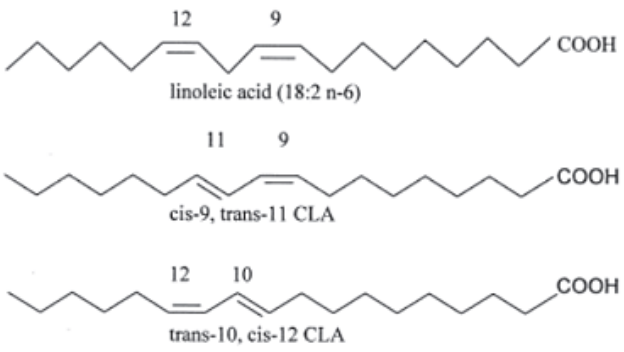

Fig. 14: Structures of linoleic acid (top); c9, 11t CLA (middle) and t10, c12 CLA (bottom)

These double bounds can either be *trans* (t) or *cis* (c) configured and a wide spectrum of isomers with variations in position (7, 9; 8, 10; 9, 11; 10, 12 or 11, 13). Nonetheless the predominant CLA isomer (85-90 %) in natural sources has c9, t11 conformation. Principal natural sources of CLA are meat and dairy products from ruminant animals (such as milk). Amount of CLA is known to vary among individuals and herds and between seasons. The greatest concentration is found during the grazing period. CLA is obtained as an intermediate product of the biohydrogenation of linoleic acid to stearic acid produced in the rumen by *Butyrivibrio fibrisolvents* (Schmid et al, 2006). Furthermore, most of the CLA found in tissues of ruminant animals is probably derived from the desaturation of trans-vaccenic acid (t11 C 18:1; Azain, 2003). Not only bacteria in the rumen, but also microorganisms in the digestive system of non ruminant can synthesize CLA from long chain FA, although the yield is very low.

In 1987, Ha et al. (1987) identified CLA as the responsible of the antitumorigenic effect reported in a previous study where mouse were fed with pan-fried hamburgers. Since then, several studies have been conducted and pleiotropic properties were attributed to CLA product. Dietary CLA has been shown to have potent anticarcinogenic and antiatherogenic effects in animal models (Kelly et al. 2007). CLA was also found to have a potent immune modulating activity characterized by increased blastogenesis and macrophage killing ability (Pariza et al. 2001). In addition to these biological properties, CLA was reported to reduce body fat content and increase lean body mass in pigs and rodents. The basis for these effects has not been fully explained, but probably involves effects of CLA on eicosanoid metabolism, cytokine production and/or gene expression. In addition, it appears that different effects are produced for t10, c12 and c9, t11 CLA isomers, and it is difficult to imagine a single mechanism that accounts for this observation. The reported effects of CLA on lipid metabolism and body composition, and at least some of the effects of CLA on the immune system, appear to be due to t10, c12 CLA (Pariza et al., 2000).

Because of the interesting biological properties, synthetic forms of CLA became available; most of them were obtained from sunflower oil (rich in c9,c12 C18:2) through catalytic dehydrogenation. The synthetic mixtures contain c9, t11 and t10, c12 isomers at about equal amounts as the major components and small amounts of other CLA isomers such as t8, c10; c11, t13. Lymphatic recovery of CLA was similar to that of C 18:2 n-6 (96 %) and all the isomers were equally absorbed by the enterocytes (Martin et al., 2000) and they can be accumulated in these tissues rich in neutral lipids, metabolized as linoleic acid (giving conjugated diene (CD) C18:3, CD C20:3 and CD C20:4 as products) influencing linoleic acid desaturation and elongation (Carta et al., 2002). Hence, studies with CLA in species other than rodents and human subjects generally fit into three categories: (1) those aimed at increasing CLA content in tissues destined for human consumption as means to increase CLA intake in human subjects (i.e. egg, meat and dairy products); (2) those aimed at using the repartitioning effect of CLA to reduce body fat in the animal; (3) those related to a direct heath benefit of CLA in the animal based on its immunomodulatory effects.

Conjugated linoleic acid studies in swine

Suggested roles of the addition of CLA into pig diets are reviewed in Table 4. One of the interests in incorporating CLA in swine diets was to reduce animal fatness and improve feed efficiency. Therefore, lean meat percentage in the carcass increases at certain slaughter weight, increasing the carcasses value. In addition, several reports found an increase in IMF which could directly impact meat quality attributes. The increase in IMF while the other fat depots are reduced suggests different actions of CLA in tissues. An increase of SFA and a reduction of MUFA, which potentially increases the product shelf life and its consistency but is against nutritional recommendations, were found in most of the reports by effect of CLA (Dikeman, 2007). The addition of CLA oil in pig diets, leads to an accumulation of CLA isomers in the different tissues, although normally the isomers (c9,t11 and

t10,c12) are incorporated into the same percentage in the diet, the isomer c9,t11 seems to be preferentially incorporated in the tissues and the highest rates of incorporation occur in adipose tissue and are lower for the rest of tissue. Hence, it makes possible to obtain CLA enriched pork for human consumption.

It has to be pointed out that when comparing different studies some discrepancies in the phenotypic characters and CLA action in different tissues are found. Thus, the effect of CLA on backfat deposition and particulary, on IMF in pigs is not clear. These differences may result from the difference between variables such as: composition of the CLA oil incorporated to the diet, percentage of inclusion of CLA, genetic of pigs, duration of the treatment, age when the experimental period is started and differences between diets composition. Tischendorf et al. (2002) observed that the response to CLA was greater in barrows that in gilts while Dugan et al. (2001) attributed a lower response in low-energy diets than with added fat.

Different mechanisms of action of CLA proposed to reduce adiposity could be:

- Reduction of lipogenesis (Brown et al., 2001). Ostrowska et al. (2002) suggested that pigs fed diets with CLA reduce *de novo* synthesis, fat accretion via decreased adipose tissue, TAG synthesis from performed FA, possibly trough reduced LPL activity.
- Increase of lipolysis and FA oxidation (Park et al., 1997), although Martin et al. (2006) found CLA highly affected activity of lipolytic enzymes in muscle but not in subcutaneous adipose tissue.
- Reduction of the preadipocyte differentiation (Kang et al., 2003). Corino et al. (2005) reported that CLA supplementation decreased adipocyte size with a consequent increase in number of adipocytes per unit area. Moreover, the number of proliferating preadipocytes was lower and the number of apoptotic adipocytes was higher in pigs fed CLA compared with control. Brodie et al. (1999) attributed the fat reduction to CLA inhibition of both proliferation and differentiation of preadipocytes while Satory and Smith (1999) only attributed it to an inhibition of stromal vascular

preadipocytes hyperplasia.

The above mentioned mechanism may be regulated at different levels:

- Modification of protein activities. The increase of SFA and reduction of MUFA is widely attributed to an inhibition of Δ9 desaturase (Lee et al., 1998), the enzyme responsible for converting the palmitate and stearate to palmitoleic and oleic acids, respectively. Furthermore, Zhong et al. (2011) concluded that CLA enhanced the abundance of proteins related to energy metabolism, fatty acid oxidation and synthesis, aminoacid defense, and transport. Conde-Aguilera et al. (2011) found that CLA decreased hepatic glucose production decreasing gluconeogenesis, whereas glycogen synthesis and degradation, TAG synthesis were not affected compared with linoleic acid in pig hepatocytes. Park et al. (1999) found that 10t, 12c CLA but not 9c, 11t reduced lipoprotein lipase activity.
- Modification at the level of gene expression. Changes in mRNA abundance often indicate changes in the protein abundance of a gene product, but the relationship of transcript to protein is not always 1:1. For example, Lengi and Corl (2010) found that CLA reduced the protein concentration of ACCα while the mRNA abundance remained unaffected. Lin et al. (2004) observed that SCD mRNA levels were not altered by dietary treatment, but the CLA isomers reduced SCD activity in liver of mice. On the other hand, while several studies had been performed on the effect of CLA in gene expression, the majority includes rodent animals or cell cultures and few of them are done in pigs. Moreover, in rodents or cell cultures the isomers effects are studied separately (c9,t11 and t10,c12) because of the low amount of product needed. In studies conducted with pigs, the mixture of both is used because the purified isomer is too expensive to be used in the large amount needed in pig diets. Studying isomers separately showed that they may have contrary actions on the same gene (Brown et al., 2003), and the resultant FA modification in the tissues by effect of CLA may also

interfere in the regulation. Ashwell et al. (2010), evaluating the expression of a large amount of genes using microarrays in liver of mice, found 5 different pathways and processes which contained a significant number of differentially expressed genes by addition of CLA respect to the control diet: (1) signal transduction (PKA signaling); (2) muscle contraction; (3) signal transduction (cAMP signaling); (4) development of angiopoietin signaling; (5) immune response and surprisingly few genes in lipid metabolism were impacted. Hence, comparing several reports conclusive results could not be found (Table 5). Possible reasons can be due to specific functions or experimental conditions. Therefore, at the moment the activity of CLA through this mechanism is not clearly explained.

- Changes in microRNA (miRNA). The miRNAs are short RNA sequences found in eukaryotic cells. They may act as post-transcriptional regulator sequences that bind to mRNA, usually resulting in translational repression or target degradation and gene silencing. Parra et al. (2010) suggested this novel level by which CLA may exert its effects.
- Apoptosis. CLA, particularly t10,c12 CLA may increase TNFα increasing apoptosis in adipose tissue (Tsuboyama-Kasaoka et al., 2000; House et al., 2005).

The different actions of CLA in live organisms and the different mechanisms of action proposed makes CLA a molecule with interesting biochemical properties, but at the same time it is difficult to understand the exact role that it plays in the different organisms.

Table 4: Summary of published reports on the effect of conjugated linoleic acid (CLA) on carcass composition in the pig

Reference	Gender	Genetic	Wt range	CLA source	Dietary ingredients	Treatments	Response CLA
Dugan et al. (1997)	Barrows, gilts n=108	Landrace x (Landrace x Large White)	62-106 kg	CLA-50	Wheat-barley-SBM	2 % SFO vs. 2 % CLA	↓ SC fat (g/kg carcass), 7 % ↑ Lean, 2 %
Ostrowska et al. (1999)	Gilts n=60	Large White x Landrace	57 -106 kg	CLA-55	Wheat-SBM-peas-blood meal-SBO	0, 0.125, 0.25, 0.5, 0.75, 1 % CLA	↑ gain to feed ratio ↓ SC fat (linear), 20 % ↑ Lean Control 23.5 mm P2 fat
Dugan et al. (1999)	Barrows, gilts n=54	Landrace x (Landrace x Large White)	62-106 kg	CLA-50	Wheat-barley-SBM	2 % sunflower oil vs. 2 % CLA	↑ IMF, 22 %; contol 1.55 % in LM
Dugan et al. (2001)	Barrows n=216	Landrace x (Landrace x Large White)	36-115 kg	CLA-65 ME	Wheat-barley-SBM-canola meal (2-5%)	0 vs. 0.25 and 0.5 % CLA	↓ SC fat, 11 % at 2% canola, 3 % at 5% canola Lean ↑, 5 % at 2 % canola
Thiel-Cooper et al. (2001)	Barrows n=40	Yorkshire x Landrace x Duroc x Hampshire	26-116 kg	CLA-60	Corn-SBM No added fat	0, 0.12, 0.25, 0.5, 1 % CLA	↑ gain (linear) ↓SC fat, quadratic , 10 % Control 28.6 mm fat
Wiegand et al. (2001)	Barrows n=60	Crossbred	40-106 kg	CLA-60	Corn-SBM	1.25 % SBO vs. 1.25 % CLA	↑ Gain:Feed ↓ SC 10^{th} rib and last rib ↑Marbling, 18 %, 19% and 17 % in negative, carrier or positive stress genotype pigs, respectively
Tischendorf et al. (2002)	Barrows, gilts n=80	Pietrain x (Landrace x Large White)	24-111 kg	CLA-55	Barley-SBM	2 % rapeseed oil vs. 2 % CLA	Barrows, ↓ SC fat, 11 % Control 26 mm fat Gilts, no change Control, 20 mm fat No effect in IMF

Reference	Gender	Genetic	Wt range	CLA source	Dietary ingredients	Treatments	Response CLA
Averette-Gatlin et al. (2002)	Gilts n=144	Lean genotype	49-113 kg	CLA-60	Corn-SBM 0 vs. 4 g added fat/100 g	1 % corn oil vs. 1 % CLA	No effect of CLA on SC fat ↑ marbling; 18.8 % ↑ SFA ↓ MUFA in LM Control, 15 mm fat
Bee et al. (2002)	Barrows and gilts n=24	Swiss Large White	25-105 kg	SELIN-CLA	Wheat-barley-oat-yeast and wheat bran	2 % lard; 2 % linoleic acid or 2 % CLA	↓ SC in LD (25 % vs. lard, 20 % vs. linoleic acid) ↑ SFA ↓ MUFA FAS and ME acivities no affected
D'Souza and Mullan (2002)	Females, surgical and immunological barrows n=116	Large White x Landrace x Duroc	5-110 kg	-	-	-	↓ SC in P2 ↑IMF in females, 24 %
Joo et al. (2002)	Gilts n=20	Landrace x Large White x Duroc	5th month-105 kg	CLA-91	Commercial diet	0, 1, 2.5, 5 % CLA	↑ IMF, 44 % ↑ SFA and ↓MUFA in LM
Smith et al. (2002)	Barrows n=18	Crossbred	5.6 kg-25.6 kg	CLA-60	Corn-Soybean	1.5 % beef tallow, 1.5 % corn oil or 1.5 % CLA	Incorporation of c9,t11 > t10,c12 (2.54 % vs. 1.62 %) ↑ SFA, ↓MUFA in SC
Wiegand et al. (2002)	Barrows n=92	(Yorkshire x Landrace) x (Duroc x Hampshire)	28-115 kg	CLA-60	Corn-SBM	1.25 % SBO vs. 1.25 % CLA	↓ SC fat, 20 %; control 26 mm fat ↑ loin muscle area, 9 %; control 39 cm^2 ↑ Marbling, 23 %; control 2.04 % ↑ SFA, ↓ MUFA and PUFA in LM and SC
Corino et al. (2003)	Barrows and gilts n=36	Large White	97-172 kg	CLA-65	Corn-barley-wheat bran-SBM-Meat meal	Lard was replaced by 0, 0.25 or 0.5 % of CLA	↓ SC 10th rib, 9 % Control 38.5 mm fat ↓ MUFA in adipose tissue ↓ ACC in adipose tissue

Reference	Gender	Genetic	Wt range	CLA source	Dietary ingredients	Treatments	Response CLA
Dugan et al. (2003)	Barrows n=216	Landrace x (Landrace x Large White)	36-115 kg	CLA-65	Wheat-barley-SBM 20 or 50 g added fat	0 v. 0.25 and 0.5 %	↑ IMF, 13 % ↑ SFA and PUFA and ↓ MUFA in SC
Ostrowska et al. (2003a)	Female n=30	(Large White x Landrace)	57 -106 kg	CLA-55	Wheat-SBM-peas-blood meal-SBO	0, 0.125, 0.25, 0.5, 0.75 or 1 % CLA	CLA is primarily incorporated into SC and to a lesser extent into IMF c9,t11 the most abundant in SC and c11,t13 in IMF tissues ↑ C14:0, C16:0 and ↓long chain PUFA in IMF and SC
Ostrowska et al. (2003b)	Female n=30	Large White x Landrace	57-106 kg	CLA-55	Wheat-SBM Peas-SBO	0, 0.125, 0.250, 0.5, 0.75, 1 % CLA	↓ Feed intake increasing dose ↓ whole body fat ↑lean mass
Migdal et al. (2004)	Barrows n=40	(Large White x Landrace) x Pietrain	70-130 kg	-	Wheat-barley-corn-soyabean	2 % sunflower oil vs. 2 % CLA	No effect on SC fat or IMF ↑ SFA and ↓PUFA in LM
Sun et al. (2004)	Barrows n=54	Duroc x Landrace x Large White	64 (6 wk)	CLA-68	Corn-SBM-wheat bran	4 % SBO, 2 % CLA and 2 % SBO, 4 % CLA	↓ SC 10^{th} rib, 10 % and last rib, 9 %; control 22.1 and 17.3 mm fat, respectively ↑ muscle area, 6 % and IMF, 29%; control 36.6 cm^2 and 2.4 %, respectively ↑ SFA and ↓MUFA and PUFA in loin and SC

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Reference	Gender	Genetic	Wt range	CLA source	Dietary ingredients	Treatments	Response CLA
Corino et al. (2005)	Barrows and gilts n=36	Goland x Hypor	106-155 kg	CLA in FFA	Corn-barley-wheat middings-SBM	0.75 CLA vs. 0.75 SBO	No effect on SC fat Control, 23.6 mm fat
Lauridsen et al. (2005)	Barrows n=100	Danish Landrace x Danish Yorkshire x Duroc	40-100/130 kg	CLA-60	Barley-wheat-SBM	1.5 % animal fat with 0.5 % sunflower oil or 0.5 % CLA	No effect on SC fat or IMF ↑ SFA, ↓ MUFA
Lo Fiego et al. (2005a)	Barrows and gilts n=36	Large White	97-172 kg	CLA-65	Corn-barley-wheat bran-SBM-Meat meal	Lard was replaced by 0, 0.25 or 0.5 % of CLA	CLA supplementation ↑ the amount of CLA in SC and IMF (*biceps femoris*) Incorporation of c9,t11 > t10,c12
Weber et al. (2006)	Female n=228	Newsham XL x (Duroc x Yorkshire Landrace)	59-112 kg	CLA-60	Corn-SBM	1 % CLA vs. 1 %SBO	↓ SC 10th rib, 7 %; Control, 18 mm fat ↓ SC last rib, 9 %; Control, 20 mm fat No effect on loin IMF ↑ SFA, ↓ MUFA in inner/outer SC layers and LM; ↓ PUFA in LM
Bee et al. (2008)	Barrows n=32	Swiss Large White	18-104 kg	CLA-60	Barley-corn-wheat-dry sugar beet pulp-SBM	2 % extruded linseed and linseed supplemented with 1 % CLA	↓ SC 10th rib, 11% ↑ SFA ↓ MUFA in LM, SM and SC Incorporation of c9,t11 > t10,c12 ↑ G6PDH ↓ SCD in SC
Morel et al. (2008)	Female n=64	Duroc x (Large White x Landrace)	29-102 kg	Sanovite (CLA + Vit E)	Barley-wheat-wheat middlings-SBM	0.614 % supplement vs. not supplemented	No effect on SC fat ↑ IMF in LM ↑ C 18:0, ↓ C 18:1

Reference	Gender	Genetic	Wt range	CLA source	Dietary ingredients	Treatments	Response CLA
Intarapichet et al. (2008)	Males and Females n=48	Duroc x (Landrace x Large White)	60 (6 wk)	CLA-30	Rice-Rice bran-soy meal-fish meal	2 % palm oil and palm oil supplemented with 0.5 or 1 % CLA	Loin: ↓ IMF, 37 %; ↓ SFA, ↓ MUFA; ↓PUFA Ham: no effect Incorporation of c9,t11 > t10,c12; c9,c11; t9,t11
Larsen et al. (2009)	Barrows n=48	Yorkshire x Landrace x Duroc x Hampshire	55-113 kg	CLA-60	Corn-SBM	1.25 % SBO vs. 1.25 % CLA	↑ saturation in belly fat
Cordero et al. (2010a)	Males and Females n=40	Iberian x Duroc	120-153 kg	CLA-60	Barley-wheat-corn-SBM	1 % SBO vs. 1 % CLA	No effect on SC fat or IMF ↑ SFA ↓ MUFA in LM and SC and↓ PUFA in SC Incorporation of c9,t11 > t10,c12 Activities of G6PDH and ME were not affected
Cordero et al. (2010b)	Female	Large White x (Large White x Landrace)	59-133 kg	CLA-60	Barley-Wheat-Corn Soya 44	1 % lard vs. 0.5, 1 , 2 % CLA	↑ loin weight, 9 %; control 4.11 % ↑ IMF in LM, 19 %; control 2.04 % ↑ SFA and ↓ MUFA in LM and SC Incorporation of c9,t11 > t10,c12
Han et al. (2011)	Barrows and gilts	Landrace x Large White	39-108 kg	CLA-73	Corn-SBM	0, 0.5, 1, 1.5 % CLA	↑ SFA, ↓ UFA in LM and SC Incorporation of c9,t11 > t10,c12
Zhong et al. (2011)	Gilts n=72	Duroc x Landrace x Large White	60-100 kg	-	Corn-SBM	2.5 % SFO vs 2.5 % CLA	↑ IMF in LM, 54 %; control 1.45 % ↑ SFA, ↓ MUFA in LM

Reference	Gender	Genetic	Wt range	CLA source	Dietary ingredients	Treatments	Response CLA
Barnes et al. (2012)	Barrows n=20	PIC 280 x Cambrough 23	52-102 kg	CLA-60	Corn-SBM	1 % SBO vs. 1 % CLA	↓ SC 10th rib, 16 %; control 30.23 mm ↓ loin area, 6 %; control 36.29 cm^2 ↑ IMF in loin, 35 %; control 2.81 % ↑SFA, ↓MUFA in LM

ACC: acetyl coenzyme A carboxylase; FFA: free fatty acids; G6PDH : glucose 6-phosphate dehydrogenase ; IMF: intramuscular fat; SBM: LM: *longissimus* muscle; ME : malic enzyme; soybean meal; SBO: soybean oil; SC: subcutaneous fat; SCD : steaoryl CoA desaturase ; SFO: sunflower oil; SM: *semimembranosus* muscle; USF: unsaturated fatty acids; Vit: vitamin; wk: week. Adapted from (Azain, 2003).

Table 5: Summary of published reports on the effect of conjugated linoleic acid (CLA) on gene expression in different tissues of several species

			Oxidation	Cellular differentiation	FA synthesis			FA uptake	FA desaturation	
Author	**Organism**	**CLA source**	**PPARα**	**PPARγ**	**SREBP1**	**FAS**	**ACC**	**LPL**	**D6D**	**SCD**
Brodie et al. (1999)	3T3-L1 preadipocytes	Mix isomers of 9,11		↓ γ2						
Choi et al. (2000)	Mouse 3T3-L1 adipocytes	10t, 12c c9,11t (no effect)		↑ γ2		↑				↓ 1 ↑ 2
Ding et al. (2000)	Porcine adipocyte	9,11 and 10,12 CLA		=				↑		
Evans et al. (2001)	3T3-L1 preadipocytes	10t,c12		↑ γ2 (acute) ↓ γ2 (chronic)						
Meadus et al. (2002)	LD barrows (↑ IMF, 14 %)	2 % CLA (9c,11t and 10t, 12c)	=	↑						
Takahashi et al. (2002)	WAT, BAT	CLA (32.9% 9c,11t/9t,11c, 33.7% 10t, 12c 3.8% others)		↓						
Brown et al. (2003)	Human preadipocytes	10t, 12c 9c,11t		↓ ↑						
Granlund et al. (2003)	3T3-L1 adipocyte	10t,c12 c9,11t (no effect)		↓						
Kang et al. (2003)	3T3-L1 adipocytes	10t, 12c		↓		↓				
McNeel and Mersmann (2003)	Porcine adipocyte	CLA, 9,11 and 10,12 CLA		↓				↓		
Meadus (2003)	LT barrows 105kg	2 % CLA (both isomers)	=	↑						
McNeel and Mersmann (2003)	Porcine adipocytes	9,11 and 10,12 CLA and CLA mix		=				=		

			Oxidation	Cellular differentiation	FA synthesis			FA uptake	FA desaturation	
Author	**Organism**	**CLA source**	**PPARα**	**PPARγ**	**SREBP1**	**FAS**	**ACC**	**LPL**	**D6D**	**SCD**
Takahashi et al. (2003)	Liver mouse	CLA (9c,11t and 10t, 12c)			↑	↑	↑		↑	
Tsuboyama-Kasaoka et al. (2003)	Liver mice	CLA (33.0% 9c,11t; 34.8% 10t, 11c; 2.4% c9,c11/c10,c12; 2% t9,c11/t10,t12)	=		↑	=	↑	=		↑ 1
Warren et al. (2003)	Liver mice	10t, 12c	↓					=		
		9c,11t	↑					=		
Brown et al. (2004)	Cultures of stromal vascular cells containing newly differentiated human adipocytes	10t, 12c		↓						
Kang et al. (2004)	Epididymal adipose tissue mice	10t, 12c		=		↑				= 1
										↑2
Lin et al. (2004)	Mammary gland mice	9c,11t and 10t, 11c				↓	↓			↓
	Liver mice	(but 10t, 12c is more potent)				=	=			=
Brandebourg and Hu (2005a)	Pig preadipocytes	10t, 12c		↓	↓1c					
Bassaganya-Riera and Hontecillas (2006)	Colonic gene expression pig	CLA (9c,11t and 10t, 12c)		↑						
Zabala et al. (2006)	Hamster adipose tissue	10t, 12c		↓	↓1a,1c	↓	↓			
Zhang et al. (2006)	Spleen chiks after fed lipopolysaccharide	CLA (both isomers)		↑						

			Oxidation	Cellular differentiation	FA synthesis			FA uptake	FA desaturation	
Author	**Organism**	**CLA source**	**PPARα**	**PPARγ**	**SREBP1**	**FAS**	**ACC**	**LPL**	**D6D**	**SCD**
Rasooly et al. (2007)	Liver mouse	10t, 12c	↓			↑	↑			
		9c,11t	=			=	=			↑
Corl et al. (2008)	Adipose tissue in neonatal pigs (↓ by CLA)	1 % CLA (9c,11t and 10t, 12c)					↓	↓		
Jose et al. (2008)	Cultrue of Adipose tissue explants of growing pigs	10t, 12c				↓				
König et al. (2008)	Liver of laying hens	CLA (9c,11t and 10t, 12c)	=		↑ 1, = 2					
Zhao et al. (2008)	Liver fish	CLA (both isomers)	↑	↑						
Zhou et al. (2008)	Adipose tissue obese rat	CLA (38.2% 9c,11t and 42.3% 10t, 11c)		↑						
Castellanos-Tapia et al. (2009)	Muscle tissue mice	CLA (34.4% 9c,11t, 35.1% 10t, 12c 4.1% others)						↑		
	Epididymal adipose tissue mice							↓		
Miranda et al. (2009)	Hamster, subcutaneous and perirenal adipose tissue	10t, 12c		=	= 1a, 1c	=		=		
Navarro et al. (2009)	Liver hamsters	10t, 12c	↑				↑			↑ 1
Tarling et al. (2009)	Liver and perirenal adipose tissue in hamsters (no effects in liver)	10t, 12c (no effects 9c,11t)			↓			↓		

			Oxidation	Cellular Differentiation	FA synthesis			FA uptake	FA desaturation	
Author	**Organism**	**CLA source**	**PPARα**	**PPARγ**	**SREBP1**	**FAS**	**ACC**	**LPL**	**D6D**	**SCD**
Wendel et al. (2009)	White adipose tissue of *ob/ob* mice	CLA (39.2% 9c,11t and 38.5% 10t, 12c)	=	↓	↓			↓		
Ashwell et al. (2010)	Liver mice	10t, 12c								
Jiang et al. (2010)	Backfat finishing pigs LD finishing pigs (↑ 54 % IMF)	0-1.25-2.5 %CLA (36.9% 9c,11t, 37.5% 10t, 12c 5.4% others)						= =		
Lengi and Corl (2010)	Bovine preadipocytes	10t, 12c		=		=				↓ 1
Zhai et al. (2010)	3T3-L1 cells	10t, 12c 9c,11t	↑↑ ↑							
Barnes et al. (2012)	LD barrows	1 % CLA (9c,11t and 10t, 12c)		=						

ACC: acetyl-CoA carboxylase; BAT: brown adipose tissue; D6D: delta-6-desaturase; FAS: fatty acid synthase; IMF: intramuscular fat; LD: *longissimus dorsi*; LPL: lipoprotein lipase; LT: *longissimus thoracis*; PPARα: peroxisome proliferator alpha; PPARγ: peroxisome proliferator gamma; SCD: steroyl-Co-A desaturase; SREBP1: sterol regulatory element-binding protein 1; WAT: white adipose tissue.

3.5.4. Vitamin intake

Meat lipids are sensible to oxidation, and incorporation of vitamin E in pig diets prevents FA oxidation influencing the organoleptic properties of meat (Lebret et al., 1999). Hence, feeding supplemental levels of vitamin E is beneficial for improving meat color and shelf-life of beef, lamb and pork.

Several studies in cattle supported that not supplementing diets with vitamin A has potential for improving marbling (Table 6). Although fewer studies were performed in pigs, some found that ommiting vitamin A in the diets increased IMF content (D'Souza et al, 2003) while some others did not or had found the opposite effect (Olivares et al., 2009). Thus, studies done in ruminants seem to have a clearer response than studies performed in pigs. These findings are in line of ESFA recommendations last 2009 of reducing the vitamin A levels in feed for livestock without producing negative effects for the animal health or development because it was detected that some of the consumers have the risk to exceed the maximum safe amount of 3,000 μg/day.

Vitamin A otherwise called trans-retinol plays a key role in vision, controlling animal growth and somehow stimulating nervous system. The vitamin can be either consumed in the diet or biosynthesized from β-carotene (Mathews and Van Holde, 1966, chapter 19). In intestinal absorptive cells, retinol derived from either source is esterified to long chain FA from retinylesters. Retinylesters are then packaged in chylomicrons, secreted through the lymphatic system into blood and are taken up by the liver, although there is some evidence that a significant amount is secreted into portal circulation probably as free retinol. The liver serves as the major storage for vitamin A in the body (Harrison, 2012). When a release is induced, retinol is mobilized from the liver bound to a protein called serum retinol binding protein (RBP). In blood, retinol-bound RBP is associated with a homotetrameric protein termed transthyretin (TTR), which, in addition to binding RBP, transports thyroxin (T_4). The ternary retinol/RBP/TTR complex is the

circulating vitamin A source for extrahepatic tissue. Uptake of retinol from blood into target cells is mediated by a protein stimulated by retinoic acid (STRA6). In target cells, retinol can be stored in form of retinylesters or it can be converted into the transcriptionally active metabolites retinoic acids (Berry and Noy, 2012). Cellular RA binding proteins types I and II (CRABP-I and CRABP-II) are found in the cytosol and in presence of RA may be translocated to the nucleus, where the complex associated directly with retinoid receptors mediates the transfer from binding protein to the receptor (Theodosiou et al., 2010; Fig. 15)

Thus, RA can activate two types of receptors of the nuclear receptor superfamily: the retinoic acid receptors (RARs), which *in vitro* bind both all-trans RA and 9-cis RA with high affinity, and the retinoid X receptors (RXRs), which *in vitro* bind specifically 9-cis RA. Since RXR is an obligate heterodimeric partner for many nuclear receptors involved in metabolism, it is reasonable to assume that vitamin A status and retinoid contribute to glucose and lipid homeostasis. RAR-RXR heterodimers control the expression of typical retinoid-target genes by binding to defined RA response elements (RAREs) in the gene promoter and modulation transcription. Several genes encoding protein in lipid metabolism appear to be up-regulated by retinoids at the transcriptional level by RAR-dependent pathways. These include the gene for phosphoenolpyruvate carboxykinase, which is the rate-limiting enzyme of glyceroneogenesis and gluconeogenesis and the gene for SCD-1 which converts the SFA into MUFA. RXR can also be activated by other nuclear receptors, such as the activation of LXR:RXR and PPAR:RXR upon retinoid binding (Bonet et al., 2012). All-trans, 9-cis RA and 13-cis RA are reported as good activators of PPARγ expression, hence can be associated to powerful agents for the promotion of bovine or rat adipogenesis (Garcia-Rojas et al., 2006; Krskova-Tybitanclova et al., 2008). Another way for retinoids to regulate lipid homeostasis is through regulation of hepatic *Srep-1c* expression. The retinoid and insulin signaling pathways can converge on the same sites in *Srebp-1c* promoter and synergize to induce its expression, and in turn the expression of its downstream lipogenic genes

(Zhao et al., 2012). Contrary to this finding Brandebourg and Hu (2005b) were the first that observed an inhibitory effect of RA on pig preadipocytes differentiation in primary cultures that is mediated by RAR and is correlated with the down regulation of PPARγ, RXR and SREBP-1c. Since then several reports observed this inhibitory effect: Ziouzenkova and Plutzky (2008) found that apocarotenal aldehydes resulting from the metabolization of ß-carotene may also reduce the expression of PPARγ and PPARα. Oki et al. (2008) found that RA inhibited adipogenic differentiation of DFAT cells, a mature adipocyte-derived preadipocytes cell line, without up-regulating the expression levels of adipogenic transcription factors and Moon et al. (2008) found that RA down-regulated expression of PPARγ in 3T3-L1 preadipocytes culture. Furthermore, nongenomic effects of RA may also contribute to its impact on lipid metabolism. These effects involve the modulation of the activity of several important protein kinases. In mice, treatment with all-trans RA results in an increase of FA oxidation in liver, skeletal muscle and adipose tissue (Bonet et al., 2012). Recently, was discovered that STRA6 is not only a vitamin A transporter but also functions as a surface signaling receptor, inducing the expression of PPARγ, which enhances lipid accumulation (Berry and Noy, 2012). Taking into account the vitamin A as a regulator for fat deposition, it is suggested that the amount of vitamin A included in pig diets may implicate differences in pig fat depots. As suggested by the studies realized by Olivares et al. (2009b) who found that when vitamin A is added in a large amount (100,000 IU/kg feed), an increase of IMF without affecting subcutaneous fat occurred. However, the role of vitamin A on fat deposition is not clear because the same group two years later found that an increase of IMF occurred when the level of dietary vitamin A was reduced from 13,000 to 1,300 IU/kg feed (Olivares et al., 2011).

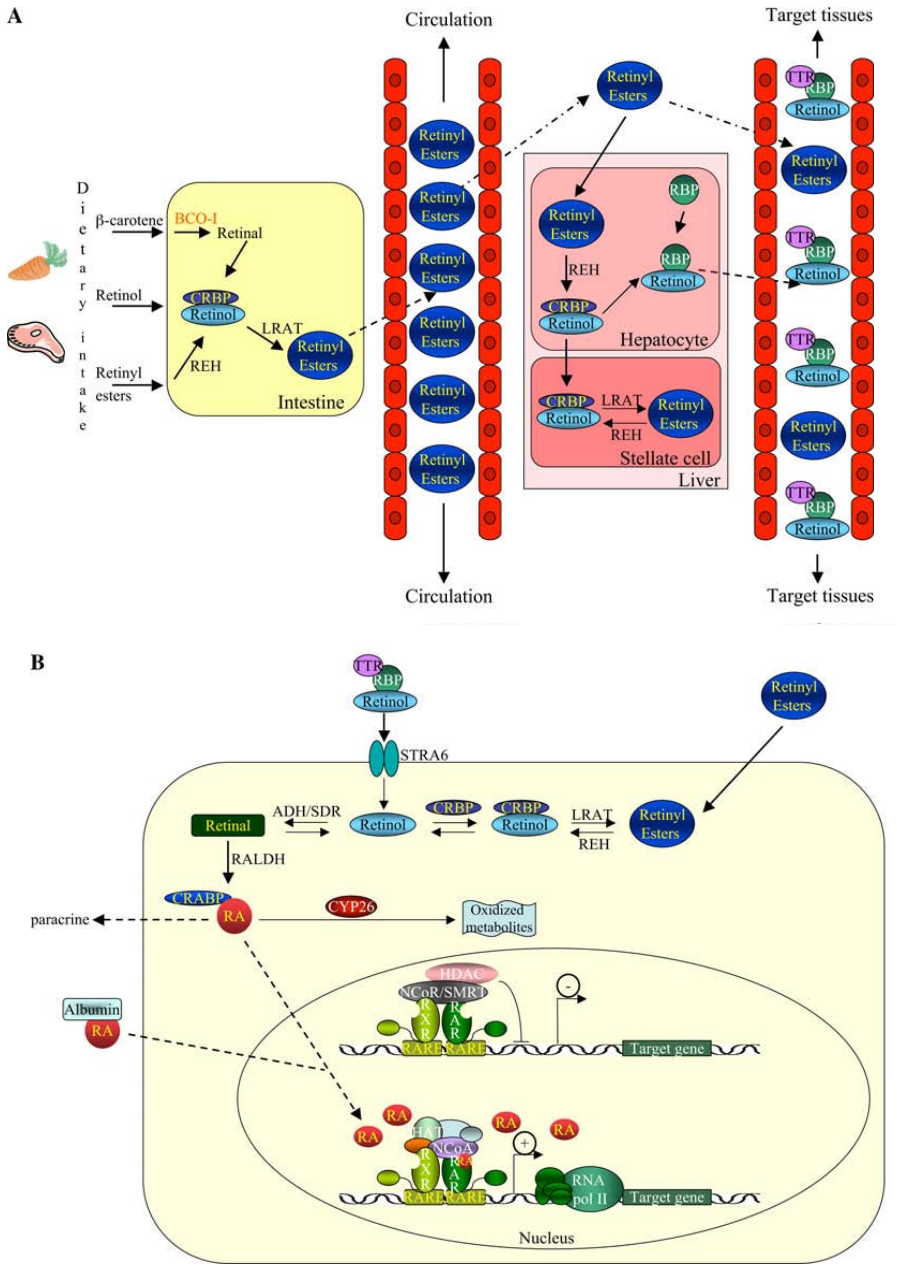
Table 6: Summary of published reports evaluating the effect of vitamin A level supplementation on carcass composition in different livestock species.

Reference	Gender	Genetic	Wt range	Supplement of vitamin A	Dietary ingredients	Response vitamin A
Studies with beef cattle						
Siebert et al. (2006)	Steers	Angus	12 month old for 268 d	0 vs. 60,000 IU/100 kg BW/d	Triticale, wheat, canola meal	No effect of Vit A SCF No Vit A ↑ IMF, 35 %
Gorocica-Buenfil et al. (2007b)	Steers n=168	Angus crossbred	295 – 579 kg	0 vs. 2,700 IU/kg	Corn, SBM	No VitA: No effect SCF ↑ Marbling, 10 % (ns) ↑cell number and ↓ diameter in LM IMF
Gorocica-Buenfil et al. (2007c)	Steers n=60	Holstein	218 – 588 kg	2,200, 0 (131 d), 0 (243 d) IU/kg	Corn, wheat, SBM	No VitA: No effect SCF ↑ IMF (243 d), 33 %
Gorocica-Buenfil et al. (2007a)	Steers n=165	Angus crossbred	300 – 555 kg	0 vs. 2,200 IU /kg	Corn, SBM, SBO	No VitA: ↑ Marbling, 6 %
Kruk et al. (2008)	Steers n=20	Angus	332 kg for 270 d	0 vs. 60,000 IU/100 kg BW/d	Standard feedlot	No effect of Vit A SCF and ST IMF No VitA: ↑ IMF, 35 % in LM
Arnett et al. (2009)	Steers n=48	Angus crossbred	233/259 – 485 kg	0 vs. 42,180 IU/d	SBM	No VitA: ↓ SCF (ns) ↑IMF (ns), 27 % or 10 % for early or traditional weaning, respectively

Reference	Gender	Genetic	Wt range	Supplement of vitamin A	Dietary ingredients	Response vitamin A
Studies with lambs						
Arnett et al. (2007)	Wethers n=40	Rambouillet x Finn ewes mated to Suffolk x Hampshire rams	29 – 61 kg	56-110 d: 6,000-6,000 IU/kg 6,000-0 IU/kg 0-6,000 IU/kg 0-0 IU/kg	Corn, SBM, cottonseed	Feeding no VitA for one period ↑ SCF Feeding no VitA ↓ IMF, 26 % (in whole period)
Arana et al. (2008)	Male n=24	Rasa Aragonesa	After birth – 17/58 kg	500,000 IU animal twice per week	Alfalfa, soybean	No effect in SCF or LT IMF
Studies with Pigs						
D'Souza et al. (2003)	Females n=50	LW x LD x DU	24 – 104 kg	0 vs. 100 IU/kg	Barley, wheat, lupins, SBM, Blood meal, fish meal, canola oil	No effect in SCF Restricted VitA: ↑ IMF, 54 %
Olivares et al. (2009a)	Barrows n=128	DU x (LW x LD)	67.9 – 125.9 kg	7,500 vs. 100,000 IU/kg	Barley, wheat, corn, SBM	No effect of SCF or IMF High VitA: ↑ SFA in SCF ↓MUFA in inner SCF ↑ SFA in neutral lipids of liver and LM ↓PUFA in neutral lipids of liver
Olivares et al. (2009b)	Barrows n=256	DU x (LW x LD) or (LW x LD) x (LW x LD)	56.4 – 114.5 kg	0 vs. 100,000 IU/kg	Corn, wheat, barley, SBO vs. palm oil	No effect SCF High VitA: ↑ IMF, 20 % in Du but not in LW x LD sires ↑ retinol content in SCF and liver but not in LM ↓ C 18:0 in inner layer SCF ↑SFA and ↓ MUFA in LD neutral lipids ↑SFA and ↓ PUFA in LD polar lipids

Reference	Gender	Genetic	Wt range	Supplement of vitamin A	Dietary ingredients	Response vitamin A
Olivares et al. (2011)	Barrows n=108	LW x (LW x LD)	55.8 – 125.7 kg	13,000; 1,300; 13,000 (6wk) + 0 (5w) IU/kg	Corn, wheat, barley, Soybean	No effect SCF High VitA: ↓ IMF in LM, 21 % ↓ SFA in outer SCF layer ↑ SFA and ↓ PUFA in liver

BW: body weight; d: day; DU: Duroc; IMF: intramuscular fat; LD: Landrace; LW: Large White; LM: *longissimus* muscle; ns: not significant; SBM: soybean meal; SBO: soybean oil; SCF: subcutaneous fat; ST: semitendinosus; VitA: Vitamin A; Wt: weight



)

Fig. 15: Metabolism of vitamin A (A) and transcriptional activation (B)

From: Theodosiou et al. (2010)

3.5.5. Cereal source

Cereals are the first energy source in the diets. The principal cereal used to formulate diets mainly depends on the cereal produced in the region. So, in areas where wheat is the major cereal grown, producers, nutritionists, and feed mills are all accustomed using formulas based on this cereal, and rather apprehensive when presented with corn-based formulas. Corn is certainly an excellent source of energy for pigs but it is low in fiber, lysine and tryptophan so it can not be the only cereal source of the diet. Wheat is the grain of choice for nursery diets in many countries around the world, even where corn is locally available. Wheat has slightly lower energy content than corn and it is also low in lysine and threonine. An added problem of the use of wheat as the main cereal source is that it has a great amount of variability among wheat varieties, and even within batches of the same variety. Although, these are the main cereals sources, normally other cereals are added to the diet in lower amounts. For example: sorghum that contains about as much energy as wheat and in protein quality it resembles to corn; rice which is grown mainly for human consumption and as a result its use in pig diets is rather limited; oats that are high in fiber and are consequently low in energy; barley that is also high in fiber and has a high β-glucan content (anti-nutritional factor); triticale that has been often blamed for unpalatability, ergot infestation, and high pentosan (anti-nutritional factor); rye that is extremely high in pentosans and other anti-nutritional factors; or cassava that has practically the same feeding value as corn but inclusions of 50% depresses weight gain and feed efficiency.

Because cereal sources vary in the nutritional composition, feeding animals with one source compared with another may interfere in the growth performance parameters and in consequence pig adiposity. Hence, higher fatness was observed in animals fed a diet based on barley while IMF was higher in animals fed corn based diets (Bout and Girard, 1988). Lampe et al. (2006) and Daza et al. (2010) found that pigs fed barley based diets will reduce costs of feeding and may increase

IMF, SFA and MUFA in subcutaneous fat compared with corn based diets. Using corn batches differing just in 0.3 % of linoleic acid content in diets can lead to significant differences in fatty acid composition of fats depot (Della Casa et al., 2010). So, it can be concluded that cereal source and the variability within the cereal variety or batch may affect the growth performance, fat deposition and composition in pigs.

OBJECTIVES

BACKGROUND AND OBJECTIVES

Several reports suggest that meat with a high IMF content is associated with a better acceptability for the consumer while some others did not find relation between IMF and meat quality traits. The environment where the study is performed may impact on that effect, because as it is reported in the introduction the perception of meat quality changes between cultures. Hence, an initial study with a representative group of Spanish population was performed (Chapter 1). The objective of this experiment was:

- To evaluate the eating and visual acceptability of pork differing in IMF content, regardless of other pork quality attributes.

In addition:

- To understand more about this acceptability by studying segments of consumers.
- To determine which fresh pork characteristics are important for consumers at the point of purchase

As consequence of the genetic selection against P2 backfat during past decades and the trend to slaugher pigs at early stages when IMF is not still completely formed, resulted in leaner genotypes with a reduced IMF content, which may negatively affect meat quality attributes. Different nutritional strategies are proposed in the literature with the aim of increasing it, but controversial results are found. Thus, the principal objective of this thesis was to increase IMF content using different nutritional strategies (if possible without increasing the other fat depots) in order to improve the acceptability of the fresh pork. Several strategies have been proposed:

- Inclusion of CLA (Chapter 2): Several reports evaluating the effect of CLA on pig fat deposition have been published in order to reduce carcass backfat. However, only some of them observed an increase of the IMF when CLA was added to the diet. The objective of this experiment was:

- To use a higher dose compared with other reports in order to increase the magnitude of the effect, if it occurs.

In addition:

- To determine if CLA affects growth performance and the distribution of fat in the carcass.
- To determine if CLA affects the fatty acid profile (FA) in several tissues.
- To determine the effect in the different lipid fractions in the muscle.
- To determine the rate at which CLA is incorporated in different tissues.
- To determine if the occurred changes are due to some modification at expression level of genes related with the lipid metabolism.

- Reduction of the level of dietary vitamin A (Chapter 3): In ruminants, several reports seem to indicate that IMF could be increased with a reduction of the dietary level of vitamin A while in pigs there are few studies with controversial results. The objective of this experiment was:
  - To study if reduction of dietary vitamin A increases IMF in pigs, for this three different treatments were used, one using the usual dose in the premix, another one using an amount close to the swine requirements recommended by NRC and a third one without inclusion.

  In addition:
  - To determine if vitamin A level affects growth performance and the distribution of fat in the carcass.
  - To determine the amount of vitamin A deposited in different tissues.
  - To determine if gene expression is affected by dietary vitamin A in the loin.

- Reduction of the level of protein, lysine or both protein and lysine (Chapter 4): Several studies reported that reduction of protein or lysine level may increase IMF, however, normally when one parameter is reduced the other one is reduced at the same proportion. In this study, lower protein levels compared with the other studies are used, in order to study the effect under conditions that would result in lower nitrogen excretion. The main objective was:
    - To determine if IMF increases due to protein, lysine or the interaction of both protein and lysine.

  In addition:
    - To determine if protein, lysine or the interaction of both protein and lysine affect growth performance and the distribution of fat in the carcass.
    - To determine if protein, lysine or the interaction of both protein and lysine affects the fatty acid profile (FA) in several tissues.
- Dietary supplementation with arginine and/or leucine in low or normal protein diets using a leaner genotype compared the other studies (Chapter 5): Only one study has been reported for each of these amino acids evaluating the effect of the supplementation of those amino acids on IMF, and because the role they develop in the organism may regulate fat deposition in the muscle. The objective of this study was:
    - To determine if the effect of the reduction of protein on IMF is genotype dependent.
    - To determine if supplementation of arginine, leucine or arginine and leucine increases IMF.

  In addition:
    - To determine if supplementation of arginine, leucine or arginine and leucine affect growth perfromance the distribution of fat in the carcass.

CHAPTER 1:

Consumer test

Chapter 1:

Do all the consumers accept marbling in the same way? The relationship between eating and visual acceptability of pork with different intramuscular fat content

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The meat quality eating traits are percieved differently depending on culture in which they are evaluated. In this chapter a consumer test is performed in order to evaluate Spanish consumer preferences in relation to IMF and/or marbling. Two different evaluations were performed, one visual and one gustative. The results from this test showed that Spanish consumers prefer pork with high values of IMF from the point of view of taste, however, two differentiated segments of population were distinguished from the point of view of visual preference. Becuase of the relationship between IMF and eating preference in the following chapters are evaluated different nutritional strategies in order to increase this parameter. On the other hand, marketing strategies should be done in order to inform the conusmer about of the relation between IMF and eating quality because amost half of the population ingnore that relation.

Do all the consumers accept marbling in the same way? The relationship between eating and visual acceptability of pork with different intramuscular fat content

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Abstract

Several reports show that intramuscular fat (IMF) and/or marbling affect the sensory acceptability of meat. The aim of the present work was to (1) investigate on Spanish consumers about the eating and visual acceptability of pork with different levels of IMF content, (2) understand more about this acceptability studying segments of consumers and (3) determine which fresh pork characteristics are important at the point of purchase. Loin section (n=40) were sorted into four IMF groups: 0.96±0.30% (G1), 2.11±0.07% (G2), 3.72±0.26% (G3), and 5.78±0.19% (G4). Consumers (n=200) evaluated the acceptability, tenderness and juiciness of cooked loin chops from each IMF group and then ranked raw chops according to visual preference. Two groups of consumers -'lean loin lovers' (55.5%) and 'marbled loin lovers' (44.5%)- were identified based on their visual preferences; however, according to their eating acceptability scores, all the consumers preferred loins with higher IMF levels. The minimum IMF content recommended is between 2.2% and 3.4%. Marketing strategies are necessary to satisfy the preferences of the different segments of consumers.

Keywords

Consumers, intramuscular fat, marbling, preferences, segments, sensory acceptability

1. Introduction

An important factor affects consumer acceptability of pork: the amount of intramuscular fat (IMF), which varies across breed, sex, diet and weight at slaughter (Cilla et al., 2006; D'Souza, Pethick, Dunshea, Pluske, & Mullan, 2003; Raj et al., 2002; Gou, Guerrero, & Arnau, 1995). The IMF is moderately related to the amount of marbling or visual fat (Faucitano, Rivest, Daigle, Lévesque, & Gariepy, 2004;

Brun, Gispert, Valero, & Font i Furnols, 2011). Some reports show positive relationships between the acceptability or the tenderness of pork and the level of IMF content and/or marbling (Bejerholm, & Barton-Gade, 1986; Berge, Culioli, & Ouali, 1993; Cannata et al., 2010; Fortin, Robertso, & Tong, 2005), due to the lubrication during chewing (Johnson, Drevjani, Allen, & Reasbeck, 1988), whereas others denoted only minor contributions of IMF/marbling on pork acceptability (Channon, Kerr, & Walker, 2004; Moeller et al., 2010; O'Mahoney Cowan, & Keane, 1991-1992; van Laack, Stevens, & Stalder, 2001) or even negative relationships (Andrighetto, Gottardo, Andreoli, & Cozzi, 1999). Some research has demonstrated that highly marbled loins were less accepted by consumers that low marbled ones (Brewer, Zhu, & McKeith, 2001; Moeller et al., 2010; Fernandez, Monin, Talmont, Mourot, & Lebret, 1999;) while this effect is not clear in other studies (O'Mahoney et al., 1991-1992). Nevertheless, Ngapo, Martin and Dransfield (2007a) reported that acceptability of the marbling depends on the country and consumers of some Asiatic countries (Japan, Taiwan, and Korea), preferred marbled meat. Furthermore, it has been demonstrated that acceptability of meat differs among consumers (Carbonell, Izquierdo, Carbonell, & Costell, 2008; Font i Furnols et al., 2009; Fortomaris, Arsenos, Georgiadis, Banos, Stamataris, & Zygoyiannis, 2006; Ngapo et al., 2007a; Ngapo, Martin & Dransfield, 2007b; Verbeke, Pérez-Cueto, & Grunert, 2011), thus it is imperative to identify these segments of consumer, and develop marketing strategies for each of these segment (Naes, KubberØd, & Siverstsen, 2001).

Therefore, the objectives of the present study were to: (1) investigate on Spanish consumers about the eating and visual acceptability of pork differing in IMF content, regardless of the other pork quality attributes, (2) understand more about this acceptability studying segments of consumers; and (3) determine which fresh pork characteristics are important for consumers at the point of purchase.

2. Materials and methods

2.1. Sample selection and preparation

One hundred loin sections (*longissimus thoracis* between the 1st and the 3rd ribs from the last rib) with subcutaneous fat were obtained from 3 different slaughter plants on multiple days to have a representation of various producers and genetic types, and to ensure a variability of intramuscular fat (IMF) content. At 24 h *post mortem,* electrical conductivity (EC) was measured using a Pork Quality Meater (PQM-Kombi, Aichach, Germany) and ultimate pH (pHu) was measured using a Crison Portable pH-Meter (Crison, Barcelona, Spain) equipped with a Xerolyt Electrode. All PSE (EC ≥ 6.0 mS) according to Barton-Gade, Warris, Brown, and Lambooij (1995), and DFD (pHu ≥ 6.0) according to Joo, Kauffman, Kim, and Kim (1995), loin sections were excluded from the study.

Marbling was determined by a trained technician according to National Pork Producers Council (NPPC, 1999) standards, ranging from 1 (devoid of marbling) to 10 (abundantly marbled), whereas IMF was measured by means of the near infrared technology FoodScan equipment (Foss Analytical, Denmark) which measures at wavelengths between 850 nm and 1050 nm. The IMF determined with this equipment correlates well (IMF_{Soxtec}=-0.270+0.997·$IMF_{FoodScan}$, R^2=0.92, RMSE=0.17%) with Soxtec reference method (Soxtec™ 2050, Foss Analytical, Denmark). Forty loins were selected to give four levels of IMF content (10 loin sections/IMF group) as defined in Table 7. At 1 day *post mortem* loins were placed in an aluminium bag and frozen at -20ºC.

Table 7: Intramuscular fat content (%) by group (n=10/group).

Group	IMF content (%)				Marbling (NPPC)*
	Mean	StdDev	Min.	Max.	
G1	0.96	0.30	0.53	1.36	1 (100%)
G2	2.11	0.07	2.01	2.19	1 (30%) and 2 (70 %)
G3	3.72	0.26	3.41	4.12	3 (100%)
G4	5.78	0.19	5.50	5.98	3 (80%) and 4 (20%)

*National Pork Producer's Council scale; % of samples of each value

For the eating evaluations, loin sections were thawed 24 hours at 4ºC. Then the central part of the section was cut into three 1.5 cm-thick slices and the subcutaneous fat was trimmed to a thickness of 3 mm. Then, slices were placed directly on the oven grill tray and cooked in a pre-heated oven (FAGOR Innovation Class A; Fagor Electrodomésticos, S. Coop., Mondragón, Spain) at 200ºC without turning them. The internal temperature of the slices was measured by means of thermocouple K probes (Beamex Oy Ab, Pietarssari, Finland) and pork slices were cooked to an endpoint internal temperature of 76ºC, which is recommended to discriminate samples when considering various sensory properties (Bejerholm, & Aalsyng, 2003). After reaching this temperature, meat was removed from the oven. The edges of the slices were trimmed and each slice was divided into 1.5 cm-thick pieces (approximately four pieces/slice) perpendicular to the subcutaneous fat. The pieces were wrapped in aluminium foil, coded, and kept warm in a heater at 55ºC until the moment of serving (maximum 10 minutes later).

Raw loin slices 1.5 cm-thick were used for visual evaluation. Four slices, one from each IMF group, were placed on a white tray, coded and covered with transparent film. All the slices were trimmed of subcutaneous fat to a similar thickness and prepared to similar shape to avoid any consumer bias based on shape.

2.2. Experimental design and consumer evaluation

Consumers (n=200) between the ages of 18 and 73 years, who lived or worked in Barcelona city or its surroundings, were selected to be representative of the Spanish population (Table 8). An average of 10 consumers participated in each of the 20 sensory evaluation sessions.

The sensory evaluation was twofold:

First, an eating analysis was performed. Consumers evaluated the overall acceptability, as well as the tenderness and juiciness, of four blind samples from each IMF groups according to a nine-point scale (1= I dislike very much/very hard/very dry to 9= I like very much/very tender/very juicy). Samples were served

monadically to the consumers following a predetermined order to avoid the first sample and carry over effect (MacFie, Bratchell, Greenhoff, & Vallis, 1989).

Table 8: Socio-demographic characteristics of consumers[+]

				Cluster 1			Cluster 2		
	Men	Women	Total	Men	Women	Total	Men	Women	Total
Total	96	104	200	41	48	89	55	56	111
Age									
18 to 25 years	16	14	30	4	2	6	12	12	24
26 to 40 years	32	30	62	11	11	22	21	19	40
41 to 60 years	33	41	74	18	21	39	15	20	35
60 to 73 years	15	19	34	8	14	22	7	5	12
Finished level of studies									
Primary school	11	17	28	5	13	18	6	3	9
Secondary school	53	58	111	24	23	47	29	35	64
University	31	29	60	12	12	24	19	17	36
Work situation									
Employed	53	53	106	20	20	40	33	33	66
Unemployed	41	50	91	20	28	48	21	22	43
Economical contribution at home									
100%	15	17	32	8	10	18	7	7	14
>50%	22	12	34	9	5	14	13	7	20
50%	31	21	52	16	9	25	15	12	27
<50%	8	24	32	2	11	13	6	13	19
0%	19	27	46	6	12	18	13	15	28
Pork consumption									
More than twice week	35	38	73	14	21	35	21	17	38
Once a week	46	59	105	20	25	45	26	34	60
Fortnightly	12	5	17	6	1	7	6	4	10
Once a month or less	1	0	1	0	0	0	1	0	1

[+]Number of consumers in each category

Secondly, a visual analysis was performed. A tray containing 4 fresh loins slices (one from each IMF fat group and from the same loin they had evaluated for cooked sensory attributes) was shown to the consumers, and they were instructed to rank the samples according to their purchasing preference. Furthermore, the consumers' explanation for their order preference was recorded. The presentation of the loin slices on the tray was changed between sessions to avoid biases.

Consumers were also asked two additional questions: (1) Which is the main factor you consider when you buy pork loins? (price, colour, marbling and area size were suggested); and (2) Why do you consume pork? (price, taste and variation of meat type variables were suggested).

2.3. Statistical analysis

Purchase preference of loin sections was studied by means of an analysis of the frequencies of each ranking with SAS software (SAS Institute Inc., Cary, NC, USA). The order of preference was analysed using the Kruskal-Wallis test with XLStat (MS Excel, 2005), and multiple comparisons were performed using Steel-Dwass-Critchlow-Fligner test (KCS, 2011). Clusters of consumers were identified depending on their purchase preference by using the PROC CLUSTER of SAS. An agglomerative hierarchical cluster analysis, using the Ward method, was performed on the square Euclidian distance matrix. The number of clusters was selected from the dendrogram.

The mixed models procedure of SAS was used to determine differences among consumers scores on the IMF content of the loin sections. It was applied to all the consumers and to each cluster. The model included IMF group as fixed effect, session as blocking effect, and consumer as random effect. Significant differences between least square means were determined using Tukey's test ($P<0.05$).

Correlations between visual marbling and IMF content were calculated using PROC CORR of SAS, and correlations between consumers' scores and IMF level of the sample were calculated individually for each consumer and globally for all the consumers together. Furthermore, the frequency tables of each answer to the two questions were obtained using the FREQ procedure of the same software.

Finally, the frequency of each answer to the question put to the consumers regarding the reason for their purchase preference was obtained.

3. Results

3.1. IMF and marbling content of loin sections

Loin IMF contents between 0.53% and 1.36% (G1) were scored as a 1 on the NPPC (1999) marbling scale, whereas samples with IMF contents between 3.41% and 4.12% (G3) were scored as 3 (Table 7). However, IMF contents of 2.01% to 2.19%

(G2) had marbling scores of 1 (30%) and 2 (70%) and IMF contents between 5.50% and 5.98% (G4) had marbling scores of 3 (80%) and 4 (20%). Part of this overlapping in marbling scores within the same IMF groups is due to the fact that not all the IMF content is visible and the groups have been created regarding the IMF content. The correlation between IMF content and marbling was quite high (r=0.89; *P*<0.001) in the conditions of this experiment, since it is dependent on the methodology used to determine IMF as well as factors related to the preparation of the samples (Fortin et al., 2005).

3.2. Consumers' visual scores vs. marbling level

When all the consumers' scores were considered together, the loin slices chosen as first option were mainly the lower marbling groups, G1 and G2 (Table 9). The loin slices chosen as 4th option were the extreme, G1 and G4. Means of the rank-order of the samples showed that samples with the highest IMF content (G4) were (*P*<0.05) less preferred than samples with the medium IMF content (G2 and G3) although marbling between G3 and G4 was not very different when evaluated by NPPC marbling scale.

Table 9: Relative frequency (%) and average of the order of the sample by intramuscular fat group for all the consumers together and by clusters.

	Preference order				
	1st	2nd	3rd	4th	Average
All consumers (n=200)					
G1 (0.96%)	29.0	23.5	17.5	30.0	2.5^{ab}
G2 (2.11%)	28.0	27.5	31.0	13.5	2.3^{b}
G3 (3.72%)	21.0	31.0	32.0	16.0	2.4^{b}
G4 (5.78%)	22.0	18.0	19.5	40.5	2.8^{a}
Cluster 1 (n=89)					
G1 (0.96%)	0.0	5.6	27.0	67.4	3.6^{a}
G2 (2.11%)	23.6	19.1	36.0	21.3	2.6^{b}
G3 (3.72%)	36.0	46.1	18.0	0.0	1.8^{c}
G4 (5.78%)	40.4	29.2	19.1	11.2	2.0^{c}
Cluster 2 (n=111)					
G1 (0.96%)	52.3	37.8	9.9	0.0	1.6^{d}
G2 (2.11%)	31.5	34.2	27.0	7.2	2.1^{c}
G3 (3.72%)	9.0	18.9	43.2	28.8	2.9^{b}
G4 (5.78%)	7.2	9.0	19.8	64.0	3.4^{a}

^{a,b,c} Within a consumer group and column, least square means lacking a common superscript letter differ (*P*<0.05)

When the order of preference was used to segment consumers, two clusters were obtained. Table 8 presents the socio-demographic characteristics of the consumers of each cluster, showing no important differences between them, in the characteristics considered.

The first cluster of consumers (44.5%) demonstrated a preference for loins with higher IMF level and marbling, and were designated as 'marbled loin lovers' (Table 9). This cluster of consumers never considered loin slices with the least IMF content (G1) as a first choice (Table 9), ranking it fourth 67.4% of the times. Furthermore, this cluster ranked loins with the highest IMF content (G4) either first or second 69.6% of the times. When the average of the order was considered, loins with the highest marbling and IMF content (G3 and G4) were placed in the first positions (P<0.05), followed by loins from G2 (P<0.05) and in the last position loins with the lowest IMF content (G1) (P<0.05).

The second cluster, consisting of 111 consumers (55.5%) ranked samples with the lowest IMF content (G1) either first (52.3% of the times) or second (37.8% of the times), and ranked the G2 samples first 31.5% of the times and second 34.2% of the times (Table 9). These consumers could be typecast as 'lean loin lovers', and this segment of consumers did not prefer the loins with higher marbling and IMF contents, ranking G3 and G4 loins last 28.8% and 64.0% of the times, respectively. This was also exhibited by the average of the order of preference, which was higher (lower preference) for loins with higher IMF content (G4), followed by those from G3, G2, and, finally, those with little IMF content (G1).

Thus, it was not surprising that 'marbled loin lovers' (cluster 1) listed 'marbling' as the main criterion used when selecting fresh pork, whereas 'lean loin lovers' listed 'without marbling' and 'without fat' as their primary criteria (Table 10) when they are asked regarding their evaluation of loins with different marbling/IMF content. It should be noted that colour was listed as one of the most important factors affecting purchasing decision by all consumers, even thought in this evaluation the most evident difference between loins was the marbling.

Table 10: Relative frequency (%) of the attributes used by consumers (globally and by clusters) as a choice criteria at the point of purchase of the loins visually evaluated.

	cluster 1 (n=89)	cluster2 (n=111)	Total (n=200)
Colour	25.0	28.2	26.9
Without marbling	1.9	16.0	10.2
Marbling	38.9	7.7	20.5
Without fat	6.5	31.4	21.2
Fat	10.2	1.9	5.3
Thickness, size or drip losses	9.3	10.9	11.0
Texture, juiciness or tenderness	8.3	3.8	4.9

3.3. Consumers' eating acceptability scores vs. intramuscular fat content

Consumers gave higher (P<0.05) eating acceptability scores to pork with an average IMF of 5.78% (G4) and 3.78% (G3) than loins with less than 2.19% IMF (G1 and G2; Table 11). No (P>0.05) differences in acceptability, tenderness or juiciness were observed between the G1 and G2 categories, which had average IMF contents of 0.96% and 2.11%, respectively.

Regarding the overall acceptability, tenderness, and juiciness scores for each cluster (based on visual preferences), the 'marbled loin lovers' (cluster 1) and the 'lean loin lovers' (cluster 2) showed the same gustative preferences (Table 11). Both clusters gave higher (P<0.05) scores for loins from G3 and G4 than from G1 and G2, indicating higher eating acceptability scores for loins with higher IMF levels.

When all the consumers were considered together, correlations between overall acceptability, tenderness, and juiciness scores with IMF content of the loin were 0.38, 0.46 and 0.40, respectively (P<0.0001). Furthermore, 81.5%, 86.0%, and 83.5% of the correlations (P<0.05) of acceptability, tenderness and juiciness with IMF were positive, and 61.0%, 71.0% and 62.0%, respectively, were between 0.5 and 1.0. This indicates that the higher the IMF content the higher the consumers' overall acceptability, tenderness and juiciness scores (Table 12).

Table 11: Least square means and standard error (SE) of the consumer's scores by intramuscular content group[1].

	Overall acceptability	Tenderness	Juiciness
All consumers (n=200)			
G1 (0.96%)	5.6^{c}	5.0^{c}	4.7^{c}
G2 (2.11%)	5.6^{c}	4.9^{c}	4.8^{c}
G3 (3.72%)	6.5^{b}	6.3^{b}	5.9^{b}
G4 (5.78%)	7.0^{a}	7.0^{a}	6.4^{a}
SE	*0.10*	*0.12*	*0.12*
Cluster 1 (n=89)			
G1 (0.96%)	5.5^{b}	5.0^{b}	4.6^{b}
G2 (2.11%)	5.8^{b}	5.1^{b}	4.8^{b}
G3 (3.72%)	6.6^{a}	6.5^{a}	6.2^{a}
G4 (5.78%)	6.9^{a}	7.0^{a}	6.4^{a}
SE	*0.15*	*0.17*	*0.17*
Cluster 2 (n=111)			
G1 (0.96%)	5.7^{c}	5.1^{c}	4.8^{c}
G2 (2.11%)	5.5^{c}	4.8^{c}	4.7^{c}
G3 (3.72%)	6.4^{b}	6.1^{b}	5.7^{b}
G4 (5.78%)	7.0^{a}	7.0^{a}	6.4^{a}
SE	*0.13*	*0.16*	*0.16*

[1]Scores from 1: dislike extremely, extremely hard or extremely dry to 9: like extremely, extremely tender or extremely juiciness.

[a,b,c] Within a consumer group and column, least squares means lacking a common superscript letter differ (P<0.05).

Table 12: Relative frequency (%) of the correlations between consumers' scores and intramuscular fat content[+].

Correlations	Overall acceptability	Tenderness	Juiciness
-1 to -0.5	12.5^{b}	5.5^{c}	8.0^{c}
-0.5 to 0	6.0^{c}	8.5^{bc}	8.5
0 to 0.50	20.5^{b}	15.0^{b}	21.5^{b}
0.50 to 1	61.0^{a}	71.0^{a}	62.0^{a}

[+] Different superscripts indicate significant differences within a column (P<0.05)

3.4. Purchase and pork consumption reasons

Frequencies of the consumers' answers to the question 'Which is the main factor you consider when you buy pork?' and 'Why do you consume pork?' are presented in Table 13. Colour was the most important reason for purchasing pork (68.5% of the consumers), followed by price, low fat content and different aspect characteristics (20.5%, 9.0% and 8.5%, respectively). The most important difference between the two clusters were 'two colours' (6.7% *vs.* 0.9%), which refers to the loin closer to the caudal part of the loin where there are two muscles with different

colour (*longissimus dorsi* and *multifidus dorsi*), and also 'marbling' that were preferred by the 'marbled loin lovers' (11.2% *vs.* 1.8%), whereas 'low fat content', was preferred by the 'lean loin lovers' (14.4% *vs.* 2.2%). These differences are logical considering that the clusters were obtained according to the visual preference of loins with different marbling/IMF content. Marbling answers could have been overestimated due to the previous visual evaluation of loins with different IMF content. People consume pork mainly because they like the taste (63.5% of the consumers) and to vary the type of meat (55.5% of the consumers). No differences were found between clusters regarding this question.

Table 13: Relative frequency (%) of answers of the consumers to the questions 'Which is the main factor you consider when you buy pork?' and 'Why do you consume pork?'.

	Cluster 1 (n=89)	Cluster 2 (n=111)	Total (n=200)
Which is the main factor you consider when you buy pork?			
Colour	65.2	71.2	68.5
Two colours[1]	6.7	0.9	3.5
Marbling	11.2	1.8	6.0
Price	16.9	23.4	20.5
Fat	4.5	7.2	6.0
Low marbling content	2.2	2.7	2.5
Low fat content	2.2	14.4	9.0
Aspect: size, thickness, freshness, texture	9.0	8.1	8.5
Others: Iberian, quality	4.5	0.0	2.0
Why do you consume pork?			
Price	11.2	18.9	15.5
Taste	65.2	62.2	63.5
To vary the type of meat	56.2	55.0	55.5
Others: easy to cook and to find, red meat, habit	4.5	2.7	3.5

[1] Two colours refers to the loin closer to the caudal part of the loin where there are two different muscles (*longissimus dorsi* and *multifidus dorsi*), one lighter than the other

4. Discussion

The purchasing decision is dependent on different appearance characteristics of the meat (e.g. colour, marbling, fat and other aspect factors), as well as other characteristics related to health concerns and nutritional aspects (Grunet, 1997). The focus of the present study was the IMF/marbling level of pork, and consumers' visual appraisal of fresh pork loin slices with different IMF/marbling levels as the

most perceptible differences, demonstrated that this was the most important characteristic affecting purchasing decision and makes possible to classify consumers according to this visual meat trait in 'marbling loin lovers' and 'lean loin lovers' (see the clusters obtained and the attributes used by consumers as the choice criteria of the different loin slices evaluated in Table 10). This concurs with the results of Brewer (1998). However, when there are other characteristics apart of marbling varying between loin slices such as colour, fat cover and drip losses, Ngapo et al (2007a) reported that marbling affects the consumers' preference in only a few number of consumers or cluster of consumers (about 17%, mainly from Korea, Taiwan and Japan), with colour being one of the most important characteristics. In fact, consumers in the present study also identified colour as the most important characteristic they consider at the point of purchase (Table 13), in accordance with others works (Verbeke, De Smet, Vackier, Van Oeckel, Warnants & Van Kenhove, 2005; Ngapo et al., 2007a). It is important to note that the marbling content can influence the perception of colour because loins with increased marbling appear lighter than those with reduced marbling (Brewer et al., 2001).

It has been reported that the amount of visible IMF or marbling negatively affects consumers purchase decision most likely due a perception of it being less healthy than lean (Issanchou, 1996; Brewer, 1998; Resurrección, 2003). Brewer et al. (2001) found that chops with less than 2.5% IMF had a higher overall appearance acceptability and purchase intent than chops with higher IMF content (3.0% to 3.5%) and Fernandez et al. (1999) also found that purchasing intention decreased with increasing IMF level but as reported by Ngapo et al. (1997a), in some countries marbling affects positively consumers' purchasing decision. The present work shows that there are different types of consumers: 55.5% of the consumers evaluated (cluster 2) were 'lean loin lovers' who preferred loins with less than 1.4% (G1) or 2.2% (G2) of IMF, *i.e*. marbling scores 1 or 2, and 'marbled loin lovers' who liked loins with more than 3.4% IMF (G3 and G4), *i.e.* marbling scores 3 or 4. Thus, care should be taken when considering all consumers together because it is highly

possible to draw conclusions that might not be true for the whole population. For this reason, the segmentation of consumers into groups with similar characteristics provides valuable information for marketing strategies. According to others, each segment provides specific challenges either for industry or the public health sector (Verbeke et al., 2011) or in this case, also for producers and breeding companies. However, results of the present study did not allow the characterization of both cluster of consumers regarding their gender, age, level of studies, pork consumption frequency, work situation, and economical contribution at home, and Verbeke et al. (2005) reported similar difficulties when trying to classify consumer's preferences for marbling by age, gender and pork consumption. Ngapo et al. (2007b) also found similar results in consumer's choice, in all the countries of their study, either when they were grouped according to age and gender or when they were considered together.

DeVol, McKeith, Bechtel, Novakofski, Shanks, and Carr (1988) found that values of IMF between 2.5 and 3.0% were optimum for tenderness, because at lower IMF levels, pork was tougher and there was very little effect on tenderness of higher IMF levels. Cannata et al. (2010) reported higher sensory tenderness and juiciness when marbling was 2.5%, or higher. Brewer (1998) also suggested that an IMF content between 1.5% and 3.5% had higher flavour intensity and juiciness even if there was no effect on tenderness. Furthermore, Bejerholm and Barton-Gade (1986) established a minimum IMF content of 2.0% for acceptable tenderness and Fortin et al. (2005) reported that the minimum level of IMF for a 'pleasing eating experience' was 1.5%. However, Moeller et al. (2010) and Rincker, Killefer, Ellis, Brewer, and McKeith (2008) reported a small influence of IMF content on consumers' sensory scores. In this study, the minimum IMF content has been found between G2 and G3, thus, between 2.2% and 3.4% IMF, or meat with NPPC marbling score higher than 3. Nevertheless it is not possible to establish the exact IMF value due to the lack of samples between these two groups. In the Spanish market, most of the pork belong to G1 and G2 regarding their IMF values, unless

when specific pork slices – from Duroc or Iberian lines- were required (Cordero et al., 2010; Gispert et al., 2010; Mas et al., 2010; Olivares, Daza, Rey, & Lopez-Bote, 2009; Ventanas, Ventanas, Jurado, & Estévez, 2006). However across all consumers or segments of consumers, loins with the higher IMF levels (from G3 and G4) produced higher acceptability, tenderness, and juiciness scores, and in almost half of the consumers higher visual preference at the point of purchase. Consequently, half of the consumers, the 'marbled loin lovers' would have difficulties to find the product they finally prefer at the point of purchase and almost all of the consumers do not eat the pork they prefer from the eating point of view. This result can help to guide pig production and marketing of pork. The present work showed a positive correlation between IMF and eating characteristics, in agreement with Enfält, Lundström, Hansson, Lundeheim and Nyström (1997), who found a highly positive correlation between IMF and tenderness (r=0.64, $P \leq 0.01$), overall acceptability (r=0.63, $P \leq 0.001$) and meat taste intensity (r=0.49, $P \leq 0.01$). Moreover Gispert, Valero, Oliver, and Diestre (1997) reported a correlation coefficient of 0.67 between acceptability and IMF content, and Heyer and Lebret (2007) observed a correlation coefficient of 0.62 ($P<0.001$) between juiciness and IMF content.

Results from the present study showed a very clear classification of two groups of consumers based on visual evaluation of the loin slices with different IMF content; however, when these consumers tasted the loins, acceptability scores were higher for loins with higher IMF content in both groups (Table 11). This indicated that, for 'marbled loin lovers', there was a concurrence between their visual preference and their sensorial preference, thus, expected quality concurred with experienced quality or preference. On the other hand, there is a discrepancy for 'lean loin lovers' as their expected quality did not correspond with experienced quality, which was also noted by Brewer et al. (2001). Thus, beliefs and acceptability did not agree, because half of the consumers do not know that fat is very necessary to have a good eating experience. This discrepancy has also been found in other products like *soppressata* when various traditional and industrial origins were

evaluated (Iaccarino, Di Monaco, Mincione, Cavella, & Masi, 2006), *pâté de campagne* produced according to various traditional or non-traditional processes (Siret, & Issanchou, 2000), or lamb meat from different feeding systems (pasture, concentrate or two mixtures between pasture and concentrate) (Font i Furnols et al., 2011).

Regarding the factors consumers consider at the point of purchase, it is possible to see that, after the colour, price is also an important characteristic affecting the consumer's purchasing decision (Lange, Rousseau, & Issanchou, 1999; Lockshin, Jarvis, d'Hauteville, & Perrouty, 2006; Font i Furnols *et al.*, 2011) although was not the most important reason to consume it. Pork flavour, in accordance with Ngapo et al. (2007b), and variation in the type of meat consumed were more important than price in the present study. Besides price, however, visual acceptability of meat is an essential component of the consumer's point-of-sales purchasing decision; so, it is important to design marketing strategies for the different segments of consumers in order to satisfy their expectations.

5. Conclusions

Spanish consumers can be classified as either 'lean loin lovers' or 'marbled loin lovers' based on their visual preference of pork loins with different intramuscular fat content, but both groups of consumers increased their cooked pork palatability score with the level of intramuscular fat. Consequently, almost half of the consumers (lean loin lovers) do not know that fat is very necessary to have a more satisfying eating experience than they currently have. Marketing strategies emphasizing the importance of marbling in eating quality would possible help consumers to improve their pork eating experience. A minimum of IMF content between 2.2% and 3.4%, is recommended to improve the eating acceptability.

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CHAPTER 2:

Conjugated Linoleic Acid

Chapter 2:

Effect of a high dose of CLA in finishing pig diets on fat deposition and FA composition in intramuscular fat and other fat depots

Submitted to Meat Science

Conjugated linoleic acid, is a widely studied product because of their pleiotropic properties. However, its role on pig fat deposition, particularly on IMF is not clear. The first paper of this chapter evaluates the effect of supplementing a high dose of CLA (4%) on IMF and pig fat deposition. Results showed that the incorporation of CLA does not increase the IMF content in Duroc x Landrace pigs. However, addition of CLA tended to reduce overall animal fatness. CLA was incorporated in the different tissues at different rates and also affected the FA composition in a tissue specific way, increasing saturation in all studied tissues. Saturated and monounsaturated FA were mainly affected in the neutral lipid fraction and polyunsaturated FA in the polar lipid fraction of IMF of *longissimus thoracis*.

Effect of a high dose of CLA in finishing pig diets on fat deposition and FA composition in intramuscular fat and other fat depots

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Abstract

Sixteen gilts were fed either control (4% of sunflower oil) or experimental diet (4% conjugated linoleic acid (CLA) oil). CLA had no effect on the intramuscular fat (IMF) content neither in *longissimus thoracis* (LT) nor in *semimembranosus* (SM) muscles but increased liver weight, reduced perirenal fat and tended to reduce backfat between the last 3^{th}-4^{th} lumbar vertebrae. Despite 9c,11t and 10t,12c CLA isomers were included in the same proportion in the diet, the 9c,11t and 9c,11c were the isomers more deposited in all tissues. Addition of CLA in diet affected fatty acid composition in a tissue specific manner, increasing SFA in all tissues, reducing MUFA in LT and LT subcutaneous fat, and PUFA in LT subcutaneous fat, liver and SM. The FA modification by dietary CLA in LT IMF was reflected in the different lipid fractions, SFA and MUFA mainly in the neutral lipid fraction, and PUFA in the polar fraction.

Keywords

Intramuscular fat; neutral lipids; polar lipids; free fatty acid; meat quality

1. Introduction

Fat and fatty acids (FA), whether in adipose tissue or muscle, contribute importantly to various aspects of meat quality and are central to its nutritional value (Wood et al., 2008). It is generally assumed that intramuscular fat (IMF) content positively influences sensory quality traits, including flavour, juiciness and tenderness of meat, whereas a low amount of this fat results in a less tasty meat. Accordingly, IMF levels should reach values between 2.2 and 3.4 % before any noticeable effects on sensory qualities could be detected (Font-i-Furnols, Tous, Esteve-Garcia, & Gispert, 2012).

Selection against fatness or P2 backfat thickness carried out during the last decades in the pig has been very successful (Kempster, Cook & Grantley-Smith, 1986).

Consequently, IMF content has been also dramatically reduced to less than 1% in some lean genotypes widely used nowadays (Wood, 1990). It is believed that IMF develops later and behaves differently from subcutaneous adipose tissue with regard to development of cellularity and metabolic capacity (Lee and Kauffmann, 1974). Hence, an increased IMF content without affecting or reducing backfat through a strategic feeding regimen would be a desirable tool in pork meat production. Several nutritional attempts had been studied during the last years and one of the most promising is the inclusion of conjugated linoleic acid (CLA) in foods for growing/finishing pigs.

The CLAs are a mixture of positional and geometric isomers of linoleic acid (9c,12c C18:2), which were first identified in rumen fluid as an intermediate of the biohydrogenation process (Bartlett & Chapman, 1961). In synthetic CLA preparations the 9c,11t and 10t,12c isomers are predominant (often in a 1:1 ratio; Larsen, Toubro, & Astrup, 2003). It appears that 9c,11t isomer has positive effects on some types of cancer by inhibiting tumorogenesis (Kelley, Hubbard & Erickson, 2007), while 10t,12c isomer could be responsible for changes in whole-body fat deposition (Pariza, Park & Cook, 2000). In first studies with pigs dietary CLA increased lean tissue deposition and decreased fat deposition (Dugan, Aalhus, Schaefer, & Kramer, 1997; Ostrowska, Muralitharan, Cross, Bauman, & Dunshea, 1999). Comprehensive reviews on the effects of CLA on growth performance and carcass fat deposition in pigs had been published by Azain (2003), Lopez-Bote, Ortiz and Menoyo (2004) and Corino, Di Giancamillo, Rossi, and Domeneghini (2005). In general, the response to CLA was not conclusive and inconsistency could be attributed to the type of pig used in studies or to dietary factors like the source of CLA, the dietary fat content or the duration of feeding. According to Azain (2003), it appears that CLA reduces carcass fat in pigs with more than 23 cm subcutaneous fat thickness at 100 kg body weight, but not when fat thickness was less than 20 cm. Moreover, the response to CLA seems to be greater in barrows than in gilts or entire males (Tischendorf, Schone, Kirchheim, & Jahreis, 2002) and in low-energy

diets (Dugan, Aalhus, Lien, Schaefer, & Kramer, 2001). On the other hand, some authors also report an increase in IMF content of CLA fed pigs (Averette Gatlin, See, Hansen, Sutton, & Odle, 2002; Dugan, Aalhus, Schaefer & Kramer, 1999, Jiang, et al., 2010; Joo, Lee, Ha & Park, 2002, Morel, Janz, Zou, Purchas, Hendriks & Wilkinson, 2008) which would be very interesting in terms of pork meat quality. However, as for backfat thickness, results are inconsistent because some other studies did not show any affect on IMF content (Corino, Magni, Pastorelli, Rossi, & Mourot, 2003; Lauridsen, Mu, & Henckel, 2005; Martin, Antequera, Muriel, Perez-Palacios and Ruiz, 2008b) and the same reasons could be evoked.

In addition, dietary CLA seems to be highly deposited in body tissues of monogastric animals (Bee, 2001; Lopez-Bote *et al.*, 2004, Corino *et al.*, 2005; Jiang *et al.,* 2010) and as a result, in pork and meat products (Schmid, Collomb, Sieber, & Bee, 2006). An increase of saturated fatty acids (SFA) and a reduction of monounsaturated fatty acids (MUFA) in subcutaneous tissue and loin were attributed due to an inhibitory effect of Δ9 desaturase (Averette Gatlin et al., 2002, Bee, Jacot, Guex, & Biolley, 2008).

Due to the inconsistency of the results of CLA on IMF and backfat thickness, one of the goals of the present study is to evaluate if the inclusion of a high dose of CLA oil (4%) as the only dietary fat source during fattening of Duroc x Landrace gilts increases IMF content while reducing backfat thickness in order to determine if the lack of consistency between studies is due to the level of CLA inclusion. Moreover, to determine if CLA is equally incorporated and has the same ability to modify FA composition in different tissues of pig. *Longissimus thoracis* (LT) was chosen as a typical portion consumed fresh, *semimembranosus* muscle (SM) as representative of ham muscle, LT subcutaneous fat as the main fat deposit where lipid metabolism takes place in pig and liver as the important role in the whole metabolism and due to implications for further human health benefits.

2. Materials and methods

2.1. Animals and diets

Sixteen 73±3 kg *Landrace* × *Duroc* gilts of commercial origin chosen from a larger group were blocked by weight and housed in adjacent individual boxes (2.25m^2) in a room provided with forced ventilation by extraction. Gilts, from 73±3 kg to slaughter at 117±5 kg live weight (54 days), were randomly assigned within block to one of the dietary treatments, eight animals were fed the control diet (4% of sunflower oil) and eight fed the diet containing CLA (4% of CLA oil) for *ad libitum* consumption. The CLA oil (IOI Group Loders Croklaan, *Wormerveer*, *Netherlands*) was chemically obtained from sunflower oil and contained a 62.7% mixture of conjugated linoleic acid isomers (30.5% 9c,11t CLA and 10t,12c 30.6% CLA). The composition of the experimental diets and their FA composition are shown in Table 14 and Table 15, respectively.

Table 14: Ingredients of the experimental diets (as-fed)

	Body weight range, kg	
Item	73 to 91	92 to 117
Ingredient, %		
Barley	53.6	60.7
Manioc	20.0	20.0
Soybean meal 44%	17.3	11.1
Sunflower seed meal	2.60	1.59
Fat source[a]	4.00	4.00
Dicalcium phosphate	1.18	1.09
Calcium carbonate	0.51	0.50
Sodium bicarbonate	0.03	0.27
Sodium chloride	0.35	0.18
Mineral-vitamine premix[b]	0.40	0.40
L-lysine HCl	0.08	0.13
L-threonine	0.01	0.02

[a] Control diet: sunflower oil; Experimental diet: CLA oil provided by IOI Group Loders Croklaan, Wornerveer, Netherlands.

[b] One kg of feed contains: 5,000 IU vitamin A; 1,000 IU vitamin D_3; 15mg vitamin E; 1.3mg vitamin B_1; 3.5mg vitamin B_2; 0.025mg vitamin B_{12}; 1.5mg vitamin B_6; 10mg calcium pantothenate; 15mg nicotinic acid; 0.1mg biotin; 0.6mg folic acid; 2mg vitamin K; 80mg Fe; 6mg Cu; 0.75mg Co; 60mg Zn; 30mg Mn; 0.75mg I; 0.10mg Se; 0.125g Ethoxyquin.

Table 15: Nutrient composition of control (C) and conjugated linoleic acid (CLA) diets

	Body weight range, kg			
	73 to 91		92 to 117	
	C	CLA	C	CLA
Chemical composition				
ME, kcal/kg[a]	3,100	3,100	3,100	3,100
NE, kcal/kg[a]	2,349	2,349	2,384	2,384
Crude Protein[b], %	15.9	15.8	12.7	12.6
Lys, g/kg[a]	8.40	8.40	7.40	7.40
Ether extract[b], %	3.98	4.81	5.50	5.39
Fatty acid composition[b], %				
C 14:0	0.18	0.16	0.15	0.15
C 16:0	12.1	11.3	10.8	11.1
C 16:1 n-9 cis	0.17	0.13	0.16	0.14
C 17:0	0.09	0.08	0.07	0.07
C 18:0	3.41	3.35	3.23	3.16
C 18:1 n-9 cis	22.3	23.8	23.4	24.1
C 18:1 n-7	0.80	0.86	0.76	0.80
C 18:2 n-6 cis	56.7	18.3	58.1	18.0
C 18:3 n-3 cis	2.54	2.00	1.87	1.76
C 20:0	0.31	0.66	0.26	0.74
9c,11t CLA	ND	16.1	ND	14.8
10t,12c CLA	ND	15.5	ND	14.2
C 20:1 n-9 cis	0.33	0.35	0.33	0.37
9c,11c CLA	ND	5.65	ND	8.86
9t,11t CLA	ND	0.75	ND	0.77
C 22:0	0.69	0.72	0.64	0.69
SFA	16.9	16.3	15.2	16.0
MUFA	23.6	25.2	24.6	25.4
PUFA	59.3	58.3	60.0	58.4

ND: under limit of detection
[a] Calculated values according to INRA tables (Sauvant, Perez &Tran, 2004).
[b] Determined

2.2. Slaughter conditions

Animals were transported for 2h to the IRTA experimental abattoir and approximately 16h before slaughter were held off feed with access to water during lairage. Pigs were weighed and slaughtered minimizing the stress using standard *ante mortem* procedures and 85% CO_2 stunning for 120s using CO_2 *Dip Lift* (Butina, Alps, Copenhaguen, Denmark). Once carcasses were eviscerated, they were split longitudinally and weighed at 45min. Liver and perirenal fat were weighed and their proportion with respect to the live animal weight before slaughter was

calculated. Liver samples were collected, vacuum packed and stored at -20ºC for chemical analysis.

2.3. Carcass measurements

Fat and muscle thicknesses were measured using Fat-O-Meat'er probe (Carometec A/S, Herlev, Denmark) between the 3rd and the 4th last ribs at 6 cm to the midline. Values obtained were used to calculate, lean meat percentage following the Spanish official equation (Lean (%) = 66.91 - 0.895 * backfat thickness + 0.144 * muscle depth; Font i Furnols & Gispert, 2009). In addition, backfat thickness between the 3rd and 4th lumbar vertebrae at 8 cm of the midline and at the last rib at 6 cm of the midline was determined with the same probe. Fat thickness in the shoulder at the level of the first rib, in the cranial position of the 1st lumbar vertebrae and the minimum fat thickness over the muscle *Gluteus medius* were measured with a ruler over the carcass midline.

After carcass refrigeration at 3ºC for 24h, carcasses were weighed, carcass length was measured as the distance between the recess of the first rib and the anterior edge of the symphysis pubic and loin length as the distance from the atlas bone to the first lumbar vertebrae. Then, the left side of each carcass was cut following a simplified European reference method (Walstra & Merkus, 1995). Some joints were combined to be considered as primary joints: ham (included hind shank and hind foot), loin, belly (included the ventral part of the belly and jowl), shoulder (with front shank, front foot and neck), tenderloin, and head (with cheek). Each joint was weighed and their proportion with respect to cold carcass weight was calculated. Furthermore, the SM was separated from the ham and the LT muscle and subcutaneous fat (with skin) from the loin, and their respective weights were recorded. The SM, the LT and the LT subcutaneous fat (between 3th and 4th ribs region) samples were taken, vacuum packaged and stored at -20ºC until determinations.

2.4. Meat quality measurements

Muscle pH was measured using a Crison portable meter (Crison, Barcelona, Spain) equipped with a xerolyt electrode in the LT between the 4th and 5th last ribs and in SM muscles at 45 min (pH45) and 24 h (pHu) *post mortem* on the left carcass side. Electrical conductivity was measured in the carcass using a Pork Quality Meater (PQM-Kombi, Aichach, Germany) at the last rib level in the LT and SM muscles at 24 h (ECu) *post mortem*. Drip loss was obtained from the LT muscle according to the methodology describe by Rasmussen and Andersson (1996).

2.5. Chemical methods

Dry matter according to (AOAC 2010, 934.01), and crude protein by Dumas method using a nitrogen analyzer FP528 Leco (AOAC 2010 method 992.15) were determined in feed.

Lipids from LT and SM muscles, subcutaneous fat and liver were extracted with chloroform-methanol according to Folch, Lees and Stanley (1957) procedures. Neutral lipids (**NL**), polar lipids (**PL**) and free fatty acids (**FFAs**) were separated from total lipids of LT samples in solid phase extraction columns (AMINO 500mg/3ml) according to the method described by Ruiz, Antequera, Andres, Petron and Muriel (2004). Lipids were transmethylated with BF_3 and methanolic KOH (Morrison & Smith, 1964) and FA were determined by gas chromatography (Hewlett Packard 6890, USA) using a capillary column DB23 (0.25mm x 0.25µm x 30m) and a flame ionization detector (**FID**). A temperature gradient with initial temperature of 170ºC followed by an increase at a rate of 2.5ºC/min until 210ºC and raised again at a rate of 5ºC/min to 240ºC where it remained for 5min was used for the whole run. Injector and detector temperatures were both set at 250ºC. Injection was in the split mode with a ratio of 100.6:1. The carrier gas was helium with a flux of 55.8 mL/min, and pressure of 10.99 psi at the column head. The standard adopted were F.A.M.E Mix C4-C24, cis-11-Vaccenic Methyl Ester (Supelco, Bellefonte, USA), cis-

13,16,19-Docosatrienoic acid methyl ester, cis-7,10,13,16-Docosatetranoic acid methyl ester, methyl all-cis-7,10,13,16,19-docosapenaenoate (Sigma, St. Louis, USA), 9(Z),11(E)-Octadecanoic acid, 10(E),12(Z)-Octadecanoic acid, 9(E),11(E)-Octadecanoic acid and 9(Z),11(Z)-Octadecanoic acid (Matreya, Pleasant Gap, USA). Nonadecanoic acid (Sigma, St.Louis, USA) was used as internal standard. Results were expressed in percentage of total fatty acids and the sum of PUFA did not include the sum of CLA isomers found in each tissue.

2.6. Statistical Analysis

The experiment was designed as a randomized complete block design with 8 blocks of initial live weight and two dietary treatments. Analysis of variance of the different variables was performed using PROC GLM procedure of SAS®. The model included treatment and blocks as fixed effects. Differences between treatment means were investigated with the value for each treatment of ANOVA table.

3. Results

3.2. Performance, carcass and meat quality parameters

No significant differences in performance parameters of gilts were found due to the replacement of sunflower oil to CLA for a period of 54 days (average daily feed intake: C: 3.16 kg/day, CLA: 3.20 kg/day; average daily weight gain: C: 0.82 kg/day, CLA: 0.82 kg/day; feed to gain ratio: C: 3.84 kg/kg, CLA: 3.94 kg/kg). Animals fed 62.5g CLA per day on average (a total of 3.37kg of CLA for whole experiment) had similar final body weight than those fed the control diet (C: 117.9 kg vs. CLA: 116.9 kg live weight).

Dietary CLA did not affect the carcass weight, yield, carcass length or loin length (Table 16). Backfat thickness only tended to be reduced between the 3^{th} and the 4^{th} lumbar vertebrae by CLA (P = 0.066), the other backfat measurements over the carcass presented the same trend but did not reach the statistical significance.

Additionally, although differences were not significant, lean meat percentage was numerically higher in the animals fed CLA. Carcasses from animals fed CLA, when stored for 24h at 2ºC had higher chilling losses ($P < 0.01$) compared with the control. The meat quality parameters such as pH, and conductivity were not affected by dietary treatment.

Table 16: Effect of diet on carcass characteristics, and meat quality measurements from *longissimus thoracis* (LT) and *semimembranosus* (SM) muscles of swine

	Diets			
Item (n=8/treatment)	Control	CLA	RMSE	P-value
Carcass characteristics				
Live weight at slaughter, kg	115.6	114.6	5.08	0.700
Hot carcass weight, kg	93.5	92.2	4.16	0.544
Carcass yield, %	80.9	80.4	0.78	0.299
Chilling losses, %	2.32	2.57	0.152	0.005
Carcass length, cm	87.6	87.2	2.62	0.815
Loin length, cm	91.1	91.6	2.45	0.689
Backfat thickness				
1st rib, mm [c]	40.9	37.9	4.76	0.228
3th-4th last ribs, mm [a]	24.1	21.3	3.59	0.146
Last rib, mm [a]	22.5	19.9	3.75	0.187
1st lumbar vertebrae, mm [c]	27.9	25.6	4.09	0.290
3th-4th lumbar vertebrae, mm [b]	31.1	26.6	4.46	0.066
Gluteus medius, mm [c, d]	24.5	22.2	4.01	0.281
Muscle depht				
3th-4th last ribs , mm [a]	47.9	48.3	3.78	0.856
Lean percentage, % [e]	52.2	54.8	3.27	0.145
Longissimus thoracis				
pH 45 min	6.36	6.47	0.184	0.284
pHu	5.57	5.58	0.114	0.796
ECu, mS	3.34	3.26	1.029	0.886
Drip loss, %	2.30	1.61	1.287	0.305
Semimembranosus				
pH 45 min	6.35	6.43	0.125	0.215
pHu	5.54	5.58	0.091	0.329
ECu, mS	6.97	5.85	1.801	0.232

RMSE: Root Mean Square Error; ECu: ultimate electrical conductivity; pHu: ultimate pH (24 h).

[a] Measurement done at 6 cm to the carcass midline using Fat-O-Meater probe.

[b] Measurement done at 8 cm to the carcass midline using Fat-O-Meater probe.

[c] Measurement done on the carcass midline with a ruler.

[d] Minimum fat thickness over the muscle with a ruler.

[e] Calculated from backfat thickness and loin depth between the 3th and 4th using the Spanish official equation (Lean percentage (%) = 66.91 - 0.895 * backfat thickness + 0.144 * muscle depth; Font-i-Furnols, & Gispert, 2009).

Dietary CLA caused a reduction in perirenal fat weight ($P < 0.05$) and an increase of liver weight as a percentage of whole body weight (Table 17). However, the proportion of the combined main carcass joints with respect to the cold carcass weight (Walstra & Merkus, 1995) only showed a trend to reduce ($P = 0.08$) the belly percentage when animals were fed 4% CLA.

Table 17: The influence of diet on liver, perirenal fat and the weight of some carcass joints.

	Diets			
Proportion (n=8/treatment)	Control	CLA	RMSE	P-value
g/kg BW				
Liver	15.5	17.2	1.30	0.025
Perirenal Fat	9.43	7.38	1.447	0.013
g/kg carcass				
Head	78.7	78.5	4.61	0.915
Shoulder	263	266	7.98	0.429
Belly	152	144	8.21	0.080
Tenderloin	11.0	11.6	0.86	0.204
Ham	305	304	7.88	0.958
SM muscle	21.5	22.7	1.66	0.196
Loin	176	180	12.7	0.539
LT muscle	55.9	59.2	4.46	0.175
Backfat + skin	63.0	57.0	7.53	0.149

Combination of some joints following the European reference method (Walstra & Merkus, 1995): head (with cheek), shoulder (with front shank, front foot and neck), belly (included the ventral part of the belly and jowl), tenderloin, ham (included hind shank and hind foot) and loin.
RMSE: Root Mean Square Error; BW: Live Weight; SM: *Semimembranosus* muscle; LT: *Longissimus thoracis* muscle.

3.4. Fatty acid composition in muscles: longissimus thoracis and semimembranosus

A wide modification of fatty acid composition of LT IMF by dietary CLA was observed (Table 18). Although C18:0 remained unaffected, C14:0, C16:0 and the total SFA acids were all increased by dietary CLA ($P < 0.05$). While C16:1 n-7 cis was increased ($P < 0.01$), 18:1 n-9 cis and total MUFA were reduced ($P < 0.001$; $P < 0.01$, respectively) by inclusion of dietary CLA. Although the sum of all PUFA was not affected by dietary treatment, the sum of n-3, 18:3 n-3 cis and C20:5 n-3 cis were increased ($P < 0.001$) in the LT IMF of animals fed CLA. However, the sum of the n-6, C18:2 n-6 and C20:4 n-6 were not affected by the addition of CLA in the diet.

Thus, the ratio n-6/n-3 was significantly decreased ($P < 0.001$). The content of CLA in this tissue was 2.64% of the total fatty acids, being 9c,11t the isomer more deposited.

In the SM muscle total SFA ($P < 0.001$), C16:0 ($P < 0.001$), C18:0 ($P < 0.05$) and C14:0 ($P < 0.001$) were increased by dietary CLA. However, neither MUFA nor C18:1 n-9 cis were modified and C16:1 n-7 cis was increased ($P < 0.01$) by CLA. PUFA ($P < 0.01$) including n-6 FA ($P < 0.001$), C18:2 n-6 cis ($P < 0.001$) and C20:4 n-6 ($P < 0.05$) were reduced by CLA, while C18:3 n-3 cis ($P < 0.05$) and C20:5 n-3 cis ($P < 0.05$) were increased. The amount of CLA deposited in this tissue was 2.12% of the total fatty acids, being the 9c,11t and 9c,11c the isomers more deposited.

Table 18: Modification of FA composition in *longissimus thoracis* (LT) and *semimembranosus* (SM) muscles by dietary CLA with respect to Control

	LT				*SM*			
	Diets				Diets			
% (n=8/treatment)	Control	CLA	RMSE	P-value	Control	CLA	RMSE	P-value
IMF	**2.06**	**1.95**	**0.358**	**0.553**	**2.07**	**2.02**	**0.665**	**0.865**
C 14:0	1.24	1.87	0.332	0.002	1.07	1.86	0.212	<0.001
C 16:0	21.7	23.6	1.222	0.008	20.0	23.6	0.65	<0.001
C 16:1 n-7 cis	2.76	3.91	0.666	0.004	2.37	3.45	0.563	0.002
C 18:0	11.2	11.2	0.838	0.995	10.3	11.6	1.19	0.049
C 18:1 n-9 cis	33.8	26.4	3.144	<0.001	32.9	31.2	2.94	0.268
C 18:1 n-7	3.51	3.19	0.342	0.084	3.43	3.38	0.339	0.772
C 18:2 n-6 cis	16.3	15.5	3.164	0.640	18.7	13.0	2.10	<0.001
C 18:3 n-3 cis	0.29	0.49	0.058	<0.001	0.40	0.46	0.056	0.037
9c,11t CLA	**ND**	**1.30**	**0.087**	**<0.001**	**0.01**	**0.80**	**0.232**	**<0.001**
10t,12c CLA	**ND**	**0.61**	**0.062**	**<0.001**	**ND**	**0.43**	**0.092**	**<0.001**
9t,11t CLA	**ND**	**0.13**	**0.015**	**<0.001**	**0.01**	**0.07**	**0.024**	**<0.001**
9c,11c CLA	**ND**	**0.60**	**0.134**	**<0.001**	**0.10**	**0.83**	**0.289**	**<0.001**
C 20:4 n-6	3.63	4.00	0.969	0.465	4.25	2.97	1.034	0.027
C 20:5 n-3 cis	0.06	0.19	0.044	<0.001	0.23	0.32	0.061	0.017
C 22:6 n-3 cis	0.06	0.06	0.013	0.849	0.08	0.08	0.027	0.590
Minor FA[a]	5.52	6.98	0.929	0.007	6.15	5.97	0.630	0.600
SFA	34.9	37.4	1.77	0.013	32.2	38.0	1.65	<0.001
MUFA	42.8	37.2	3.41	0.006	41.4	41.1	3.32	0.871
PUFA	22.3	25.3	4.66	0.216	26.3	20.8	3.30	0.005
CLA	ND	2.64	0.202	<0.001	0.12	2.12	0.148	<0.001
n-3	0.86	1.52	0.202	<0.001	1.25	1.19	0.269	0.669
n-6	21.4	21.2	4.405	0.899	24.9	17.5	3.26	<0.001
n-6/n-3	24.8	13.9	1.123	<0.001	21.3	14.8	4.75	0.016

RMSE: Root Mean Square Error; ND: value under limit of detection in this case the RMSE corresponds to the treatment with the numeric value.

[a] Minor FA include: C 10:0; C 12:0; C 14:1 n-9 cis; C 15:0; C 15:1 n-5 cis; C 17:0; C 17:1 n-7 cis; C 18:1 n-9 trans; C 18:2 n-6 trans; C 18:3 n-6 cis; C 20:0; C 20:1 n-9 cis; C 20:2 n-6 cis; C 21:0; C 20:3 n-6; C 20:3 n-3 cis; C 22:0; C 22:1; C 23:0; C 22:4 n-6; C 22:3 n-3; C 22:5n-3; C 24:1 n-9 cis.

3.4.1. Fatty acid composition of neutral, polar and free fatty acid fractions of intramuscular fat of longissimus thoracis

The total SFA, C14:0 and C16:0 acids were also increased by dietary CLA NL fraction ($P < 0.001$) of LT muscle while C18:0 was unaffected by addition of CLA in the diet (Table 19). The sum of MUFA and 18:1 n-9 cis were reduced in NL fraction (all $P < 0.001$) even though C16:1 n-7 cis was increased by dietary CLA ($P < 0.01$). Whereas a low percentage of PUFA compared with the other fractions was observed, a reduction of total PUFA, n-6 FA and n-3 by dietary CLA was observed in the NL fraction ($P < 0.001$; $P < 0.001$; $P < 0.05$). Taking into account the individual fatty acids, C18:2 n-6, C20:4 n-6 and C22:6 n-3 cis were all reduced when CLA was incorporated to the diet ($P < 0.001$; $P < 0.001$; $P < 0.01$). NL fraction contained a 1.59 % of CLA of total fatty acids.

The main saturated fatty acid (C16:0), was unaffected by dietary CLA in PL fraction besides an increase of the sum of SFA, C14:0 and C18:0 ($P = 0.05$; $P < 0.001$; $P < 0.01$) was observed. Although C16:1 n-7 cis and 18:1 n-9 cis were increased by dietary CLA ($P = 0.001$; $P < 0.001$, respectively), the sum of total MUFA was not affected. PUFA percentage tended to be reduced by inclusion of CLA in the diet ($P < 0.1$), and a reduction of C18:2 n-6 cis and n-6 fatty acids (all $P < 0.001$) while C18:3 n-3 cis ($P < 0.001$) increased was observed. PL fraction contained a 3.80 % of CLA of total FA.

Table 19: Modification of FA composition in neutral lipid fraction (NL), polar lipid fraction (PL) and free fatty acid (FFA) of *longissimus thoracis* (LT) muscle by dietary CLA with respect to Control

	NL				PL				FFA			
	Diets				Diets				Diets			
FA, % (n=8/treatment)	Control	CLA	RMSE	P-value	Control	CLA	RMSE	P-value	Control	CLA	RMSE	P-value
C 14:0	1.64	2.92	0.494	<0.001	0.12	0.27	0.054	<0.001	0.54	0.70	0.137	0.105
C 16:0	23.4	28.2	0.74	<0.001	18.7	20.0	1.75	0.194	22.2	17.4	7.144	0.315
C 16:1 n-7 cis	3.89	6.62	0.936	<0.001	0.03	0.32	0.131	0.001	0.12	1.15	0.235	<0.001
C 18:0	10.1	10.4	1.48	0.750	9.14	10.4	0.64	0.002	9.18	10.3	3.048	0.652
C 18:1 n-9 cis	46.0	38.8	1.12	<0.001	8.12	11.8	0.973	<0.001	8.46	12.9	2.233	0.011
C 18:1 n-7	4.26	4.31	0.402	0.851	4.56	2.16	1.532	0.012	9.77	2.54	2.623	0.002
C 18:2 n-6 cis	7.06	4.29	0.593	<0.001	31.3	27.1	1.59	<0.001	21.1	30.2	5.065	0.018
C 18:3 n-3 cis	0.29	0.26	0.036	0.214	0.20	0.73	0.126	<0.001	0.18	0.85	0.238	0.001
9c,11t CLA	**ND**	**0.58**	**0.096**	**<0.001**	**ND**	**1.52**	**0.293**	**<0.001**	**ND**	**0.05**	**0.114**	**0.479**
10t,12c CLA	**ND**	**0.23**	**0.027**	**<0.001**	**ND**	**0.77**	**0.134**	**<0.001**	**ND**	**0.05**	**0.108**	**0.479**
9t,11t CLA	**ND**	**0.20**	**0.018**	**<0.001**	**ND**	**0.06**	**0.069**	**0.148**	**ND**	**0.18**	**0.110**	**0.029**
9c,11c CLA	**ND**	**0.58**	**0.062**	**<0.001**	**ND**	**1.46**	**0.381**	**<0.001**	**ND**	**2.14**	**0.860**	**0.003**
C 20:4 n-6	0.24	0.12	0.050	<0.001	6.20	5.17	2.434	0.444	5.29	9.02	1.571	0.022
C 20:5 n-3 cis	0.01	ND	0.011	0.408	0.07	0.11	0.094	0.412	0.04	0.54	0.153	<0.001
C 22:6 n-3 cis	0.04	ND	0.020	0.006	0.24	0.31	0.074	0.108	0.17	0.11	0.168	0.607
Minor FA[a]	2.83	2.39	0.292	0.016	20.7	17.3	2.463	0.026	21.2	10.9	4.56	0.006
SFA	36.1	42.4	1.68	<0.001	29.8	32.1	1.84	0.038	36.2	30.2	9.60	0.347
MUFA	55.3	50.9	1.63	<0.001	26.1	25.9	3.35	0.954	28.1	19.7	5.01	0.026
PUFA	8.60	6.68	0.720	<0.001	44.0	41.9	2.17	0.092	35.7	50.0	7.02	0.010
CLA	ND	1.59	0.061	<0.001	ND	3.80	0.236	<0.001	ND	2.43	1.075	0.006
n-3	0.46	0.32	0.104	0.032	2.43	3.02	2.705	0.694	1.83	3.74	0.756	0.003
n-6	8.14	4.76	0.687	<0.001	41.6	35.1	2.69	<0.001	34.0	43.9	6.71	0.044
n-6/n-3	18.9	15.3	4.48	0.161	29.9	14.7	8.98	0.008	18.3	12.6	4.85	0.082

RMSE: Root Mean Square Error; ND: value under limit of detection in this case the RMSE corresponds to the treatment with the numeric value under limit of detection.

[a] Minor FA include: C 12:0; C 14:1 n-9 cis; C 15:0; C 15:1 n-5 cis; C 17:0; C 17:1 n-7 cis; C 18:1 n-9 trans; C 18:2 n-6 trans; C 18:3 n-6 cis; C 20:0; C 20:1 n-9 cis; C 20:2 n-6 cis; C 21:0; C 20:3 n-6; C 20:3 n-3 cis; C 22:0; C 22:4 n-6; C 22:5n-3; C 24:1 n-9 cis.

The SFA in the FFA fraction were not affected by the addition of CLA in the diets. Nonetheless, a reduction of MUFA was observed ($P < 0.05$) while C16:1 n-7 cis and 18:1 n-9 cis were increased ($P < 0.001$; $P < 0.05$) by effect of CLA. Total PUFA, n-6, C18:2 n-6 cis, C20:4 n-6 ($P < 0.05$), n-3, C18:3 n-3 cis and C20:5 n-3 cis ($P < 0.01$; $P < 0.01$; $P < 0.001$, respectively) were increased by dietary CLA in FFA fraction. Additionally, FFA contained a 2.43 % of total FA.

3.6. FA composition in liver

Changes in liver chemical composition were not observed despite the increase of liver weight (Table 20). The total SFA ($P < 0.001$) including C14:0, C16:0, and C18:0 were increased ($P < 0.05$) by dietary CLA in the liver. No significant differences of MUFA percentage ($P < 0.1$) were found between treatments. A tendency to reduce C18:1 n-9 cis by CLA ($P < 0.1$), a significant reduction of PUFA ($P < 0.001$), n-6 ($P < 0.001$), C18:2 n-6 cis ($P < 0.001$) and C20:4 n-6 ($P < 0.001$) were found when CLA was included in the diet. An increase of C18:3 n-3 cis ($P < 0.05$), C20:5 n-3 cis ($P < 0.001$) and n-3 ($P < 0.01$) was observed by the same treatment. The amount of CLA in this tissue was 4.15 % of the total fatty acids, being the 9c,11t and 9c,11c the isomers more deposited.

Table 20: Modification of fatty acid composition in liver by dietary CLA with respect to Control

	Diets			
% (n=8/treatment)	Control	CLA	RMSE	P-value
Lipids[a]	3.44	3.47	0.260	0.795
C 14:0	0.24	0.39	0.112	0.018
C 16:0	12.7	14.2	1.27	0.034
C 16:1 n-7 cis	0.41	0.39	0.103	0.739
C 18:0	25.9	28.0	1.73	0.033
C 18:1 n-9 cis	11.4	9.62	1.820	0.071
C 18:1 n-7	1.25	1.03	0.128	0.005
C 18:2 n-6 cis	20.2	17.2	1.32	<0.001
C 18:3 n-3 cis	0.32	0.43	0.077	0.011
9c,11t CLA	**0.03**	**1.42**	**0.331**	**<0.001**
10t,12c CLA	**0.02**	**0.64**	**0.104**	**<0.001**
9t,11t CLA	**ND**	**0.08**	**0.015**	**<0.001**
9c,11c CLA	**0.02**	**2.00**	**0.264**	**<0.001**
C 20:4 n-6	19.4	13.7	2.18	<0.001
C 20:5 n-3 cis	0.29	0.91	0.125	<0.001
C 22:6 n-3 cis	0.18	0.10	0.051	0.006
Minor FA[b]	7.50	9.73	0.872	0.001
SFA	40.6	45.6	0.87	<0.001
MUFA	14.4	14.5	1.99	0.970
PUFA	44.8	39.8	1.71	<0.001
CLA	0.08	4.15	0.390	<0.001
n-3	1.54	2.19	0.356	0.003
n-6	43.2	33.5	1.36	<0.001
n-6/n-3	29.0	15.7	4.39	<0.001

RMSE: Root Mean Square Error; ND: value under limit of detection in this case the RMSE corresponds to the treatment with the numeric value.
[a] Lipid content extracted by Folch, Lees and Stanley (1957)
[b]Minor FA include: C 15:0; C 15:1 n-5 cis; C 17:0; C 17:1 n-7 cis; C 18:1 n-9 trans; C 18:2 n-6 trans; C 18:3 n-6 cis; C 20:0; C 20:1 n-9 cis; C 20:2 n-6 cis; C 21:0; C 20:3 n-6; C 20:3 n-3 ; C 22:0; C 22:1; C 22:2 n-6; C 23:0; C 22:4 n-6; C 22:3 n-3; C 22:5 n-3; C 24:1 n-9 cis

3.7. FA composition in subcutaneous fat

Lipids percentage of LT subcutaneous fat was decreased ($P < 0.001$) by CLA (Table 21). The total SFA including C16:0 ($P < 0.001$), C18:0 ($P < 0.001$) and C14:0 ($P < 0.001$) were increased in subcutaneous fat by dietary CLA. However, the MUFA ($P < 0.001$) including C18:1 n-9 cis ($P < 0.001$) and PUFA ($P < 0.01$) were reduced by CLA. A reduction of n-6 FA ($P < 0.001$) caused by a reduction of C18:2 n-6 cis ($P < 0.001$) which also caused a reduction of the ratio n-6/n-3 ($P < 0.001$) was observed. The percentage of n-3 FA of this tissue was lower compared to the other tissues examined and C20:5 n-3 cis and C22:6 n-3 cis were both below detection limits. The

proportion of CLA in this tissue was 7.72 % of the total fatty acids, being the 9c,11t the isomer more deposited.

Table 21: Modification of fatty acid composition in the subcutaneous fat of the *longissimus thoracis* (LT) by dietary CLA with respect to Control

	Diets			
% (n=8/treatment)	Control	CLA	RMSE	P-value
Lipids[a]	74.3	68.4	2.89	0.002
C 14:0	1.52	2.75	0.364	<0.001
C 16:0	21.2	25.1	1.33	<0.001
C 16:1 n-7 cis	1.52	1.34	0.213	0.110
C 18:0	13.0	17.1	1.99	0.001
C 18:1 n-9 cis	36.0	28.0	2.79	<0.001
C 18:1 n-7	1.86	1.68	0.184	0.070
C 18:2 n-6 cis	19.5	10.9	2.60	<0.001
C 18:3 n-3 cis	0.72	0.75	0.042	0.259
9c,11t CLA	**0.49**	**3.09**	**1.042**	**<0.001**
10t,12c CLA	**0.33**	**2.00**	**0.631**	**<0.001**
9t,11t CLA	**0.01**	**0.13**	**0.051**	**<0.001**
9c,11c CLA	**0.17**	**2.49**	**0.710**	**<0.001**
C 20:4 n-6	0.44	0.35	0.136	0.240
Minor FA[b]	3.28	4.31	0.538	0.002
SFA	36.5	46.1	3.55	<0.001
MUFA	40.7	33.5	2.81	<0.001
PUFA	22.7	20.5	1.27	0.003
CLA	1.00	7.72	2.014	<0.001
n-3	0.80	0.90	0.053	0.002
n-6	21.0	11.9	2.81	<0.001
n-6/n-3	26.3	13.2	3.76	<0.001

RMSE: Root Mean Square Error; ND: value under limit of detection in this case the RMSE corresponds to the treatment with the numeric value.
[a] Lipid content extracted by Folch, Lees and Stanley (1957)
[b] Minor FA include: C 10:0; C 12:0; C 15:0; C 15:1 n-5 cis; C 17:0; C 17:1 n-7 cis; C 18:1 n-9 trans; C 18:2 n-6 trans; C 20:0; C 20:1 n-9 cis; C 20:2 n-6 cis; C 20:3 n-6; C 20:3 n-3 cis; C 22:4 n-6.

4. Discussion

A higher dose of CLA oil (4%) was used than in previous studies in order to amplify the possible response to CLA in IMF content. Wiegand, Parrish, Swan, Larsen & Baas (2001) speculated that if body fat were decreased by CLA supplementation then less energy would be required to maintain animal growth, thus making them more efficient. No modification of performance parameters by dietary CLA was observed, which is in agreement with Bee (2001), although Dugan, Aalhus, Schaefer

and Kramer (1997), Ostrowska, Muralitharan, Cross, Bauman and Dunshea (1999) and Thiel-Cooper, Parrish, Sparks, Wiegand and Ewan (2001) observed an increase of feed efficiency in pigs fed dietary CLA.

A reduction of carcass fat was observed in animals fed dietary CLA although not all the measurements reached significance. The weight of perirenal fat was significantly reduced by 22%, while LT subcutaneous fat was only reduced by 11%, and not statistically significant. The same trend was observed on the other measurements done on the carcass. In contrast, IMF was not affected by dietary treatment in any of the muscles studied. The results suggest that the effect of CLA is more effective in perirenal fat than subcutaneous or IMF. No significant differences in backfat depth and *longissimus* muscle thickness by supplemental CLA were observed by Gatlin, See, Larick, Lin and Odle (2002). In contrast, Dugan *et al.* (2001), D'Souza and Mullan (2002) and Ostrowska, Cross, Muralitharan, Bauman and Dunshea (2002) found a backfat reduction and an increase of lean content in pigs fed CLA. Similar discrepancies occur for IMF: while Migdal, Pasciak, Wojtysiak, Barowicz, Pieszka and Pietras (2004) and Ostrowska *et al.* (1999) did not find any effect of dietary CLA, Dugan, Aalhus, Jeremiah, Kramer and Schaefer (1999), Martin, Antequera, Muriel, Perez-Palacios and Ruiz (2008) and Joo *et al.* (2002) observed an increase of IMF in pigs fed CLA diet. A leaner genotype was used in the studies in which a response to CLA was observed. Differences in the results can be attributed to the source of CLA, the level of fat in the diet, gender, breed and percentage of lean and duration of the feeding program (Azain, 2003). This study shows that feeding a high dose of CLA to Landrace x Duroc gilts from around 73 to 117 kg of live weight does not produce changes in IMF content, and comparing with other studies it seems that the effectiveness of CLA may be higher in leaner genotypes. It cannot be discarded that the effect of CLA may occur at early stages and be manifested later. It has been shown that CLA affects the expression of PPARγ (Brandebourg & Hu, 2005), hence the effect of CLA may be stronger until the second month when development of the adipose tissue is due mainly to

hyperplasia and not at later stages (between second and fifth month) when hyperplasia diminishes and hypertrophy is the major component (Anderson & Kauffman, 1973).

In pigs, lipid synthesis mainly occurs in adipose tissue. Still, liver plays an important role in whole animal metabolism. An increase in liver weight was found in pigs fed the diet containing CLA oil suggesting higher metabolic activity. These results are in agreement with Yuan, Sun, Sinclair and Li (2009) in mice and Navarro *et al.*, (2009) who associated the liver enlargement of hamsters receiving CLA to an increase of metabolic potential to process fatty acids from mobilised adipose stores.

The tissue where CLA had the highest percentage of incorporation was LT subcutaneous fat (7.72%) and the lowest percentage was found in the muscles (LD: 2.64% and SM: 2.12%). Hence, from the point of view of the consumer, eating a portion of 100g of loin which has 2% IMF, the sum of CLA isomers ingested would only represent 53 mg of which 12.2 mg would be 10t,12c CLA, the isomer expected to produce changes in the lipid metabolism. López Bote, Ortiz and Menoyo (2004) suggested that the minimum quantity to observe some effect in humans is approximately 3g/day, hence eating a 100g portion of loin would be a small fraction of the amount need to observe any effect in humans. Although diets contained similar quantities of both isomers (9c,11t CLA and 10t,12c CLA) the quantity deposited in all tissues was higher for the isomer 9c,11t than for 10t,12c as was observed by Lo Fiego, Macchioni, Santoro, Pastorelli and Corino (2005) and Bee *et al.* (2008). It is known that the CLA may contain at least 28 different isomers, but only the two major isomers (9c,11t CLA and 10t,12c CLA) are evaluated in most studies performed in pigs, and the rest may also have some important roles in metabolism. In the present study the percentage of the isomer 9c,11c was evaluated and its percentage was similar to 9c,11t in all the tissues analyzed except in LD. The appearance of the 9c,11c isomer could be a product of the metabolism of some of the other CLA isomers incorporated in the diet.

The effect of CLA in FA composition was different between muscles, LT and SM. Intarapichet, Maikhunthod and Thungmanee (2008) also found an effect of CLA in FA profile of LT muscle but not in a muscle from ham. The increase of SFA (mainly C16:0) by dietary CLA was not reflected in more IMF neither in LT nor SM, in agreement with Cordero, Isabel, Menoyo, Daza, Morales & Piñeiro, (2010). This shift toward to saturation in all tissues decreasing MUFA in LT muscle and subcutaneous fat could decrease lipid oxidation of the pork fat (Larsen, Wiegand, Parrish, Swan & Sparks, 2009) and improve the meat technological properties, but could have an hypercholesterolemic effect for the consumer. These changes in saturation reflect a reduction of Δ-9 desaturase activity by CLA (Smith, et al., 2002). However, oleic acid was more reduced by CLA than palmitoleic acid, indicating that the inhibition of Δ-9 desaturase may be less pronounced for palmitic acid (Emken, Adlof, Rohwedder & Gulley, 1993). The reduction of linoleic acid and the consecutive n-6 FA could be a result of the replacement of the sunflower oil (rich in linoleic acid) by CLA oil in the experimental diet, which caused an increase in the n-3 elongation and desaturation pathway, increasing the percentage of EPA and reducing the percentage of AA i.e. in the liver. These modifications could not be detected in LT subcutaneous fat due to the low presence of long n-3 fatty acids. The reduction of linoleic acid caused by the replacement of sunflower oil to CLA oil could also be the cause of the reduction of the total PUFA content in the liver and in the LT subcutaneous fat. The reduction of PUFA in LT subcutaneous fat is in agreement with Wiegand, Sparks, Parrish and Zimmerman (2002) and Sun, Zhu, Qiao, Fan and Li (2004). However, Dugan, Aalhus, Rolland and Jeremiah (2003) showed the opposite effect when canola oil was used as control. Thus, CLA has a high impact in FA composition and this effect seems to be tissue specific; while SFA were increased in all tissues (a 2.5 % in LT, a 5.8 % in SM, a 5 % in the liver and a 9.6 % in LT subcutaneous fat), MUFA were only reduced in LT (5.6 %) and LT subcutaneous fat (7.2 %), and PUFA in SM (5.5 %), liver (5 %) and LT subcutaneous fat (2.2 %).

Taking into account the percentage of FA in the different lipid fractions of LT muscle, the highest percentage of CLA was found in PL fraction which does not mean that PL is the fraction with the highest amount of CLA respect to TFA, because it has to be considered that NL account for 60-70 % while PL only accounts 15-20 % of the TFA. The modification of SFA and MUFA was similar in NL fraction to what observed in TFA. While NL fraction had the lowest percentage of PUFA, the PL fraction showed a marked reduction in the proportions of n-6 FA, without changes in the proportion of saturated to unsaturated fatty acids. Hence, it could be concluded that the n-6/n-3 modification observed in LT muscle was basically due to a modification in the PL fraction. FFA (1-5 % of TFA) shows little changes due to CLA supplementation. In contrast, Martin, Muriel, Antequera, Andres and Ruiz (2009) found an increase of PUFA, mainly linoleic acid, in NL, while PL and FFA remained unaffected by the inclusion of CLA in the diet.

5. Conclusions

The inclusion of a high dose of dietary CLA oil (4%) in gilts of a conventional genotype (*Landrace* x *Duroc*) does not increase IMF content. Growth performance was not affected by CLA inclusion but tended to reduce fat deposition, particularly perirenal and increased liver weight. CLA acted in a tissue specific way, increasing SFA in all studied tissues, reducing the MUFA in LT and LT subcutaneous fat, and PUFA in LT subcutaneous fat, liver and SM. The effect of CLA on FA profile was different in LT and SM muscles, and it was similar in SM and liver. Additionally, SFA and MUFA were mainly modified in NL and PUFA in PL fraction of LT IMF.

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Chapter 2:

Dietary conjugated linoleic acid modify gene expression in liver, muscles and fat tissues of finishing pigs

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The mode of action of CLA in the different tissues is not fully understood. Several studies were performed in rodents and cultures, however because experimental conditions are different, effects are not well correlated with gene expression. Furthermore, there are few studies evaluating the effect of CLA in pigs. In the second paper, in order to understand the mode of action of CLA in pig, the effect of a high dose of CLA in the expression of some genes related with lipid metabolism in different pig tissues was evaluated. From the results obtained it could be concluded that the effect of CLA on the transcription of genes related with lipid metabolism is different according the tissue studied and that all the changes observed in the transcription are not reflected in fat deposition, suggesting that other post-transcriptional mechanisms influence fat deposition.

Running head: CLA affects lipid metabolism in pigs

Dietary conjugated linoleic acid modify gene expression in liver, muscles and fat tissues of finishing pigs[1]

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ABSTRACT: The aim of this study was to investigate underlying mechanisms of dietary conjugated linoleic acid (CLA) on lipid metabolism in various tissues of pigs. Sixteen gilts (73 ± 3 kg) were fed a control (containing sunflower oil- SFO) or an experimental diet in which 4% of SFO was replaced by CLA and slaughtered at an average BW of 117 ± 4.9 kg. Transcription of *peroxisome proliferator-activated receptor alpha* (PPARα*), peroxisome proliferator-activated receptor gamma* (PPARγ***),*** fatty acid synthase (FAS), sterol regulatory element binding protein (SREBP1), acetyl-CoA carboxylase (ACC), lipoprotein lipase (LPL), delta-6-desaturase (D6D), and stearoyl CoA desaturase (SCD) were determined by real-time PCR in *longissimus thoracis* (LT) and *semimembranosus* (SM) muscles, LT subcutaneous and SM intermuscular fat and in liver. Fatty acid composition was analyzed using gas chromatography in the same tissues except SM intermuscular fat. Dietary CLA increased PPARγ in LT muscle ($P < 0.05$), whereas CLA reduced PPARα transcription in all tissues studied ($P < 0.05$) except in intermuscular fat. Transcription of genes related with FA synthesis were reduced by CLA in SM muscle and liver (SREBP1,

both $P < 0.1$; ACC, $P < 0.01$ in SM; and FAS, $P < 0.01$ in liver), whereas CLA reduced ($P < 0.05$) LPL and D6D transcriptions in SM muscle and reduced ($P < 0.05$) SCD in liver but increased ($P < 0.05$) SCD in LT muscle and intermuscular fat. Saturated fatty acids were increased in all studied tissues ($P < 0.01$), while monounsaturated and polyunsaturated were reduced in a tissue specific way by CLA. It was concluded that dietary CLA affected transcription of genes and the fat metabolism in a tissue specific manner.

Keywords: CLA, intramuscular fat, lipid metabolism

INTRODUCTION

Genetic selection has resulted in leaner pig carcasses, and more knowledge is needed to improve the eating quality of pork meat, which is associated with high levels of intramuscular fat (IMF). Addition of conjugated linoleic acid (CLA) in feed is a potential way to increase IMF of pork meat while concomitantly reducing the subcutaneous fat (Morel et al., 2008). In a previous study, a reduction of whole body fatness without modification of IMF was observed by inclusion of CLA in pig diets and may potentially be caused by altered gene expression.

The hypothesis of the present study was that dietary CLA affects transcription of genes related to lipid metabolism, and thereby influence fat metabolism and fat deposition within the carcass. At slaughter, FA composition and transcription of selected genes was quantified as potential markers for lipid oxidation (*proliferator-activated receptor alpha*: PPARα)*, de novo* fat synthesis (sterol regulatory element binding protein: SREBP1; fatty acid synthase: FAS; acetyl-CoA carboxylase: ACC), FA uptake (lipoprotein lipase: LPL), cell differentiation (*peroxisome proliferator-activated receptor gamma*: PPARγ) and conversion of fatty acids (delta-6-desaturase: D6D, and steroyl-CoA-desaturase: SCD) in two different types of muscle (*longissimus thoracis* [**LT**] and *semimembranosus* [**SM**]), two different types of fat (LT subcutaneous and SM intermuscular fat) and in liver.

MATERIALS AND METHODS

Sixteen crossbred gilts (Landrace × Duroc) weighing 73 ± 3 kg were randomly assigned either to control diet (C) containing 4% sunflower oil or experimental diet in which 4 % CLA oil (CLA) replaced sunflower oil. The CLA oil (IOI Group Loders Croklaan, Wormerveer, Netherlands) contained 56% of CLA isomers (equal amounts of c9, t11 and t10, c12). Pigs were slaughtered at 117 ± 4.9 kg BW and after slaughter liver, LT, SM, LT subcutaneous fat and SM intermuscular fat were removed, immediately frozen in liquid N and then stored at -75°C for gene expression analyses. The procedures for RNA extraction, cDNA synthesis and qPCR were described by Theil et al., 2006. The RNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Inc., Wilmington, DE) and RNA quality evaluated based on A_{260}/A_{280} and A_{260}/A_{240} ratios. Primer pairs used to detect LPL (5´-ctaccaaagtgccatcaaagt and 5´-ggtttgttgtacagctgagtct; accession no. NM_214286) and for PPARγ (5'-cccagcagcattatccaatatct and 5´-ccgccatccagtcgataaac; accession no. NM_214379) were designed specifically for this study. The other primer pairs and probes used and the method used for FA determination were fully described by Duran-Montgé et al. (2009) along with a full description of the qPCR methodology. Delta Ct values (Ct target - Ct housekeeping gene) were calculated and analyzed statistically using GLM procedure of SAS, and the delta delta method (ΔCt of **CLA** treatment - ΔCt of **C** diet) was calculated to derive the relative transcription corrected for PCR efficiencies of the target genes as described by Theil et al. (2006).

RESULTS AND DISCUSSION

Dietary effects of CLA on gene expression have previously been studied in both cultures and rodent animals, but discrepancies are reported likely due to CLA isomer, dietary inclusion level of CLA, treatment period, *in vitro* conditions, animal species or the house keeping genes used. Transcription of β-actin was not affected

by dietary CLA in the present study in any of the studied tissues ($P > 0.15$ for all tissues) and hence considered a suitable housekeeping gene.

The lower PPARα expression (Table 22) observed in all studied tissues suggests that CLA reduced FA oxidation ($P < 0.05$). Dietary CLA reduced FA *de novo* synthesis preferably in oxidative tissues (i.e. liver and SM muscle), as indicated by expression of lipogenic genes (SREBP1 $P < 0.1$; FAS $P < 0.01$; and ACC $P < 0.01$). However, no significant fat reduction was observed in all carcass locations in the present study (Tous et al., unpublished), which could be explained by simultaneous reduction on both FA oxidation and FA *de novo* synthesis.

Table 22: Relative transcription of lipogenic genes in CLA pigs for each tissue and gene[1]

Gene[2]	Suggested role	LT	SM	SCF	ITMF	Liver
PPARα	***FA oxidation***	0.62*	0.67*	0.30*	0.71	0.66*
PPARγ	Cellular differentiation	1.56*	0.66	1.07	0.87	1.12
SREBP1	FA synthesis	1.49	0.54†	1.31	2.41†	0.46†
FAS	FA synthesis	1.13	0.77	1.36	0.89	0.28**
ACC	FA synthesis	1.17	0.50**	0.95	0.90	0.81
LPL	FA uptake	1.01	0.65*	0.83	1.14	0.78
D6D	ω-3 synthesis	1.27	0.46*	0.71	0.99	1.07
SCD	MUFA synthesis	3.14**	0.69	2.47	14.9**	0.22**

[1] CLA increased (> 1.0) or decreased (< 1.0) transcription relative to pigs fed control diet (†; $P < 0.1$; *; $P < 0.05$; **; $P < 0.01$) in *longissimus thoracis* muscle (LT), *semimembranosus* muscle (SM), subuctanous fat (SCF), intermuscular fat (ITMF) and liver.

[2] Acetyl-CoA carboxylase (ACC), Delta-6-desaturase (D6D), Fatty acid synthase (FAS), Lipoprotein lipase (LPL), peroxisome proliferator-activated receptor alpha (PPARα), Peroxisome proliferator-activated receptor gamma (PPARγ), Stearoyl CoA desaturases (SCD), Sterol regulatory element binding protein (SREBP1).

The reduced SCD expression in the liver ($P < 0.01$) is consistent with the higher content of SFA (Table 23) and the reduced oleic acid, the main SCD product in CLA fed pigs (C: 11.4%, CLA: 9.62% of total FA; $P < 0.1$). In LT muscle, SCD expression was increased ($P < 0.01$), but surprisingly MUFA content was reduced. The primers/probe designed for the SCD gene targeted both SCD1 and SCD2 and the discrepancy between SCD expression and MUFA content suggests that different isoforms of SCD may be expressed in the various tissues.

Table 23: Effects of dietary CLA on fatty acid saturation in different tissues

		∑ SFA[1], %	*∑ MUFA[2], %*	*∑ PUFA[3], %*
LT	**C**	34.9	42.8	22.3
	CLA	37.4	37.2	25.3
	RMSE	1.77	3.41	4.66
	P-value	0.013	0.006	0.216
SM	**C**	32.2	41.4	26.3
	CLA	38.0	41.1	20.8
	RMSE	1.65	3.32	3.3
	P-value	<0.001	0.871	0.005
Subcutaneous fat	**C**	36.5	40.7	22.7
	CLA	46.1	33.5	20.5
	RMSE	3.55	2.81	1.27
	P-value	<0.001	<0.001	0.003
Liver	**C**	40.6	14.4	44.8
	CLA	45.6	14.5	39.8
	RMSE	0.87	1.99	1.71
	P-value	<0.001	0.97	<0.001

[1] Sum of saturated (SFA), mono unsaturated (MUFA) and poly unsaturated fatty acids (PUFA) in *longissimus thoracis* muscle (LT), *semimembranosus* muscle (SM), subcutaneous fat and liver.

A lower expression of D6D by CLA was observed in SM muscle ($P < 0.05$), in concordance with low PUFA contents. The effects of CLA observed in pigs were opposite to those observed in the liver of hamsters (Navarro et al., 2009).

A reduced LPL expression was observed in SM muscle ($P < 0.01$), suggesting a reduction in the uptake of FA. In contrast, no effect of CLA in LPL expression was observed in the muscle and backfat of pigs by Jiang et al. (2010). Differences observed between tissues could be due to the presence of different isozyme forms in the different tissues, having different modes of action (in adipose tissue LPL is insulin dependent which is different from heart and skeletal muscles).

Dietary CLA increases percentage of intramuscular fat (Meadus, 2003). In agreement with this, PPARγ expression was increased in LT muscle ($P < 0.05$) indicating increased differentiation of preadipocytes to mature adipocytes and higher IMF in LT muscle, although it was not supported by (Lauridsen et al., 2005; Tous et al., unpublished). The discrepancy between higher PPARγ expression and similar IMF content in LT muscle may be due to an inhibition of PPARγ activity beyond the transcription level.

In conclusion, expression of genes involved in regulating fat metabolism and fatty acid composition were affected by CLA in a tissue-specific manner. Furthermore, CLA reduces the de novo synthesis and oxidation of FA in oxidative tissues (i.e liver and SM muscle). Nonetheless, the changes in gene expression were not always reflected in changes in fat deposition, which suggests that regulatory mechanism of fat metabolism beyond transcription may be affected by dietary CLA level and that observed changes in gene expression is of minor biological relevancy.

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CHAPTER 3:

Vitamin A

Chapter 3:

Effect of vitamin A depletion on fat deposition in finishing pigs, liver retinol content and gene expression in the *longissimus* muscle

Several studies in ruminants reported that the reduction of dietary vitamin A increases IMF. Few studies have been performed in pigs and the results are not conclusive. In this chapter, a study evaluating the effect of 3 different levels of dietary vitamin A in pig diets was performed: (1) containing a typical amount added in the vitamin-mineral premix (5000 IU/kg), (2) the amount of vitamin A close to the requirement according to NRC (1998; 1250 IU/Kg), and (3) without vitamin A (0 IU/kg). The IMF content was not significantly affected by dietary treatment, although the trend observed was the contrary to he initial hypothesis. As a consequence, animals which received diets without vitamin A had more muscle and less fat in the carcass.

Effect of vitamin A depletion on fat deposition in finishing pigs, liver retinol content and gene expression in the *longissimus* muscle

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ABSTRACT: The content of intramuscular fat (IMF) influences meat quality. In the last decades, animals with a lower fat deposition have been selected for pork production, which resulted in a reduction of IMF. Nutritional strategies as reduction of dietary vitamin A have been proposed with the aim of increasing IMF and in turn the meat quality. Accordingly, the purpose of the study was to evaluate if a reduction of dietary vitamin A would influence IMF, backfat deposition and pig performance parameters. Forty eight barrows were fed diets with different vitamin A levels: without supplemental vitamin A (0 IU vitamin A /kg; n=16), an amount close to the recommended by NRC (1998) (1,250 IU vitamin A/kg; n=16) or the amount used in a commercial formulation (5,000 IU vitamin A/kg; n=16). The feed without dietary vitamin A did not affect growth performance parameters. However, reduced perirenal fat ($P < 0.05$) was observed in the animals fed the diet with no supplementation of vitamin A and numerical reductions of IMF and subcutaneous fat were also observed, suggesting a reduction of fatness when vitamin A was omitted in the diet, contrary to the initial hypothesis. The diet without vitamin A, thus, resulted in a higher proportion of muscle ($P < 0.1$). The content of retinol in the liver was increased when the animals were fed higher

levels of dietary vitamin A but animals fed without vitamin A diet also produce retinol, although in a reduced amount, which could explain no dietary effects on performance. When comparing 5,000 IU/kg with 0 IU/kg diets, only a trend in reduced expression of PPARα without impaired modification on fat content was observed in *longissimus* muscle. From this study, it can be concluded that omitting vitamin A does not affect performance, decreases perirenal fat and possibly overall fat deposition including IMF, contrary to the original hypothesis.

INTRODUCTION

Recently, it was reported that the level of vitamin A may affect fat deposition in different livestock species, because retinoids may act as ligands for various nuclear receptors that can promote or repress adipogenesis (Krskova-Tybitanclova et al., 2008) or they may change the cellularity toward a tissue formed by smaller cells but in greater numbers (Arana et al., 2008). A higher amount of intramuscular fat (**IMF**) has a positive influence on the sensory experience associated with eating pork (Font-i-Furnols et al. 2012). Hence, the challenge for the pork industry is to produce a product with enough IMF to ensure a pleasant eating experience and at the same time low enough to alleviate the health concerns associated with high fat products (Fortin *et al.*, 2005).

Vitamin A is a lipophilic compound, also known as retinoic acid which may be obtained from some carotenoids from feed stuff by animal and fish through an enzymatic reaction. According to National Research council (1998) the nutritional requirement for vitamin A in growing-finishing pigs is 1,300 IU/kg diet. However, commercial diets are normally supplemented with this and other vitamins and contain a higher concentration. In 2009, EFSA (European Food Safety Authority) recommended to establish new maximum limits in the feed of animals for food production (pigs, cattle and poultry) hence, avoiding the high levels of vitamin A ingested by the consumers which could be a risk in terms of health.

In beef cattle, low vitamin A does not affect average daily gain (ADG), average daily dry matter intake (DMI), or gain to feed ratio (G:F); however, quality grade tends to be greater increasing marbling scores without affecting backfat (Arnett et al., 2009). Additionally, Gorocica-Buenfil et al., 2007 reported that low vitamin A diet induces hyperplasia in the intramuscular but not in the subcutaneous fat depot of beef. The effect of the level of vitamin A on IMF or backfat deposition in pigs seems less clear. D'Souza et al. (2003) and Olivares et al. (2011) showed an increase of IMF when the level of vitamin A was reduced. While D'Souza et al. (2008) did not observe any effect or an increase of IMF was observed when the dietary vitamin A was increased (Olivares et al. 2009b).

The objective of the study was to evaluate the effect of reduced dietary vitamin A levels on IMF, backfat deposition and growth performance parameters. To analyze the expression of some genes related with lipid metabolism in *longissimus* muscle and to measure the retinol stored in the liver in order to observe if the dietary modifications produced some metabolic changes in the main retinol storage place.

MATERIAL AND METHODS

Live animal care and measurements

Forty eight *Landrace x Duroc* barrows of 35.7±2.85 kg live weight were chosen from a larger group and were housed individually in adjacent pens in 3 different rooms provided with forced ventilation by extraction. Pigs were ranked by weight and randomly assigned to one of three treatments (16 pigs per treatment) avoiding the maternal effect. The three dietary treatments were: diet without vitamin A (0 IU/kg diet), the same basal diet with the supplementation of an amount close to the recommended requirement of vitamin A by NRC (1,250IU/kg diet) or the usual vitamin-mineral premix (5,000 IU/kg diet). Pigs received the diets *ad libitum* and had free access to water. The composition of the diets is shown in Table 24. The ß-carotene content was estimated using the information available in INRA tables

(Sauvant et al., 2002), and considering that 1IU of vitamin A is equivalent to 0.6 µg of ß-carotene. Thus, for the first period the ß-carotene was 43.5 µg/kg feed and for the second and third periods it was 47.1 µg/kg. The analyzed vitamin A content for 0 IU diet was 654 ± 41 IU/ kg feed; for 1,250 IU was 2,078 ± 145 IU/ kg feed and for 5,000 IU was 6,991 ± 242 IU/ kg feed.

Table 24: Ingredients and chemical composition of experimental diets

Treatment	***1st Period*** *(0-21 days)*	***2nd Period*** *(21-47 days)*	***3rd Period*** *(47-53 days)*
Ingredient, %			
Sorgum	31.6	36.8	36.8
Manioc	25.0	25.0	25.0
Wheat	20.0	20.0	20.0
Soybean meal 44%	19.8	14.4	15.0
Dicalcium phosphate	1.16	1.49	1.25
Lard	0.50	0.50	0.50
Calcium carbonate	0.41	0.36	0.39
Mineral-Vitamin premix[a]	0.40	0.40	0.40
L-lysine HCl	0.39	0.35	0.21
Sodium Chloride	0.26	0.12	0.18
L-Threonine	0.18	0.15	0.06
Sodium bicarbonate	0.16	0.35	0.26
DL-methionine	0.16	0.11	0.04
L-tryptophan	0.03	0.02	-
Chemical composition			
ME (kcal/kg)	3129	3124	3127
Protein (%)	14.9	13.0	13.0
Lys (g/kg)	10.0	8.40	7.40

[a] One kg of feed contains: 5,000 IU vitamin A, 1,250 IU vitamin A or 0 IU vitamin A depending on the treatment for each period; 1,000 IU vitamin D_3; 15 mg vitamin E; 1.3mg vitamin B_1; 3.5 mg vitamin B_2; 0.025 mg vitamin B_{12}; 1.5 mg vitamin B_6; 10 mg calcium pantothenate; 15 mg nicotinic acid; 0.1 mg biotin; 0.6 mg folic acid; 2 mg vitamin K; 80 mg Fe; 6 mg Cu; 0.75 mg Co; 60 mg Zn; 30 mg Mn; 0.75 mg I; 0.10 mg Se; 0.125 g Ethoxiquin.
ME: Metabolizable Energy

Slaughter conditions and carcass quality measurements

Animals were transported to IRTA experimental abattoir (about 10 min trip) and approximately 16h before slaughter were held off feed but free access to water during lairage. Pigs slaughtered minimizing the stress using standard *ante mortem* procedures and 85 % CO_2 stunning for 120 s using CO_2 Dip Lift (Butina, Alps, Copenhagen, Denmark). Once carcasses were eviscerated, they were split

longitudinally and weighed. Liver and perirenal fat were weighed and their proportion with respect to the live animal weight before slaughter was calculated. Liver samples were collected, vacuum packed and stored at -20ºC for chemical analysis.

Slaughter conditions

Fat and muscle thickness were measured with the Fat-O-Meat'er (FOM, Carometec A/S, Herlev, Denmark) between the 3rd and the 4th last ribs at 6 cm off the midline. From these measurements lean meat percentage was calculated using the Spanish official equation (lean percentage (%) = 61.56 - 0.878 x backfat thickness 3th-4th last ribs + 0.157 x muscle depth 3th-4th last ribs; Gispert & Diestre, 1994). In addition, backfat thickness at the last rib at 6 cm off the midline and between the 3rd and 4th lumbar vertebrae at 8 cm off the midline was determined with the same probe. The minimum fat thickness over the muscle *Gluteus medius* and fat thickness in the cranial position of the 1st lumbar vertebrae were measured with a ruler over the carcass midline.

After carcass refrigeration at 3 ºC for 24 h, carcasses were weighed. Then, the left side of each carcass was cut following a simplified European reference method (Walstra & Merkus, 1995). The primary joints obtained were: ham (included hind shank and hind foot), loin, belly (included the ventral part of the belly and jowl), shoulder (with front shank, front foot and neck), tenderloin, and head (with cheek). Each joint was weighed and their proportion with respect to cold carcass weight was calculated. Furthermore, the SM was separated from the ham and the LT muscle and subcutaneous fat (with skin) from the loin, and their respective weights were recorded. Samples of LT between 3rd and 4th ribs region were taken, vacuum packaged and stored at -20 ºC until determinations.

Meat quality measurements

Muscle pH was measured in the left carcass side using a Crison portable meter (Crison, Barcelona, Spain) equipped with a Xerolyte electrode in the LT between the 4th and 5th last ribs and in SM muscles at 45 min (pH45) and 24 h (pHu) *post mortem*. Electrical conductivity was measured in the carcass using a Pork Quality Meater (PQM-Kombi, Aichach, Germany) at the last rib level in the LT and SM muscles at 24 h (ECu) *post mortem*. Instrumental colour (CIE, 1976) was measured at the level of the last rib on a cross section of the LT muscle after 15 min of blooming time with Colorimeter CR-400 (using D65 as illuminant and standard observer of 10º; Minolta Co., Ldt., Osaka, Japan) and luminosity (L*), redness (a*) and yellowness (b*) were recorded. Drip loss was obtained from the LT muscle according to the methodology describe by Rasmussen and Andersson (1996).

Chemical methods

Lipids from LT were extracted with chloroform-methanol according to Folch, Lees and Stanley (1957). Dry matter was determined according to (AOAC 2010, 934.01), and crude protein by Dumas method using a nitrogen analyzer FP528 Leco (AOAC 2010 method 992.15). The retinol content from the liver was hydrolyzed using methanol-KOH solution, extracted with hexane and determined with HPLC using a C18 column and eluted with a methanol/water gradient.

Gene expression

Approximately 30 mg of LT muscle was homogenized in a tube containing TriReagent (Molecular Research Center, Cincinnati, OH, USA), 1-bromo-3-chloropropane (**BCP;** Molecular Research Center, Cincinnati, OH, USA) was added in order to separate the upper phase containing the RNA upon centrifugation of the homogenate. RNA was precipitated in isopropanol and the precipitate was washed twice in ethanol 75%. The RNA concentration was determined using NanoDrop ND-

1000 spectrophotometer (Nano-Drop Technologies, Inc., Wilmington, DE) and RNA quality was considered when A_{260}/A_{280} was between 1.9 and 2.1 and A_{260}/A_{240} between 1.2 and 1.6.

Purified RNA was reverse-transcribed with oligo-dT and random primers using the Superscript III RNAse H reverse transcriptase kit (Invitrogen, Taastrup, Denmark) according to the manufacturer's protocol. Reverse-transcribed material (1 µl) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden) using primer pairs specific for each gene and the signal was detected quantitatively by SYBR Green (SREBP-1, PPARγ, LPL and PPAR-α), or probes labeled with carboxyfluorescein (FAM) on the 5' end. Primer pairs used to detect LPL (5´-ctaccaaagtgccatcaaagt and 5´-ggtttgttgtacagctgagtct; accession no. NM_214286) and for PPARγ (5'-cccagcagcattatccaatatct and 5´-ccgccatccagtcgataaac; accession no. NM_214379) were designed specifically for this study using Primer Express software, version 2.0 (Applied Biosystems Inc.). The other primer pairs and probes used and the method used for FA determination were fully described by Duran-Montgé et al. (2009).

β-actin, GAPDH and HPRT1 were tested as possible endogenous control. Transcription of HPRT1 was not affected by vitamin A level and was considered as control gene. The samples were analyzed using an ABI 7900HT detection system (Applied Biosystems, Stockholm, Sweden). A selected sample was serially diluted and analyzed in triplicate to test the linearity and efficiency of the PCR amplifications. Furthermore, control wells with water and genomic pig DNA, were used as negative controls. For RT-PCR, 40 cycles were needed at 95 °C for 15 s and 60 °C for 60 s to amplify the PCR products. All the target genes were analyzed by duplicate and the housekeeping genes in triplicate and the response was quantified as the number of PCR cycles required to reach a certain threshold.

Statistical Analysis

The different variables studied except gene expression were analyzed by two way ANOVA with 16 blocks corresponding to initial body weight and location in the farm and three dietary treatments corresponding to the three levels of vitamin A (0, 1,250 or 5,000 IU/kg feed). Differences among treatment means were investigated with a set of orthogonal linear contrast comparing no vitamin A (0 IU/kg) *vs.* vitamin A (1,500 and 5,000 IU/kg) and comparing the two vitamin A levels (1,500 IU/kg *vs.* and 5,000 IU/kg) using the estimate function of GLM procedure of SAS (SAS System for Windows 9.2). Differences were considered different when $P < 0.05$ and considered a trend with $P < 0.1$.

For the gene expression data, delta Ct values (Ct target - Ct housekeeping gene) were calculated and analyzed statistically using GLM procedure of SAS, and the delta delta method (ΔCt of **5,000 IU/kg** diet - ΔCt of **0 IU/kg** diet) was calculated to derive the relative transcription corrected for PCR efficiencies of the target genes as described by Theil et al. (2006).

RESULTS

No significant differences among dietary treatments were found in average daily feed intake (2.93, 2.82, 2.94; $P_{0\ vs.vit} = 0.432$ and $P_{1,500\ vs.\ 5,000} = 0.133$), average daily gain (1.05, 1.00, 1.03; $P_{0\ vs.vit} = 0.197$ and $P_{1,500\ vs.\ 5,000} = 0.267$), final live weight (118.6, 114.3, 117.0; $P_{0\ vs.vit} = 0.089$ and $P_{1,500\ vs.\ 5,000} = 0.179$) or feed to gain ratio of barrows (2.81, 2.82, 2.86; ; $P_{0\ vs.vit} = 0.680$ and $P_{1,500\ vs.\ 5,000} = 0.660$) for 0, 1,250 and 5,000 IU/kg, respectively.

Carcass characteristics, quality measurements in *longissimus thoracis* (LT) or *semimembranosus* (SM) muscles, carcass backfat thickness or lean meat percentage were not significantly affected by the level of dietary vitamin A in any location (Table 25). A trend to increase of muscle depth ($P < 0.1$) and a numerical reduction of backfat thickness was observed when dietary vitamin A was omitted in

the diet. The same trend was observed when carcass dissection or perirenal fat weight was evaluated (Table 26). Perirenal fat weight was reduced ($P < 0.05$) and subcutaneous fat weight was numerically reduced when vitamin A supplementation in the diet was omitted. The opposite trend occurred in muscles, as tenderloin tended to be increased ($P < 0.1$) and LT and SM were numerically increased by the omission of the dietary vitamin A.

Table 25: Effect of dietary vitamin A level on carcass characteristics, *longissimus thoracis* (LT) and *semimembranosus* (SM) meat quality parameters

	0 IU	1,250 IU	5,000 IU	RMSE	P-value 0 vs. Vit	P-value 1,500 vs. 5,000
Carcass characteristics						
Carcass yield, %	79.9	80.0	80.2	1.15	0.594	0.692
Chilling losses, %	2.54	2.50	2.60	0.25	0.839	0.277
Backfat thickness, mm						
3th-4th last ribs[d]	29.6	29.2	30.2	4.64	0.914	0.574
Last rib[d]	26.5	26.7	28.0	4.51	0.548	0.419
3th-4th lumbar vertebrae[e]	33.8	34.7	36.6	5.32	0.261	0.329
Gluteus medius[f, g]	29.1	28.7	29.1	4.48	0.893	0.816
1st lumbar vertebrae[f]	32.4	33.2	34.2	4.86	0.387	0.567
1st rib, mm[f]	45.3	44.1	44.7	5.16	0.558	0.735
Muscle depth, mm						
3th-4th last ribs[d]	51.2	47.9	49.0	5.00	0.067	0.518
Lean meat percentage, %[h]	43.6	43.4	42.7	4.15	0.654	0.665
Semimembranosus						
pH 45 min	6.39	6.30	6.36	0.18	0.287	0.349
pHu	5.51	5.49	5.48	0.07	0.217	0.639
ECu, mS	6.54	6.41	6.84	1.44	0.855	0.398
Longissimus thoracis						
pH 45 min	6.46	6.42	6.48	0.17	0.803	0.357
pHu	5.56	5.57	5.56	0.10	0.785	0.986
ECu, mS	4.01	4.01	3.77	0.94	0.699	0.481
Drip loss, %	1.64	1.44	1.70	1.09	0.680	0.435
Colour						
Lightness, L*	48.3	48.5	48.3	1.97	0.851	0.865
Redness, a*	5.84	6.05	6.06	0.73	0.342	0.966
Yellowness, b*	0.79	0.98	0.97	0.64	0.347	0.933

RMSE: Root Mean Square Error; ECu: ultimate electrical conductivity (24 h *post mortem*); pHu: ultimate pH (24 h *post mortem*).

[d] Measurement done at 6 cm to the carcass midline with Fat-O-Meat'er.

[e] Measurement done at 8 cm to the carcass midline with Fat-O-Meat'er.

[f] Measurement done on the carcass midline with a ruler.

[g] Minimum fat thickness over the muscle.

[h] Calculated from backfat thickness and loin depth between the 3th and 4th using the Spanish official equation (Lean percentage (%) = 61.56 - 0.878 * backfat thickness 3th-4th last ribs + 0.157 * muscle depth 3th-4th last ribs; Gispert & Diestre, 1994).

Table 26: Effect of dietary vitamin A level on the weight of some carcass cuts, liver and perirenal fat

	0 IU	1,250 IU	5,000 IU	RMSE	P-value 0 vs. Vit	P-value 1,500 vs. 5,000
g/kg LW						
Liver	16.0	16.6	16.3	1.17	0.212	0.507
Perirenal Fat	8.94	9.87	9.93	1.31	0.018	0.100
g/kg carcass[1]						
Head	75.8	76.3	75.9	4.02	0.829	0.736
Shoulder	265	263	262	7.68	0.670	0.656
Belly	142	140	140	8.33	0.554	0.844
Tenderloin	10.8	10.5	10.1	0.94	0.090	0.328
Ham	298	299	297	10.8	0.996	0.665
SM muscle	19.4	18.6	18.4	2.78	0.314	0.861
Loin	180	178	181.9	13.4	0.975	0.421
LT muscle	49.8	47.9	47.5	5.72	0.241	0.837
Backfat + skin	72.0	73.1	78.2	11.68	0.319	0.228

RMSE: Root Mean Square Error; LW: Live Weight; SM: *Semimembranosus* muscle; LT: *Longissimus thoracis* muscle

[1]Carcass joints following the European reference method (Walstra, & Merkus, 1995) avoiding some of the cuts: ham (included hind shank and hind foot), loin, belly (included the ventral part of the belly and jowl), shoulder (with front shank, front foot and neck), tenderloin and head (with cheek).

The chemical composition of LT muscle was not affected by dietary treatment (Table 27). Thus, the initial hypothesis that diets without supplementation of vitamin A increases IMF was not accomplished. The same was found in other fat depots, and although no significant, the IMF content was numerically lower in the animals fed the treatment without vitamin A supplementation. A reduction of PPARα was observed when no vitamin A was supplemented while the expression of other genes studied was not affected by dietary treatment.

The retinol content in the liver was significantly affected by the dietary supplementation of vitamin A (Fig. 16). As the content of dietary vitamin A in the diet increased, the retinol content in the liver increased. Concomitantly, the content of retinol in the liver was the lowest in the animals fed the diets without vitamin A, and although the vitamin was not supplied in the diet those animals were still able to synthesize vitamin A. Attempts to analyze vitamin A in LT muscle were done but the retinol levels were below detection limits even in the high vitamin A treatment.

Table 27: Chemical analysis and lipid gene expression of *longissimus thoracis* (LT)

	0 IU	1,250 IU	5,000 IU	RMSE	P-value 0 vs.Vit	P-value 1,500 vs. 5,000
Dry matter, %	27.9	27.9	28.1	0.98	0.665	0.465
Protein, %	24.3	23.9	24.0	1.12	0.370	0.832
IMF[1], %	2.29	2.70	2.62	0.86	0.167	0.795
Gene expression[2]						
PPARα	*0.75 (0.82-1.22)*	-	1.00 (0.62-0.92)	0.148	*0.073*	-
PPARγ	*0.92 (0.74-1.35)*	-	1.00 (0.68-1.25)	0.221	*0.721*	-
SREBP1	1.00 (0.83-1.20)	-	1.00 (0.83-1.21)	0.137	0.993	-
FAS	1.05 (0.80-1.25)	-	1.00 (0.83-1.31)	0.167	0.785	-
ACC	1.05 (0.84-1.20)	-	1.00 (0.88-1.26)	0.131	0.698	-
LPL	0.96 (0.82-1.21)	-	1.00 (1.17-0.79)	0.142	0.793	-
D6D	0.88 (1.29-0.78)	-	1.00 (1.13-0.68)	0.185	0.491	-
SCD	0.55 (3.17-0.32)	-	1.00 (1.77-0.18)	0.849	0.493	-

RMSE: Root Mean Square Error; IMF: lipid content in the muscle (intramuscular fat); PPARα: peroxisome proliferator-activated receptor alpha; PPARγ: peroxisome proliferator-activated receptor gamma; SREBP1: sterol regulatory element binding protein; FAS: fatty acid synthase; ACC: acetil-CoA carboxilase; LPL: lipoprotein lipase; D6D: Delta-6-desaturase; SCD: Stearoyl CoA desaturase.

[1]Lipids were analyzed according Folch et al. (1957).

[2]Results are relative to the diets with 5,000 IU/kg of vitamin A (1.00) for each gene and are compared with 0 IU/kg. Between brackets are expressed the confidence limits (lower – upper) for each gene and treatment.

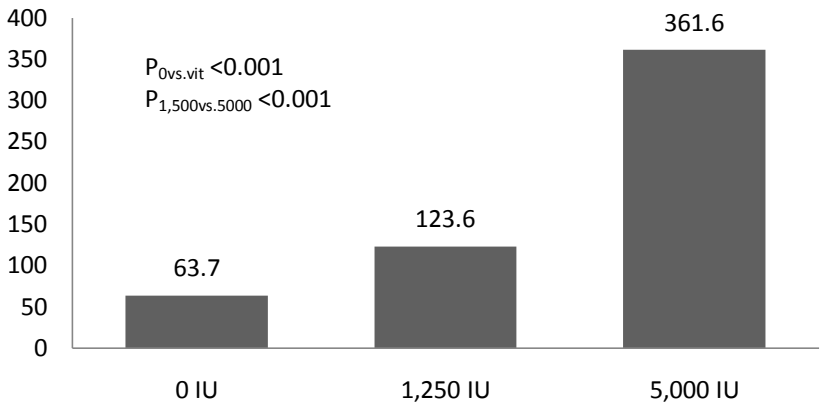

Fig. 16. Retinol content in the liver of animals fed diets with different vitamin A content

DISCUSSION

This experiment was started at an early stage (35.7±2.85 kg live weight) in order to eliminate the possible storage of vitamin A from the pre-experimental period. The three different levels of dietary vitamin A were chosen with the aim of having a diet without vitamin A added (animals can only produce the vitamin from β-carotene present in the different cereals of the diet, 0 IU/kg diet), a diet with the amount close to the NRC recommendation (1,250 IU/kg diet), and a diet with the level of vitamin A normally used for finishing pig diets (5,000 IU/kg diet).

The growth parameters, which were very good for the standard of the breed, were not affected by dietary vitamin A level, which suggest that animals not receiving dietary vitamin A, can convert β-carotene originating from the diet in sufficient amounts to produce vitamin A and compensate the absence of this vitamin in the diet. Furthermore, this results support the ESFA recommendation, that the levels of dietary vitamin A can be reduced without affecting the growth of the animals. In line with our study, D'Souza et al (2003) and Olivares et al (2009a) did not observe effects on the growing parameters when the level dietary vitamin A was reduced although the lowest levels supplemented of vitamin A used by Olivares et al 2009a were higher (7,500 IU vitamin A/kg diet) than the doses supplemented in the present study (0, 1,250 or 5,000 IU vitamin A/kg).

Reduction of dietary vitamin A, although only significant for perirenal fat, tended to reduce whole body fat around 10 % (subcutaneous fat thickness, subcutaneous fat and perirenal fat weight, IMF content) and increase muscle content around 5 % (loin depth, tenderloin, LT and SM weight) when the two extreme diets were compared. These results are contrary of the initial hypothesis, that reduction of vitamin A increases IMF and suggest the opposite effect, namely higher values of IMF can be achieved by increasing the level of dietary vitamin A. D'Souza et al. (2003) and Olivares et al. (2009a) did not find differences in backfat thickness or loin depth. The results observed in the IMF are in agreement with Olivares et al.

(2009b) who observed an interaction between the level of vitamin A and genotype, producing a 20 % increase of IMF when Duroc sires were fed 100,000 IU vitamin A/kg feed vs. 0 IU vitamin A/kg feed but this increase was not produced in Landrace x Large White sire.

The content of retinol in the liver reflected the level of vitamin A added to the diet. However, animals which did not receive vitamin A supplementation still contained some vitamin A in the liver. This could be explained by the ability of those animals to synthesize vitamin A from the ß-carotene present in the diet. Accordingly Olivares et al. (2011), pigs that received the high dietary vitamin A level (13,000 IU/kg) showed higher retinol concentrations in liver than those receiving a low vitamin A level (1,300 IU/kg).

The results in gene expression did not represented large dietary effects on the studied genes. A numeral reduction of SCD was observed in the muscle when animals were fed diets without vitamin A which usually is accompanied with a low fat synthesis, those results might have been more clear in the adipose tissue, because the muscle contains less than 3 % of fat. Furthermore, a trend to reduce the PPARα was observed in animals fed the diets without the addition of vitamin A, suggesting lower oxidation. So, it seems that the trend to reduce IMF may be principally due to a reduction of fat synthesis and not to an increased fat oxidation.

The results from this experiment indicate that the level of dietary vitamin A can be reduced to NRC levels or even more, without affecting growing parameters or having impact in carcass yield, very likely because animals fed without vitamin A supplementation were able to synthesize the vitamin A from the ß-carotene present in the diet. Reduction of vitamin A in the diet tended to increase muscle depth, reducing perirenal fat, and numerically overall fat deposition including IMF and retinol content in the liver was increased when vitamin A in the diet was increased.

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CHAPTER 4:

Protein and lysine

Chapter 4:

Influence of dietary protein and lysine levels on growth performance, carcass characteristics, lipid content and fatty acid profile of finishing pigs

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The reduction of dietary protein has been evaluated in several studies in order to reduce the amount of nitrogen excreted to the environment. Furthermore, reduction of dietary protein or lysine may also increase the IMF content, but usually, when one parameter is reduced so is the other. In this study the two parameters were separately evaluated in 4 different diets. Results showed an interaction between the level of protein and lysine, being the animals fed low protein, high lysine or high protein low lysine the ones with higher IMF content. However, when both parameters were reduced the lowest values of IMF content were obtained. This study suggests that reduction of dietary protein may be applied in order to increase the IMF content in Duroc x Landrace pigs without affecting the performance parameters.

Influence of dietary protein and lysine levels on growth performance, carcass characteristics, lipid content and fatty acid profile of finishing pigs

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Abstract

One hundred and four barrows (Landrace x Duroc) were fed one of four experimental diets: high protein high lysine, high protein low lysine, low protein high lysine or low protein low lysine to evaluate the effects of the diet on fat deposition in pig. Animals fed high protein low lysine had an increase in feed to gain ratio ($P < 0.1$). Intramuscular fat (**IMF**) increased when protein or lysine were independently reduced ($P < 0.05$), but reduction of both parameters simultaneously resulted in the lowest value. Dietary protein reduction increased backfat thickness ($P < 0.1$), saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) ($P < 0.05$), reducing polyunsaturated (PUFA) in *longissimus thoracis* muscle and subcutaneous fat ($P < 0.05$) while in liver SFA remained unaffected and only reduction of n-3 FA was observed in *semimembranosus* muscle ($P < 0.05$). These results suggest that the relationship impacts on growth and pig fat content including IMF, which may improve meat quality.

Keywords

amino acid, backfat, dietary protein, fatty acid composition, intramuscular fat, lysine

1. Introduction

During the last decades, selection for leaner genotypes has resulted in reduced intramuscular fat (IMF, less than 1%) in some genotypes, and this was associated with tougher pork meat (Kempester, Cook & Grantley-Smith, 1986). Acceptability of pork perceived by consumer improved as IMF percentage increased (Font-i-Furnols, Tous, Esteve-Garcia, & Gispert, 2012). Hence, the challenge for the pork industry is to achieve enough IMF to ensure a pleasant eating experience but low enough to alleviate the health concerns associated with high fat pork (Fortin, Robertson, & Tong, 2005).

Different nutritional strategies are proposed to increase the content of IMF. Apparently, dietary protein reduction may reduce pig growth and increase the fatness of whole animal. Costa, McGillivray, Qianfan, Wood, Evans and Chang (2004) suggest that IMF increases when pigs were fed a low protein diet, because it restricts muscle growth, resulting in surplus energy being converted into fat. On the other hand, lysine is the major limiting amino acid in pig diets and plays a pivotal role in energy metabolism in porcine muscle and determining growth rate. A reduction of dietary lysine decreased lean meat percentage in pig carcasses with a consequent increase in fat tissue (Szabo, Jansman, Babinszky, Kanis, & Verstegen, 2001). However, in most of the studies both parameters (protein and lysine) were reduced at the same time, which makes it difficult to attribute the effect observed to lysine or to protein (Conde-Aguilera, Lachica, Nieto, & Fernández-Fígares, 2011; Costa et al., 2004; D'Souza, Pethick, Dunshea, Pluske, & Mullan, 2008; Gondret, & Lebret, 2002; Wood, Richardson, Nute, Fisher, Campo, & Kasapidou, 2004).

The objective of this experiment was to determine if IMF can be increased with the reduction of dietary protein level, the reduction of dietary lysine or the interaction between both parameters and to evaluate if these dietary modifications affect performance, carcass characteristics, lipid content and fatty acid profile in different tissues.

2. Materials and methods

The experiment was conducted in compliance with the Spanish guidelines for human care and use of animals in research and the protocol was approved by the Ethical Animal Committee of IRTA.

2.1. *Animals and diets*

One hundred and four 62±5 kg live weight (LW) Landrace x Duroc barrows chosen from a larger group were blocked by weight avoiding the effect of maternal origin and were housed in adjacent pens (2 or 3 pigs per pen). Pigs were randomly

assigned to one of the dietary treatments in a 2 x 2 factorial design: high protein, high lysine (HPHL, n=26) which was considered the control, low protein, high lysine (LPHL, n=26), high protein, low lysine (HPLL, n=26) and low protein, low lysine (LPLL, n=26). Diet formulation was based on digestible lysine maintaining net energy and the ratio of the essential amino acids to digestible lysine constant. Ingredients and nutritional details are shown in Table 28. The feeding program consisted of two diets in relation with the protein content, one from 62±5 to 97±7 kg LW and the other one from 97±7 to slaughter weight. During the experimental period, pigs received the diets for *ad libitum* consumption and had free access to water. Individual body weights were determined at the beginning of the trial (62±5 kg BW) on day 36 (97±7 kg BW) and before slaughter. Sixty eight pigs were selected in two different days (day 56: thirty five; day 70: thirty three, respectively) based on the ending weight 48h before slaughter (124±5 kg LW) and were transported to the abattoir in order to slaughter all animals at similar body weight. Pen feed intake and feed efficiency were calculated until the day before the first shipment of the animals to the abattoir (day 57).

2.2. *Slaughter conditions*

Animals were transported to the IRTA experimental abattoir (2 h) in two different days (day 56 or 70) and they stayed approximately 16h fasting time before slaughter but with free access to water. Pigs were weighed and slaughtered minimizing the stress using standard *ante mortem* procedures and stunned with 85% CO_2 for 120 s using CO_2 Dip Lift (Butina, Alps, Copenhaguen, Denmark). Once carcasses were eviscerated, they were split longitudinally and weighed before 45min. Perirenal fat and liver were weighed and liver samples for its role in the metabolism of whole animal were collected, vacuum packed and stored at -20 ºC for chemical analysis.

Table 28: Ingredient and chemical composition of experimental diets

	Body weight range							
	62±5 to 97±7 kg BW				97±7 to 112±8 kg BW			
Protein level	High		Low		High		Low	
Lysine level	High	Low	High	Low	High	Low	High	Low
Ingredient, %								
Corn	30.7	28.8	77.7	72.9	49.9	48.1	49.5	47.6
Barley	59.6	61.5	5.50	3.26	45.7	47.5	5.15	7.06
Soybean meal 44%	3.35	3.35	10.4	10.6	-	-	8.88	8.88
Cassava/Manioc	-	-	3.14	10.1	1.09	1.09	32.9	32.9
Soybean oil	3.47	3.65	0.50	0.50	0.50	0.67	0.50	0.68
Dicalcium phosphate	0.84	0.84	0.93	1.01	0.72	0.72	0.97	0.97
Calcium carbonate	0.58	0.58	0.49	0.40	0.55	0.55	1.00	1.00
Sodium bicarbonate	0.58	0.58	0.38	0.30	0.70	0.70	0.06	0.06
Sodium chloride	-	-	0.13	0.18	-	-	0.18	0.18
Mineral-vitamin premix[a]	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
L-lysine HCl	0.33	0.21	0.30	0.18	0.30	0.17	0.24	0.11
L-threonine	0.08	0.08	0.09	0.09	0.05	0.05	0.08	0.08
L-tryptophan	0.01	0.01	0.04	0.04	0.01	0.01	0.03	0.03
DL-methionine	0.01	0.01	0.02	0.02	-	-	0.02	0.02
Chemical composition								
ME, kcal/kg [b]	3,188	3,206	3,217	3,199	3,099	3,099	3,103	3,103
NE, kcal/kg [b]	2,499	2,499	2,511	2,500	2,440	2,440	2,440	2,440
Ether extract, % [c]	6.00	6.20	3.90	3.30	3.00	3.30	2.70	2.60
CP, % [c]	13.1	13.0	12.1	11.9	10.6	10.6	9.80	9.80
Digestible amino acids[b], g/kg								
Lys	6.50	5.54	6.50	5.54	5.20	4.24	5.20	4.24
Thr	4.30	4.34	4.38	4.38	3.42	3.44	3.48	3.51
Met	1.97	1.98	2.03	2.03	1.70	1.71	1.61	1.62
Met/Cys	4.43	4.38	4.03	4.01	3.91	3.94	3.14	3.17
Trp	1.22	1.27	1.32	1.31	0.98	1.00	1.03	1.05

[a] One kg of feed contains: 5,000IU vitamin A; 1,000IU vitamin D_3; 15mg vitamin E; 1.3mg vitamin B_1; 3.5mg vitamin B_2; 0.025mg vitamin B_{12}; 1.5mg vitamin B_6; 10mg calcium pantothenate; 15mg nicotinic acid; 0.1mg biotin; 0.6mg folic acid; 2mg vitamin K; 80mg Fe; 6mg Cu; 0.75mg Co; 60mg Zn; 30mg Mn; 0.75mg I; 0.10mg Se; 0.125g Ethoxiquin.
[b] Calculated values according to INRA tables (Sauvant D., et al. 2004)
[c] Analyzed values

2.3. *Carcass quality measurements*

Fat and muscle thickness were measured with the Fat-O-Meat'er (Carometec A/S, Herlev, Denmark) between the 3rd and the 4th last ribs at 6 cm off the midline. From these measurements, lean meat percentage was calculated using the Spanish official equation (Lean percentage (%) = 66.91 – 0.895 * backfat thickness + 0.144 * muscle depth; Font-i-Furnols, & Gispert, 2009). In addition, backfat thickness at the

last rib at 6 cm off the midline and between the 3rd and 4th lumbar vertebrae at 8 cm off the midline were determined with the same probe. The minimum fat thickness over the muscle *Gluteus medius*, fat thickness in the cranial position of the 1st lumbar vertebrae and in the shoulder at the level of first rib were measured with a ruler over the carcass midline.

After carcass refrigeration at 3 ºC for 24 h, carcasses were weighed. Then, the left side of each carcass was cut following a simplified European reference method (Walstra, & Merkus, 1995). The primary joints obtained were: ham (included hind shank and hind foot), loin, belly (included the ventral part of the belly and jowl), shoulder (with front shank, front foot and neck), tenderloin and head (with cheek). Each joint was weighed and their proportion with respect to cold carcass weight was calculated. Furthermore, the *semimembranosus* muscle (**SM**) was separated from the ham and the *longissimus thoracis* (**LT**) muscle and subcutaneous fat (with skin) from the loin, and their respective weights were recorded. Samples of SM as a mainly oxidative muscle, LT between 3th and 4th ribs region (starting from the caudal part) as a mainly glycolitic muscle and LT subcutaneous fat samples from the same location as a primary site of fatty acid (**FA**) synthesis and storage were taken, vacuum packaged and stored at -20 ºC until determinations.

2.4. *Carcass quality measurements*

Muscle pH was measured in the left carcass side using a Crison portable meter (Crison, Barcelona, Spain) equipped with a Xerolyte electrode in the LT at the last rib level and in SM muscles at 45 min (**pH45**) and at 24 h (**pHu**) *post mortem*. Electrical conductivity was measured in the carcass using Pork Quality Meater (PQM-Kombi, Aichach, Germany) also at the last rib level in the LT and SM muscles at 24 h *post mortem*.

Instrumental colour (CIE, 1976) was measured at 24 h post mortem at the level of the last rib on a cross section of the LT muscle after 15 min of bloom time with Colorimeter CR-400 (using D65 as illuminant and standard observer of 10º; Minolta

Co., Ldt., Osaka, Japan) and luminosity (L*), redness (a*) and yellowness (b*) were recorded. Drip loss was obtained from the LT muscle according the methodology described by Rasmussen and Andersson (1996).

2.5. *Chemical methods*

Dry matter according to (A.O.A.C, 2010; method 934.01) and crude protein by Dumas method using a nitrogen analyzer FP528 Leco (A.O.A.C, 2010; method 992.15) were determined in feed and biological samples.

Lipids from LT, SM, subcutaneous fat and liver were extracted with chloroform-methanol according to Folch, Lees and Stanley (1957), transmethylated with BF_3 and methanolic KOH (Morrison, & Smith, 1964). FA were determined by gas chromatography (Hewlett Packard 6890, USA) using a capillary column DB23 (0.25 mm x 0.25 µm x 30 m) and flame ionization detector (**FID**). A temperature gradient with initial temperature of 170 ºC followed by an increase at rate of 2.5 ºC/min until 210 ºC and raised again at a rate of 5 ºC/min to 240 ºC where it remained for 5 min was used for the whole run. Injector and detector temperatures were both 250 ºC. Injection was in split mode with a ratio of 100.6:1. The carrier gas was helium with a flux of 55.8 mL/min, and pressure of 10.99 psi at the column head. The standards adopted were F.A.M.E Mix C4-C24, cis -11-Vaccenic Methyl Ester (Supelco, Bellefonte, USA), cis-13,16,19-Docosatrienoic acid methyl ester, cis-7,10,13,16-Docosatetranoic acid methyl ester, methyl all-cis-7,10,13,16,19-docosapenaenoate (Sigma, St. Louis, USA). Nonadecanoic acid (Sigma, St.Louis, USA) was used as internal standard. Results were expressed as percentage of total FA.

2.6. *Statistical Analysis*

The study performed was a randomized complete block design with a 2x2 factorial arrangement (2 levels of protein and 2 levels of lysine). Statistical analyses were performed using GLM procedure of SAS (SAS System for Windows 9.2). The model

accounted for the effects of protein level (**Prot**), lysine level (**Lys**) and their interaction (**Prot*Lys**). Results are expressed as least-square means and root means square. Significance of interaction and main effects were obtained from the ANOVA table. In case a significant interaction was found, significance of differences between treatment means was obtained using the PDIFF option of SAS. $P < 0.05$ was considered statistically significant and $P < 0.1$ as a tendency. Pen was used as experimental unit for performance data, whereas individual pig data serve as the experimental unit for the other measurements. Day of slaughter was initially introduced in the model for carcass measurements, but as the effect was not significant, it was removed from the final analysis. Carcass weight was used as covariate for backfat thickness and muscle depth between 3^{th}-4^{th} last ribs, lean percentage, minimum backfat thickness over *Gluteus medius* and backfat thickness in the 1^{st} lumbar vertebrae.

3. Results

3.1. *Performance parameters*

Growth performance parameters of barrows fed diets with different amount of protein or lysine in diet are presented in Table 29. During the first period, from 62±5 to 97±7 kg of LW an interaction ($P < 0.05$) between protein and lysine was observed for feed to gain ratio (**FGR**); the highest values were for the animals receiving HPLL and not different among the other treatments. During the second period, from 97±7 to 112±8 kg LW performance parameters were not significantly ($P > 0.1$) affected by dietary treatment. However, taking into account the overall period, the same trends as for the first period were observed. Results indicate that animals receiving the HPLL diets tended to have a higher FGR ($P < 0.01$), and animals fed low protein diets tended to have lower FGR although it did not reach statistical significance ($P > 0.1$).

Table 29: Effects of dietary protein and lysine level on the performance of growing pigs from 62±5 kg LW to 112±8 kg LW (56 days of experimental feeding period)

Protein	Normal		Low					
Lysine	High	Low	High	Low	RMSE[4]	Prot	Lys	Prot*Lys
From 62 to 97 kg LW								
Initial weight, kg	62.6	62.6	62.4	62.5	4.68	0.946	0.989	0.982
ADFI[1], kg/day	3.04	3.26	3.12	2.97	0.340	0.344	0.733	0.091
ADG[2], kg/day	0.96	0.92	0.99	0.96	0.114	0.392	0.341	0.962
FGR[3]	3.17[b]	3.55[a]	3.15[b]	3.13[b]	0.268	0.015	0.042	0.023
From 97 to 112 kg LW								
Intermediate weight, kg	97.2	95.8	98.1	96.9	7.30	0.659	0.595	0.967
ADFI[1], kg/day	3.30	3.22	3.34	3.24	0.339	0.781	0.398	0.914
ADG[2], kg/day	0.74	0.72	0.79	0.73	0.112	0.319	0.298	0.593
FGR[3]	4.53	4.52	4.27	4.46	0.440	0.264	0.504	0.482
From 62 to 112 kg LW								
Final weight, kg	111.9	110.2	113.9	111.6	8.41	0.515	0.459	0.914
ADFI[1], kg/day	3.13	3.25	2.20	3.07	0.308	0.566	0.931	0.217
ADG[2], kg/day	0.88	0.85	0.92	0.88	0.091	0.261	0.223	0.844
FGR[3]	3.56[b]	3.83[a]	3.48[b]	3.51[b]	0.211	0.005	0.033	0.076

[a,b] Within a row, means without a common superscript differ ($P < 0.05$)
[1]ADFI: average daily feed intake; [2]ADG: average daily gain; [3]FGR: feed to gain ratio; [c]RMSE: Root Mean Square Error

3.2. *Carcass and meat quality measurements*

Carcass characteristics, LT and SM meat quality parameters are shown in Table 30. Carcass yield, chilling losses or carcass weight were not affected by dietary treatment ($P > 0.1$). Backfat thickness over the Gluteus *medius* muscle and on the carcass 1st lumbar vertebrae tended to increase ($P < 0.1$) when the level of dietary protein was reduced. Meat quality parameters measured in SM or colour parameters of LT and were not affected by dietary treatment. However, pH 45 min in LT tended to increase when the level of dietary lysine was reduced, but it did not result in a classification of meat as DFD (dark, firm, dry) or PSE (pale, soft, exudative). Drip loss in LT samples was lower when animals fed low protein compared with those of the high protein diets ($P < 0.05$).

The effect of the diet in the different carcass joins is presented in Table 31. A reduction of the level of dietary protein caused a significant reduction of the

proportion of liver with respect to LW ($P < 0.05$) while the other carcass joints remained unaffected by dietary treatment ($P > 0.1$).

Table 30: Effect of dietary protein and lysine level on carcass characteristics, *longissimus thoracis* (LT) and *semimembranosus* (SM) meat quality parameters

Protein	High		Low					
Lysine	High	Low	High	Low	RMSE[3]	Prot	Lys	Prot*Lys
Carcass characteristics[c]								
Carcass weight, kg	100.2	98.3	100.4	98.7	4.75	0.815	0.119	0.965
Carcass yield, %	80.4	79.7	80.3	80.2	1.39	0.606	0.221	0.390
Chilling losses, %	2.23	2.24	2.24	2.18	0.30	0.701	0.683	0.631
Backfat thickness, mm								
3rd-4th last ribs[d]	27.4	26.4	27.7	26.4	3.96	0.943	0.289	0.773
Last rib[d]	23.9	23.1	23.6	23.3	4.75	0.607	0.580	0.677
3rd-4th lumbar vertebrae	31.9	33.4	32.9	31.9	5.77	0.866	0.870	0.361
Gluteus medius[f, g]	24.1	24.0	26.5	26.3	5.20	0.064	0.703	0.937
1st lumbar vertebrae[f]	27.4	28.1	30.6	30.4	5.83	0.056	0.459	0.741
1st rib, mm [f]	44.9	42.2	44.6	44.0	4.55	0.497	0.420	0.305
Muscle depth, mm								
3th-4th last ribs[d]	50.4	49.2	50.1	50.6	3.22	0.458	0.664	0.287
Lean meat percentage, % [h]	49.6	50.4	49.3	50.6	3.77	0.943	0.289	0.773
Semimembranosus								
pH 45 min	6.58	6.63	6.62	6.67	0.13	0.260	0.107	0.902
pHu[2]	5.49	5.52	5.51	5.55	0.11	0.381	0.277	0.849
ECu[1], mS	5.16	5.30	4.83	5.42	1.40	0.753	0.294	0.510
Longissimus thoracis								
pH 45 min	6.53	6.68	6.63	6.65	0.18	0.485	0.064	0.173
pHu[2]	5.58	5.55	5.59	5.61	0.15	0.346	0.907	0.480
ECu[1], mS	3.16	3.07	3.02	3.17	0.81	0.926	0.885	0.538
Drip loss, %	2.83[a]	2.19[ab]	1.96[b]	1.49[b]	1.38	0.031	0.126	0.809
Colour								
Lightness, L*	48.3	48.0	48.7	47.7	2.34	0.950	0.272	0.571
Redness, a*	8.03	8.09	8.18	7.94	1.01	0.998	0.716	0.528
Yellowness, b*	1.35	1.49	1.67	1.32	0.84	0.729	0.612	0.239

[a,b] Within a row, means without a common superscript differ ($P < 0.05$)

[1]ECu: ultimate electrical conductivity (24 h *post mortem*); [2]pHu: ultimate pH (24 h *post morem*); [3]RMSE: Root Mean Square Error;.
[c]Animals were slaughtered when they reached 124±5 kg LW (56 or 70 days of experimental feeding period)
[d] Measurement done at 6 cm off the carcass midline with Fat-O-Meat'er.
[e] Measurement done at 8 cm off the carcass midline with Fat-O-Meat'er.
[f] Measurement done on the carcass midline with a ruler.
[g] Minimum fat thickness over the muscle.
[h] Calculated from backfat thickness and loin depth between the 3th and 4th using the Spanish official equation (Lean percentage (%) = 66.91 - 0.895 * backfat thickness 3th-4th last ribs + 0.144 * muscle depth 3th-4th last ribs; Font i Furnols & Gispert, 2009).

Table 31: Effect of dietary protein and lysine level on the weight of some carcass cuts, liver and perirenal fat

Protein	High		Low					
Lysine	High	Low	High	Low	RMSE[3]	Prot	Lys	Prot*Lys
g/kg LW[1]								
Liver	16.5[a]	16.2[a]	15.5[ab]	15.2[b]	1.70	0.021	0.472	0.899
Perirenal Fat	11.3	11.5	10.6	11.1	2.24	0.313	0.549	0.797
g/kg carcass[+]								
Head	76.3	77.9	79.2	78.6	4.47	0.104	0.633	0.308
Shoulder	276	274	278	272	10.2	0.977	0.137	0.365
Belly	145	143	145	149	10.7	0.274	0.850	0.300
Tenderloin	12.2	12.0	11.6	12.2	1.30	0.521	0.527	0.210
Ham	300	306	299	299	10.0	0.158	0.195	0.221
SM[4] muscle	22.3	22.1	20.7	22.0	2.14	0.115	0.326	0.152
Loin	184	183	183	181	9.66	0.540	0.605	0.805
LT[2] muscle	58.1	58.2	56.4	59.7	6.11	0.928	0.271	0.280
Backfat + skin	66.7	67.8	70.4	67.7	10.5	0.492	0.759	0.459

[a,b] Within a row, means without a common superscript differ ($P < 0.05$)
[1]LW: Live weight; [2]LT: *Longissimus thoracis* muscle; [3]RMSE: Root Mean Square Error; [4]SM: *Semimembranosus* muscle
[+]Carcass joints following the European reference method (Walstra, & Merkus, 1995) avoiding some of the cuts: ham (included hind shank and hind foot), loin, belly (included the ventral part of the belly and jowl), shoulder (with front shank, front foot and neck), tenderloin and head (with cheek).

3.3. *Chemical composition*

The chemical composition of LT, SM, and liver is shown in Table 32. In LT muscle, a higher protein percentage was found in animals fed a low protein diet ($P < 0.01$). Additionally, an interaction between protein and lysine was observed for IMF percentage ($P < 0.05$), and the animals that received the diets LPHL or HPLL showed the highest IMF values. In SM, an interaction between the level of protein and lysine was observed for dry matter and IMF percentages (both $P < 0.05$). The highest values observed were for the animals fed HPLL and the lowest values were for LPLL treatments. In the liver, an interaction between the level of protein and lysine was also observed for protein and lipid percentage ($P < 0.05$ and $P < 0.1$, respectively). Pigs fed LPHL or HPLL diets had the lowest protein percentages ($P < 0.05$) and tended to have lower lipid percentage ($P < 0.1$).

Table 32: Chemical analysis of *longissimus thoracis* (LT), *semimembranous* (SM), and liver

Protein	High		Low					
Lysine	High	Low	High	Low	RMSE[2]	Prot	Lys	Prot*Lys
Longissimus thoracis								
Dry matter, %	27.8	27.9	28.2	28.1	1.06	0.270	0.794	0.686
Protein, %	23.4[b]	23.4[b]	24.0[a]	24.1[a]	0.86	0.006	0.794	0.796
IMF[1,+], %	2.24[b]	2.58[a]	2.60[a]	2.19[b]	0.73	0.936	0.836	0.042
Semimembranosus								
Dry matter, %	27.8[ab]	28.6[a]	28.4[a]	27.4[b]	1.48	0.432	0.826	0.015
Protein, %	23.7	22.9	23.6	23.7	1.07	0.130	0.223	0.100
IMF[1,+], %	3.29[ab]	4.48[a]	3.56[ab]	2.61[b]	1.75	0.066	0.783	0.014
Liver								
Dry matter, %	27.9	28.1	27.0	28.0	1.56	0.191	0.133	0.323
Protein, %	20.2[a]	19.1[b]	19.8[ab]	20.3[a]	1.50	0.254	0.442	0.028
Lipids[+], %	3.35	3.13	3.12	3.28	0.42	0.708	0.781	0.070

[a,b] Within a row, means without a common superscript differ ($P < 0.05$)
[1]IMF: lipid content in the muscle (intramuscular fat); [2]RMSE: Root Mean Square Error;
[+]Lipids were analyzed according Folch et al. (1957).

3.4. *Fatty acid composition in LT muscle*

The main FAs were affected by the reduction of protein level in the diet in LT muscle (Table 33). Total saturated fatty acids (SFA) and total monounsaturated fatty acids (MUFA) including C 14:0, C 16:0, C 18:0 and C 18:1 n-9 cis were

increased when the protein level of the diet was reduced ($P < 0.05$). When dietary protein was reduced, PUFA ($P < 0.01$), n-3 ($P < 0.001$) and n-6 ($P < 0.01$) including C 18:3 n-3 cis ($P < 0.01$), C 20:5 n-3 ($P < 0.001$), C 18:2 n-6 ($P < 0.001$) and C 20:4 n-6 ($P < 0.05$) were decreased. The modifications observed in PUFA led to an increase in the ratio n-6/n-3 in animals fed diets with the lowest protein level ($P < 0.05$).

Table 33: Effects of dietary protein and lysine levels on FA composition in *longissimus thoracis* (LT) muscle

Protein	High		Low					
Lysine	High	Low	High	Low	RMSE[3]	Prot	Lys	Prot*Lys
C 14:0	1.20^{b}	1.19^{b}	1.31^{a}	1.26^{ab}	0.153	0.021	0.432	0.528
C 16:0	22.2^{b}	22.2^{b}	23.2^{a}	22.7^{ab}	1.14	0.005	0.369	0.362
C 16:1 n-7 cis	3.14	2.93	3.23	3.08	0.469	0.326	0.127	0.794
C 18:0	10.7^{c}	11.0^{bc}	11.6^{a}	11.4^{ab}	0.76	0.001	0.982	0.145
C 18:1 n-9 cis	34.7^{b}	36.8^{ab}	38.1^{a}	37.6^{a}	3.61	0.022	0.375	0.147
C 18:1 n-7	3.90	3.83	3.90	3.93	0.296	0.484	0.822	0.532
C 18:2 n-6 cis	14.5^{a}	13.2^{ab}	11.0^{c}	11.6^{bc}	3.00	0.001	0.648	0.183
C 18:3 n-3 cis	0.48^{a}	0.47^{a}	0.31^{b}	0.34^{b}	0.070	<0.001	0.767	0.269
C 20:0	0.14^{b}	0.15^{ab}	0.17^{a}	0.16^{ab}	0.030	0.007	0.974	0.104
C 20:1 n-9 cis	0.46^{b}	0.53^{a}	0.52^{a}	0.54^{a}	0.092	0.096	0.031	0.222
C 20:2 n-6 cis	0.42^{a}	0.42^{ab}	0.35^{c}	0.38^{bc}	0.072	0.004	0.571	0.390
C 20:3 n-6	0.42^{a}	0.37^{ab}	0.32^{b}	0.37^{ab}	0.118	0.126	0.986	0.073
C 20:4 n-6	3.75^{a}	3.28^{ab}	2.67^{b}	3.04^{ab}	1.161	0.024	0.849	0.145
C 20:5 n-3 cis	0.13^{a}	0.10^{b}	0.07^{c}	0.09^{bc}	0.037	<0.001	0.481	0.040
C 22:4 n-6	0.65^{a}	0.57^{ab}	0.50^{b}	0.56^{ab}	0.156	0.031	0.816	0.061
C 22:5 n-3	0.04	0.06	0.07	0.09	0.072	0.127	0.253	0.877
C 24:1 n-9 cis	0.12^{ab}	0.15^{a}	0.10^{b}	0.10^{b}	0.051	0.002	0.205	0.307
C 22:6 n-3 cis	0.03	0.04	0.04	0.02	0.026	0.549	0.639	0.103
Minor FA^{+}	3.01^{a}	2.76^{ab}	2.50^{b}	2.75^{ab}	0.542	0.051	0.993	0.067
SFA[4]	35.0^{b}	35.2^{b}	37.0^{a}	36.2^{ab}	1.70	<0.001	0.414	0.217
MUFA[1]	44.3^{b}	46.1^{ab}	47.4^{a}	47.1^{a}	3.53	0.022	0.417	0.224
PUFA[2]	20.7^{a}	18.7^{ab}	15.5^{c}	16.7^{bc}	4.51	0.002	0.744	0.156
n-3	0.74^{a}	0.74^{a}	0.53^{b}	0.57^{b}	0.129	<0.001	0.533	0.410
n-6	19.9^{a}	18.0^{ab}	15.0^{b}	16.2^{b}	4.42	0.003	0.724	0.155
n-6/n-3	26.6^{ab}	24.9^{b}	28.3^{a}	28.3^{a}	4.46	0.025	0.412	0.439

^{a,b,c} Within a row, means without a common superscript differ ($P < 0.05$)

[1]MUFA: monounsaturated fatty acids; [2]PUFA: polyunsaturated fatty acids; [3]RMSE: Root Mean Square Error; [4]SFA: saturated fatty acids

$^{+}$Minor FA include: C 10:0, C 12:0, C 14:1, C 15:0, C 15:1, C 17:0, C 18:1 n-9 trans, C 18:2 n-6 trans, C18:3 n-6 cis, C 21:0, C 20:3 n-3 cis, C 20:5 n-3 cis

3.5. *Fatty acid composition in SM*

Minor changes were observed in SM due to dietary treatment (Table 34). The SFA and MUFA including their main FA were not modified by the reduction of the level

of protein or lysine in the diet (all $P > 0.1$). PUFA and n-6 were neither affected by dietary treatment (all $P > 0.1$). Nonetheless, n-3 FA and the main n-3 FA, the C 18:3 n-3 cis were reduced when pigs were fed the diets with the lowest protein level (all $P < 0.001$). An interaction between dietary protein and dietary lysine level was observed for 22:6 n-3 and the animals that received the HPLL diet had the lowest values. The reduction observed in the n-3 FA was accompanied by an increase in the ratio n-6/n-3.

Table 34: Effect of dietary protein and lysine level on FA composition in *semimembranosus* (SM) muscle

Protein	High		Low					
Lysine	High	Low	High	Low	RMSE[3]	Prot	Lys	Prot*Lys
C 14:0	1.00	1.02	0.97	0.99	0.150	0.381	0.503	0.987
C 16:0	20.7[ab]	20.1[b]	20.5[ab]	20.9[a]	0.996	0.296	0.692	0.052
C 16:1 n-7 cis	2.49	2.35	2.46	2.53	0.418	0.458	0.717	0.327
C 18:0	10.7	10.6	11.1	10.7	0.784	0.161	0.204	0.574
C 18:1 n-9 cis	32.9	34.9	33.3	33.8	4.18	0.774	0.230	0.452
C 18:1 n-7	3.80[a]	3.60[b]	3.78[a]	3.83[a]	0.252	0.098	0.223	0.040
C 18:2 n-6 cis	18.5	18.1	18.1	17.6	2.63	0.532	0.493	0.916
C 18:3 n-3 cis	0.55[b]	0.61[a]	0.44[c]	0.43[c]	0.076	<0.001	0.231	0.069
C 20:0	0.14	0.13	0.13	0.12	0.023	0.617	0.125	0.852
C 20:1 n-9 cis	0.51	0.54	0.51	0.53	0.086	0.822	0.279	0.758
C 20:2 n-6 cis	0.64[a]	0.63[a]	0.60[ab]	0.55[b]	0.087	0.006	0.185	0.359
C 20:3 n-6	0.51	0.48	0.54	0.54	0.108	0.088	0.561	0.539
C 20:5 n-3 cis	0.14[a]	0.13[ab]	0.12[b]	0.13[ab]	0.031	0.121	0.581	0.218
C 20:4 n-6	4.95	4.58	5.08	5.14	1.201	0.245	0.603	0.472
C 22:4 n-6	0.68[ab]	0.62[b]	0.72[a]	0.70[ab]	0.120	0.067	0.146	0.534
C 24:1 n-9 cis	0.17[ab]	0.19[a]	0.14[b]	0.15[b]	0.046	0.003	0.207	0.607
C 22:6 n-3 cis	0.07[a]	0.04[b]	0.06[ab]	0.07[a]	0.024	0.322	0.125	0.014
Minor FA[+]	1.55[a]	1.35[ab]	1.34[ab]	1.22[b]	0.316	0.030	0.040	0.615
SFA[4]	33.3	32.5	33.4	33.4	1.56	0.208	0.297	0.316
MUFA[1]	40.3	41.9	40.6	41.2	4.55	0.830	0.323	0.621
PUFA[2]	26.4	25.5	25.9	25.4	4.01	0.801	0.478	0.861
n-3	0.86[a]	0.89[a]	0.69[b]	0.69[b]	0.090	<0.001	0.431	0.451
n-6	25.5	24.6	25.2	24.7	3.97	0.947	0.462	0.847
n-6/n-3	29.7[b]	27.8[b]	36.9[a]	35.6[a]	4.75	<0.001	0.170	0.797

[a,b,c] Within a row, means without a common superscript differ ($P < 0.05$)

[1]MUFA: monounsaturated fatty acids; [2]PUFA: polyunsaturated fatty acids; [3]RMSE: Root Mean Square Error; [4]SFA: saturated fatty acids

[+]Minor FA include: C 10:0, C 12:0, C 14:1, C 15:0, C 15:1, C 17:0, C 17:1 n-7 cis, C 18:1 n-9 trans, C 18:2 n-6 trans, C18:3 n-6 cis, C 21:0, C 20:3 n-3 cis, C 20:5 n-3 cis, C 22:0

3.6. *Fatty acid composition in liver*

The effects of the level of protein and lysine are presented in Table 35. Total SFA were not affected by dietary treatment. Nonetheless, C 14:0 was increased when the level of dietary lysine was reduced ($P < 0.05$), C 16:0 had the highest values when both dietary protein and lysine levels were reduced ($P < 0.01$) and C 18:0 was reduced when the low level of dietary lysine was used ($P < 0.05$). MUFA including C 18:1 n-9 cis were increased in LPLL diets ($P < 0.05$ and $P < 0.001$, respectively). PUFA, n-6 were reduced in LPLL ($P < 0.001$), C 18:2 n-6 was reduced when the level of dietary protein was reduced ($P < 0.001$) and C 18:3 n-3 was reduced in the LPHL diets ($P < 0.001$). LPHL diets had the lowest percentage of n-3 FA ($P < 0.01$) which resulted in an increase in the ratio n-6/n-3 ($P < 0.01$).

3.7. *Fatty acid composition in LT subcutaneous fat*

Lipid percentage tended to increase in LT subcutaneous fat of animals fed the low protein diets ($P < 0.1$; Table 36). Dietary protein level was the parameter that most affected the fatty acid composition of subcutaneous fat. In diets with the lowest level of dietary protein the SFA and C 18:0 tended to increase ($P < 0.1$) but the main SFA (C 16:0) did not reach significance ($P > 0.1$). Additionally, MUFA and C 18:1 n-9 cis (both $P < 0.001$) were significantly increased by the same treatment. The reduction of dietary protein also produced a reduction of PUFA including both, n-3 (C 18:3 n-3; $P < 0.001$) and n-6 (C 18:2 n-6; $P < 0.001$). The C 20:4 n-6 was reduced in all the treatments with respect to the HPHL. The modification of the PUFA resulted in a higher n-6/n-3 ratio ($P < 0.001$).

Table 35: Effect of dietary protein and lysine level on FA composition in liver

Protein	High		Low					
Lysine	High	Low	High	Low	RMSE[3]	Prot	Lys	Prot*Lys
C 14:0	0.20^{b}	0.21^{ab}	0.19^{b}	0.25^{a}	0.056	0.329	0.023	0.094
C 16:0	12.9^{b}	13.5^{b}	13.7^{b}	14.6^{a}	1.18	0.002	0.015	0.548
C 16:1 n-7 cis	0.46^{ab}	0.42^{b}	0.45^{ab}	0.54^{a}	0.134	0.128	0.375	0.073
C 18:0	27.9^{a}	27.4^{ab}	27.7^{a}	26.7^{b}	1.34	0.167	0.030	0.347
C 18:1 n-9 cis	11.5^{b}	11.2^{b}	12.1^{b}	13.1^{a}	1.33	<0.001	0.234	0.067
C 18:1 n-7	1.35^{ab}	1.33^{b}	1.32^{b}	1.45^{a}	0.151	0.229	0.141	0.037
C 18:2 n-6 cis	17.0^{a}	17.0^{a}	16.0^{b}	15.8^{b}	1.15	<0.001	0.732	0.824
C 18:3 n-3 cis	0.33^{a}	0.34^{a}	0.24^{b}	0.29^{a}	0.074	<0.001	0.080	0.249
C 20:0	0.13^{ab}	0.13^{ab}	0.12^{b}	0.15^{a}	0.025	0.873	0.108	0.044
C 20:1 n-9 cis	0.18	0.18	0.18	0.19	0.024	0.180	0.675	0.833
C 20:2 n-6 cis	0.84^{a}	0.85^{a}	0.85^{a}	0.77^{b}	0.092	0.111	0.152	0.095
C 20:3 n-6	1.07^{a}	0.96^{ab}	0.90^{b}	0.73^{c}	0.233	0.001	0.023	0.593
C 20:4 n-6	19.9	19.8	20.0	19.3	1.44	0.671	0.278	0.387
C 22:4 n-6	1.30^{b}	1.29^{b}	1.66^{a}	1.68^{a}	0.191	<0.001	0.964	0.677
C 20:5 n-3 cis	0.41	0.41	0.38	0.39	0.092	0.283	0.592	0.824
C 24:1 n-9 cis	1.44^{b}	1.77^{a}	1.00^{c}	1.03^{c}	0.384	<0.001	0.063	0.113
C 22:6 n-3 cis	0.13^{ab}	0.13^{ab}	0.12^{b}	0.16^{a}	0.049	0.775	0.094	0.092
Minor FA^{+}	3.04	3.07	3.08	2.85	0.743	0.663	0.545	0.526
SFA[4]	43.0	43.1	43.6	43.2	1.05	0.228	0.572	0.310
MUFA[1]	15.5^{b}	15.5^{b}	15.7^{b}	16.9^{a}	1.48	0.029	0.089	0.111
PUFA[2]	41.5^{a}	41.4^{a}	40.7^{ab}	39.9^{b}	1.21	<0.001	0.111	0.273
n-3	1.31^{ab}	1.35^{a}	1.17^{b}	1.41^{a}	0.214	0.398	0.009	0.054
n-6	40.2^{a}	40.0^{a}	39.5^{a}	38.5^{b}	1.22	<0.001	0.042	0.153
n-6/n-3	31.9^{ab}	30.2^{b}	34.6^{a}	27.9^{b}	5.66	0.875	0.004	0.081

^{a,b,c} Within a row, means without a common superscript differ ($P < 0.05$)

[1]MUFA: monounsaturated fatty acids; [2]PUFA: polyunsaturated fatty acids; [3]RMSE: Root Mean Square Error; [4]SFA: saturated fatty acids

$^{+}$Minor FA include: C 10:0, C 12:0, C 15:0, C 15:1, C 17:0, C 17:1 n-7 cis, C 18:1 n-9 trans, C 18:2 n-6 trans, C18:3 n-6 cis, C 20:3 n-3 cis, C 20:5 n-3 cis, C 22:0, C 23:0, C 22:3 n-3

Table 36: Effect dietary protein and lysine level on lipid content and FA composition in LT subcutaneous fat

Protein	High		Low					
Lysine	High	Low	High	Low	RMSE[3]	Prot	Lys	Prot*Lys
Lipids, %	68.4	70.5	71.3	71.5	4.03	0.054	0.235	0.359
C 14:0	1.39^{ab}	1.31^{b}	1.43^{a}	1.42^{a}	0.147	0.054	0.217	0.314
C 16:0	20.2	19.9	20.7	20.1	1.50	0.381	0.259	0.739
C 16:1 n-7 cis	1.95^{a}	1.61^{b}	1.98^{a}	1.91^{a}	0.324	0.040	0.013	0.100
C 18:0	11.4^{b}	12.2^{ab}	12.3^{a}	12.4^{a}	1.16	0.055	0.172	0.220
C 18:1 n-9 cis	39.9^{b}	39.9^{b}	41.8^{a}	42.6^{a}	1.64	<0.001	0.343	0.314
C 18:1 n-7	2.29^{bc}	2.14^{c}	2.37^{ab}	2.45^{a}	0.224	<0.001	0.570	0.040
C 18:2 n-6 cis	17.8^{a}	17.8^{a}	14.9^{b}	14.4^{b}	1.82	<0.001	0.611	0.616
C 18:3 n-3 cis	1.24^{a}	1.26^{a}	0.77^{b}	0.75^{b}	0.150	<0.001	0.997	0.656
C 20:0	0.18	0.19	0.19	0.20	0.074	0.545	0.703	0.743
C 20:1 n-9 cis	0.78^{b}	0.80^{b}	0.90^{a}	0.90^{a}	0.123	0.001	0.747	0.742
C 20:2 n-6 cis	0.77^{a}	0.83^{a}	0.96^{b}	0.70^{b}	0.115	<0.001	0.212	0.295
C 20:3 n-6	0.10	0.08	0.08	0.06	0.072	0.204	0.345	0.800
C 20:4 n-6	0.44^{a}	0.39^{ab}	0.34^{b}	0.37^{ab}	0.116	0.036	0.670	0.162
C 22:4 n-6	0.19^{a}	0.09^{b}	0.10^{b}	0.08^{b}	0.093^{b}	0.028	0.014	0.121
Minor FA[+]	1.37	1.55	1.46	1.65	0.431	0.380	0.086	0.968
SFA[4]	33.9	34.2	35.3	34.7	2.19	0.069	0.783	0.391
MUFA[1]	45.3^{b}	45.1^{b}	47.5^{a}	48.6^{a}	1.81	<0.001	0.384	0.152
PUFA[2]	20.8^{a}	20.8^{a}	17.1^{b}	16.7^{b}	2.09	<0.001	0.643	0.728
n-3	1.36^{a}	1.41^{a}	0.84^{b}	0.85^{b}	0.180	<0.001	0.426	0.583
n-6	19.5^{a}	19.3^{a}	16.3^{b}	15.9^{b}	1.95	<0.001	0.569	0.745
n-6/n-3	14.6^{b}	13.7^{b}	19.5^{a}	18.8^{a}	2.04	<0.001	0.106	0.837

[a,b,c] Within a row, means without a common superscript differ ($P < 0.05$)
[1]MUFA: monounsaturated fatty acids; [2]PUFA: polyunsaturated fatty acids; [3]RMSE: Root Mean Square Error; [4]SFA: saturated fatty acids
[+]Minor FA include: C 10:0, C 12:0, C 15:0, C 17:0, C 17:1 n-7 cis, C 18:1 n-9 trans, C 18:2 n-6 trans, C18:3 n-6 cis, C 20:3 n-3 cis

4. Discussion

4.1. *Interaction between dietary parameters, protein and lysine*

The feed conversion ratio was negatively influenced in barrows fed HPLL diets during the first period and also reflected during the whole experiment. These animals needed to eat more to achieve the body weight of the group control, although the reduction in weight gain did not reach significance in our trial. These results suggest a marginal deficiency of this essential amino acid. The increase in FGR in animals fed HPLL and not in the animals fed LPLL, suggests that the relation between lysine and protein determines the deficiency of this amino acid, rather

than the level *per se*. Jin et al., (2010) and Witte, Ellis, McKeith, and Wilson (2000) also found an increase in feed to gain ratio without significant modification of ADFI or ADG when dietary lysine was reduced and the level of protein was kept at the same level as the control diet.

Backfat thickness, muscle depth, carcass lean percentage and carcass cuts, liver or perirenal fat proportions were not affected by the interaction between dietary protein and lysine levels. Because the interaction was not significant, the results obtained of those parameters are discussed below as separated effects.

The IMF percentage was increased with respect to the control diet in both muscles studied (SM and LT) when protein was reduced and the dietary lysine level was the same as the control (9.1 % and 16.1 %, respectively) and when dietary lysine level was reduced but the percentage of dietary protein was the same as the control (36.2 % and 15.2 %, respectively). This indicates that IMF is increased by the reduction of lysine or protein but not both at the same time indicating the reduction of both compounds has the opposite effect. Thus, if a higher amount of IMF is desired, one of the studied parameters (protein or lysine) has to be kept in a high amount while the other has to be reduced. Results also showed that IMF deposition is differently regulated in other fat depots because the interaction did not occur in backfat. The increase in IMF was also reported by authors who had reduced the level of dietary lysine keeping the level of dietary protein equal to the control diet (Bidner et al., 2004; Jin et al., 2010; Witte et al., 2000; Zhang, Yin, Zhou, Li, Ni, & Dong, 2008). More studies had reported an increase in IMF when the protein or lysine were reduced, but the two parameters were reduced in the same proportion (Conde-Aguilera et al., 2011; Costa et al., 2004; D'Souza et al., 2008; Gondret and Lebret, 2002; Wood et al., 2004). The increase in IMF observed in the present study may be of great interest because a previous study showed that the meat eating acceptability by consumers increases with the IMF content in meat (Font-i-Furnols et al., 2012). Additionally to the increase in IMF, the FA composition

of the different muscles studied, subcutaneous adipose tissue was not affected by the interaction between the dietary protein and lysine levels.

The lipid content of liver was reduced with respect to the control diet, when protein was reduced and the dietary lysine level was the same as the control and when dietary lysine level was reduced but the percentage of dietary protein was the same as the control by 63 % and 66 %, respectively. These results show that in barrows fed low protein high lysine or high protein low lysine diets, fat was preferentially deposited in the muscle and reduced in the liver. Additionally, almost no variation in the FA composition was observed in liver due to an interaction between protein and lysine.

4.2. *Reduction of dietary protein*

Dietary protein restriction while keeping lysine at requirement levels improved the feed conversion without impairment in LW mainly during the first period of the study suggesting that when protein is reduced but the level of lysine is maintained the growth of the animal is not retarded by protein restriction while the reduced cost of nitrogen excretion could be responsible for the improvement in feed efficiency. Le Bellego, Milgen, and Nobelet (2002) reported that barrows fed low protein diets (R2 and R3; R1: 19.7 %; R2: 15.3 % and R3: 16.4 % for growing phase all with a digestible lysine of 0.85 g/MJ NE and R1: 17.5 %; R2: 12.5 % and R3: 13.3 % for finishing phase all with a digestible lysine of 0.70 g/MJ NE) resulted in lower ADFI, and the differences could be explained because a higher percentage of protein was used compared with the present study.

The restriction of dietary protein caused an increase in backfat thickness at *Gluteus medius* and 1st lumbar vertebrae without modification of carcass lean percentage or a reduction of muscle depth. This increase in fat deposition was also reflected in an increase of lipids in the LT subcutaneous fat. A possible explanation for this increase in backfat could be that the replacement of the protein source of the diets by carbohydrates which are more easily converted to fat than dietary protein,

hence, the main pig fat depot was increased. Also, the reduced cost of nitrogen excretion would increase the energy available for fat synthesis. An increase in backfat thickness was observed when dietary protein was replaced by starch or dietary fat Lizardo, Bellego, Nobelet, Milgen and Mourot (2002).

The FA composition was mainly affected by the dietary protein reduction in LT and SM muscles, and subcutaneous fat. However, the LT muscle was more strongly modified than SM (only the n-3 FAs were reduced). From these results it could be concluded that the FA profile of fat (including IMF) from pigs fed diets with low protein level was more saturated and monounsaturated and less polyunsaturated compared with diets with high protein. The reduction of n-3 FA and n-6 FA observed in the subcutaneous fat could be explained because, although not significant, the animals fed low protein diets had a lower ADFI, hence a lower amount of those FA was incorporated through the diet. These results are in agreement with Doran, Moule, Teye, Whittington, Hallett, and Wood (2006) who also found an increase in C16:0, C16:1, C18:0 and C18:1 n-9 cis when dietary protein was reduced from 21 to 18% and who suggested an increase in *de novo* fatty acid synthesis in muscle under the experimental conditions. Teye, Sheard, Whittington, Nute, Stewart, and Wood (2006) also observed an increase in SFA and MUFA and a reduction of PUFA in subcutaneous fat of pig fed low protein diets (20.9% vs. 18.1%), however, the level of dietary lysine was also reduced, from 10 g/kg to 7g/kg.

It is fairly common to have excess protein in practical diets, particularly when the price of protein sources is low. Partially, this nitrogen excess is retained by the body (30%), excreted in the feces (15%) or excreted in the urine (50%; Dourmad, Guillou, & Noblet, 1992). Considering that protein catabolism takes place in the liver, the reduction of liver weight with respect to carcass weight in animals fed low protein could be a result of reduced nitrogen catabolism. The reduction of dietary protein to values closer to ideal protein, could lead the aminoacids in the right portions for maintenance, lean tissue growth and a reduction of nitrogen excretion. The

difference of liver size was also reflected in a modification of the FA profile. While SFA were not affected by reduction of dietary protein in liver, MUFA were increased and PUFA (mainly the n-6 PUFA) were reduced.

4.3. *Reduction of dietary lysine*

Backfat thickness and muscle depth were not significantly affected in animals fed diets with the low levels of dietary lysine. These results are opposite to those of Bidner, Ellis, Witte, Carr, and McKeith (2004) and Witte et al. (2000) who observed an increase in backfat thickness and a reduction of loin eye area. The differences between the reported results could be explained by the differences between the ratio protein/lysine, the level of other essential amino acids, and the net energy of the diets.

The level of dietary lysine had no effect on the FA composition of the different muscles studied (LT and SM) or subcutaneous fat, which is not surprising because the level of dietary lysine did not affect the proportions of fat and protein of the different tissues studied. However, Katsumata, Kobayashi, Matsumoto, Tsuneishi, and Kaji (2005) observed a reduction of PUFA including linoleic acid in the *longissimus dorsi* muscle of (Landrace x Large White) x Duroc pigs fed diets containing a lower level of dietary lysine (during the growing phase: C = 0.65 % *vs.* LL = 0.43 % and during finishing phase: C = 0.68 *vs.* LL = 0.40).

Dietary lysine level had no effect in the different carcass joints, liver or perirenal fat proportions. Although the liver lipid content was not affected by dietary lysine level, an increase in n-3 FA and a reduction of n-6 FA was observed even thought total PUFA were not affected.

The modification of one of the two parameters (protein or lysine) without keeping the other constant makes difficult to attribute the real cause of the modification observed in fat deposition. In the present study the experimental diets only differed in one of the parameters studied (lysine or protein level), allowing to

determine the effects of these factors independently and the interaction between them.

5. Conclusion

Based in the results obtained from this study it can be concluded that dietary protein and lysine level impact fat deposition and composition. The interaction between protein and lysine levels is relevant in terms of growth performance and meat quality. The IMF content was increased in both muscles studied (the LT and the SM) while the lipid content in the liver was reduced, when protein or lysine were independently reduced. However, reduction of dietary lysine in normal protein diets resulted in less efficient pigs and the reduction of protein, independently low or high lysine diets, produced an increase in pig fatness, accompanied with an increase in SFA and MUFA and a reduction of PUFA, which suggest an increase in *de novo* FA synthesis. Hence, the relation between dietary protein and lysine contents has to be considered in diet formulation for performance, meat and carcass quality.

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CHAPTER 5:

Protein, arginine and leucine

Chapter 5:

Effect of the addition of arginine and leucine in diets with different levels of protein in performance and carcass quality of finishing pigs

In this chapter the last experiment performed is presented. The genetic type was changed to a leaner one, Duroc x Landrace x Pietrain in order to evaluate if a low protein diet with the normal lysine showed the same effect on IMF in a leaner breed. Furthermore, two recent studies evaluating the effect of supplementation of arginine and leucine reported an increase of IMF content. Therefore, this study also evaluated the supplementation with each of the two amino acids. The reduction of protein in this genetic line had no effect on the IMF content, suggesting that the effect of dietary manipulations may vary depending on the genetic background of the animals or their ability to deposit fat. Supplementation of diets with leucine had no effect on IMF content, however, the supplementation with arginine produced a reduction of IMF and the whole animal fatness, contrary to the initial hypothesis. Leucine caused depressed performance in the low protein diet. Care should be taken when leucine is supplemented in a higher amounts in order to prevent an antagonism with the other branched chain amino acids.

EFFECT OF THE ADDITION OF ARGININE AND LEUCINE IN DIETS WITH DIFFERENT LEVELS OF PROTEIN IN PERFORMANCE AND CARCASS QUALITY TRAITS OF FINISHING PIGS

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ABSTRACT: This study was conducted to test the effect of adding arginine (Arg) and leucine (Leu) to diets for finishing pigs differing in protein level in terms of performance and fat deposition, especially intramuscular fat (IMF). One-hundred and eight castrated pigs (Landrace *x* Duroc) *x* Pietrain were randomly assigned to one of six experimental diets (n=18). Diets were divided in two groups in terms of protein level. Four diets were control (16 % CP from 60-90 kg LW; 13 % CP from 90-115 kg LW), one without the addition of the amino acids (C), one with 1% Arg (CA), one with 2% Leu (CL) and the other with 1% Arg and 2% Leu (CAL). The other two were low protein (14 % CP from 60-90 kg LW; 11.8 % CP from 90-115 kg LW), one without the addition of amino acids (LP) and the other with 1% Arg and 2% Leu (LPAL). Glutamic acid was added in replacement of Arg or Leu to maintain the protein level constant. Animals fed LPAL had a reduction of ADFI ($P<0.05$), ADG ($P<0.01$) and an increase of FGR ($P<0.1$) compared with the other diets suggesting an antagonism of leucine with the other branched chain amino acids. Animals fed CA or CL tended to have increased ADG suggesting that these amino acids were limiting in the C diet. Backfat thickness and belly were reduced when arginine was added or protein was reduced, while lean meat percentage and tenderloin (only for low protein diets) were increased. Marbling and IMF content in loin were reduced when the arginine was added to the diet, contrary to the initial hypothesis. The other treatments did not affect the IMF content, even when protein was reduced.

Keywords: amino acid, intramuscular fat, subcutaneous fat, loin depth

INTRODUCTION

One of the characteristics that influence the quality of meat as perceived by the consumer is the amount of intramuscular fat (IMF), the fat located within the structure of muscle, which in terms of taste is well correlated with pork acceptability (Font-i-Furnols et al. 2012). The threshold level of IMF needed for optimal eating quality proposed in the study adobe mentioned is between 2.2 and 3.4 %. In recent years, the trend has been to increase the carcass lean content reducing backfat and consequently IMF. Some studies indicate that for some modern genotypes IMF can be as low as 1% (Channon et al 2001). The challenge for nutritionists is to increase the level of IMF without increasing the amount of backfat.

In a previous study, using a breed with a high predisposition to fat deposition *(DurocxLandreace),* dietary protein or lysine reduction produced an increase of IMF (Tous et al. submitted). Some recent studies have shown that supplementing diets with leucine (leu) or arginine (arg) can also be achieved an increase of IMF. Hyun et al. (2007) observed that the supplementation of pig diets with 2% leucine increased significantly IMF in *longisimus dorsi* by 42 %. In addition, Tan et al. (2009) observed that the supplementation with 1% arginine increased IMF content by 70 % and skeletal muscle content by 5.5 % while reducing the body fat content by 11%. Leucine as a branched-chain amino acid (BCAA) is essential and therefore must be continuously available for protein synthesis. Nevertheless, leucine is special among the BCAAs. Leucine promotes global protein synthesis by signaling an increase in translation, promoting insulin release and inhibiting autophagic protein degradation. However, the leucine's effects are self-limiting because leucine promotes its own disposal by oxidative pathway, thereby terminating its positive effects on body protein accretion (Harris et al. 2003). On the other hand, recent

studies have demonstrated that dietary arginine may also stimulate protein synthesis in young pigs while increasing fat accretion (Kim and Wu, 2004; Yao et al. 2008). Those modifications may be due to the evidence that arginine regulates the metabolism of energy substrates (fatty acids, glucose, amino acids) partly through production of nitric oxide (NO; Tan et al. 2009).

The objective of this study was to determine if using a leaner genotype, reduction of dietary protein also increases IMF as in a fatter genotype. In addition, to test whether the addition of arginine or leucine to the diet can also increase the IMF content.

MATERIAL AND METHODS

The experiment was conducted in compliance with the Spanish guidelines for human care and use of animals in research and the protocol was approved by the Ethical Animal Committee of IRTA.

Animals and diets

One hundred and eight 67±4 kg life weight (LW) (*Landrace x Duroc) x Pietrain* barrows chosen from a larger group were blocked by weight avoiding the effect of maternal origin and were housed in adjacent pens (3 pigs per pen). Pigs were randomly assigned to one of the dietary treatments up to 107±7 kg LW: control diet (C), control diet + 1 % arg (CA), control diet + 2 % leu (CL), control diet + 1 % arg + 2 % leu (CAL), low protein diet (LP), low protein + 1 % arg + 2 % leu (LPAL). Diets were based on corn and soybean 44%. With the aim of preserving the same nitrogen content between diets, glutamic acid replaced the additions of arg or leu (Table 37 and Table 38). During the experimental period, pigs received the diets for *ad libitum* consumption and had free access to water.

Table 37: Ingredient composition of experimental diets

	1st period (60-90Kg)						2nd period (90-115kg)					
Ingredient (%)	C	CA	CL	CAL	LP	LPAL		CA	CL	CAL	LP	LPAL
Corn	72.9	72.8	71.8	72.8	77.0	77.2	78.6	78.7	77.8	78.7	81.1	81.3
Soyabean meal 44%	18.7	18.7	18.7	18.7	11.3	11.3	11.3	11.4	11.3	11.4	6.97	7.04
Glutamic	3.20	1.57	1.75	-	4.13	-	2.70	1.14	1.60	-	3.20	-
Leucine	-	-	1.40	1.39	-	1.56	-	-	1.03	1.02	-	1.12
Arginine	-	0.60	-	0.60	-	0.86	-	0.53	-	0.53	-	0.68
Starch	2.55	3.67	3.62	3.84	4.31	5.81	4.80	5.61	5.58	5.74	5.69	6.77
Dicalcium phosphate	0.78	0.78	0.78	0.78	0.87	0.87	0.63	0.63	0.64	0.63	0.69	0.68
Calcium carbonate	0.55	0.55	0.55	0.55	0.55	0.55	0.57	0.57	0.57	0.57	0.57	0.57
Lard	0.50	0.50	0.50	0.51	0.50	0.51	0.50	0.50	0.50	0.50	0.50	0.50
Mineral-vitamin premix	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Sodium chloride	0.33	0.33	0.32	0.33	0.05	0.05	0.02	0.02	0.02	0.02	-	-
L-lysine HCl	0.08	0.08	0.08	0.08	0.29	0.29	0.12	0.12	0.12	0.12	0.25	0.24
L- threonine	-	-	-	-	0.08	0.08	-	-	-	-	-	-
Sodium bicarbonate	0.03	0.03	0.04	0.03	0.43	0.43	0.35	0.34	0.35	0.34	0.58	0.58
DL- methionine	-	-	-	-	0.02	0.02	-	-	-	-	-	-
L-tryptophan	0.01	0.01	0.01	0.01	0.04	0.04	0.02	0.01	0.02	0.01	0.04	0.04

[a] One kg of feed contains: 5,000 IU vitamin A; 1,000IU vitamin D_3; 15mg vitamin E; 1.3mg vitamin B_1; 3.5mg vitamin B_2; 0.025mg vitamin B_{12}; 1.5mg vitamin B_6; 10mg calcium pantothenate; 15mg nicotinic acid; 0.1mg biotin; 0.6mg folic acid; 2mg vitamin K; 80mg Fe; 6mg Cu; 0.75mg Co; 60mg Zn; 30mg Mn; 0.75mg I; 0.10mg Se; 0.125g Ethoxiquin.
ME: Metabolizable Energy

Table 38: Nutritional composition of the experimental diets

	1st period (60-90Kg)						2nd period (90-115kg)					
	C	CA	CL	CAL	LP	LPAL	C	CA	CL	CAL	LP	LPAL
Net energy (kcal/kg)	2400	2400	2400	2400	2400	2400	2400	2400	2400	2400	2400	2400
Crude Protein (%)	16.0	16.0	16.0	16.0	14.0	14.0	13.0	13.0	13.0	13.0	11.8	11.8
Digestible amino acids (g/kg)												
Lys	6.50	6.50	6.50	6.50	6.50	6.50	5.20	5.20	5.20	5.20	5.20	5.20
Leu	12.2	12.2	26.0	26.0	10.5	26.0	10.6	10.7	20.8	20.8	9.64	20.8
Arg	8.15	13.0	8.12	13.0	6.08	13.0	6.11	10.4	6.10	10.4	4.89	10.4
Thr	4.55	4.55	4.52	4.55	4.36	4.36	3.60	3.63	3.59	3.62	3.46	3.46
Met	2.18	2.18	2.16	2.18	1.99	1.99	1.85	1.86	1.84	1.86	1.64	1.65
Trp	1.28	1.28	1.28	1.28	1.29	1.29	1.03	1.03	1.03	1.03	1.03	1.03
Ile	5.24	5.24	5.21	5.24	4.04	4.05	4.07	4.10	4.06	4.10	3.37	3.39
Val	6.02	6.02	5.98	6.02	4.82	4.82	4.86	4.89	4.84	4.89	4.16	4.18
Phe	6.31	6.30	6.27	6.30	5.01	5.02	5.06	5.09	5.04	5.09	4.30	4.33
His	3.51	3.51	3.49	3.51	2.83	2.83	2.85	2.87	2.84	2.87	2.45	2.47

[b] Calculated values according to INRA tables (Sauvant D., et al. 2004)

Slaughter conditions

Animals were transported to the IRTA experimental abattoir (2 h) in two different days (day 56 or 70) and they stayed approximately 16h fasting time before slaughter but with free access to water. Pigs were weighed and slaughtered minimizing the stress using standard *ante mortem* procedures and stunned with 85% CO_2 for 120 s using CO_2 Dip Lift (Butina, Alps, Copenhaguen, Denmark). Once carcasses were eviscerated, they were split longitudinally and weighed before 45min and perirenal fat and liver were weighed.

Carcass quality measurements

Fat and muscle thickness were measured with the Fat-O-Meat'er (Carometec A/S, Herlev, Denmark) between the 3rd and the 4th last ribs at 6 cm of the midline. From these measurements, lean meat percentage was calculated using the Spanish official equation (Lean percentage (%) = 66.91 – 0.895 * backfat thickness + 0.144 * muscle depth; Font-i-Furnols, & Gispert, 2009). In addition, backfat thickness at the last rib at 6 cm of the midline and between the 3rd and 4th lumbar vertebrae at 8 cm of the midline were determined with the same probe. The minimum fat thickness over the muscle *Gluteus medius*, fat thickness in the cranial position of the 1st lumbar vertebrae and in the shoulder at the level of first rib were measured with a ruler over the carcass midline.

After carcass refrigeration at 3 ºC for 24 h, carcasses were weighed. Carcass length was measured as the distance between the recess of the first rib and the anterior edge of the symphesis pubic and loin length as the distance from the atlas bone to the first lumbar vertebrae. Then, the left side of each carcass was cut following a simplified European reference method (Walstra, & Merkus, 1995). The primary joints obtained were: ham (included hind shank and hind foot), loin, belly (included the ventral part of the belly and jowl), shoulder (with front shank, front foot and neck), tenderloin and head (with cheek). Each joint was weighed and their

proportion with respect to cold carcass weight was calculated. Furthermore, the *semimembranosus* muscle (**SM**) was separated from the ham and the *longissimus thoracis* (**LT**) muscle and subcutaneous fat (with skin) from the loin, and their respective weights were recorded. The IMF content was determined with a near infrared transmitter (NIT, Infratec, TACATOR, Denmark).

Muscle pH was measured in the left carcass side using a Crison portable meter (Crison, Barcelona, Spain) equipped with a xerolyt electrode in the LT at the last rib level and in SM muscles at 45 min (**pH45**) and at 24 h (**pHu**) *post mortem*. Electrical conductivity was measured in the carcass using Pork Quality Meater (PQM-Kombi, Aichach, Germany) also at the last rib level in the LT and SM muscles at 24 h *post mortem*.

Instrumental colour (CIE, 1976) was measured at 24 h *post mortem* at the level of the last rib on a cross section of the LT muscle after 15 min of bloom time with Colorimeter CR-400 (using D65 as illuminant and standard observer of 10º; Minolta Co., Ldt., Osaka, Japan) and luminosity (L*), redness (a*) and yellowness (b*) were recorded. Drip loss was obtained from the LT muscle according the methodology described by Rasmussen and Andersson (1996).

Statistical Analysis

A preliminary statistical evaluation was performed using GLM procedure of SAS (SAS System for Windows 9.2) with initial weight as covariable and day of slaughter as block for the carcass quality measurements. The LPAL treatment presented a large standard deviation compared to the other treatments, indicating that variance was not homogeneous for all treatments. Consequently, it was eliminated from the overall data set. A student t-test for not homogeneous variances was conducted to compare the LP and LPAL treatments. For the five remaining dietary treatments (C, CA, CL, CAL, and LP), the different variables studied were analyzed by two way ANOVA with 6 blocks corresponding to body weight and location within the house. Differences among treatment means were investigated with a set of

contrasts in four different comparisons: C + CAL *vs*. CA + CL (evaluating interaction between arginine and leucine: Int), CA + CAL *vs*. C + CL (evaluating the effect of supplementing arginine: Arg), CL + CAL *vs*. C + CA (evaluating the effect of supplementing leucine: Leu) and C *vs*. LP (evaluating the effect of reducing dietary protein: Prot). Differences were considered significant at $P < 0.05$ and $P < 0.1$ was considered as tendency.

RESULTS AND DISCUSSION

Animals fed CA and CL diets tended ($P < 0.1$; Table 39) to have a higher final weigh and average daily gain (ADG). Results suggest that these two amino acids may be limiting because when they were independently added to the diet an increase of ADG was observed. However, when both arginine and leucine were added in excess in normal protein diets ADFI and ADG were slightly, but not significantly reduced, which suggests an imbalance of amino acids as ADFI was reduced without affecting feed efficiency. Furthermore, the addition of the amino acids, arginine and leucine in the low protein diets reduced the ADFI (average daily feed intake; $p<0.05$), reducing ADG ($p<0.01$), final weight ($p<0.01$) and tending to increase the FGR (feed to gain ratio, $p<0.1$). A possible explanation of the effects of both amino acids in the low protein group may be an antagonism between leucine and other branched chain amino acids (BCAA). In that case, leucine may be in excess while the other amino acids (isoleucine and valine) may become limiting, as leucine increases activity of enzymes involved in BCAA catabolism (Oestmer and Handson, 1973). Hyun et al. 2003 reported reduction of ADG when leucine was supplemented in the diet, which may be also due to a BCAA antagonism caused by leucine, as in the LPAL diet in our experiment.

Table 39: Means and root mean-square (RMSE) of performance parameters

	C	CA	CL	CAL	LP	LPAL	RMSE	Sig* Int[1]	Sig* Arg[2]	Sig* Leu[3]	Sig* Prot[4]	LP *vs*. LPAL
Final weight (Kg)	107.7	109.0	110.6	106.9	110.0	98.2	3.49	0.083	0.519	0.978	0.192	0.006
ADFI (Kg/day)	2.86	2.86	2.95	2.77	2.94	2.47	0.21	0.149	0.183	0.964	0.331	0.020
ADG (Kg/day)	0.91	0.95	0.97	0.90	0.97	0.70	0.08	0.072	0.538	0.958	0.179	0.001
FGR	3.13	3.02	3.06	3.08	3.03	3.56	0.26	0.326	0.560	0.942	0.334	0.056

C: control; CA: control + arginine; CL: control + leucine; CAL: control + arginine + leucine; LP: low protein; LPAL: low protein + arginine + leucine; ADFI: average daily feed intake; ADG: average daily weight gain; FGR: feed to gain ratio; RMSE: root means square

[1] C and CAL *vs*. CA and CL

[2] C and CL *vs*. CA and CAL

[3] C and CA *vs*. CL and CAL

[4] C *vs*. LP

In order to avoid the animal weight effect all animals were slaughtered at similar body weight. A significant reduction ($P < 0.05$, Table 40) of backfat thickness was observed on the 3^{th}-4^{th} last ribs by effect of arginine supplementation or on the last rib by effect of protein reduction. A tendency to reduce the minimum backfat thickness over the muscle *Gluteus medius* was observed in LPAL fed animals compared with the fed the LP diet ($P < 0.1$). Muscle depth at 3^{th}-4^{th} last ribs showed a significant interaction between Arg and Leu, as it was increased in CA and CL diets with respect to C and CAL. This parameter was also reduced in LPAL ($P < 0.05$) with respect to LP. Lean meat percentage was significantly increased in CA ($P < 0.05$) respect to C and CL. This parameter and tended to be increased in LP ($P < 0.1$) respect to C. Tan et al. 2008 also found an increase of the muscle and reduction of backfat in the carcass and they attributed this change to the effect of arginine on NO metabolism.

A reduction of pH and increase of conductivity and drip loss was observed in LPAL diets as compared to LP in both muscles studied, LT and SM. Leucine (CL and CAL) also reduced pH and increased conductivity in LT accompanied by an increase of drip loss with respect to the C and CA diets suggesting that meat from animals fed these treatments was more exudative. Marbling or IMF were not affected by reduction of dietary protein, which was different from what was observed in the previous experiment with the Duroc x Landrace cross, suggesting that the genetic background influences the changes in IMF and fat deposition as affected by dietary factors. Furthermore, contrary to what was expected, addition of arginine (CA and CAL) produced a slight reduction of IMF ($P < 0.1$) or marbling ($P < 0.05$) with respect to C and CL. These results are opposite to the results of Hyun et al. (2003) and Tan et al. (2008) when supplementing diets with 2 % leu or 1 % arg, respectively, which showed an increase of IMF. Color was also affected by dietary treatment, addition of arginine (CA and CAL *vs.* C and CL) or reduction of protein (LP *vs.* C) produced meat with a lower L* ($P < 0.01$) and b* ($P < 0.05$ and $P < 0.1$, respectively), suggesting a more pale color, while the supplementation with leucine (CL and CAL

vs. C and CA) or both amino acids in low protein diets (LP *vs.* LPAL) produced an increase of a* ($P < 0.01$).

An interaction between Leu and Arg was found for liver which was reduced ($P < 0.01$), and a trend ($P < 0.1$) to reduce head and increase ham was observed in animals fed CA and CL *vs.* C and CAL (Table 41). Supplementation with arginine (CA and CAL *vs.* C and CL) tended to increase shoulder ($P < 0.1$) and significantly reduced ($P < 0.05$) the belly (join with a high fat content). Reduction of dietary protein (LP *vs.* C) also tended to reduce belly ($P < 0.1$) and increased tenderloin ($P < 0.05$). The remaining carcass joints were not affected by dietary treatment.

In conclusion, the results from present experiment did not confirm the results previously obtained by Tan et al. (2008) and Hyun et al. (2003) in terms of IMF. In the conditions of the present study the addition of Leu in pig finishing diets with normal or low protein level did not increase the IMF content. However, the addition of arginine in normal protein diets produced a reduction of IMF and marbling, contrary to what was expected. Protein reduction did not increase the IMF content, suggesting that leaner genotypes (containing Pietrain) do not respond to dietary modifications. It is possible that differences between this experiment and those of Tan et al. (2008) and Hyun et al. (2003), in which supplemental arginine and leucine caused an increase of IMF deposition, can be explained by the genetic origin of the pigs. Furthermore, arginine seems to reduce the whole animal fatness an increase the lean content. Attention must be paid when leucine is added in low protein diets in order to avoid the possible antagonism between leucine and the other two BCAA and have negative effects on performance.

Table 40: Effect of dietary arginine, leucine and protein level on carcass characteristics, *longissimus thoracis* (LT) and *semimembranosus* (SM) meat quality parameters

	C	CA	CL	CAL	LP	LPAL	RMSE	Sig* Int[1]	Sig* Arg[2]	Sig* Leu[3]	Sig* Prot[4]	LP *vs.* LPAL
Carcass characteristics												
Carcass yield, %	82.1	82.1	82.7	82.1	83.0	82.4	1.53	0.419	0.526	0.458	0.090	0.059
Chilling losses, %	2.21	2.14	2.18	2.18	2.19	2.23	0.11	0.236	0.223	0.902	0.673	0.505
Carcass length, cm	83.7	83.1	83.0	82.0	83.0	81.4	2.212	0.685	0.117	0.121	0.401	0.008
Loin length,cm	85.9	85.2	84.5	82.9	84.4	83.3	3.084	0.555	0.109	0.019	0.156	0.216
Backfat thickness, mm												
3^{th}-4^{th} last ribs[a]	20.2	18.5	20.3	18.9	18.7	19.9	3.42	0.907	0.046	0.735	0.227	0.376
Last rib[a]	18.0	16.2	16.4	16.4	15.5	17.0	3.23	0.244	0.213	0.383	0.031	0.319
3^{th}-4^{th} lumbar vertebrae[b]	23.2	20.9	22.0	22.2	21.1	20.8	4.14	0.223	0.218	0.974	0.145	0.238
Gluteus medius[f, d]	19.2	18.8	19.5	18.7	18.2	17.1	3.54	0.722	0.422	0.922	0.456	0.096
1^{st} lumbar vertebrae[c]	23.1	23.9	22.4	22.4	21.9	21.1	4.82	0.646	0.767	0.318	0.555	0.207
1^{st} rib, mm[c]	35.7	36.8	35.9	36.9	35.9	36.2	3.86	0.922	0.247	0.846	0.849	0.271
Muscle depth, mm												
3^{th}-4^{th} last ribs[a]	59.8	62.4	61.1	59.5	61.3	56.9	4.16	0.034	0.655	0.482	0.265	0.015
Lean meat percentage, %[e]	57.3	59.4	57.5	58.5	59.5	57.3	3.15	0.546	0.037	0.702	0.054	0.926
Semimembranosus												
pH 45 min	6.35	6.23	6.24	6.27	6.29	6.21	0.180	0.112	0.356	0.458	0.362	0.027
pHu	5.48	5.48	5.46	5.48	5.51	5.47	0.071	0.589	0.525	0.590	0.342	0.001
ECu, mS	7.22	7.81	7.72	8.68	7.00	8.54	1.789	0.686	0.081	0.129	0.765	0.759
Longissimus thoracis												
pH 45 min	6.25	6.14	6.11	6.14	6.21	6.09	0.156	0.062	0.245	0.057	0.515	0.004
pHu	5.47	5.48	5.45	5.48	5.47	5.45	0.067	0.578	0.267	0.359	0.582	0.053
ECu, mS	4.79	5.50	6.02	5.70	5.17	6.67	1.507	0.149	0.642	0.033	0.401	0.069
Drip loss, %	4.16	4.32	5.59	4.87	3.73	4.91	1.896	0.315	0.593	0.030	0.478	0.088
Marbling	2.20	1.86	2.13	1.87	1.97	1.83	0.632	0.862	0.032	0.814	0.328	0.291
IMF, %	2.23	1.87	2.28	2.16	1.99	2.19	0.576	0.425	0.057	0.184	0.248	0.228
Colour												
Lightness, L*	49.1	48.2	49.2	47.5	47.3	47.1	2.125	0.425	0.008	0.463	0.008	0.644
Redness, a*	6.97	6.91	7.33	7.76	7.25	7.71	0.913	0.262	0.403	0.009	0.391	0.008

	C	CA	CL	CAL	LP	LPAL	RMSE	Sig* Int[1]	Sig* Arg[2]	Sig* Leu[3]	Sig* Prot[4]	LP *vs*. LPAL
Yellowness, b*	1.64	1.31	1.86	1.44	1.23	1.35	0.716	0.740	0.015	0.300	0.055	0.785

C: control; CA: control + arginine; CL: control + leucine; CAL: control + arginine + leucine; LP: low protein; LPAL: low protein + arginine + leucine; ECu: ultimate electrical conductivity (24 h *post mortem*); pHu: ultimate pH (24 h *post morem*); RMSE: Root Mean Square Error.

[1] C and CAL *vs*. CA and CL

[2] C and CL *vs*. CA and CAL

[3] C and CA *vs*. CL and CAL

[4] C *vs*. LP

[a] Measurement done at 6 cm to the carcass midline with Fat-O-Meat'er.

[b] Measurement done at 8 cm to the carcass midline with Fat-O-Meat'er.

[c] Measurement done on the carcass midline with a ruler.

[d] Minimum fat thickness over the muscle.

[e] Calculated from backfat thickness and loin depth between the 3th and 4th using the Spanish official equation (Lean percentage (%) = 66.91 - 0.895 * backfat thickness 3th-4th last ribs + 0.144 * muscle depth 3th-4th last ribs; Font i Furnols & Gispert, 2009).

Table 41: Effect of dietary arginine, leucine and protein level on the weight of some carcass cuts, liver and perirenal fat

	C	CA	CL	CAL	LP	LPAL	RMSE	Sig* Int[1]	Sig* Arg[2]	Sig* Leu[3]	Sig* Prot[4]	LP *vs.* LPAL
g/kg LW												
Liver	14.3	13.8	13.5	14.4	14.0	14.2	1.06	0.008	0.413	0.840	0.519	0.433
Perirenal Fat	8.85	8.78	9.04	8.06	9.00	8.37	1.71	0.203	0.147	0.528	0.670	0.386
g/kg carcass[a]												
Head	75.1	73.9	73.0	75.6	75.8	78.6	4.73	0.089	0.539	0.901	0.713	0.206
Shoulder	276	277	273	279	281	282	8.1	0.153	0.095	0.863	0.112	0.875
Belly	147	139	141	139	142	140	8.9	0.173	0.016	0.149	0.089	0.158
Tenderloin	13.2	13.7	14.2	13.6	14.3	13.9	1.43	0.170	0.898	0.227	0.045	0.262
Ham	312	319	316	314	314	309	9.7	0.073	0.259	0.859	0.544	0.667
SM muscle	29.5	31.1	30.6	30.5	30.4	28.9	2.59	0.168	0.221	0.637	0.311	0.995
Loin	173	173	178	173	173	172	9.0	0.321	0.342	0.184	0.912	0.254
LT muscle	62.2	64.3	63.4	64.1	64.3	61.7	4.91	0.622	0.147	0.707	0.236	0.633
Backfat + skin	48.6	50.6	50.0	46.5	46.1	46.1	10.8	0.284	0.736	0.620	0.537	0.354

C: control; CA: control + arginine; CL: control + leucine; CAL: control + arginine + leucine; LP: low protein; LPAL: low protein + arginine + leucine; LW: Live Weight; LT: *Longissimus thoracis* muscle; SM: *Semimembranosus* muscle; RMSE: Root Mean Square Error.

[1] C and CAL *vs*. CA and CL

[2] C and CL *vs*. CA and CAL

[3] C and CA *vs*. CL and CAL

[4] C *vs*. LP

[a]Carcass joints following the European reference method (Walstra, & Merkus, 1995) avoiding some of the cuts: ham (included hind shank and hind foot), loin, belly (included the ventral part of the belly and jowl), shoulder (with front shank, front foot and neck), tenderloin and head (with cheek).

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DISCUSSION

GENERAL DISCUSSION

The principal objective of the study was to increase the amount of fat in muscles (IMF) with the aim of improving the meat quality traits perceived by the consumer. Several decades ago selection of pigs started to concentrate more against carcass fatness. This occurred for several reasons: the first reason was economic, to improve animal feed efficiency in order to obtain carcasses with a higher lean percentage which have a higher economical value, and the second but not less important because a high proportion of saturated fat (provided from animal food) was associated with a risk for human health in terms of coronary diseases. The genetic selection against carcass fatness also resulted in a reduction of IMF content, which is closely associated to meat quality traits. Pork with a high IMF content is associated with significantly higher juiciness and flavor scores of pork (Fernandez et al., 1999a) while low IMF is associated with more tough meat. Some authors ventured to suggest a minimum limit of IMF to ensure a pleasing eating experience for consumer. Those values move from 1 to 4 % of IMF (Gandemer et al., 1990; Wood, 1990). However, the new genotypes obtained in the process of selection normally have values below 1 %, which negatively affects meat quality sensory attributes. For this reason, researchers working in animal nutrition proposed several nutritional attempts to increase these parameters.

In the first study of our group (Chapter 1) the visual and eating preference of loin IMF was evaluated for a representative group of Spanish population. The results showed two differentiated segments of consumers with opposite opinions (ones preferring marbling and ones preferring lean) from the point of view of purchasing decision. However, according their acceptability scores all consumers preferred the loins with higher IMF content. According the results obtained it appears that to ensure good taste values, between 2.2 and 3.4 % IMF are necessary.

This study demonstrated that almost half of the Spanish consumers do not realise that fat is necessary to achieve a good eating experience, suggesting that

marketing strategies informing of the importance of marbling are required to help consumers to improve their pork eating experience. Furthermore, as IMF is related with pork eating quality different strategies in order to increase this parameter should be evaluated.

In this thesis, some dietary manipulations are evaluated in different studies (Chapters):

1. CLA supplementation (Chapter 2)

Since CLA was recognized as a fat reducer nutrient, up to 30 studies have been performed in swine in order to evaluate the effect of CLA on pig fat deposition, some were more focused on subcutaneous fat while some also evaluated the fat content in the muscle (more than 15 studies reviewed in the introduction). Despite the large amount of studies published, discrepancies in the effect CLA on backfat and IMF are found. It is difficult to find the factor which causes the difference between studies because an increase or no effect of CLA on IMF are found in different genders (barrows/females), genetic origins (from Duroc crosses which are more prone to accumulate fat to Landrace or Large White which are more prone to accumulate muscle), different weights range (from starting at 20 – 60 kg and ending at 90-130 kg), fat sources in the control diet (mainly soybean oil, sunflower oil), different ingredients or nutritional composition in the diet formulation (barley, wheat or corn as principal ingredients), or amount of CLA included in the diet. In order to test out if the discrepancy between studies is due to an insufficient inclusion of CLA, a high level of CLA was used to maximize the possible effect.

The supplementation of pig diets with CLA produced several metabolic changes. From those, animal fat deposition was affected in the principal fat tissue depots but even at the high dose of CLA used, IMF content was not affected. The most internal depot (perirenal fat) was more reduced than the most external fat depot -subcutaneous fat- when a high dose of CLA was incorporated in pig diets. The supplementation of diets with CLA also resulted in an increase of CLA isomers in meat, although the highest incorporation was in subcutaneous fat, both muscles

studied contained values around 2 %, which may be of interest for the production of functional food. However, it has to be considered that the two main isomers incorporated in the diet were not equally incorporated in the tissues being the 9c,11t the one incorporated with the highest rates. Supplementation of pig diets with CLA also resulted in a wide modification of the FA composition and the expression of genes related with lipid metabolism in the different tissues. An increase of SFA was observed in all tissues while MUFA, PUFA were reduced in a and tissue specific manner, as the modification of the regulation of the genes related with lipid metabolism by effect of CLA. The increase of saturation when the MUFA content was reduced by CLA may be associated to a down-regulation of SCD in liver, and several reports also demonstrated an inhibition of this enzyme in different tissues (Lee et al., 1998). Furthermore, the elongation or desaturation of n-3 and n-6 FA may be altered by the competence among linolenic, linoleic and the elongated or desaturated CLA products. Studying the different lipid fractions in *longissimus* muscle showed that CLA modification of SFA and MUFA mainly occurred in the neutral lipid fraction while the modification of PUFA mainly occurred in the polar lipid fraction. Liver weight was increased but its chemical composition was not affected. When gene expression was analyzed, several genes normally used as control (HPRT1, GAPDH) were also affected by CLA treatment in some tissues. Despite all these changes, the results obtained did not always correlate well with the phenotypic characters, which suggests that the role of CLA may go beyond regulation of lipid metabolism and that other metabolic regulations i.e. post-transcriptional modifications (modulation of different enzyme activities) micro RNAs or apoptosis may occur. Further research needs to be done because although there are several studies on the effect of CLA, it is still difficult to achieve a conclusion of how it is acting.

This study demonstrated that even the inclusion of a high dose of CLA (4%) in Duroc x Landrace pig diets does not increase IMF content, while a slight reduction of body fatness was observed. Supplementation of pig diets with CLA may be an

alternative to supplement meat with this product and the changes occurred in FA composition may be helpful for meat processed products. Further research to understand the role of CLA in the different organisms needs to be done i.e. doing exhaustive studies of transcriptomics (microRNA) and proteomics (evaluating protein activities) because it seems that CLA is acting in different aspects of the metabolism in a tissue specific manner.

2. Reduction of the level of vitamin A (Chapter 3)

An increase of IMF content was reported by reduction of inclusion of vitamin A in the diet in beef cattle studies, while few studies have been conducted in pigs, the same promising results seem to occur (Table 7). To test the effect of the reduction of vitamin A level three levels were chosen: (1) using the amount in the vitamin premix normally used in pig diets, (2) one that includes the amount of vitamin A close to the requirements proposed by (NRC, 1998) and (3) the last diet without supplementation (animals only could convert the β-carotene present in the diet to obtain the retinol). Furthermore, the start of the experiment was at an early stage (20 kg LW) with the aim of avoiding the accumulation of vitamin A in the liver to overlap with the results. Contrary to what was expected from the initial hypothesis, the animals that received diets with no supplemental vitamin A had the lowest values for fat deposition, and as occurred for CLA, the difference was significant in perirenal fat, but no significant in the backfat thickness measurements or IMF in *longissimus* muscle. Furthermore, the depletion of dietary vitamin A did not negatively affect the growth performance parameters. This study clearly shows that dietary vitamin A level do not behave in the same way as in beef cattle (Siebert et al., 2006; Gorocica-Buentfil et al., 2007 (2); Arnett et al., 2009), where the reduction of dietary vitamin A produced an increase of IMF content. It is not surprising to observe differences between ruminants and swine, because their digestive system and the place for the FA synthesis are different. The amount of retinol in the liver (the main storage place for this metabolite), was well correlated with the amount of vitamin A ingested by the animals. Thus, the animals feeding

the practical level of vitamin A had the highest values. Although the amount observed was low, animals fed diets with no supplemented vitamin A presented a small amount of retinol in liver, which must have originated from the β-carotene present in the ingredients of the diet. The attempt to analyze the retinol content in the muscle ended without success, this occurred because muscle has a low amount of fat where retinol could be stored, and makes difficult to analyze its content. The expression of genes related with lipid metabolism in the *longissimus* muscle was not strongly modified, only a trend to reduce of PPARα was found when dietary vitamin A was deleted from the diet compared with the diet with the highest dose studied, but this change not appeared to be phenotypically significant.

This study indicated that the reduction of the dietary vitamin A level in Duroc x Landrace pigs does not increase IMF, even though not significant, the effect seems to be the contrary, also in other fat depots. Comparing the present study with studies done in ruminants seems that dietary vitamin A has not the same effect on fat deposition. Studies evaluating higher limits than those in relation fat depsotition could be evaluated, but should also be considered that European legislation limits the levels of vitamin A intake.

3. Reduction of the level of protein, lysine or both protein and lysine in the diet (Chapter 4)

Several studies had been performed studying the effect of the reduction of protein and lysine in the diet. Some of them intended to increase IMF content and others aimed to reduce the nitrogen excretion which has become a problem for the environment. In most of the studies that evaluated the response of the reduction of dietary protein or lysine on pig fat deposition, when protein was reduced, lysine was reduced in the same proportion and *vice versa*. In the present study, the effect of the reduction of protein or lysine and the interaction between them were evaluated independently. Furthermore, the protein level of the control diet was low compared to other studies, in line with the recommendations to reduce nitrogen excretion to the environment. Only when dietary protein was reduced an

increase of backfat content was observed, and in this case perirenal fat was not affected by the dietary treatment. Because the diets had the same net energy level, the energy that replaced protein came from carbohydrates that can be more easily converted to fat than protein, resulting in an increase of backfat content. Among all the studies, the present one was the only study where the IMF content was significantly increased by effect of the diet, and it was attributed to the interaction between the level of dietary protein and lysine. Thus, when protein was reduced but lysine was maintained as in the control diet or when lysine was reduced and the protein level was maintained as in the control diet, an increase of IMF content in the different muscles studies occurred, but when both parameters were reduced IMF showed its lowest content. This finding is contrary to some of the studies reported in the introduction and could be due to the fact that in the present study the level of dietary protein is lower compared to those used in other studies. The level at which protein or lysine are included in the diet has to be considered if is desired an increase of IMF. The reduction of lysine in high protein diets resulted in less efficient pigs, thus in order to increase IMF content without depression in the growth performance parameters, a reduction of protein without reducing the level of lysine has to be applied. When dietary protein was reduced a reduction of liver weight also occurred, and this reduction may be related to the reduced amount of ingested nitrogen, resulting in a lower amount of excreted nitrogen. Thus, using low protein diets with the amount of lysine necessary for the optimum growth leads to an increase of IMF content which is associated in a better acceptance of meat by the consumer and to a reduction of the excretion of nitrogen which is of great importance from the point of view of the environment.

This study demonstrated that the levels of dietary protein and lysine affect IMF content in Duroc x Landrace pigs. When protein was reduced but the amount of lysine was added at requirement level, and when lysine was reduced and the protein was maintained an increase of IMF occurred. These results suggest that the modification of the relation between protein and lyisine in the diet may be a

good strategy to increase the IMF content. Furthermore, reduction of dietay protein caused an increase of animal fatness and the reduction of lysine in high protein diets increased feed to gain ratio. Thus, very low protein and normal lysine could be considered in swine production in order to increase IMF content without affecting growth performance parameters.

4. Supplementation with arginine, leucine or arginine and leucine to a diet with low or normal protein levels in a leaner breed compared with the above mentioned studies (Chapter 5)

Duroc x Landrace crossbreed was used in the first three experiments, because Duroc is known as a breed with high IMF deposition. Thus, it was expected that using a breed with certain preference to fat deposition the differences observed through dietary manipulation could be easily achieved. Only two treatments out of six applied in the three first experiments resulted in an increase of IMF content, when the dietary protein was reduced keeping the level of dietary lysine as in the control diet or when the level of dietary lysine was reduced keeping the level of dietary protein as in the control diet. In this last experiment a leaner genotype was used (Duroc x Landrace) x Pietrain in order to test if reduction of dietary protein keeping dietary lysine in normal levels would show the same effect in this leaner genotype. Only the reduction of dietary protein keeping the lysine as the control diet was used, and not the reduction of the level of lysine because the latter resulted in poor performance. In addition, two studies in the literature in pigs showed that modification of some other nutrients such as the supplementation with arginine or leucine (one study for each amino acid) increased the IMF content (Hyun et al., 2003; Tan et al., 2009). The addition of both amino acids separately or together was also evaluated in this experiment in normal or low protein diets. The change to a leaner crossbreed did not cause the increase of IMF as was observed previously with a fatter breed when the level of dietary protein was reduced and lysine was added to diet in normal levels. The results obtained, suggests that different feeding strategies have to be considered depending on the breed,

because the strategy that increased IMF in one breed does not work in the other. In view of the lack of effect of reduction of protein, it can be hypothesized that dietary effects depend on the breed, and that leaner genotypes may be less responsive.

The inclusion of 2 additional amino acids (arginine and leucine) in low protein diets caused a reduction of feed intake growth and feed efficieny. Results suggest that an amino acid antagonism may have occurred, because an excess of one branched chain amino acid may cause the deficiency of the other two, as excess leucine induced the enzymes that catabolize branched chained amino acids. Therefore, care must be taken to avoid this antagonism when leucine is added, mainly in low protein diets. The supplementation of leucine did not have any effect on IMF content while supplementation with arginine tended to reduce it.

This study demonstrated that reduction of dietary protein in diets with the requirement of lysine does not increase the IMF content in Duroc x Landrace x Pietrain pigs suggesting that the effect of dietary modification on IMF content depend on the genotype used. Contrary to the initial hypothesis leucine does not have influence on IMF content while arginine tended to reduce it. Arginine also reduced the overall animal fatness. Supplementation with leucine low protein diets negatively affected the growth performance parameters, probably due to an antagonism between leucine and the other branched chain amino acids. Thus, supplementation with arginine or leucine is not a good strategy to increase IMF. Studies evaluating the same nutritional strategy (i.e. reduction of dietary protein) in several genetic types should be conducted in order to evaluate the effect on fat an particularly IMF content.

Taking together these results seem to indicate that from the dietary strategies tested, reduction of protein level maintaining the lysine level was the most successful as it significantly increased IMF without negatively affecting performance. However, the response seems to vary depending on genotype. It is possible that the magnitude of the response and possible effects of the other

nutritional strategies tested could vary depending on the genotype. Some of the nutritional strategies such as supplementation with CLA, reduction of vitamin A and arginine tended to reduce overall fattening in some cases in parallel with IMF content.

CONCLUSION

CONCLUSION

Consumer's panel

- In Spain there are two groups of consumers, the ones who purchase pork with a high IMF content (marbling loin lovers) and the ones who purchase pork with a low IMF content (lean loin lovers).
- When consumers tested the pork portions, they all found that meat with high IMF was the tastier.
- It should be clearly distinguished between marbling (amount of visible fat) and IMF (chemically analyzed fat, not has to be visible) because a part of the population visually prefers less marbling, but when consumed they prefer high IMF content.

Supplementation of CLA

- The inclusion of a high dose of dietary CLA oil (4%) in gilts of a conventional genotype (Landrace x Duroc) does not increase IMF deposition.
- CLA inclusion tends to reduce fat deposition, particularly perirenal fat without affecting growth performance.
- CLA acts in a tissue specific way, increasing SFA in all studied tissues, reducing the MUFA in LT and LT subcutaneous fat, and PUFA in LT subcutaneous fat, liver and SM.
- The highest percentage of incorporation of CLA is in LT subcutaneous fat (7.72%) and the lowest percentage is in the muscles (LD: 2.64% and SM: 2.12%). The isomer more deposited is 9c,11t CLA.
- The effect of CLA on FA profile is different in LT and SM muscles, and is similar in SM and liver.
- SFA and MUFA are mainly modified in the NL and PUFA in the PL fraction of LT IMF by effect of CLA.
- Expression of genes involved in regulation of fat metabolism and fatty acid composition were affected by CLA in a tissue-specific manner.

- CLA reduced the genes involved in *de novo* synthesis and oxidation of FA in oxidative tissues i.e liver and SM muscle.
- Changes in gene expression were not always reflected in changes in fat deposition, which suggests that regulatory levels beyond transcription may be also affected by dietary CLA.

Reduction of the level of dietary vitamin A

- Level of dietary vitamin A can be reduced to NRC levels or even more, without affecting growing parameters or having impact in carcass yield.
- Retinol content in the liver increased as increased the level of vitamin A in the diet.
- Animals fed the diets without vitamin A supplementation still have some content of retinol in in the liver.
- Omiting vitamin A in the diet possibly reduces overall carcass fatness increasing lean deposition.
- The effect of the vitamin A on the IMF seems to be the contrary to the initial hypothesis, the percentage of IMF was higher when the levels of dietary vitamin A were over the NRC recommendations, although differences did not reach statistical significance.

Reduction of protein, and lysine

- There was an interaction between protein and lysine. IMF content was increased in both muscles studied (the LT and the SM) when protein or lysine were independently reduced, but not when they were reduced simultaneously.
- Reduction of dietary lysine in high protein diets resulted in less efficient pigs.
- Reduction of protein, independently of low or high lysine diets, produced an increase of pig fatness, accompanied with an increase of SFA and MUFA and a reduction of PUFA, suggesting an increase of *de novo* FA synthesis.

Supplementation of arginine, leucine or both arginine and leucine in low and normal protein diets using a leaner genotype compared with other studies

- Supplementation of diet with both arginine and leucine in low protein diets resulted in low feed intake and growth, suggesting an antagonism between leucine and other branched chain amino acids.
- Supplementation with arginine or leucine normal protein diets resulted in an increase of weight gain, suggesting that these amino acids were limitant in the control diet.
- Addition of arginine or reduction of protein resulted in an reduction of subcutaneous fat or belly and increase of carcass lean meat percentage.
- Addition of arginine reduced IMF, however addition of leucine in finishing pig diets did not affect the amount of IMF or subcutaneous fat.
- Reduction of protein in a leaner genotype compared with the previous study did not modify the IMF content.

Global conclusion:

- From the different nutritional strategies evaluated, growth performance parameters were negatively affected in animals fed with low lysine high protein or low protein diets supplemented with leucine.
- Although several nutritional strategies were tested only in two cases (reduction of protein or reduction of lysine) IMF was significantly increased in Landrace x Duroc pigs, suggesting that dietary effects on fat deposition depend on genetic background.
- Supplementation with CLA or leucine had not effect on IMF content, while addition of arginine or reduction of dietary vitamin A (not significant) tended to reduce it, contrary to the initial hypothesis.
- Animal fatness was slightly reduced by dietary supplementation with CLA, arginine or the reduction of the level of vitamin A. However, reduction of dietary protein caused an increase of whole animal fatness. The effects

observed for overall fatness seems to be in parallel with the effect observed on IMF content.

- Fatty acid profile in different tissues is easly modified through the diet i.e changing the fat source.
- For practical uses, the most promising nutritional strategy to increase IMF in Duroc x Landrace pigs is the reduction of dietary protein keeping the levels of dietary lysine as the requirement for the animal development. If another genetic line is desired a previous evaluation of the response should be done.

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ANNEX

Annex

Do all the consumers accept marbling in the same way? The relationship between eating and visual acceptability of pork with different intramuscular fat content