UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA, ALIMENTARIA Y DE BIOSISTEMAS

CONSEQUENCES OF DIETARY SUPPLEMENTATION WITH n-3 POLYUNSATURATED FATTY ACIDS ON REPRODUCTIVE, ENDOCRINE AND METABOLIC PARAMETERS OF RABBIT DOES AND ON CARCASS QUALITY OF GROWING RABBITS

TESIS DOCTORAL

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Ingeniero Agrónomo

2017

UNIVERSIDAD POLITÉCNICA DE MADRID

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DEPARTAMENTO DE PRODUCCIÓN AGRARIA

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This research was supported by project AGL2011-23822, AGL2015-65572-C2, funding from the Spanish Ministry of Economy, Industry, and Competitiveness, and MEDGAN-CM S2013/ABI-2913 funding from the Community of Madrid.

"Para empezar un gran proyecto, hace falta valentía.
Para terminar un gran proyecto, hace falta perseverancia"

AGRADECIMIENTOS

Ya está. Con este documento finaliza una etapa. Una etapa muy dura por un lado, pero más que gratificante por otro. Por supuesto, tampoco hubiera sido posible hacerlo sola, por lo tanto, no puedo terminar sin dedicar unas palabras de agradecimiento a quien ha formado parte de ella.

En primer lugar, gracias a Pilar. Por absolutamente todo… Por permitirme formar parte en tu equipo. Por todo lo que me has enseñado (menuda diferencia de la primera presentación a la última eh?). Por cada una de las charlas en tu despacho, que ya sea en un tema u otro siempre han dado sus frutos. Madre mía, quien me iba a decir a mí que iba a tener la posibilidad de ir a China? Y no sólo fuimos, sino que conquistamos!! Nosotras podemos, claro que podemos! Podemos con todo! GRACIAS

A mis padres y a mi hermano. Porque ha habido momentos duros, desesperantes, con ganas de dejarlo… pero siempre habéis estado ahí mostrándome vuestro apoyo incondicional. GRACIAS

A cada uno de los alumnos que han pasado por la nave estos años. Todos me habéis permitido aprender. GRACIAS

A Javi. Menudos días largos eh? Anda que no nos hemos quejado nada el uno y el otro! Por todas las charlas de desahogo. GRACIAS

A quien compone el grupo de investigación CONEREPRO y al departamento de Producción Animal. Cada uno a vuestra manera habéis colaborado en que esto salga adelante. GRACIAS

A Ana, Orozco, Tere y Trini. Una vez más, como dice la canción, 5 años después "ya no queda casi nadie de los de antes". Vosotros seguís ahí. GRACIAS

A Héctor. Por las mil veces que te pude repetir lo mismo. Por las mil horas donde Antonio hablando en inglés, o intentándolo. Por los bares de intercambio de idiomas. Por los ratos de desconexión. Por las dos piedras que me dieron toda la suerte del mundo. Porque no hace falta decirnos nada… GRACIAS

A Bea. Porque la lié en la primera práctica. Por cambiarme los peces, no una, sino 4 ó 5 veces. Por la cara de susto que pusiste cuando te enteraste que venía para quedarme una temporada ;) Por todos los ratos en la nave. Porque me has salvado el culo ni sé las veces. Pero sobre todo por el sexo culinario. GRACIAS

A Nora. Porque eres lo mejor que me ha pasado en estos 4 años, creo que eso lo resume todo. Te puedes creer que aún no hemos ido a Mirasierra??? GRACIAS

A Katie. Por las pintas que terminan en dibujos y dibujos que terminan en English Lessons en un Pub. Porque no te puedes hacer a la idea de lo que me has ayudado. Dime, que preferirías? Otra tesis o el "good afternoon everyone" todos los días de tu vida??? Y no vale elegir pizza con moho!! GRACIAS

*A Lucía. Y a ti que te digo? Por animarme. Por confiar siempre en mí. Por corregirme el inglés en cualquier momento…Y por lo poco que te gusta la producción animal y lo mucho que has aprendido sólo por ayudarme. Porque con unas pintas allí y unas cañas aquí arreglamos el mundo. *gambita* GRACIAS*

A todos los que habéis compartido conmigo esta etapa

GRACIAS

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ABBREVIATION LIST

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AA	Araquidonic acid
ADF	Acid detergent fiber
ADFI	Average daily feed intake
ADG	Average daily gain
ADL	Acid detergent lignin
AI	Artificial insemination
ALA	α-linolenic acid
AMPK	Adenosine monophosphate protein kinasa
BAT	Brown adipose tissue
BPD	Biparietal diameter
CP	Crude protein
CRL	Crown-rump length
DGLA	Dihomo-γ-linoleic acid
DHA	Docosahexaenoic acid
DM	Dry matter
DPA	Docosapentaenoic acid
dpp	Days postpartum
DRM	Dried *Schizochytrium sp*. microalgae
EPA	Eicosapentaenoic acid
FA	Fatty acid
G:F	Gain/feed ratio
GLUT	Glucose transporter
GnRH	Gonadotropin-releasing hormone
H_2O_2	Hydrogen peroxide
HDL-c	High density lipoprotein cholesterol

HPA	Hypothalamic-pituitary-adrenal
IgG	Immunoglobulin G
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid
LDL-c	Low lipoprotein density cholesterol
LH	Luteinizing hormone
LM	*Longissimus dorsi* muscle
MUFA	Monounsaturated fatty acid
NDF	neutron detergent fiber
NEFA	Non-esterified fatty acid
ONL	Occipito-nasal length
PG	Prostaglandin
PPAR	Peroxisome proliferator-activated receptors
PTGS	Prostaglandin endoperoxide synthase
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SGLT	Sodium-dependent glucose transporter
SFA	Saturated fatty acid
SR-B1	Scavenger receptor class B, type 1
SREBP	Sterol regulatory element-binding proteins
STAR	Steroid acute regulator
TD	Thoracic diameter
TG	Triglycerides
UCP	Uncoupling protein
VFA	Volatile fatty acid
VLDL-c	Very low density lipoprotein cholesterol
WAT	White adipose tissue

SUMMARY

SUMMARY

The n-3 polyunsaturated fatty acids (**PUFA**) play a decisive role in the development of the brain and the nervous, immune and reproductive systems of animals. These essential fatty acids (**FA**) are particularly important with regard to high production animals; where their greater energetic demands may not be covered with a "basic" alimentation, and consequently they need a direct supplementation through the diet. Focusing on reproductive level, PUFA are precursor molecules of different hormones (steroid and prostaglandin) that regulate the ovarian activity, the embryo implantation or the parturition time among other physiological processes. Moreover, dietary PUFA can alter the lipidemic profile affecting the gene expression that regulates the metabolism and increases peroxidation. When PUFA are included in diets, carcasses with a healthy lipidemic profile are obtained. Most of studies assessed in rabbits have used PUFA from vegetal origin. Nevertheless, there is a relatively low number of researchs which have used PUFA from marine origin, relating reproductive, metabolic, and digestive, as well as growing and carcass quality parameters.

The first specific aim was to evaluate the productive, endocrine, and metabolic responses as well as oxidative stress of rabbit does and their offspring when fed a diet supplemented with n-3 PUFA (15 g/kg) during their first productive cycle. To this purpose, a total of 105 rabbit does were fed *ad libitum* from d 60 to 172 of age 2 isoenergetic and isoproteic diets differing in FA composition. The control diet (n = 52 does) contained 45.9 g/kg of n-3 of the total FA and the enriched diet (n = 53 does) contained 149.2 g/kg of n-3 of the total FA. Both experimental groups had similar feed intake during rearing, pregnancy, and lactation. The enrichment of diet had no effect on ultrasonographic assessment of does on d 9 and 16 of pregnancy, with an embryonic vesicle number, foetus and placenta size similar between groups ($P > 0.05$). Even though there were no major effects ($P > 0.05$) on fertility, duration of gestation, and number born alive and stillborn kits at parturition, live kits from enriched does were longer (71.6 ± 2.42 *vs.* 79.5 ± 2.13 mm; $P < 0.05$) and tended to be heavier (42.5 ± 3.94 *vs.* 50.8 ± 3.47 g; $P = 0.07$) than those from control does ($P < 0.05$). The 2 groups had similar milk production and mortality values during lactation; consequently, there were no differences between diets in average daily gain (**ADG**), litter weight, and number of

weaned kits (P > 0.05). In enriched does, higher plasma leptin and estradiol concentrations than in control does (P < 0.05) were observed. In addition, enriched females also had lower total and high-density lipoprotein cholesterol (**HDL-c**) than control females during lactation (P < 0.05). Regarding offspring, the enrichment of diet with PUFA caused a hyperlipidemic status (greater values of plasma triglycerides, total cholesterol, and HDL-c; P < 0.05) at 1 d postpartum (**dpp**), compared with the control group, that disappeared at 32 dpp. Supplemented does before parturition and their offspring at 1 dpp had greater oxidative stress than those in the control group.

The second specific aim was to investigate the effect of continuing the same dietary supplementation during growing period. For this purpose, we evaluated growth performance, carcass characteristics and FA profile of muscle and fat tissues (at slaughter), as well as cecal fermentation and ileal mucosa morphology of growing rabbits. Similar diets to those used in the first objective were provided each to 24 does (12 per diet) and their offspring during pregnancy and lactation. From weaning (30 d of age) to slaughter (60 d), the litters (12 per diet; 8 kits each) continued fed the corresponding experimental diet. There were no differences (P > 0.05) between groups in average daily feed intake, ADG and G:F ratio during the growing period. At slaughter, body weight, full gastrointestinal tract weight, carcass yield, meat colour and pH, drip loss percentage, content of scapular fat and tissue composition of the left hind leg were similar between groups (P > 0.05), but perirenal fat was lower (P = 0.020) and skin weight and abdominal fat tended to be lower (P = 0.055 and P = 0.063, respectively) in enriched rabbits than in control ones. Total PUFA content in both *Longissimus dorsi* muscle (**LM**) and perirenal fat was greater (P = 0.021 and < 0.001, respectively) in enriched rabbits, that also showed lower n-6/n-3 ratios in LM (1.61 *vs.* 5.80; P < 0.001) and perirenal fat (4.71 *vs.* 12.0; P < 0.001) than those fed the control diet. Cecal concentrations of total volatile fatty acids (**VFA**) were greater (P < 0.001) in enriched than in control group at 30, 45 and 60 d of age, but diet did not affect (P ≥ 0.332) VFA profile, with the exception of a lower (P = 0.013) proportion of minor VFA (sum of isobutyrate, isovalerate, and valerate) in control group. Diet did not affect (P > 0.255) either pH and NH_3-N concentrations in the cecum or ileal morphology (crypt depth and villi length).

Taking into account, the results obtained, the third specific aim of this thesis was to confirm the effect of an enriched diet with n-3 PUFA in a 4-folder higher level of inclusion (60 g/kg) than previous experiments. A total of 127 rabbit does were fed *ad libitum* throughout their two first cycles with two diets with different fat sources (in terms of n-3 of the total FA): the control diet had 31.9 g/kg (n = 63 rabbit does) and the enriched diet had 287.7 g/kg (n = 64 rabbit does). Feed intake was similar between groups ($P > 0.05$). Dietary treatment affected plasma progesterone concentration, which was higher than in control group on Days 7 (30.9 ± 2.18 *vs.* 23.9 ± 2.30 ng/ml, respectively; $P = 0.029$) and 14 (38.7 ± 2.18 *vs.* 28.2 ± 2.30 ng/ml, respectively; $P = 0.001$) of first gestation. Reproductive parameters of mothers (fertility, duration of gestation and prolificacy) and litter parameters (weight at parturition and weaning, mortality and ADG of kits during lactation) were similar in both groups. However, individual measurements of neonates of enriched group improved 5.87%, 7.10% and 18.01% ($P < 0.05$) in terms of crown-rump length, biparietal and thoracic diameters, respectively, compared to control ones at first parturition. It is noteworthy that at the second insemination, critical point in rabbit does, fertility rate of enriched group did not decline as sharply as in the control group (89.7 *vs.* 76.6%, respectively; $P = 0.067$), although ADG and littler weight were slightly lower at the second lactation after PUFA enrichment ($P < 0.05$). Moreover, total PUFA and unsaturated index of milk of enriched does group were significantly elevated than in control one (33.3 ± 0.02 *vs.* 23.2 ± 0.02 g/100g and 1.20 ± 0.00 *vs.* 0.86 ± 0.00, respectively; $P < 0.05$). Finally, plasma progesterone, ovulation rate and embryo development at 3.5 days after the artificial insemination were similar between diets ($P > 0.05$), but embryo apoptosis rate was almost twice higher in control group than in supplemented one (31.1 ± 4.56 *vs.* 17.1 ± 3.87%, respectively; $P < 0.05$).

In conclusion, an increase of n-3 PUFA concentration in the diet of rabbit does and, consequently, of their offspring during a productive cycle alters their lipid profile and the indicators of oxidative stress, without major endocrine modifications or improvements in the productive variables. The fact of continuing with the same diet during growing period showed that dietary fish oil supplementation enhanced beneficial long-chain n-3 FA and decreased n-6/n-3 ratio in rabbit meat and fat, being healthier for human consumption, without having negative effects on growth performance, cecal fermentation, and ileal morphology

or carcass characteristics. Moreover, increasing the level of inclusion of the same supplement previously administrated from the rearing and throughout the first two productive cycles improved plasma progesterone during pregnancy, fertility, milk fatty acid profile and neonates development of primiparous does supporting the accumulative long-term beneficial effect of n-3 PUFA supplementation in rabbit does.

RESUMEN

RESUMEN

Los ácidos grasos poliinsaturados n-3 (por sus siglas en inglés **PUFA** (polyunsaturated fatty acids)) juegan un papel decisivo en el desarrollo del cerebro y de los sistemas nervioso, inmune y reproductivo de los animales. Estos ácidos grasos (**AG**) esenciales tienen particular importancia en animales de alta producción, donde sus grandes demandas energéticas podrían no estar cubiertas por una alimentación "básica", y en consecuencia, necesiten una suplementación directa a través del pienso. A nivel reproductivo, los PUFA pueden ser precursores de distintas hormonas (esteroides y prostaglandinas) que regulan la actividad ovárica, la implantación del embrión o el momento del parto entre otros procesos fisiológicos. Además, el consumo de PUFA puede alterar el perfil lipidémico cambiando la expresión de genes que regulan el metabolismo y aumentan la peroxidación. Su consumo permite obtener canales con un perfil lipídico mucho más saludable. La mayoría de los trabajos en conejos se han realizado con PUFA de origen vegetal. Sin embargo, son escasos los que han utilizado PUFA de origen marino, relacionando parámetros reproductivos, metabólicos, digestivos, de crecimiento y de calidad de la canal.

El primer objetivo específico de esta tesis fue evaluar la respuesta productiva, endocrina y metabólica, así como el estrés oxidativo de conejas reproductoras y sus camadas cuando se alimentan con un suplemento comercial rico en PUFA n-3 (15 g/kg) durante su primer ciclo productivo. Para ello, un total de 105 conejas consumieron *ad libitum* desde el día 60 al 172 de edad dos piensos isoenergéticos e isoprotéicos que diferían únicamente en la composición de AG. El pienso control (n = 52 conejas) contenía 45,9 g/kg de n-3 del total de AG y el pienso enriquecido (n = 53 conejas) que contenía 149,2 g/kg de n-3 del total de AG. Ambos grupos experimentales tuvieron un consumo similar durante el periodo de recría, gestación y lactación. El enriquecimiento del pienso no tuvo efecto en el ensayo ecográfico realizado los días 9 y 16 de gestación, ya que se observó el mismo número de vesículas y el tamaño de embriones y placentas también fue similar entre grupos ($P > 0,05$). Aunque no hubo un efecto importante ($P > 0,05$) en la fertilidad, duración de la gestación y número de gazapos nacidos vivos y muertos al nacimiento, los gazapos recién nacidos del grupo enriquecido fueron más largos (71,6 ± 2,42 *vs.* 79,5 ± 2,13 mm; $P < 0,05$) y tendieron a pesar

más (42,5 ± 3,94 *vs.* 50,8 ± 3,47 g; *P* = 0,07 que los del grupo control (*P* < 0,05). Los dos grupos tuvieron valores similares de producción de leche y mortalidad durante la lactación, y consecuentemente, no hubo diferencias entre piensos en la ganancia media diaria (**GMD**), el peso de la camada y el número de gazapos destetados (*P* < 0,05). Las conejas que comieron el pienso enriquecido tuvieron concentraciones plasmáticas de leptina y estradiol mayores que las hembras controles (*P* < 0,05). Además, durante la lactación tuvieron también menor colesterol total y lipoproteínas de alta densidad ligadas al colesterol (**HDL-c**) que las hembras controles (*P* < 0,05). En cuanto a la descendencia, el enriquecimiento del pienso con PUFA causó un estado hiperlipidémico (mayores valores plasmáticos de triglicéridos, colesterol total y HDL-c; *P* < 0,05) el día 1 postparto (**dpp**), comparado con el grupo control, que desapareció a 32 dpp. Las hembras suplementadas antes del parto y sus gazapos a 1 dpp tuvieron mayor estrés oxidativo que el grupo control.

El segundo objetivo específico fue investigar el efecto de continuar con la misma suplementación del pienso en el periodo de cebo. Para ello, se evaluó el crecimiento, las características de la canal y el perfil de AG del tejido muscular y adiposo (al sacrificio), así como la fermentación cecal y la morfología ileal de gazapos de cebo. Se utilizaron los mismos piensos que en el primer objetivo proporcionándoselos a 24 conejas reproductoras (12 por pienso) y sus camadas durante la gestación y la lactación. Desde el destete (30 días de edad) al sacrificio (60 días de edad), las camadas (12 por pienso; 8 gazapos cada una) continuaron comiendo el correspondiente pienso experimental. No hubo diferencias entre grupos (*P* > 0,05) en el consumo medio diario, GMD y eficiencia alimenticia durante el periodo de cebo. Al sacrificio, el peso vivo, el peso del tracto gastrointestinal lleno, el rendimiento de la canal, el color y el pH de la carne, las pérdidas por goteo, el contenido de la grasa escapular y la composición tisular de la pata izquierda trasera fue similar entre grupos (*P* >0,05), pero la grasa perirrenal fue menor (*P* = 0,020) y el peso de la piel y la grasa abdominal tendieron a ser menores (*P* = 0,055 y *P* = 0,063, respectivamente) en los gazapos enriquecidos comparado con los controles. El contenido total de PUFA tanto en el músculo *Longissimus dorsi* (**LM**) como en la grasa perirrenal fue mayor (*P* = 0,021 and < 0,001, respectivamente) en gazapos enriquecidos, que también mostraron un menor ratio n-6/n-3 en el LM (1,61 vs. 5,80; *P* < 0,001) y en la grasa perirrenal

(4,71 *vs.* 12,0; *P* < 0,001) que aquellos alimentados con un pienso control. La concentración cecal de ácidos grasos volátiles (**VFA**) fue mayor (*P* < 0,001) en el grupo enriquecido que en el control los días 30, 45 y 60 de edad, sin embargo, el pienso no afectó (*P* ≥ 0,332) al perfil de VFA, con excepción de una menor proporción (*P* = 0,013) de los VFA minoritarios (suma de isobutirato, isovalerato y valerato) en el grupo control. El pienso no afectó ni al pH ni a las concentraciones de NH_3-N en el ciego ni a la morfología ileal (profundidad de las criptas y longitud de los villi).

Teniendo en cuenta los resultados obtenidos, el tercer objetivo específico de esta Tesis fue confirmar el efecto de enriquecer el pienso con el mismo suplemento basado en PUFA n-3 con un nivel de inclusión cuatro veces superior (60 g/kg) a los experimentos anteriores. Un total de 127 conejas se alimentaron *ad libitum* a lo largo de sus dos primeros ciclos con dos piensos con diferentes fuentes de grasa (en términos de AG n-3 del total de AG): el pienso control tenía 31,9 g/kg (n = 63 conejas) y el pienso enriquecido tenía 287,7 g/kg g (n = 64 conejas). El consumo de alimento fue similar en ambos grupos (*P* > 0.05). La suplementación del pienso afectó a la concentración plasmática de progesterona durante la primera gestación, que fue mayor en el grupo suplementado con respecto al grupo control en los días 7 (30,9 ± 2,18 *vs.* 23,9 ± 2,30 ng/ml, respectivamente, *P* = 0,029) y 14 (38,7 ± 2,18 *vs*. 28,2 ± 2,30 ng/ml, respectivamente, *P* = 0,001). Los parámetros reproductivos de las madres (fertilidad, duración de la gestación y prolificidad) y los parámetros de la camada (peso al parto y al destete, mortalidad y GMD de los gazapos durante la lactación) fueron similares en ambos grupos. Sin embargo, las medidas individuales de los recién nacidos del grupo enriquecido mejoraron un 5,87%, 7,10% y 18,01% (*P* < 0,05) en términos de longitud y diámetros biparietal y torácico, respectivamente, en comparación con los controles en el primer parto. Cabe señalar que en la segunda inseminación, punto crítico en la coneja, la tasa de fertilidad del grupo enriquecido no disminuyó tan drásticamente como en el grupo control (89,7 *vs.* 76,6%, respectivamente; *P* = 0,067), aunque en la segunda lactación la GMD y el peso de la camada fueron ligeramente inferiores con el pienso enriquecido con PUFA (*P* < 0,05). Además, la concentración total de PUFA y el índice de insaturación de la leche del grupo enriquecido fueron significativamente mayores

que en el grupo control (33,3 ± 0,02 *vs*. 23,2 ± 0,02 g/100 g y 1,20 ± 0,00 vs 0,86 ± 0,00, respectivamente, $P < 0,05$). Por último, la progesterona plasmática, la tasa de ovulación y el desarrollo embrionario a los 3,5 días después de la inseminación artificial fueron similares entre las dietas ($P > 0,05$), aunque la tasa de apoptosis fue casi dos veces mayor en el grupo control que en el suplementado (31,1 ± 4,56 *vs.* 17,1 ± 3,87%, respectivamente, $P < 0,05$).

En conclusión, un incremento de la concentración de PUFA n-3 en el pienso de conejas reproductoras, y consecuentemente de sus gazapos, durante un ciclo productivo altera su perfil lipídico y los indicadores de estrés oxidativo, sin mayores modificaciones endocrinas o mejoras en los parámetros productivos. El hecho de continuar con el mismo pienso durante el periodo de cebo muestra que la suplementación del pienso con aceite de pescado mejora los beneficios de los PUFA n-3 de cadena larga y reduce el ratio n-6/n-3 de la carne y grasa de conejo, haciéndola más saludable para el consumo humano, sin efectos negativos en el crecimiento, la fermentación cecal, la morfología ileal o en las características de la canal. Además, aumentar el nivel de inclusión del mismo suplemento previamente administrado desde la recría y a lo largo de los dos primeros ciclos productivos mejora la progesterona plasmática durante la gestación, la fertilidad, el perfil de AG de la leche y el desarrollo de los recién nacidos de conejas primíparas, confirmando el efecto beneficioso acumulativo a largo plazo de la suplementación con PUFA n-3 en conejas.

CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

Nowadays, unhealthy food habits in western society are characterised by a high consumption of meat, seed oils, fast food and snacks food, which contain a large amount of saturated fatty acids (**SFA**) and low proportion of polyunsaturated fatty acids (**PUFA**) (Fernández-SanJuan, 2000). A lot of scientific studies have demonstrated the positive effects on health from PUFA for insulin resistance, adult-onset diabetes mellitus (Oliveira *et al.*, 2015), hypertension (Barbosa *et al.,* 2017), arthritis (Rajaei *et al.,* 2015), atherosclerosis (Mosca *et al.*, 2017), depression (Pompili *et al.*, 2017), thrombosis (Reiner *et al.*, 2017), and some cancers (Lin *et al*., 2017) and cognitive decline (Horrocks *et al.,* 1999). For that reason, nutritionists recommend the consumption of fish and green vegetable to prevent diseases.

Also in animals, several studies carried out corroborate that PUFA are essentials as membrane compounds in the cells of all tissues, reason by which are, for example, important for the growth of the foetus (Innis, 2007), playing a vital role particularly in retina, brain, neuronal and reproductive tissues development (Jensen *et al*., 1996), live body weight and survival rate at birth (Rooke *et al*., 2001a, Rebollar *et al*., 2014). These fatty acids (**FA**) are also involved in intracellular signalling mechanisms (Kurlak and Stenphenson, 1999) and they are precursors of bioactive lipid mediator's synthesis like prostaglandins, thromboxane, leukotrienes, lipoxins and resolvins (Wathes *et al*., 2007). Long chain PUFA (**LCPUFA**) have an effect on immunity through their role in the synthesis of eicosaenoids, have an anti-inflammatory role (Schmitz and Ecker, 2008) and they are related with intestinal health (Menni *et al*., 2017). One of the most important tools for good gut health is to offer the best feed possible that meets the nutritional needs for the specific age and stage of production. Appropriate feed for the different physiological stages of development is essential and livestock need dietary supplements such as organic acids, prebiotics, probiotics, and enzymes, which are also valuable tools in antibiotic-free production systems.

The LCPUFA are essential FA since they cannot be synthetized by mammalian cells, hence, they must to be obtained from diet. The LCPUFA synthesis from their precursors involves a complex mechanism of desaturation-elongation, therefore, their direct inclusion avoids all these steps and supposes a greater efficiency supplementation. Fish oil usually presents higher amounts of n-3 LCPUFA

than seed oils or microalgae, thus it is the natural source of n-3 FA to be incorporated into conventional food products (Mataix *et al*., 2003). Many products enriched with n-3 have been developed to supplement the human diet and reach a good n-6/n-3 PUFA ratio. In this regard, there is a wide variety of commercial food products related to animal production enriched with n-3, as milk and derivate, eggs, as well as meat and poultry products (Kolanowski and Laufenberg, 2006).

2. CHEMICAL STRUCTURE AND PHYSICAL PROPERTIES OF FATS

The word fat is commonly misused to indicate all lipids, a complex group of organic substances composed of carbon, hydrogen and oxygen, and characterized by solubility in organic apolar solvents. Lipid can be divided into simple lipids, which do not contain FA, and complex lipids, which are esterified with FA (Image 1.1) (Xiccato, 2010).

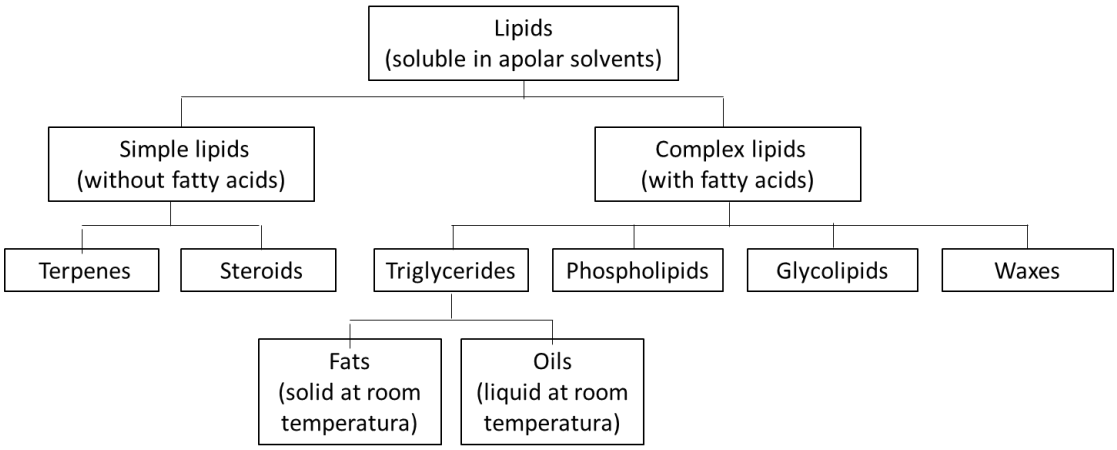

Image 1.1. Classification of lipids. (Xiccato, 2010)

Triglycerides (**TG**) can be considered “true” fats because they represent the most typical form of energy accumulation in animal and vegetable organisms. Therefore, only these lipids have real nutritional importance. The TG are the highest energy-yielding components of feeds, yielding an average of 2.25 times more energy than other components. They are formed by one glycerol molecule, a trihydric alcohol, to which three FA are esterified. The physical, chemical and nutritive properties of TG depend on the characteristics of their FA (Xiccato, 2010).

Fatty acids are organic compounds formed by a hydrocarbonated chain and a carboxylic group that is normally bounded with glycerol forming acylglycerides (mono-, di- or triglycerides). Depending on the nature of the hydrocarbonated chain, FA can be saturated or unsaturated, which in turn can be monounsaturated or PUFA.

Polyunsaturated fatty acids have more than one double bond present within the molecule and are further classified into three groups on the basis of their chemical structure: n-3, n-6 and n-9, where the first double bond is located 3, 6 or 9 carbons from the methyl end of the molecule. The position of the double bonds in

the carbon chain determines the ability of PUFA to act as precursors of others essential compounds such hormones.

Animals cannot synthesise n-6 or n-3 PUFA *de novo* as they lack the appropriate FA desaturase enzymes. The parent n-6 PUFA is linoleic acid (**LA**; C18:2n−6) and the parent n-3 PUFA is α-linolenic acid (**ALA**; C18:3n−3), and both of them need to be provided in the diet as they are absolutely necessary for numerous processes, including growth, reproduction, vision and brain development (Gurr *et al*., 2002). Mammals and other higher animals are able to elongate the carbon chain (from C18 to C22), but are unable to insert double bonds between the carbon atoms in position 1 of the chain and the carbon in position 9 (Xiccato, 2010). According to suggestion of nutritional experts, a dietary ratio of n-6/n-3 PUFA of 5:1 or less is desired (WHO/FAO, 1994). In that sense, functional food products enriched with n−3 PUFA, have been the type of functional foods whose production in Europe and USA has increased the most in the last years. There is nowadays a wide variety of commercial food products enriched with “omega-3”, as bread and bakery products, milk and derivatives, spreadable fats, eggs, juices and soft drinks, meat and poultry products, etc. (Kolanowski and Laufenberg, 2006) which are enriched from animal and vegetable sources. Current daily intake of n-3 FA is 0.1 % of total energy while the Acceptable Macronutrient Distribution Range recommends an intake level of 0.6-1.2% of energy (Trumbo *et al*., 2002). The need for n-3 LCPUFA cannot be met by only increasing ALA consumption and individuals may obtain the recommended level of n-3 FA intake from the consumption of n-3 LCPUFA content fish twice weekly (USDA, 2005), however many people do not reach this minimal intake level.

2.1. Source of fatty acids

The precursor of n-3 PUFA, ALA, is present in widely-distributed and commonly-consumed oils and foods, such as rapeseed and soyabean oils used in many manufactured foods, green leafy vegetables and nuts such as walnuts (Williams and Burdge, 2006). Linoleic acid, precursor of n-6 PUFA, is abundant in vegetable oils as corn, safflower, sunflower and rapeseed oils (Gurr *et al*., 2002).

Linoleic acid and ALA are converted in the liver to LCPUFA by desaturation and elongation enzyme systems common to both pathways. The complex mechanism of elongation and desaturation to convert LA and ALA in LCPUFA has

several competitive limiting steps and this frequently leads to suboptimal concentration of them in cell membranes. n-6 LCPUFA as arachidonic acid (**AA**; C20:4n−6) can be synthesized from LA, and n-3 LCPUFA, as eicosapentaenoic acid (**EPA**; C20:5n−3), docosapentaenoic acid (**DPA**; C22:5n−3) and docosahexaenoic acid (**DHA**; C22:6n−3), from ALA; however, the conversion of ALA in EPA, DPA and DHA is low and these n-3 LCPUFA are considered essential FA too (Image 1.2). With the exception of the final reaction that results in the formation of DHA all reactions occur in the endoplasmic reticulum of the liver cell. First, an introduction of a double bond by the action of Δ6-desaturase converts ALA to stearidonic acid (18:4n-3) and is the rate-limiting reaction of the pathway. Then, the addition of C2 by elongase activity is followed by desaturation by Δ5-desaturase to form EPA. Docosapentaenoic acid (22:5n-3) is synthesised from EPA by the addition of C2. The final step in the n-3 pathway involves a series of reactions that include: (1) the addition of C2 by elongase to form 24:5n-3; (2) desaturation at the Δ6 position to form 24:6n-3; (3) β-oxidation with the loss of C2 to form 22:6n-3 (DHA). The 24:6n-3 is translocated from the endoplasmic reticulum to the peroxisome where acyl chain shortening (loss of C2) is achieved by one cycle of the β-oxidation pathway to form DHA. The DHA is then translocated back to the endoplasmic reticulum.

The first step in the pathway involving Δ6-desaturation is shared between ALA and LA for its conversion to its longer chain products. Although the affinity of Δ6-desaturase is higher for ALA than for LA, the typically higher concentrations of LA in cellular pools result in greater conversion of LA to n-6 LCPUFA. As Δ6-desaturation is the rate-limiting step in the pathway, high dietary intakes of n-6 PUFA have been proposed to be a limiting factor in the conversion of ALA to its long chain products EPA and DHA. Thus, the direct inclusion of these n-3 LCPUFA in the diet could be a way to improve the reproduction and productivity functions of animals.

The n-3 LCPUFA (EPA and DHA) are bioactive components of fish and flaxseed oil, and are routinely consumed by the public as food additives or supplements. With animal studies, researchers have commonly used ∼5% (weight/weight) fish or flaxseed oil as intervention, corresponding to approximately 2–6% of total energy as n-3 PUFA (Kim *et al*., 2010). This dose, especially of fish oil, is often selected to model n-3 PUFA intake of Greenland Eskimos that consume n-3 PUFA in the range of 1–6% of total energy (Bang *et al*., 1980).

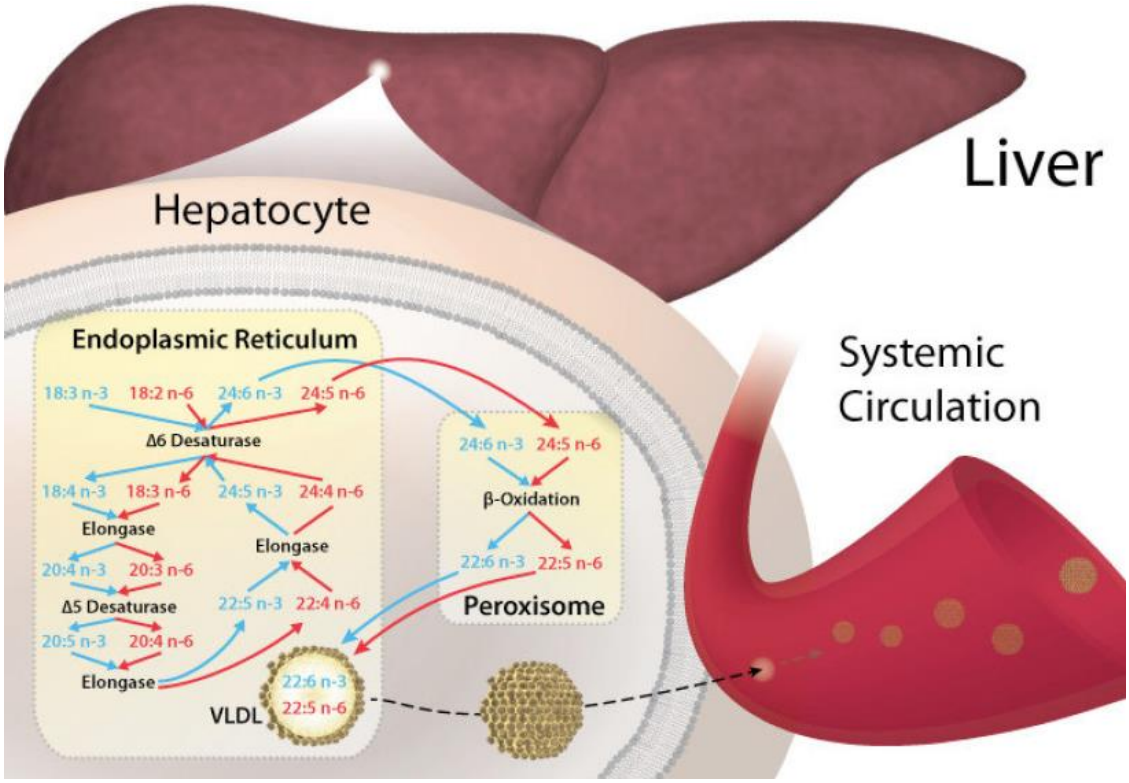

Image 1.2. Desaturations and elongations reactions taking place in the liver in order to synthesise long-chain polyunsaturated fatty acids. VDLD: very low density lipoprotein. (Domenichiello *et al*., 2015)

As new sources of LCPUFA are needed, several alternatives sources of n-3 LCPUFA have been proposed in the last years, such as marine microalgaes, algaes or transgenic plants. For example, *Schizochytrium limacinum* is a thraustochytrid that can grow in marine and estuarial environment and its strategic feature is the heterotrophic production of n-3 LCPUFA, particularly DHA or EPA (Jung and Lovitt, 2010). Dried *Schizochytrium sp*. microalgae (**DRM**) has also been determined to be generally recognized as safe for use as a DHA-rich ingredient in broiler chicken and laying hen feed at levels up to 2.8 and 4.3%, respectively. The DHA-enriched eggs from hens fed a diet containing approximately 1% DRM are now commercially marketed in some countries including Spain (Hammond *et al*., 2001).

There have been some successes using cultivation of marine microorganisms which biosynthesise DHA, but this approach is technically demanding and costly (Tonon *et al*., 2002). This is due to the LCPUFA content of microalgae depends not only on the species, but also on factors related to culture condition including composition of the medium, aeration, light intensity, temperature and age of culture (Dunstan *et al*., 1993; Tonon *et al*., 2002). Guo *et al*. (1999),

isolated 23 yeast strains and nine of them were capable of quickly assimilating the scrap fish oil a sole carbon source, producing larger cell masses, and incorporating exogenous EPA and DHA into their cellular lipids. Conchillo *et al*. (2006) published a study comparing microalgae oil and fish oil composition, and concluded that both oils presented a similar amount of n-3 LCPUFA, although microalgae oil has the disadvantage of presenting an unpleasant odour.

Some advances in biotechnology have resulted in plants that have been genetically altered to create new compounds. There have been studies based on the genetic modification of FA metabolism in order to produce economically valuable oils for food and non-food industrial uses in oilseed crops (Ohlrogge, 1994; Thelen and Ohlrogge, 2002). The alternative of transgenic plants was studied by Napier (2006) that published a review with the last advances in the production of PUFA enriched vegetables species through genetic modifications. However, nowadays the use of transgenic plants in agriculture is not well-accepted in many parts of the world, especially Europe.

Marine organisms (fish, seafood, algae...) are fed, directly or indirectly, from marine phytoplankton, the primary produced of n-3 in the trophic chain. Fish is the most common source of n-3, and *Scombridae*, *Clupeidae* and *Salmonidae* families are the fish species with the highest percentage of EPA and DHA in the foodstuff portion (Table 1.1). Moreover, fish oil usually presents higher amounts of n-3 LCPUFA than seed oils (Table 1.2). However, fish have some drawbacks: they are a declining resource, accumulate pollutants, present an unpleasant odour and the proportion of specific FA in their lipids are difficult to control.

Table 1.1. Content of eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) in some species of fish (adapted from Rubio-Rodríguez *et al*., 2010).

Species	Common name	g/100 g of foodstuff portion	
		C20:5n-3 (EPA)	C22:6n-3 (DHA)
Scomber scombrus	Mackerel	1.10	2.56
Mullus surmuletus	Red mullet	0.91	1.66
Sardina pilchardus	Sardine	0.62	1.12
Salmo salar	Salmon	0.50	1.00
Thunnus thinnus	Ton	0.24	0.98
Engraulis encrasicolus	Fresh anchovy	0.14	0.80
Pagellus bogaraveo	Sea bream	0.12	0.61
Gadus morrhua	Cod	0.23	0.47
Merluccius merluccius	Hake	0.10	0.54
Conger conger	Conger eel	0.15	0.43
Luvarus imperialis	Swordfish	0.15	0.30
Galeorhinus geleus	Dogfish	0.04	0.30

Table 1.2. Lipids (g/100 g oil) in different kinds of seed and fish oils (adapted from Rubio-Rodríguez *et al*., 2010).

Oil	SFA[1]	MUFA[2]	PUFA[3]	n-3	n-6	n-6/n-3 ratio
Sunflower	12.0	20.5	67.5	0.10	63.2	632
Corn	14.5	29.9	55.6	0.90	50.4	56.0
Soya	15.6	21.2	63.2	7.30	51.5	7.05
Palm	47.8	37.1	15.1	0.30	10.1	33.7
Olive	14.3	73.0	12.7	0.70	7.80	11.1
Cod liver	22.6	20.7	56.8	19.8	0.90	0.04
Herring	21.3	56.6	22.1	11.9	12.0	1.01
Salmon	19.9	17.0	63.1	35.3	1.06	0.03
Sardine	30.4	14.5	55.1	28.1	2.20	0.07

[1]SFA: saturated fatty acids. [2]MUFA: monounsaturated fatty acids. [3]PUFA: polyunsaturated fatty acids.

One method of increasing n-3 LCPUFA intake is through the use of fish oil supplements, which, in humans, may be particularly useful in individuals who are unwilling to make dietary changes to increase their dietary n-3 intake. Harris *et al*. (2007) demonstrated that consumption of encapsulated fish oils resulted in similar FA patterns to the intake of fish. In addition, Foran *et al*. (2003), have found that fish oils have reduced mercury contamination compared to fish and may provide a better long term source of n-3 LCPUFA.

In humans, fish oil supplement use may require the consumption of multiple capsules daily depending on the concentration of the product and the desired dose. An easier, potentially more palatable way to obtain fish oil supplementation is the use of a concentrated, flavoured emulsified fish oil preparation. Emulsification of fish oils has the potential to improve the digestion and absorption of EPA and DHA (Garaiova *et al*., 2007) due to a modification in the solubility of the supplement. Emulsified fish oil has physical and chemical characteristics that differ from capsular fish oil. The emulsified and water soluble state increases exposure to lipase and diminishes the gastric clearance time.

In animal nutrition, any supplementation is often incorporate to the diet. Whilst in pet nutrition these PUFA supplement are usually administrate in caps like in human nutrition, in feeds intended to livestock, all raw materials and supplement that form diets need to be processed by a combination of different treatments to produce an acceptable final feed. As it has been commented, the main sources of EPA and DHA are presented in seed form or different oils. The incorporation of these raw materials to animal feed is usually made adding the ingredients in different forms such as seed, pellets, powder or liquids. Gridding is the main step of the feed manufacturing process in order to adequate the particle size and facilitate the mixing and subsequent pelleting process. It is important to notice that when the fat level in rabbit diet is major to 20-30 kg/t, it is advisable to use mechanical devices to add the extra fat after pelleting, because high fat addition in the mixer impairs pellet quality (Acedo-Rico *et al*., 2010). Another way to incorporate EPA and DHA in animal diets is by means commercial supplements that have a thorough quality control of their raw materials and achieve a good knowledge of their composition and nutritive value.

Several fish origin supplements rich in LCPUFA have been tested in animal production. For example, Rooke *et al*. (2001b) used tuna fish oil in sows or Coelho

et al. (1997) and Mattos *et al*. (2002) used menhaden fish meal in cows. In that sense, one of the most frequented supplement is salmon oil that has been added to many animal diets (bulls: Gholami *et al*., 2010 and 2011; sheeps: De la Fuente *et al*., 2013; chickens: Pilevar *et al*., 2011; laying hens: Tarltona *et al*., 2013). Considering all information, the same commercial supplement, which guarantees LCPUFA enrichment from an animal source such as salmon oil, has been chosen to enrich rabbit diets in the present Thesis work.

2.2. Fatty acids digestion, absorption and transport

Lipid emulsification in the stomach is a fundamental step in fat digestion through the generation of a lipid-water interface essential for the interaction between water-soluble lipases and insoluble lipids (Ikeda, 2000). The ultimate bioavailability of dietary fat is dependent on this lipid water interface. For this reason, compared to standard fish oil, consumption of an emulsified fish oil supplement can result in an enhanced rate and extent of absorption of total n-3 FA (Raatz *et al*., 2009) due to the improvement of the action of intestinal pancreatic lipase on LCPUFA (Amand *et al*., 1994).

The small intestine is the primary site for digestion and selective absorption of nutrients and other food constituents. The FA included into TG and ingested in the diet are submitted to rather complex processes of digestion and absorption (Image 1.3). These molecules require emulsification, and therefore fat digestion occurs only in the small intestine. In this light, it should be taken into account that the absorption of PUFA is a complex process too. In order to PUFA be absorbed when provided as ethyl esters and ultimately to reach hepatocytes, the ethyl ester bond must undergo hydrolysis by pancreatic lipase enzymes to be converted into free FA for intestinal absorption.

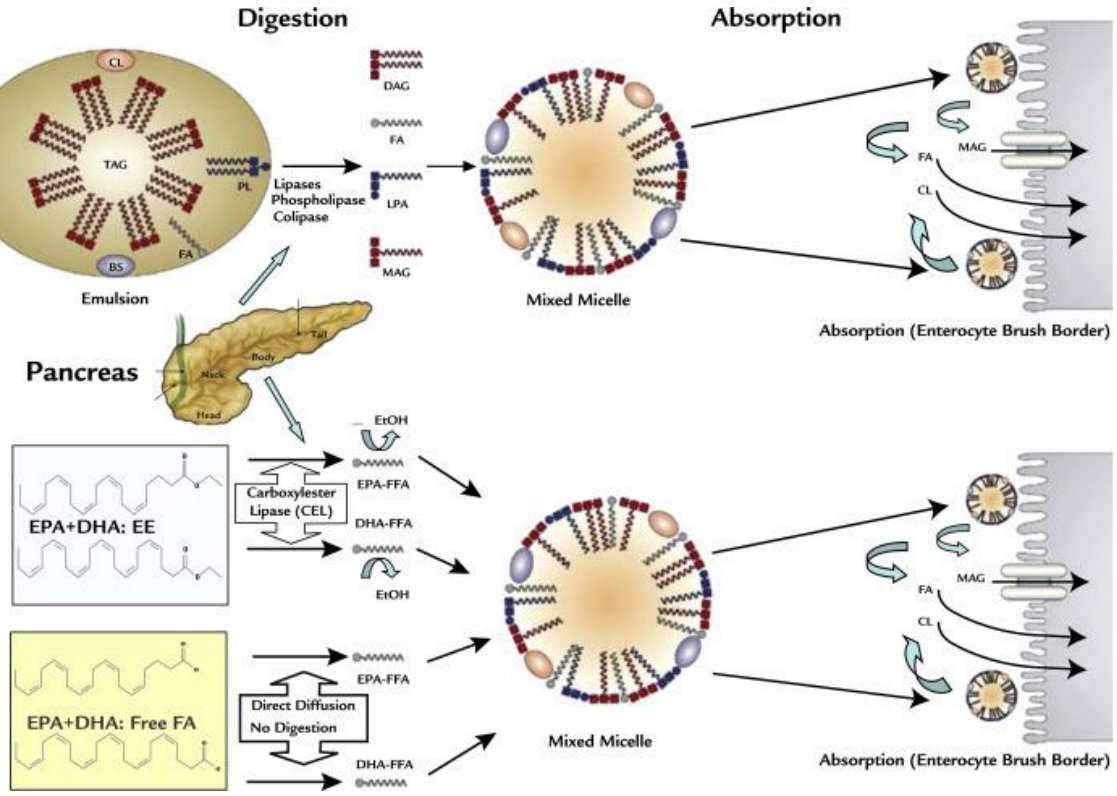

Image 1.3. Schematic showing digestion and absorption of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from ethyl ester and carboxylic acid (free fatty acid (FFA)) formulations of n-3 FA concentrate pharmaceutical products. BS: bile salts; CL: cholesterol; DAG: diacylglyceride; EE: ethyl ester; EtOH: ethanol; LPA: lysophosphatidic acid; MAG: monoacylglyceride; PL: phospholipid; TAG: triacylglyceride. (Davidson *et al*., 2012).

As Xiccato (2010) describes, fat emulsification is promoted by bile salts secreted by the liver. In this process, bile salts mix fat with fat droplets, breaking them down into minute globules than can be easily hydrolysed by pancreatic lipase and other lipolytic enzymes (colipase, sterol ester hydrolase and phospholipase). The enzymatic hydrolysis of TG leads to separation of glycerol, free FA and monoglycerides, which remain emulsified with bile, forming microscopic micelles. The intake of EPA and DHA as free FA facilitates the direct inclusion of these molecules to the micelles, without the need to involve the pancreatic lipase and the others lipolytic enzymes.

These structures move to the microvilli on the duodenum and jejunum where each fraction is absorbed. Bile salts are left in the intestinal lumen, and are then absorbed lower down the tract (distal ileum). When absorbed into enterocytes, glycerol and short-chain FA go directly in to the blood, where they circulate as non-esterified FA (**NEFA**). On the other hand, monoglycerides and medium- and LC FA

are re-synthetized as TG. Droplets of synthetized TG are then covered by a lipoproteic membrane, forming chylomicrons that pass to the lymph circulation system (Image 1.4).

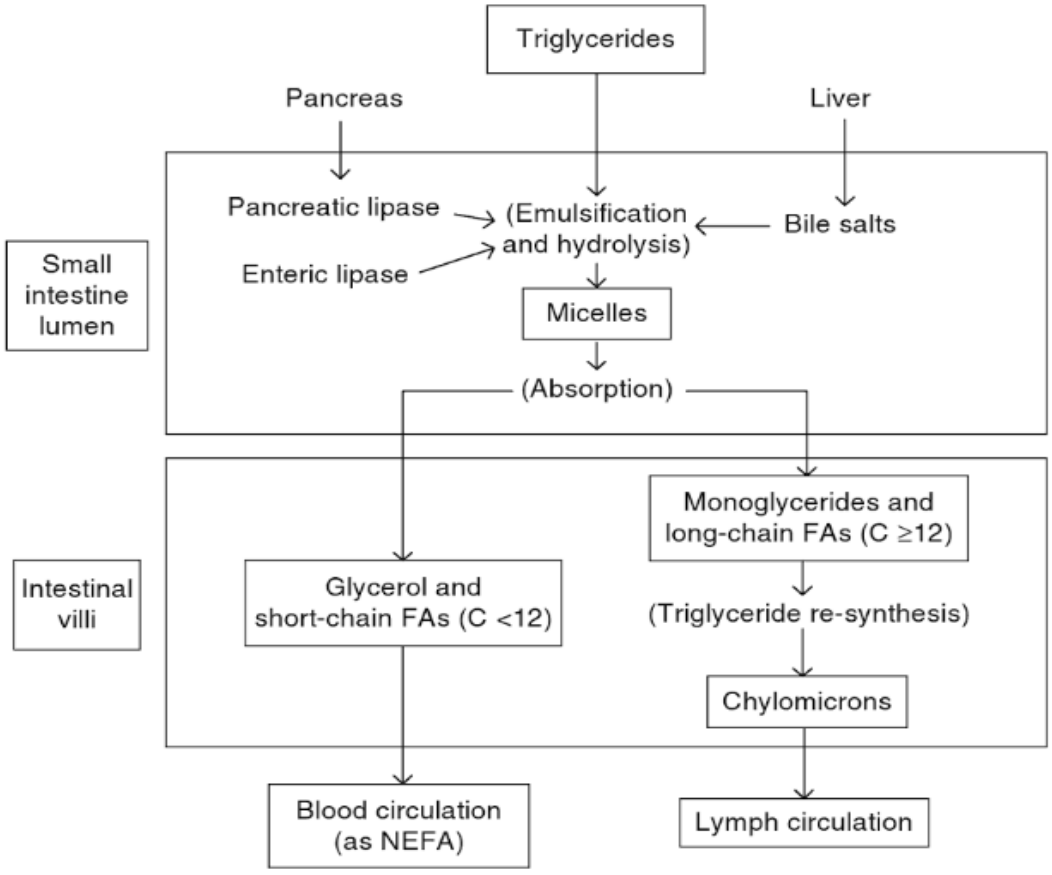

Image 1.4. Digestion and absorption of triglycerides in non-ruminants. NEFA: non-esterified fatty acids. (Xiccato, 2010).

The delivery of n-3 LCPUFA to the liver will also depend on their re-esterification to TG in the enterocytes with subsequent incorporation into chylomicron particles that enter the blood through the lymphatic system. So, the delivery of lipids absorbed can be carried out by different lipoproteins (Image 1.5).

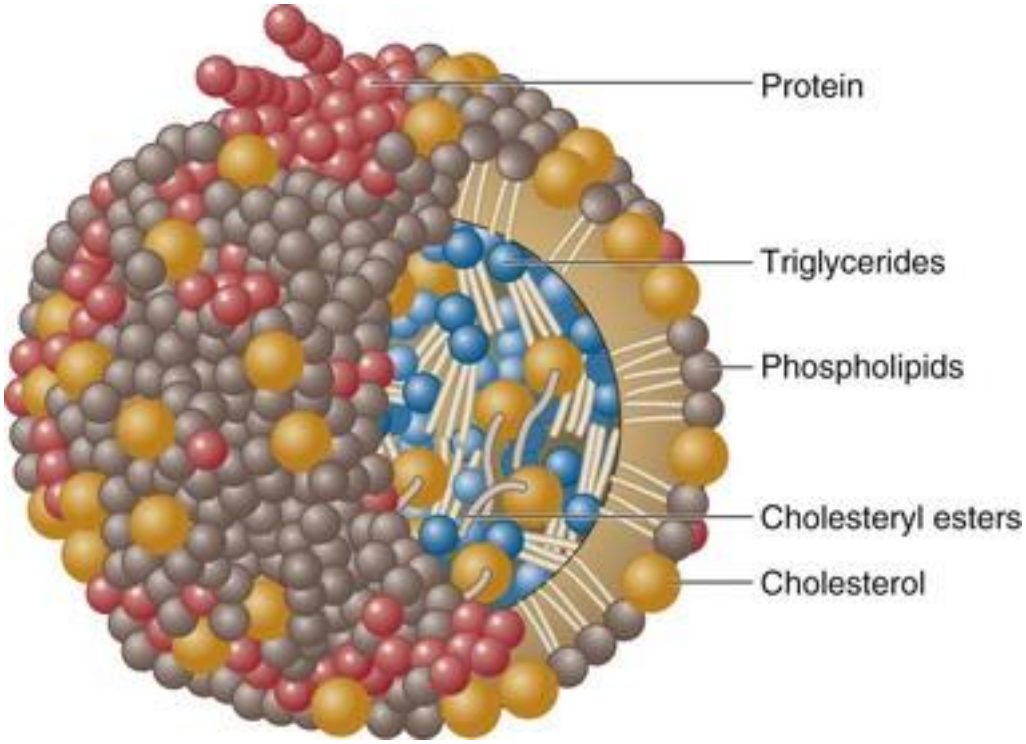

Image 1.5. Constituents of lipoproteins. (Genest *et al*., 2011)

These lipoproteins are classified according to their density, from low to high, such as (Image 1.6):

Chylomicrons are synthesized by enterocytes from lipids absorbed in the small intestine.

Very low-density lipoprotein cholesterol (**VLDL-c**) is synthesized in the liver.

The function of these two lipoproteins is to deliver energy-rich TG to cells in the body. The TG are stripped from chylomicrons and VLDL-c through the action of lipoprotein lipase, an enzyme that is found on the surface of endothelial cells. This enzyme digests the TG to FA and monoglycerides, which can then diffuse into the cell to be oxidized, or in the case of adipocytes, to be re-synthesized into TG and stored in the cell.

Low-density lipoprotein cholesterol (**LDL-c**) delivers cholesterol to cells in the body. As VLDL-c particles are stripped of TG, they become denser. These particles are remodelled at the liver and transformed into LDL-c. The function of LDL-c is to deliver cholesterol to cells, where it is used in membranes, or for the synthesis of steroid hormones. Cells take up cholesterol by receptor-mediated endocytosis. The LDL-c binds to a specific LDL-c receptor and is internalized in an endocytic vesicle.

Receptors are recycled to the cell surface, while hydrolysis in an endolysosome releases cholesterol for use in the cell.

High-density lipoprotein cholesterol (**HDL-c**) is involved in reverse cholesterol transport. Excess cholesterol is eliminated from the body via the liver, which secretes cholesterol in bile or converts it to bile salts. The liver removes LDL-c and other lipoproteins from the circulation by receptor-mediated endocytosis. Additionally, excess cholesterol from extrahepatic tissues is brought back to the liver by HDL-c in a process known as “reverse cholesterol transport”. The HDL-c precursor is synthesized and secreted by the liver and small intestine. It travels in the circulation where it gathers cholesterol to form mature HDL-c, which then returns the cholesterol to the liver.

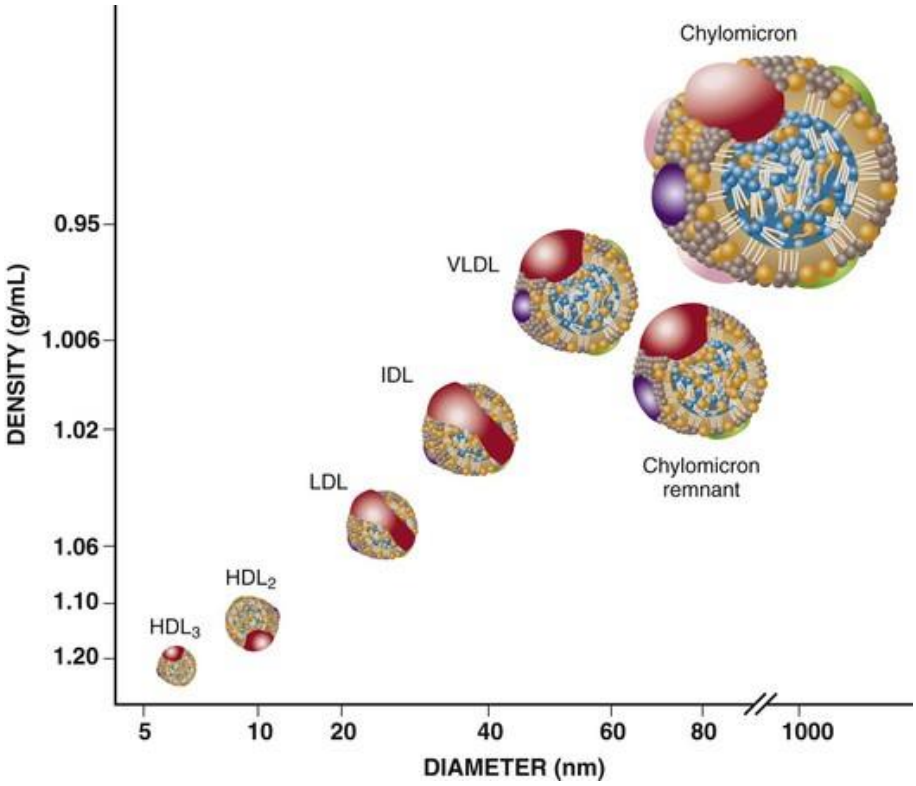

Image 1.6. Classification of the lipoproteins according to their density. VLDL: very low density lipoprotein; IDL: intermediate density lipoprotein; LDL: low density lipoprotein; HDL: high density lipoprotein. (Genest *et al*., 2011)

A scheme of the entire transport process of lipoproteins between intestine, bloodstreams, liver and extrahepatic tissues is shown in Image 1.7.

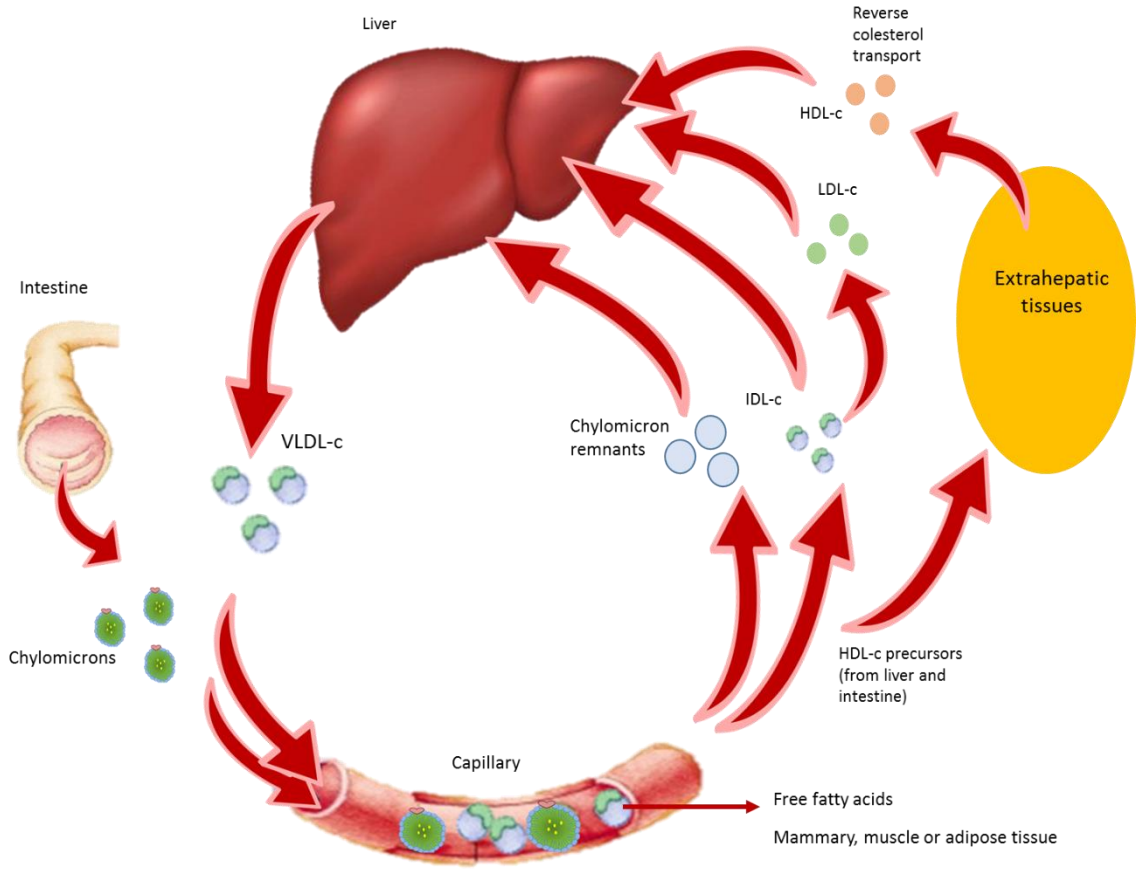

Image 1.7. Entire transport process of the different lipoproteins throughout the body. VLDL: very low density lipoprotein; IDL: intermediate density lipoprotein; LDL: low density lipoprotein; HDL: high density lipoprotein.

In consequence, LDL-c/HDL-c ratio reflects the balance between two completely opposite processes. One is the transport of cholesterol to peripheral tissues by LDL-c with its subsequent arterial internalization, and the other the reverse transport from the extrahepatic tissues to the liver by HDL-C (Thompson and Danesh, 2006). Thus, a larger ratio will implies higher amount of cholesterol from atherogenic lipoprotein circulating through the plasma compartment and likely to induce endothelial dysfunction and trigger the atherogenic process. On the other hand, a lower ratio will indicate less vascular aggression by plasma cholesterol and increased more effective reverse transport of cholesterol to the liver, as well as other beneficial effects, thereby reducing the risk of cardiovascular disease (Dobiasova and Frohlich, 1998; Casteli *et al*., 1986; Howard *et al*., 1986). Therefore, atherogenic

lipoprotein profile of plasma is an important risk factor for coronary artery disease, and it is characterized by high LDL-c/HDL-c ratio and increased level of TG (Rapaport *et al*., 1993). According to the model of Grover (Grover *et al*., 1999) either the ratio of LDL-c/HDL-c or TG/HDL-c or a low level of HDL-c are the best lipid related predictors of future cardiovascular events (Assmann and Schulte, 1992; Tanne *et al*., 1997; Castelli *et al*., 1992; Amarenco *et al*., 2004, 2007). It is, therefore, important to observe not only HDL-c or LDL-c alone but also their ratio. Nonetheless, it should be noted that a high LDL-c/HDL-c ratio does not necessarily correspond to a high serum level of cholesterol.

As it has been mentioned and it was represented in the Image 1.2, once ALA or LA reach the liver and once inside of hepatocyte, the synthesis of n-3 or n-6 LCPUFA takes place, respectively. In this regard, DHA synthesis enzymes are most highly expressed in the liver as compared to heart or brain (Leonard *et al*., 2002), corresponding to more than 30-fold higher rates of DHA synthesis in this organ (Igarashi *et al*., 2007).

3. PHYSIOLOGICAL ACTIONS OF PUFA

3.1. PUFA and lipid metabolism in liver

As it is shown in Image 1.8, n-3 LCPUFA regulation is not only conducting in hepatocytes, but also integrates effects of EPA and DHA on liver, skeletal muscle, and adipose tissue metabolism. Kalupahana *et al*. (2011) have described that these FA increase adipose tissue FA oxidation, the secretion of several hormones such as adiponectin, leptin, and visfatin, and they also alleviate adipose tissue inflammation via G protein-coupled receptor 120 and resolvins/protectins. In the skeletal muscle, EPA and DHA promote FA oxidation, thereby preventing accumulation of FA intermediates.

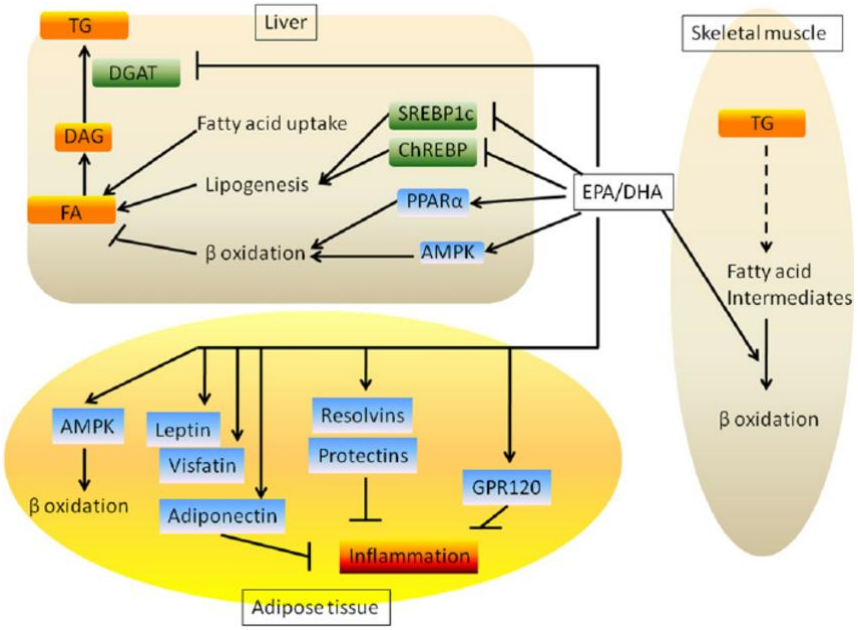

Image 1.8. Integrated effects of EPA and DHA on liver, skeletal muscle, and adipose tissue metabolism. TG: triglycerides; DAG: diacylglycerol; FA: fatty acids; SREBP: sterol regulatory element-binding proteins; ChREBP: carbohydrate responsive element-binding protein; AMPK: adenosine monophosphate protein kinase; GPR: G protein-coupled receptor. (Kalupahana *et al*., 2011)

Effects of n-3 PUFA on lipid metabolism are widely reported in rodents (Rustan *et al*., 1992; Flachs *et al*., 2006) and in human subjects (Woodman *et al*., 2002; Balk *et al*., 2006), with various findings on LDL-c, HDL-c, total cholesterol and TG depending on the type of study and animal species. Results obtained in different clinical studies have helped to understand the mechanisms whereby EPA and DHA

reduce plasma TG, all of which rely on the actions of these FA in the liver. These actions include:

(1) the reduction of hepatic lipogenesis and TG formation;

(2) increase of FA β-oxidation;

(3) the decline in the formation and degree of TG enrichment of VLDL-c particles; (Harris and Bulchandani, 2006; Bays *et al*., 2008).

The molecular mechanisms mediating these effects involve modulation of several nuclear receptors and proteins, including peroxisome proliferator-activated receptors (**PPAR**), sterol regulatory element-binding proteins (**SREBP**), nuclear liver X receptor α, and retinoid X receptor α (Harris and Bulchandani, 2006; Bays *et al*., 2008). The net result is less entry of VLDL-TG into the circulation from the liver and enhanced clearance of TG in circulation (Image 1.9).

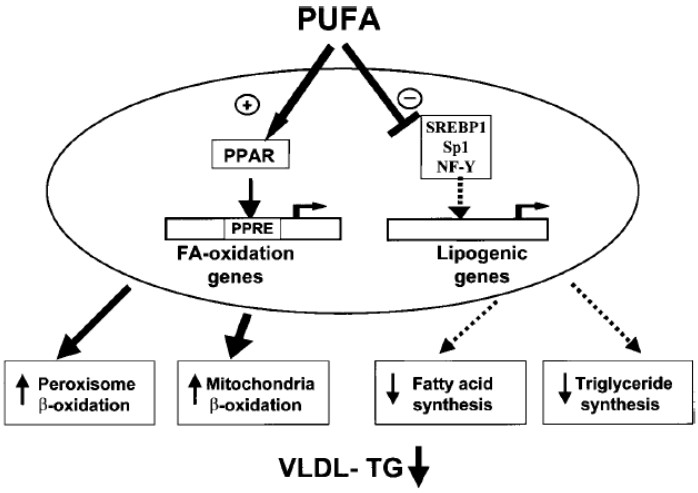

Image 1.9. Nuclear mechanism of polyunsaturated fatty acids (PUFA) regulation of gene expression. FA: fatty acids; NF-Y: nuclear factor Y; PPAR: peroxisome proliferator-activated receptor; PPRE: peroxisome proliferator-activated receptor response element; Sp1: stimulatory protein-1; SREBP-1: sterol regulatory element binding protein-1; TG: triglycerides; VLDL: very low-density lipoprotein. (Clarke, 2001).

A wide range of studies support the triglyceride lowering properties of marine origin n-3 PUFAs supplementation (Kalupahana *et al*., 2010; Horakova *et al*., 2012; Sato *et al*., 2010; Rossmeisl *et al*., 2012; Janovska *et al*., 2013; Flachs *et al*., 2011). According to previous works, Chadli *et al*. (2012) confirmed the

hypotriacylglycerolaemic effect of n-3 PUFA and they also showed a lower liver TG content related to a decrease in VLDL-c secretion that may be explained by both a decrease in lipid synthesis and catabolism. These authors measured the gene expression of SREBP-1c, the main transcriptional factor controlling lipid synthesis in the liver and observed a decrease in their expression in the presence of n-3 PUFA. They also noted that n-3 PUFA reduced diacylglycerol O-acyltransferase 2, an enzyme that catalyses the final step of TG synthesis. Nevertheless, there is some controversy in the reports. The expression of SREBP-1c in mice fed with a DHA (Sun *et al*., 2011) and EPA (Tanaka *et al*., 2010) supplemented diet was limited. By contrast, no effects were observed in SREBP-1c expression in rats fed with EPA (Perez-Echarri *et al*., 2009). Furthermore, CPT-1, an enzyme involved in the transport of FA into the mitochondria, was higher in individuals fed with a n-3 PUFA diet, suggesting an active mitochondrial β-oxidation (Chadli *et al*., 2012).

Earlier works carried out in hamsters showed that EPA and DHA decrease fasting and postprandial plasma HDL-c (Chadli *et al*., 2012). In this connection, the decrease of total cholesterol that has been reported in other studies in hamsters (Lin *et al*., 2005; Mast *et al*., 2010) and mice (Le Morvan *et al*., 2002) has been associated with a decrease in HDL-c, and related likewise with an overexpression of scavenger receptor class B, type 1 (**SR-B1**) (Le Morvan *et al*., 2002; Spady *et al*., 1999). The SR-B1 is an integral membrane protein found in numerous cell types/tissues, including the liver. It is best known for its role in facilitating the uptake of cholesteryl esters from HDL-c in the liver. Therefore, n-3 PUFA stimulate the flow of cholesterol ester transport from peripheral tissues to the liver, where cholesterol can either be secreted via the bile duct or be used to synthesise steroid hormones (Rhainds and Brissette, 2004).

However, there is controversy in the results obtained studying the SR-B1 activity. A study in obese rats reported an increase in SR-B1 action, but with no change in HDL-c level (Sheril *et al*., 2009). Also, Chadli *et al*. (2012) working with hamsters fed with a cholesterol-enriched diet observed a high HDL-c and low SR-B1 gene expression associated, in this case, with a decrease in reverse cholesterol transport efficiency (Treguier *et al*., 2011). These authors found an increase of SR-B1 expression in liver.

In that line, several reports evidence that the effect of fish oil on LDL-c concentration is also inconsistent (Wijendran abd Hayes, 2004). For example,

normolipidaemic subjects show reduction in plasma LDL-c concentrations following intake of fish oil diet (Illingworth *et al*., 1984; Neste, 1986), however, fish oil supplementation to hyperlipidemic subjects causes an increase in LDL-c concentrations (Sullivan *et al*., 1986; Hsu *et al*., 2000; De Silva *et al*., 2005).

Several studies confirmed that total and LDL-c levels increase with age (Steinberg, 1988; Levine *et al*., 1995; Heiss *et al*., 1980; Abbott *et al*., 1983; Parini *et al*., 1999), as does the incidence of cardiovascular disease (Castelli *et al*., 1989). The age-related changes in cholesterolemia were attributed to an age-related-increase in 3-hydroxy-3-methylglutaryl coenzyme A reductase activity (Marino *et al*., 1998, 2002), the key enzyme of cholesterol biosynthesis (Geelen *et al*., 1986) and to a decrease in the expression of LDL-c receptors on the liver cell membrane (Field and Gibbons, 2000). The LDL-c receptors increase in the total lysate older rat liver, but the LDL-c receptors on the membranes of liver cells (involved in the removal of LDL-c particles from blood stream (Stryer, 1995)) are known to decrease with increasing age (Pallottini *et al*., 2006). Works of Straniero *et al*. (2008) in rats confirmed that the PUFA enriched diet prevented the age related changes in total, LDL-c and HDL-c plasma levels. The beneficial effects were clearly visible by the age of 24 months, when the rats fed a PUFA enriched diet exhibited juvenile plasma lipid content.

3.2. PUFA and lipid metabolism in adipose tissues

White adipose tissue (**WAT**) is the major site for storage of excess energy in the body (principally TG). Adipose tissue have an important physiological role beyond their lipid storage capacity, it secretes numerous bioactive peptides collectively known as adipokines (Kim and Moustaid-Moussa, 2000; Olefsky and Glass, 2010) which vary widely in both their function and mechanisms of control. One such mechanism of control is the FA composition of adipose tissue which as it has been already mentioned, can affect cellular signalling, FA trafficking, gene expression and, consequently, metabolism (Gertow *et al*., 2006). Adipose tissue composition varies based on two main effectors: energy balance, which regulates the metabolism of free FA within the adipose tissue, and diet, which will alter the FA profile of the adipose tissue.

An important adipokine that could be stimulated by changes in FA profile is leptin. It was first discovered as the protein encoded by the obese gene, named for

the phenotype of the double knockout mouse. These mice experience no satiety, and thus eat continuously when fed *ad libitum*, leading to severe diet-induced obesity. The role of leptin in hypothalamic-mediated appetite suppression in response to caloric intake is not its only function. Previous studies demonstrated that lipid composition of the diet can have an important role to play in adipokine expression, PUFA may act as ligands for PPARγ to alter adipokine expression (Yu *et al*., 2011). Fatty acids might regulate transcription of leptin and several adipocyte-specific genes by changing the regulation pattern of the nuclear receptor PPARγ (Kallen and Lazar, 1996). A study in rats fed with a fish oil-enriched diet found that by increasing activation of PPARγ, the epididymal leptin mRNA levels decrease. In addition, a reduction in PPARγ expression in cultured cells treated with EPA and DHA was to approximately 70% of that in the control (Reseland *et al*., 2001). Besides, the n-3 PUFA had an influence on leptin gene expression that was accompanied by a reduction in PPARγ and SREBP-1 mRNA levels Reseland *et al*., 2001).

Brown adipose tissue (**BAT**) is a thermogenic organ that protects the body from cold environment via dissipating chemical energy (lipid and glucose) as heat. The BAT is characterised as possessing large amounts of the unique uncoupling protein (**UCP**) 1 which when activated enables the free-flow of protons across the inner mitochondria membrane, resulting in the rapid dissipation of chemical energy as heat. The primary energy source for this process comes from NEFA that are released from lipid at the same time as UCP1 is activated. Brown adipose tissue thermogenesis is predominantly governed by the sympathetic nervous system via the adrenergic receptor signalling pathways. Upon stimulation, sympathetic nerve releases norepinephrine that binds to β3 adrenergic receptors in the membrane of brown adipocytes to activate a cascade of signalling pathways, leading to increases in FA β-oxidation and heat production (Cannon and Nedergaard, 2004). However, previous studies have reported that neurons in the dorsomedial hypothalamus/dorsal hypothalamic area also participate in the regulation of BAT activity (Nakamura and Morrison, 2011). Moreover, it has been described that leptin receptor-expressing neurons in the dorsomedial hypothalamus increase sympathetic outflow to BAT and vice versa. Thus, the injection of leptin into the dorsomedial hypothalamus/dorsal hypothalamic area normalises body temperature in leptin deficient ob/ob mice (Enriori *et al*., 2011), suggesting that leptin controls

energy expenditure by inducing BAT activation and thermogenesis via neurons in the dorsomedial hypothalamus/dorsal hypothalamic area. Nonetheless, it is unlikely that leptin signalling in dorsomedial hypothalamus neurons only regulates BAT thermogenesis, but rather initiates a coordinate response that involves FA release from adipose tissue which is the major substrate for thermogenesis in BAT.

BAT is principally located in the interscapular and perirenal fat depots (Sidossis and Kajimura, 2015), and larger amounts of this tissue are present in newborns even though it decrease with age (Harms and Seale, 2013; Pfeifer and Hoffmann, 2015). The onset of nonshivering thermogenesis in BAT is a prerequisite for effective adaptation to the cold challenge of the extrauterine environment in a majority of mammals but especially in species that are precocial (Symonds *et al*., 1995). For example, altricial offspring, such as mice, rats and rabbits, which are born after a short gestation with an immature hypothalamic-pituitary-adrenal axis. Consequently their BAT matures postnatally in parallel with maturation of this axis (Giralt *et al*., 1990).

Interestingly, subcutaneous WAT also has the ability to differentiate into brown-like adipocytes and may potentially contribute to increased thermogenesis (Pahlavani *et al*., 2017). Previous works have reported that EPA reduces high-fat diet-induced obesity and insulin resistance in mice. Whether BAT mediates some of these beneficial effects of EPA has been less investigated. In that sense, a recent study showed increased expression of some representative BAT genes in the subcutaneous WAT and suppressed expression of genes related to the WAT phenotype with EPA (Zhao and Chen, 2014). However, only few studies have focused on mechanisms involving EPA activation of BAT as a potential mean to reduce bodyweight. Sneddon *et al*. (2009) showed that conjugated linoleic acid plus n-3 PUFA increases food intake without affecting body weight (**BW**) potentially by inducing BAT size and UCP1 level in rats.

3.3. PUFA and oxidative stress

Oxidative stress is a general term that describes the imbalance between the relative levels of reactive oxygen species (**ROS**) and intra- and extracellular antioxidants wherein the amount of ROS is in excess (Reuter *et al*., 2010). Low and moderate quantities of ROS are beneficial to some physiological processes, including pathogen elimination, tissue repair, and wound healing. However,

abundant quantities of ROS will lead to the loss of cell functions through oxidative damage of several cell constituents, including DNA, RNA, lipids, PUFA and proteins, and can result in organ and pathway-specific toxicity related to processes such as alteration of membrane permeability, promotion of inappropriate apoptosis, and reduction of the antioxidant defence ability of the body (Veskoukis *et al*., 2012; Zhu *et al*., 2012; Jones, 2006). Numerous factors, such as weaning, environmental factors, infection, and pro-oxidants, can induce oxidative stress, resulting in tremendous economic loss during livestock production (Liu *et al*., 2014; Yin *et al*., 2013).

In that sense, during certain physiological situations, as the rapid passage of the mammalian neonates from the intrauterine to the extrauterine environment, a drastic exposure to environment oxygen is induced, which imply a sudden systemic and pulmonary adaptation to the post-natal condition (Mutinati *et al*. 2013). Thus, the first breaths of the neonate imply also an increase in ROS generation (Jain *et al*. 1996; Saugstad 2003; Wiedemann *et al*. 2003; Gaál *et al*. 2006), which make newborns more prone to develop oxidative stress than adults. During parturition, in fact, the foetus is transferred from an intrauterine hypoxic environment with 20–25 mm Hg oxygen tension (PO_2) to an extrauterine normoxic environment with approximately 100 mm Hg PO_2 (Gitto *et al*. 2009). This oxidative stress situation also results from their constitutive deficiency in antioxidants (Saugstad 2005; Gitto *et al*. 2009). It is true that in comparison with adults, increased concentrations of some antioxidants such as ascorbate and bilirubin are found in newborns (Gopinathan *et al*. 1994; Wiedemann *et al*. 2003), but only for a short period after birth (Stevens and Autor 1980). Furthermore, it is important to notice that other major antioxidants such as vitamin E, β-carotene, melatonin and sulphhydryl groups, of plasma metal binding proteins such as ceruloplasmin and transferrine and of erythrocyte superoxide dismutase show reduced activity in newborns (Gitto *et al*. 2009).

Several investigators studied the relationship between the oxidative state of the mother and the newborn, at parturition. In this connection, Auguelles *et al*. (2006) measured oxidative stress markers (carbonyl groups, lipid peroxides and total antioxidant capacity) in human blood and found a good correlation between the oxidative status of the mother and that of the neonate, with higher maternal oxidative stress correlating with an even higher oxidative stress of the newborn based on

measurements in the umbilical cord blood. Rizzo *et al*. (2008) performed a study in pregnant ewes, around parturition and found a significant increase in serum ROS concentrations between 36 h before and 24 h after lambing. This is the first study to report serum ROS levels in sheep around lambing, explaining the increase in ROS generation as both a consequence and a cause of the acute inflammatory process accompanying parturition. In addition, others report confirmed an increased risk of oxidative stress in newborn calves showing higher levels of pro-oxidants than their mothers (Gaál *et al*. 2006; Albera and Kankofer 2011).

Oxidation is an accelerated chain reaction, where small amounts of peroxides in the source oils or exposure to oxidative conditions may dramatically influence the rate of oxidation of n-3 PUFA. The susceptibility of FA to oxidation is thought to be directly dependent on their degree of unsaturation, and subsequently, supplementations with highly unsaturated n-3 PUFA have been reported to increase oxidative damage (Di Nunzio *et al*., 2011). Therefore, the higher unsaturation level of DHA may increase the susceptibility of the molecule to be oxidized compared to EPA, rending to a higher level of free radicals. Nevertheless, Richard *et al*. (2008) investigated the free radical-scavenging potential of n-3 and n-6 supplements in cell cultures. They observed that supplemented cells with n-3 PUFA produced lower amounts of ROS than cells fed with n-6 (AA and LA) despite the lower unsaturation level of the molecules. It was shown that n-6 series were more susceptible to oxidize than n-3 ones. Therefore, it was suggested that the susceptibility to oxidation of PUFA molecules is not as straightforward as hypothesized. Not only the lower degree of unsaturation leads to higher antioxidant activity, but the chemical structure may play an important role in the different antioxidant aptitude.

Furthermore, some n-3 PUFA products contain camouflaging or deodorising substances to make them more palatable which may further accelerate secondary oxidation (Shahidi and Zhong, 2010; Albert *et al*., 2013). Recent human studies have suggested that the levels of oxidation can negatively affect the efficacy of an n-3 PUFA supplement, limiting the beneficial effects on circulating TG and cholesterol levels (Garcia-Hernandez *et al*., 2013). Similarly, animal models provide evidences that suggest that chronic exposure to oxidised n-3 PUFA may lead to growth retardation, increased inflammation, cardiomyopathy and carcinogenesis (Esterbauer, 1993; Bartsch and Nair, 2006). Thus, the levels of oxidation may

dramatically affect the efficacy and safety of using n-3 PUFA nutritional supplements, which may counteract the intended use of these products.

As a result, a correct diet formulation (including the administration of antioxidant vitamins) of the mother during pregnancy and close to delivery, besides exerting a positive impact on pregnancy itself, can directly improve the survival of the newborn through an increase in colostrum/milk both production and quality, and consequently, an increase the birthweight and growth rate of the newborn (Sciorsci and Rizzo, 2012).

3.4. PUFA and glycaemic index

Glucose absorption in the small intestine is mediated by the apical sodium-dependent glucose transporter (**SGLT**) 1 and the basolateral glucose transporter (**GLUT**) 2 in the brush-border membrane of the enterocyte (Farquharson *et al*., 1995; Gabler *et al*., 2009), both of which are regulated by a number of factors. One of them is the n-3 LCPUFA, which through diet have been shown to normalise intestinal glucose absorption in rat pups from mothers fed diets that are low in unsaturated FA (Vicario *et al*., 2005). However, the mechanism by which n−3 PUFA may augment intestinal glucose uptake is not fully understood (Gabler *et al*., 2007). For example, in vitro studies carried out with the “Ussing chamber technique”, Gabler *et al*., (2009) obtained an approximate 70% increase in active glucose uptake when short-term exposure to DHA was applied. This finding clearly indicates the potential for an acute regulation of glucose uptake by DHA. Due to the indications of n−3 PUFA activate adenosine monophosphate protein kinase (**AMPK**) in other tissues (Suchankova *et al*., 2005), these authors cautiously speculate that the acute effect of DHA is mediated by AMPK. However, further studies need to be carried out to allude to the exact mechanism of action of DHA on AMPK. Regarding to these thoughts, Walker *et al*. (2005) concluded that the activation of AMPK results in enhanced glucose uptake by increasing the levels of GLUT2 in the brush-border membrane of mice jejunum. Since dietary n−3 PUFA enhance rat hepatic AMPK activity in vivo (Suchankova *et al*., 2005), Gabler *et al*., (2009) cautiously suggested that it is the DHA or n−3 LCPUFA that may be acutely signalling glucose transporter proteins through AMPK. Thus, the increase in glucose uptake appears to be orchestrated by two separate mechanisms: first, increased total jejunum SGLT1 protein content due to protected fish oil feeding; and, second, in an acute manner,

potentially through the n−3 PUFA activation of jejunum AMPK, resulting in the increased translocation of GLUT2 to the brush-border membrane of the enterocyte.

As it has been mentioned, the phenotypic expression of the intestinal digestive and transport functions may be modified by the lipid content of the diet (Thomson *et al*., 1989). The absorption of fructose across the enterocyte brush-border membrane is mediated by a sodium-independent facilitative transporter, GLUT5, and among the seven members able to transport fructose, GLUT5 is the sole transporter specific for fructose with no ability to transport glucose (Burant *et al*., 1992). Both SGLT1 and GLUT5 are restricted to the brush-border membrane domain of the villus, but there were different patterns of fructose uptake compared with glucose uptake. Studies conducted by Jarocka-Cyrta *et al*. (1998) elucidated that maternal dietary FA consumption during gestation had different effects on the adaptability of jejunal and ileal fructose transport. For example, although continuous feeding of the high n-6/n-3 diet to the mother was associated with higher rates of glucose uptake, the rates of fructose uptake were lower. The differences observed in the adaptation of the intestinal glucose and fructose transport in response to the various dietary lipids as a consequence of exposure to different FA during gestation suggests that SGLT1 and GLUT5 are influenced in a different manner by various dietary FA.

3.5. PUFA and reproductive system

Nutrition acts at various points of the hypothalamus–pituitary–ovarian axis to control ovarian function and follicular environment (Armstrong *et al*., 2001, Garnsworthy *et al*., 2008a). More specifically, maternal nutrition affects the circulating concentrations of metabolites and hormones, it also influences in growth factors presenting in the follicular fluid, as well as in the oviduct and in the uterus (Webb *et al*., 2007).

Several works have proved that dietary PUFA supplementation can influence biosynthetic pathways involved in both prostaglandin (**PG**) synthesis and steroidogenesis that have multiple roles in the regulation of reproductive functions (for review see Wathes *et al*., 2007). The PUFA have also important effects on reproductive processes including ovarian follicular growth, oocyte maturation, ovulation, corpus luteum function as well as progesterone production, fertilization, maintenance of pregnancy and parturition (Abayasekara and Wathes, 1999).

3.5.1. Oocytes and follicles

Oocytes of many species contain high levels of FA, which are used as an energy source during their maturation and the extended period of embryo development before implantation. Dietary PUFA alter the FA composition of cumulus cells, granulosa cells, and oocytes which may be relevant for oocyte quality (Kim *et al*., 2001), maturation and subsequent competence (Ferguson and Leese, 2006). Previous works have deep in this topic; Zeron *et al*. (2002) collected higher numbers of high-quality oocytes from n-3 PUFA supplemented ewes and align with them, Kim *et al*. (2001) observed differences in FA composition between high- and low-quality oocytes. Even though these differences did not directly influence oocyte maturation, their effects on oocyte competence cannot be discounted and may be responsible for differences in fertilization rate and developmental potential (Kim *et al*., 2001). Also in cows, dietary supplementation with LCPUFA (both n-3 and n-6) induced changes in several aspects of folliculogenesis, including both an increase in total follicular numbers and in the size of the dominant or pre-ovulatory follicles (Bilby *et al*., 2006). Recent works with fish oil supplemented goats (Mahla *et al*., 2017) obtained a higher number of large follicles and pre-ovulatory follicles than a control group. These findings are supported by earlier reports where increased number of medium size follicles was found in cows supplemented with high doses of fish meal (Moussavi *et al*., 2007). In contrast, Petit *et al*. (2002 and 2004) found little or no effect of linseed, fish oil, flax, and sunflower seed supplemented diets on follicular dynamics.

3.5.2. Ovulation

Ovulatory process in mammals share many characteristic of an inflammation-like response. In this regard, in a model proposed by Espey (1980), as an inflammatory reaction, briefly the ovulation mechanism acts as follow: an ovulatory surge of LH before ovulation induces a rise in production of cyclic AMP, steroidogenesis, and release of histamine as well as related compounds that mediate the initial phases of inflammation. During the intermediate stages of ovulation, PG enhance inflammatory reactions and activate thecal fibroblasts. In the final stages of ovulation, pre-ovulatory follicle-secreted serine proteases activate

local collagenases, resulting in a multitude of proteolytic enzymes that degrade follicular connective tissue and thus induce ovulation.

As it has been commented, the position of the double bonds in the carbon chain determines the ability of PUFA to act as precursors of others essential compounds such hormones. Specifically, twenty carbons PUFA are the direct precursors of large physiologically active compounds called eicosanoids, which include PG, thromboxanes, leukotrienes and lipoxins (Needleman *et al*., 1986). The synthesis of PG in body tissues, included ovary, is a highly regulated process. Excess of PUFA are stored in most cells within phospholipids in the membranes in an esterified form. The proportion of different PUFA in the diet alters cell membrane of phospholipid composition and this become quantitatively significant because the precursors of each group of PG compete for the same enzyme systems for metabolism. Therefore, the initial step is the generation of the PUFA substrate within the cell. The free PUFA (Dihomo-γ-linoleic acid (**DGLA**), AA or EPA) generated are then metabolized by prostaglandin endoperoxide synthase (**PTGS**) enzymes (Image 1.10).

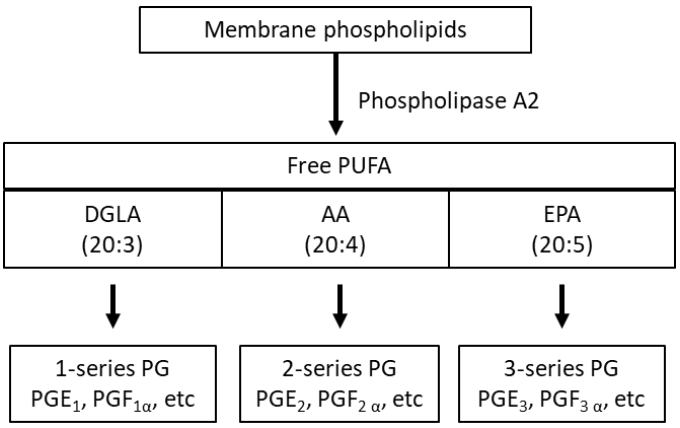

Image 1.10. Scheme to illustrate the generation of 1-, 2-, and 3-series prostaglandins (PG) from dietary PUFA. DGLA: dihomo-γ-linoleic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid. (Adapted from Wathes *et al*., 2007)

The 1- and 2-series PG are derived from the n-6 PUFA DGLA and AA respectively, whereas the 3-serie PG is derived from EPA (Needleman *et al*., 1986). The PGE synthase is the enzyme responsible for series 1, 2, and 3 of PGE, where

the most important is PGE_2 that is responsible output in inflammatory situations (Murakami and Kudo, 2004). On the other hand, the PGF synthase is the responsible for series 1, 2 and 3 of PGF, related with different biological process. The 2-series PG are generally considered to be more biologically active than the 1- and 3-series PG. For example, 2-series PG are thought to act as luteotrophic factors in the early luteal phase. PGE_2 stimulates progesterone secretion by cyclic corpora lutea whereas in the corpora lutea of pregnancy, PGE_1 is more potent than PGE_2 in stimulating progesterone secretion (Weems *et al*., 1997). Later in the cycle, $PGF_{2\alpha}$ is the main luteolytic agent (Poyser, 2005).

Diets high in n-6 PUFA increase the availability of AA for eicosanoid synthesis (Kinsella *et al*., 1990). Therefore, any variable that reduces the availability of AA reduces biosynthesis of two-series PGs and has the potential to alter ovulation. The ingestion of n-3 PUFA alters eicosanoid production by replacing AA in tissue phospholipids (Gurr and James, 1975). A work of Trujillo and Broughton (1995) elucidated that oocyte ovulation is inversely related to ovarian phospholipid AA incorporation, with the high n-3 PUFA consuming group having the highest oocyte production and the lowest AA concentrations in ovarian phospholipids. In contrast, the n-6 PUFA consuming group had the lowest oocyte production and the highest ovarian incorporation of AA. The possible explanation of these results was that n-3 PUFA intake resulted in increased production of 3-series PG. The diet causing the lowest ovulatory rate was the n-6 PUFA-enriched diet, an effect that may have been induced through overwhelming production of PGE_2 and suppression of ovulation in the same manner that high PGE_2 concentrations suppress the immune response (Kinsella *et al*, 1990).

Previous studies have demonstrated that the administration of high concentrations of PGE_2 before ovulation in rabbits and hamsters reduces production of oocytes (Hamada *et al*., 1977; Martin and Talbot, 1981; Schmidt *et al*., 1986). Conversely, in isolated rat ovaries, ovulation is inhibited by administering indomethacin (nonsteroidal anti-inflammatory agent) resulting in a reduction of PGE_2 and $PGF_{2\alpha}$ (Sogn *et al*., 1987). Ovulation is restored by the addition of exogenous PGE_2. On the other hand, consumption of the high n-3 PUFA diet resulted in greater oocyte release, possibly reflecting in an increase, although less biologically potent, in the production of a PGE_3 or a decrease in PGE_2 biosynthesis.

Hence, both resulted in an enhancement of ovulation, induced through alleviation of the suppressive effect of PGE_2.

3.5.3. Early embryo development

Thangavelu *et al*. (2007) examined the effect of PUFA on in vivo embryo development of cattle 7 days after insemination and did not find influence of treatment on fertilization rate or number of embryos, although PUFA increased total cell numbers in embryos at the expanded blastocyst stage, which the authors speculated might be due to higher circulating progesterone concentrations in cows offered PUFA. Changes to the progesterone profile during the estrous cycle would be likely to affect early embryo development indirectly. In this regard, Mann *et al*. (1998) suggested that decreased progesterone concentrations in the early luteal phase in cattle reduce embryo survival, an effect thought to be mediated via changes in uterine secretions. Nonetheless, as it is known, progesterone not only prepares the uterus for implantation of the embryo but also helps maintain pregnancy by providing nourishment to the conceptus. Furthermore, progesterone has a major role in the endometrium in preparation for implantation of a fertilized oocyte, and in many species a decrease in circulating progesterone at the time of fertilization is sufficient to delay implantation (Rothchild, 1983).

In sows, Webel *et al*. (2004) proved that non-supplemented group had an embryonic survival of only 59% and n-3 LCPUFA supplementation improved embryonic survival to 71% a day 30 of gestation, and consequently an increase in the litter size was observed. Both, the decrease in early embryonic mortality and consequently, the improves in the fertility rate observed in several species dietary supplemented with n-3 PUFA (Burke *et al*., 1997; Nazir *et al*., 2013) are explained by a reduction the 2-series PG, precisely in the uterine $PGF_{2\alpha}$ secretion and/or decrease in the sensitivity of the corpus luteum to $PGF_{2\alpha}$ during critical stage of embryonic development, preventing the onset of luteolysis and facilitating the establishment of pregnancy (Mattos *et al*., 2000), thus giving the conceptus longer to develop before the possible onset of luteolysis (Mattos *et al*., 2004).

3.5.4. Placental transfer

During pregnancy, mammal mothers adapt her metabolism to support the continuous draining of substrates by the foetus. Her increase in net BW (free of the

conceptus) corresponds to the accumulation of fat depots during the first two-thirds of gestation, switching to an accelerated breakdown of these during the last third (Herrera, 2002). Transfer between the maternal and the foetal blood takes place in the placenta. During pregnancy, the placenta grows rapidly and exhibits marked changes in morphological structure according to foetal development. Even though the placenta is a temporally organ, performs a number of important functions throughout the gestation, including anchor of the developing foetus to the uterine wall, mediate in maternal immune tolerance, O_2/CO_2 exchange, provide nutrients for the foetus and remove waste products during embryonic development (Bauer *et al*., 1998).

PUFA exert a direct effect thought different moments of the gestation. One of most important is that n-3 PUFA increase the prostacyclin:thromboxane ratio, thereby promoting vasodilatation and reducing blood viscosity (Leaf and Weber, 1988), both of which would facilitate placental blood flow and thus improve foetal growth.

However, maternal TG do not directly cross the placenta (Herrera, *et al*., 1998). In this context, placental tissue from different species has been shown to express lipoprotein lipase activity (Elphick and Hull, 1977; Rotherwell and Elphick, 1982; Bonet *et al*., 1992) as well as phospholipase A2 (Farrugia *et al*., 1993; Rice *et al*., 1998) and intracellular lipase activities (Biale, 1985; Kaminsky *et al*., 1991; Mochizuki *et al*., 1975). Through this mechanism, maternal plasma TG are hydrolysed and taken up by the placenta, where reesterification and intracellular hydrolysis facilitates diffusion of the released FA to the foetus, and their subsequent transport to foetal liver (Image 1.11). Those FA are rapidly taken up by foetal liver, where they are esterified and released back into circulation as TG. Thus, a significant linear correlation is developed for certain LCPUFA between maternal plasma and cord plasma TG during late gestation (Berghaus *et al*., 2000). Furthermore, the foetus also benefits from other product of maternal metabolism. Despite ketogenesis is not active in the foetus, ketone bodies easily cross the placenta, therefore, they reach the same level in foetus plasma than in maternal plasma. Once in foetal plasma, ketone bodies may be used not only as fuels but also as lipogenic substrates.

Furthermore, during prenatal stage, the ingestion of a fluid compound of a mixture of amniotic fluid, lung liquid and oral/nasal secretions may be an important

regulator of gastrointestinal tract development of the foetus (Mulvihill *et al*., 1985). Foetal intestinal epithelium has the capacity to absorb carbohydrates, protein, and lipids in the last third of human pregnancy (Charlton-Char and Rudoph, 1979; Lev and Orlic, 1972; Levy *et al*., 1992; Ritkin and Reynolds, 1975). Consequently, amniotic fluid may be a potential source of foetal nutrition. Therefore, maternal nutrition during critical period as gestation is intimately linked to gastrointestinal development of the foetus.

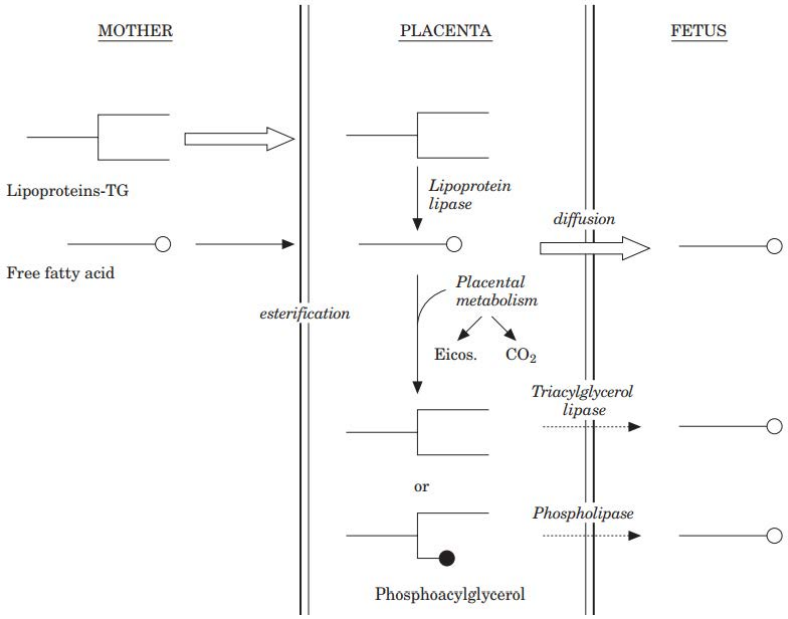

Image 1.11. Schematic representation of the placental transfer of fatty acids to the foetus. TG: triglycerides. (Herrera, 2002)

These studies show the importance of maternal dietary FA controlling the availability of LCPUFA to the foetus and newborn. For example, in tissues such as the brain, lipids constitute around 50 per cent dry weight and almost half of the total lipid content is composed of PUFA (Gurr, 1993), but the degree to which the foetus of different species are capable of desaturation and elongation is not clear. Studies carried out in sows confirm that DHA increased in embryos at early gestation (Brazle *et al*., 2009; Smit *et al*., 2013). These results evidence that either DHA is taken up through the placenta into the embryo during early pregnancy or that more EPA was taken up by the embryo and then converted to DHA. In addition, tissues of piglets from n-3 PUFA supplemented sows showed higher EPA and DHA levels at birth than those from control sows (Rooke *et al*., 2001b and 2001c), thus, it is likely that n-3 PUFA uptake continues throughout gestation and not only at early stage of it.

Nonetheless, it is important to highlight that maternal PUFA supplementation could be responsible for foetal damage during pregnancy in rats (Viana *et al*., 1996; Simán and Eriksson, 1997; Viana *et al*., 2000), due to the fact that excess intake of PUFA may reduce antioxidant capacity (Cho and Choi, 1994), enhancing susceptibility to oxidative damage (Mazière *et al*., 1998). Hammond *et al*. (2001) studied the effect of several oral doses of Dried *Schizochytrium sp*. microalgae (DRM) containing oil rich in EPA and DHA compared with fish oil, composed of 100% total (crude) fat, which was used as a negative control. Treatments were dosed orally by gastric intubation during 13 consecutive days (day 6 to 18 of pregnancy). As a possible consequence of the high-fat content of the fish oil and DRM, reductions in food consumption and BW gain and a slight increase in abortions occurred in the fish oil control groups and the groups with highest dose of DRM. No indication of developmental toxicity was observed in foetuses at any dose level at the morphological evaluation.

3.5.5. Neonatal transfer

In most eutherian mammals, it is accepted that changes in the hypothalamus-pituitary-adrenal axis are responsible for initiating the onset of parturition (Challis *et al*., 2000). In this connection, PUFA may thus be able to influence the timing of parturition through alterations to PG or adrenal steroid synthesis. This was originally demonstrated in sheep, where a rise in foetal cortisol initiates a chain of events that culminates in the birth of the offspring. The main action of cortisol regarding to parturition is to raise levels of 2-series PGs (Challis *et al*., 2002; Olson, 2003). Both foetal and maternal tissues, including amnion, chorion, and decidual endometrium, synthesise PG in vitro (Challis *et al*., 2000). The levels of PG along with their synthetic enzymes (mainly PTGS2) increase either before or at the time of labour. The onset of normal parturition is associated with increased levels of both LA and AA in blood (Ashby *et al*., 1997). For example, sheep fed a diet rich in n-6 PUFA during late gestation had higher circulating concentrations of 2-series PG (PGE2 and PGF2a), produced more PG in uterine and placental tissues, and appeared to be more susceptible to preterm parturition (Elmes *et al*., 2005; Cheng *et al*., 2005). Similar observations have been reported in human subjects, where women who delivered prematurely were shown to possess higher n-6 PUFA concentrations in erythrocytes (Reece *et al*., 1997). Also in rats, diets containing high n-6 PUFA

resulted in a reduction in the length of gestation that might have been the result of high AA in the plasma as a disturbance of the endometrial lining system and interferes with foetal development. In contrast, a positive correlation has been shown between prolonged dietary intake of n-3 PUFA in late pregnancy and gestation length, possibly because these FA alter the balance between stimulatory and inhibitory prostaglandins in the parturition process (Olsen *et al*., 1991). These results have been previously observed in ewes (Baguma-Nibasheka *et al*., 1999; Capper *et al*., 2006; Pickard *et al*., 2008) and sows (Rooke *et al*., 2001a), however could not confirmed recently either in sows (Yao *et al*., 2012), rabbits (Rebollar *et al*., 2014) or goats (Mahla *et al*., 2017).

The nutritional status of the mother during gestation has been related to foetal growth, and, in general, reduced nutritional status with respect to n-6 and n-3 PUFA has been correlated with reduced neonatal growth and head circumference in humans (Jumpsen *et al*., 1997). Studies in sows have shown benefits of n-3 PUFA supplementation on postnatal growth (Rooke *et al*., 2000 and 2001b; Mateo *et al*., 2009) and pre-weaning mortality of the litter (Rooke *et al*., 2001a). Others report an increase in born alive pigs of gilts (Edwards and Pike, 1997; Spencer *et al*., 2004) and sows (Webel *et al*., 2003 and 2004; Smits *et al*., 2011) when supplementing with n-3 PUFA at various stages of gestation, lactation and/or during rearing. In the same line, in rabbits, Rebollar *et al*. (2014) observed a reduction in the number of stillborn and longer kits at parturition time.

Several mechanisms have been suggested as mediating effects of n-3 PUFA in relation with these results of growth and survival, either through direct incorporation of n-3 PUFAs in tissues of offspring, or through expression of lipogenic enzymes in those tissues, which affects the biosynthesis of PUFA from dietary precursors. A mechanism through which n-3 PUFA can influence growth performance and survival is by improving the immune system. Immunoglobulin G (**IgG**) in colostrum is the main source of antibodies that boosts the neonatal pigs' passive immune system, and colostral IgG concentrations were greater in sows fed a n-3 LCPUFA rich diet (Mateo *et al*., 2009) and FA influenced the expression of immune related genes (Jump and Clarke, 1999; Kitajka *et al*., 2004).

Overall, therefore, mammals can benefit from n-3 PUFA supplementation to the mother in two ways; prenatally, when developing embryos have access to DHA, and postnatally, when litters consume colostrum and milk containing elevated

concentrations of EPA and DHA. Gabler *et al*. (2009) reported increased ex vivo active glucose uptake by the proximal jejunum of 21-day old pigs from sows fed n-3 PUFA supplements during gestation, during lactation or both. Moreover, Rooke *et al*. (2001c) reported that when sows were only fed with n-3 PUFA from salmon oil during the last part of gestation, piglets still grew faster after birth. On this subject, working with sows, Mateo *et al*. (2009) confirmed that BW from parturition until weaning tended to be higher in litters from n-3 PUFA fed sows, again consistent with earlier studies (Rooke *et al*., 2000 and 2001b). By contrast, also in sows, Taugbol *et al*. (1993) and Fritsche *et al*. (1993) as well as Smits *et al*. (2011) did not find an effect on weaning weight when n-3 PUFA supplementation started at the end of pregnancy, may be because longer periods of supplementation are needed in gestation to produce positive effects on litter weaning weight.

Studies carried out by Lin *et al*. (1991) demonstrated that in the milk of control rabbits, PUFA represented 34% of total FA, almost all of which was LA and ALA, with only traces of DHA. However, the same authors observed that the dietary enrichment of rabbits with fish oil resulted in a higher total level of PUFA in the milk, principally by increasing the proportion of n-3 LCPUFA from trace levels to more than 10%. When sows were supplemented with n-3 LCPUFA in gestation and lactation, n-3 PUFA levels were also higher in colostrum and milk (Taugbol *et al*., 1993; Rooke *et al*., 2000 and 2001a). Nevertheless, Amusquivar *et al*. (2010) showed that n-3 PUFA levels were increased in sow milk and plasma when animals were only supplemented with n-3 PUFA during the first half of gestation. Thus, these results suggested that the n-3 PUFA can be stored in maternal adipose tissue and mobilized during milk production.

3.6. PUFA and digestive system

The integrity of the intestinal morphology is often considered as a basis for evaluating the normal physiological functions of the small intestine. Intestinal epithelial cells are derived from the crypt and move along the villus surface up to the villus tip. Longer and wider villi are closely related to activate cell mitosis and can improve nutrient absorption in the small intestine because of the large surface area available for this function (Samanya and Yamauchi, 2002; Ilsley *et al*., 2005). Inversely, shorter intestinal villi impair absorption in the intestine, decrease the absorption ability of an animal, and result in low performance (Ilsley *et al*., 2005).

Crypt depth enhancement is associated with fast tissue turnover and a high energy demand for intestine maintenance relative to other organs (Xu *et al*., 2003). Any additional tissue turnover must improve nutrient requirements for maintenance and will result in poor feed efficiency in livestock.

The intestinal mucosa is a dynamic structure that undergoes biochemical, ultrastructural, and morphological changes throughout life (Thomson and Wild, 1997a,b). The absorptive cells of the small intestine have the capability of adapting their structure and function in response to changes in the intraluminal content of nutrients (Henning, 1981). The FA composition of membrane phospholipids responds to changes in the dietary FA consumed by animals and the balance between the n-6 and n-3 PUFA (Farquharson *et al*., 1995; Keelan *et al*., 1986). Thus, the phenotypic expression of the intestinal digestive and transport functions may be modified by the lipid content of the diet (Thomson *et al*., 1989).

Recent studies confirmed that the composition of the gut microbiota is influenced by environmental factors such as diet, antibiotic therapy, and environmental exposure to microorganisms (Vernocchi *et al*., 2016). In addition, other animal studies suggesting that the effect of n-3 PUFA on the gut microbiota may also play an important role in the effects of these FA on clinical parameters (Noriega *et al*., 2016; Kaliannan *et al*., 2015; Yu *et al*., 2014). In this regard, analysis of gut microbiota and faecal transfer in mice has revealed that elevated tissue n-3 PUFA enhance intestinal production and secretion of intestinal alkaline phosphatase, which induces changes in the gut bacteria composition resulting in decreased lipopolysaccharide production and gut permeability, and ultimately, reduced metabolic endotoxemia and inflammation (Kaliannan *et al*., 2015). Moreover, dietary EPA and DHA exert anti-inflammatory properties (Mori and Beilin, 2004) by altering cytokine production (Calder, 2001; Browning, 2003), often at the expense of AA, whereby pro-inflammatory eicosanoids derived from AA, such as PGE_2 and leukotriene B4, are replaced by anti-inflammatory eicosanoids derived from EPA and DHA (Calder, 2009).

Studies carried out in mice by Patterson *et al*. (2014) elucidated that a flaxseed/fish oil diet was the only one to significantly increase the intestinal populations of *Bifidobacteriaceae* and *Bifidobacterium*, at the family and genus levels, respectively, possibly through the increased ability of flaxseed/fish oil to increase the adhesion of bifidobacteria to the intestinal wall. Thus, PUFA

(flaxseed/fish oil) may have a bifidogenic effect on the intestinal microbiota composition of the host by increasing the levels of *Bifidobacterium*, while also positively influencing the composition of host tissues with n-3 PUFA-derived health-promoting FA. However, there is controversy in the results in this regard.

Whilst Delgado (2017) found no effects on mortality after reduce the n-6/n-3 PUFA ratio with the addition of linseed oil to growing rabbit diet, Maertens *et al*. (2005) in an experimental farm naturally affected by epizootic rabbit enteropathy reported a decrease of mortality in rabbits weaned from does fed an n-3 rich diet and fed the n-3 rich diet after weaning (n-6/n-3 PUFA ratio = 1.0, obtained with extruded linseed), compared with a control diet (n-6/n-3 PUFA ratio = 4.8). A similar positive effect on mortality was reported by Casado *et al*. (2013), when decreased the n-6/n-3 PUFA ratio from 5.9 to 0.8 using linseed oil. It might be related to the enhanced immune status in rabbits fed linseed oil than in those fed hydrogenated soybean oil, safflower oil or menhaden oil (Kelley *et al*., 1988). Previous works have been reported that medium-chain FA (caprilyc and capric) acids can alter the intestinal microbiota, thus affecting cecal fermentation (Marounek *et al*., 2002) and the development of pathogenic strains (Skřivanová *et al*., 2009).

3.7. PUFA and productive performance during growing period

Focusing on growth performance, previous works have investigated about the rabbit dietary FA supplementation with several sources from vegetable and animal origin. An overview of the results of these studies is shown in Table 1.3. In general, the incorporation of different FA to the rabbit diet decreases their feed intake with conflicting results regarding to average daily gain (**ADG**) or BW at slaughter time.

Table 1.3. Results of productive performance or rabbits after the dietary fatty acid supplementation with several sources from vegetable and animal origin

Authors	FA Source	Parameter			
		Feed intake	Average daily gain	Body weight[1]	Feed conversion index
Carraro *et al*. (2005)	Olive stone meal	-	=	=	-
Pla *et al*. (2008)	Animal fat	-	↑	-	-
	Sunflower oil	-	↑	-	-
	Linseed oil	-	↓	-	-
Eiben *et al*. (2010)	Sunflower oil	↓	↑	↑	=
	Linseed oil	↓	=	=	=
Dal Bosco *et al*. (2012)	Olive pomace[2]	↓	↓	↓	-
Al-Nouri *et al*. (2012)	Soy bean oil	-	↑	↑	↑
	Sesame oil	-	↑	↑	↑
	Microalgae oil	-	↓	↓	↓
	Fish oil	-	=	=	=
Vizzarri *et al*. (2017)	*Lippia citriodora*	=	=	=	-
Delgado (2017)	Linseed oil	=	↑	↑	↑

[1] Body weight determined at slaughter time.

3.8. PUFA and meat

Blasco and Ouhayoun (2010) defined rabbit hot carcass weight as weight of the carcass 15-30 min after slaughter. The carcass does not include blood, skin, distal parts of the tail, fore and hind legs, gastrointestinal and urogenital tracts. It includes head, liver, kidneys and organs located in the thorax and neck (lungs, oesophagus, trachea, thymus and heart). (Image 1.12).

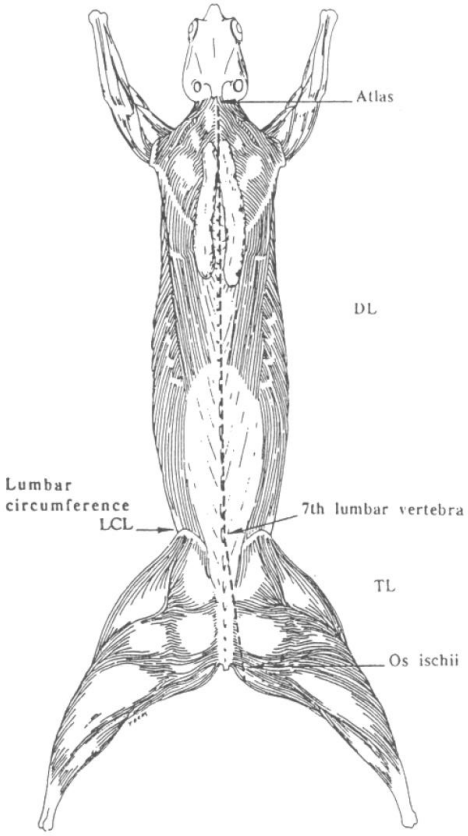

Image 1.12. Linear measurements of rabbit carcass. DL: Dorsal length (interval between the atlas vertebra and the 7th lumbar vertebra); TL: thigh length (interval between the 7th lumbar vertebra and the distal part of ischia); LCL: lumbar circumference (carcass circumference at the level of the 7th lumbar vertebra). Blasco and Ouhayoun (2010)

The same authors also defined chilled carcass weight as the weight of above carcass after chilling for 24 hours in a ventilated cold room (0-4ºC) about one hour from the slaughter. Washing carcass (i.e. with water) is to avoid. It is recommended to hang the carcass during chilling with sufficient air around it. Taking it into account, there are some parameters that define the quality of rabbit carcass. Meat quality consists of nutritional properties, sensory characteristics, and health and

technological factors and is significantly influenced by dietary factors including FA. Both, meat and carcass quality are complex traits and are influenced by many ante mortem and post-mortem factors (Sellier and Monin, 1994; Huff-Lonergan *et al*., 2002). In this regard, the effects of the FA on firmness are due to the different melting points of the FA in the meat (Enser *et al*., 1984), and many researchers have studied the effects of diets supplemented with different sources or levels of fat on animal performance and FA composition.

In goat kid, DHA milk enrichment did not affect meat pH and colour (L*, a*, b*, Chroma, Hue), either at the time of slaughter (15 min) or after 24 h of chilling. Muscle pH is considered to be a measure of post-mortem muscle metabolism, so any qualitative or quantitative change on diet could affect this parameter (Solomon *et al*., 1986). In pigs, Li *et al*. (2015) found the maintaining of the dietary n-6/n-3 PUFA ratios between 1:1 and 5:1 would facilitate the absorption and utilization of FA and free amino acids, and result in improved muscle and adipose composition of meat.

Focusing on rabbits, regarding carcass traits, Eiben *et al*. (2010) used different dietary ratios of sunflower and linseed oils and observed that empty, chilled as well as reference carcasses of enriched rabbits were similar and 1–2% heavier but contained 16–27% more perirenal fat than the control rabbits. In addition, no differences were found in the weights of valuable carcass parts and cuts (liver, intermediate and hind part, hind leg, loin and hind leg meat). The same authors, based on tactile evaluations, found depot fat of rabbits on oil-rich diets to be oily and soft whilst it was firm in the controls. This is important as it reveals different FA composition (Dalle Zotte, 2002), thus providing information on product quality if fresh whole carcasses are purchased. By contrast, the oily and soft looking carcass could be undesirable for the buyer, assuming higher risk of lipid oxidation and resulting organoleptic and storability problems (Hernández and Pla, 2008; Pla *et al*., 2008).

Other works such as that of Pla *et al*. (2008) compared the carcass value of 63-day-old rabbits fed with 3% sunflower oil or 3% linseed oil diets and reported similar reference carcass weights but higher dressing out percentage with linseed oil diet. In the same line, Bianchi *et al*. (2006) used an 8% linseed diet in rabbits between 65 and 87 days of age resulted in a smaller and fatter carcass than the controls without supplementation. More recently, Dabbou *et al*. (2017) also studied the effect of a vegetable origin in carcass characteristics on growing rabbits. These

authors included bilberry pomace (rich in ALA) in rabbit diet and reported that liver weight was lower in these animals; however all other parameters measured (weight of skin, full gastrointestinal tract, chilled and reference carcass, head as well as perirenal fat) were not affected by bilberry pomace dietary inclusion.

Regarding meat quality traits, pH24 and colour of the *longissimus thoracic et lumborum* muscle measured by Dabbou *et al*. (2017) were no affected after the inclusion of bilberry pomace and fell within standard ranges for rabbit meat, consistent results with those of Abdel-Khalek (2013) who reviewed that dietary supplementation including antioxidants had no clear trend in the physical and chemical characteristics of rabbit meat. Li *et al*., (2012) observed that dietary LA supplementation had no significant influence on dressing out percentage, L* of muscle colour of LL, pH value, shear force and drip loss percentage of the experimental rabbits. These results are align with previous of Dal Bosco *et al*., (2004) were the proximate composition of the fresh muscle was not significantly affected by the dietary treatment and Peiretti and Meineri (2008), who corroborated that the diets did not significantly influence the carcass yield and the proportion of various carcass parts and organs of the rabbits.

As it has been mentioned, the composition of dietary fat, as a result of the use of various fat sources, can modify the FA composition of different rabbit tissue. In this sense, Tres *et al*. (2014) used two different dietary fish oil in rabbit and confirmed that meat FA composition reflected most of the differences found between diets. One of the purposes of adding fish oil to animal feed is to produce n-3 enriched food products. The amounts of EPA and DHA in the meats obtained in their study were actually quite high compared with other experimental feeding diets (Bernardini *et al*., 1999; Bou *et al*., 2004; Tres *et al*., 2008), and were much higher than those obtained when vegetable oils rich in linolenic acid, such as linseed oil, were added to feeds (Bou *et al*., 2009; Dal Bosco *et al*., 2004; Rymer and Givens, 2005; Tres *et al*., 2008; Benatmane *et al*., 2011; Dabbou *et al*., 2017).

It must be highlighted that, although high levels of PUFA could increase the rancidity and the colour deterioration of the meat during storage, it has also been associated with improvement of the flavour development of the meat during cooking (Wood *et al*., 2004).

The supply of n-3 PUFA through animal diet is one of the most successful approaches used to increase them in their products (Bou *et al*., 2009; Kouba and

Mourot, 2011; Dalle Zotte and Szendro, 2011). By providing a high amount of ALA, it is expected that the animal will biosynthesise and accumulate EPA and DHA. However, as it has been mentioned, this pathway is not very efficient, particularly with regards to DHA synthesis, for most animal species (Burdge and Calder, 2005). In contrast, higher amounts of EPA and DHA are attained in animal tissues even when lower levels of marine origin products such fish, algae or krill oils are added to diets (Burdge and Calder, 2005; Rymer and Givens, 2005; Bou *et al*., 2009).

In this regard, rabbit meat is considered a Mediterranean food (Gai *et al*., 2009), particularly appreciated by consumers for its low lipid content, even though rich of PUFA, for its high biological value proteins content (20–21%), as well as because of its low cholesterol content (Dalle Zotte and Szendro, 2011). In addition, the manipulation of the diet of rabbits is very effective in producing “enriched meat”. For example, some bioactive compounds such as n-3 PUFA, conjugated linoleic acid (CLA), and vitamin E can be easily incorporated into the meat (Dalle Zotte and Szendro, 2011).

The nutritional quality of fat for human consumption is usually evaluated in terms of the PUFA/SFA ratio (optimal values ≥0.45), the n-6/n-3 PUFA ratio (optimal values ≤4), and the atherogenicity and thrombogenicity indexes (both as low as possible) (Lazzaroni *et al*., 2009). However, despite being a meat that offers excellent nutritive and dietetic properties (Hernández and Gondret, 2006), in ordinary dietary conditions, the n-6/n-3 PUFA ratio in rabbit meat is set at around 10 (Dalle Zotte, 2002), making relatively easy to achieve optimal values though alimentation.

Recent studies of Dabbou *et al*. (2017) have researched about the inclusion of bilberry pomace (rich in ALA) in rabbit diet, and have observed lower atherogenic and thrombogenic index in *Longissimus thoracic et lumborum* muscle as well as a reduction of n-6/n-3 ratio from 9.3 to 2.88 in these animals. Thus, these authors concluded that bilberry pomace inclusion in rabbit diets may be of particular significance for the related nutritional benefits associated to human consumption of rabbit meat. Optimal values in n-6/n-3 PUFA ratio were also observed using a dietary supplementation of *Lippia citriodora* (rich in ALA) as vegetable source in rabbits during growing period (Vizzarri *et al*., 2017). However, lower information exist about enrichment in rabbit diets with sources of animal origin as fish oil in order to obtain "enriched meat".

CHAPTER 2

OBJECTIVES

The purpose of this PhD Thesis is to determine the effects of a dietary enrichment with a supplement based on n-3 PUFA in the productive/reproductive performance and physiological responses of rabbit does and their offspring during lactation and growing period.

To accomplish with this general aim, three main objectives have been carried out:

1. **To study the influences on metabolic and endocrine responses of rabbit does and their offspring.**

Productive, endocrine, and metabolic responses as well as oxidative stress of rabbit does and their offspring when fed a diet supplemented with n-3 PUFA during their first productive cycle will be evaluated.

2. **To know the effects on performance, meat quality, and cecal fermentation of growing rabbit.**

The effect of continuing in young rabbits the same dietary supplementation with fish oil consumed by their mothers will be investigated. For this purpose, we will evaluate growth performance (during all fattening period), carcass characteristics and FA profile of muscle and fat tissues (at slaughter), as well as cecal fermentation and ileal mucosa morphology of growing rabbits (at 30, 45, and 60 d of age).

3. **To evaluate the improvements on reproductive performance, milk composition and kit viability of primiparous rabbit does.**

The effect of an enriched diet with n-3 PUFA in a 4-folder higher level of inclusion than previous experiments will be confirmed.

CHAPTER 3

A diet supplemented with n-3 polyunsaturated fatty acids influences the metabolic and endocrine response of rabbit does and their offspring

1. INTRODUCTION

In mammals, conversion of essential FA into the more biologically active n-3 LCPUFA, such as EPA (C20:5n-3) and DHA (C22:6n-3) is inefficient. Specifically during pregnancy, EPA and DHA are of critical significance for fetal development (Innis, 2007). Most of fetal lipids are derived from maternal circulation via the placenta and are either obtained from the diet or lipolysis in the last phases of pregnancy (Herrera *et al.*, 2014), constituting the fat deposits on which the survival of the newborn depends. In rabbit production, does are selected by high prolificacy reducing the uterine space to the fetus and decreasing the BW of newborn (Cifre *et al.*, 1998). Moreover, rabbit kits are altricial, and their degree of development at birth has decisive consequences on their survival (Bautista *et al.*, 2015), which has been already enhanced in previous studies using EPA and DHA supplementation (Rebollar *et al.*, 2014). However, many epidemiological and dietary interventions have shown that consumption of n-3 PUFA significantly alters the serum lipid profile (Harris, 1997a, b). It also may change the expression of genes related to lipid metabolism, enhance peroxidation and reduce antioxidant capacity, impairing fetal and newborn homeostasis (Jump and Clarke, 1999; Chow, 2007; Jones *et al.*, 2014; Prostek *et al.*, 2014). The benefit-to-risk ratio of increasing n-3 PUFA intake during gestation and lactation has not been completely established.

To our knowledge, no studies exist on the effects of dietary n-3 PUFA supplementation on rabbit does and the possible consequences on their offspring. Therefore, the specific objective of the present study was to evaluate the influence of a supplementation with EPA and DHA on: 1) reproductive variables, 2) related endocrine profiles of does, and 3) metabolic profiles and systemic oxidative status of rabbit does and their offspring.

2. MATERIALS AND METHODS

2.1. Animals, housing and experimental design

The study was performed according to the Spanish Policy for Animal Protection RD53/2013. The experiment was specifically assessed and approved by the Animal Ethics Committee of the Community of Madrid (Ref. PROEX 302/15).

A total of 105 New Zealand x California white rabbit does were fed for *ad libitum* consumption experimental diets from rearing (60 d of age) to their first weaning (172 d of age). Each dam and their kits were housed in the same flat-deck-cage (700 x 500 x 330 mm) with 16 h of light and 8 h of darkness and room-temperature ranged from 18 to 23°C throughout the trial. They were individually identified at the beginning of the experiment and remained in their cages throughout the entire trial.

Two isofibrous, isoenergetic, and isoproteic diets were formulated following the nutritional recommendations for breeding does issued by de Blas and Mateos (2010). Both diets had the same basal mixture of ingredients and only varied in the type of fat added: either 7.5 g/kg lard (Control group, n = 52 does) or 15.0 g/kg of a commercial supplement (Optomega-50; Optivite International Ltd., Spain) containing a 50% of ether extract and 35% of n-3 PUFA and 2,500 mg/kg of vitamin E (Enriched group, n = 53 does).

The ingredients and chemical composition of diets are given in Table 3.1 and Table 3.2, respectively, and the FA profile of experimental diets is shown in Table 3.3. Only one batch of each diet was used during the study. Diets were vacuum-packet and stored at 5ºC protecting them of lipid oxidation and other degradative processes. Samples of both diets were collected weekly and composited for the further analysis. Rabbit does feed intake was determined at the end of rearing, pregnancy, and lactation periods.

Table 3.1. Ingredient and chemical composition of a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 15.0 g/kg (g/kg, as fed basis unless otherwise indicated)

	Diet[1]	
Ingredient	Control	Enriched
Wheat bran	300	300
Barley grain	111	111
Sunflower meal 280 g/kg CP	199	199
Palmkernel 160 g/kg CP	60.0	60.0
Lucerne meal	100	100
Barley sprouts	50.0	50.0
Sugarbeet pulp	57.0	57.0
Sugarcane molasses	30.0	30.0
Wheat straw	42.0	42.0
Lard	7.5	-
Sepiolite	7.5	-
Optomega 50 [2]	-	15
Calcium carbonate	19.0	19.0
Sodium chloride	6.0	6.0
Lysine, 500 g	1.7	1.7
Choline clorhide	0.3	0.3
Organic acids	0.7	0.7
Min-vitpremix [3]	3.0	3.0
Antioxidants [4]	3.3	3.3
Zinc bacitracin premix [5]	2.0	2.0

[1] Only one batch of each diet was used during the study. Samples of both diets were collected weekly and composited for the further analysis. [2] Optivite International Ltd. (Spain). Contained salmon fish oil, 100%; ether extract, 50%; n-6, 8%; n-3, 35%; CP, 4%, ME, 5254 kcal/kg; and Vitamin E, 2500 mg/kg. [3] Mineral and vitamin premix supplied per kg of complete diet: Vitamin A 9999.9 IU; Vitamin D 1080 IU; Vitamin E, 200 mg/kg; vitamin K3: 1.7 mg; Tiamine:1.7 mg; Riboflavin: 4.3 mg; Pantithenic acid: 13.6 mg; Pyrodoxine: 1.7 mg; Mn:22.7 mg; Co:595 µg; Se: 140 µg; I: 1.2 mg. [4] Supplied per kg of diet: [E320 Butilhidroxianisol (BHA)+E324 Etoxiquina+E321 Butilhidroxitolueno (BHT) 30.000 mg; E562 sepiolite 910.000 mg] (Trow Nutrition Spain SA); Luctanox 3000 mg (Lucta, Barcelona, Spain) [5]Contained 100 mg Zinc-bacitracin/kg (APSA, Reus, Spain).

Table 3.2. Chemical composition of a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 15.0 g/kg (g/kg, as fed basis unless otherwise indicated)

	Diet[1]	
Item	Control	Enriched
Chemical composition analysed		
Gross Energy, MJ/kg	16.4	16.5
Dry matter	906	904
Ash	81.1	77.8
Crude protein	16.0	16.0
Ether extract	31.6	31.4
aNDFom[2]	332	335
ADFom[3]	161	163
ADL[4]	39.8	41.0
Chemical composition calculated		
Lysine	7.0	7.0
Methionine + Cystine	2.6	2.6
Threonine	5.5	5.5
Calcium	12	12
Phosphorus	5.6	5.6

[1] Only one batch of each diet was used during the study. Samples of both diets were collected weekly and composited for the further analysis. [2]aNDFom = amylase neutral detergent fiber organic matter basis. [3]ADF = acid detergent fiber organic matter basis. [4]ADL = acid detergent lignin.

Table 3.3. Fatty acid composition (g/kg total FA methyl esters) of a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 15.0 g/kg

	Diet[1]	
Item	Control	Enriched
Total SFA[2]	351.1	316.6
C12:0	63.3	63.6
C14:0	53.2	61.0
C16:0	182.9	166.2
C18:0	54.2	27.6
Total MUFA[3]	267.4	205.9
C16:1n-7	13.4	16.8
C18:1n-9	240.9	177.0
C20:1n-9	13.1	12.1
Total PUFA[4]	380.9	477.2
C18:2n-6	327.4	315.1
C18:3n-3	40.8	44.5
C18:4 n-3	5.1	21.6
C20:5n-3	0.0	33.9
C22:5n-3	0.0	9.2
C22:6n-3	0.0	40.0
n-9	254.0	189.3
n-6	335	328.0
n-3	45.9	149.2
n-6/n-3 ratio	7.3	2.2
UI[5]	115	127

[1]Only one batch of each diet was used during the study. Samples of both diets were collected weekly and composited for the further analysis. [2]SFA = saturated fetty acids. [3]MUFA = monounsaturated fatty acids. [4]PUFA = polyunsaturated fatty acids. [5]UI = unsaturation index.

The experimental design is shown in Figure 3.1. All females were inseminated at 110 d of age and with an average weight of 4,062 ± 30.5 g. Artificial insemination (**AI**) was performed with seminal doses with at least 20 million spermatozoa in 0.5 mL of diluent (Magapor S.L., Zaragoza, Spain), prepared using a pool of fresh heterospermic semen from a group of rabbit bucks selected for high growth-performance. To induce ovulation, does were given an intramuscular injection of 20 µg gonadorelin (Inducel-GnRH, Lab. Ovejero, León, Spain).

To asses placental and fetal development a SonoSite S-Series ultrasound machine equipped with a multifrequency (5–8 MHz) lineal array probe (SonoSite, Inc., Bothell, WA, USA) was used. Due to the complexity, the duration of the procedure as well as the need to be carried out by the same experienced technician, a random subsample of 16 pregnant females (8 control and 8 enriched does) were taken from a total of does. Rabbits were shaved in the abdominal area and manually restrained in dorsal recumbence during 20 min approximately, remaining calm and relaxed during the examination. The number and diameter of all chorionic vesicles, embryo length, and width and placental thickness were measured on d 9 of gestation (Image 3.1). Placental thickness, crown-rump length (**CRL**), occipito-nasal length (**ONL**), biparietal diameter, (**BPD**) and thoracic diameter (**TD**) of fetuses were determined on d 16 of gestation (Image 3.2).

At parturition, fertility [(number of does giving parturition/total number of does inseminated) × 100], total number of newborn, born alive, and stillborn kits were recorded monitoring the nests every 12 h. Furthermore, to more precisely determine the effect of PUFA on individual growth of kits born alive, 30 litters (15 control and 15 enriched does) with 11.0 ± 0.53 kits born alive and 1.42 ± 0.35 stillborn were sampled to determine individual weights and measurements of CRL, ONL, BPD, and TD (Image 3.3). After that, in all rabbit does, litter size was subsequently standardized to 10–12 pups by removing or adding kits within each dietary treatment. Litter weight at 21 and 32 days postpartum (**dpp**) (weaning), as well as ADG of kits were measured. Milk production was estimated by weighing all the litters at 21 d of age and using the regression equation developed by De Blas *et al.* (1995), as follows: milk production (kg) = 0.75 + 1.75 LBW21 (kg); where LBW21 corresponds to BW of the litter at 21 d of lactation. The mortality of kits during lactation was recorded and expressed as the percentage of animals dead at weaning with respect to the number of kits after standardizing the litter size.

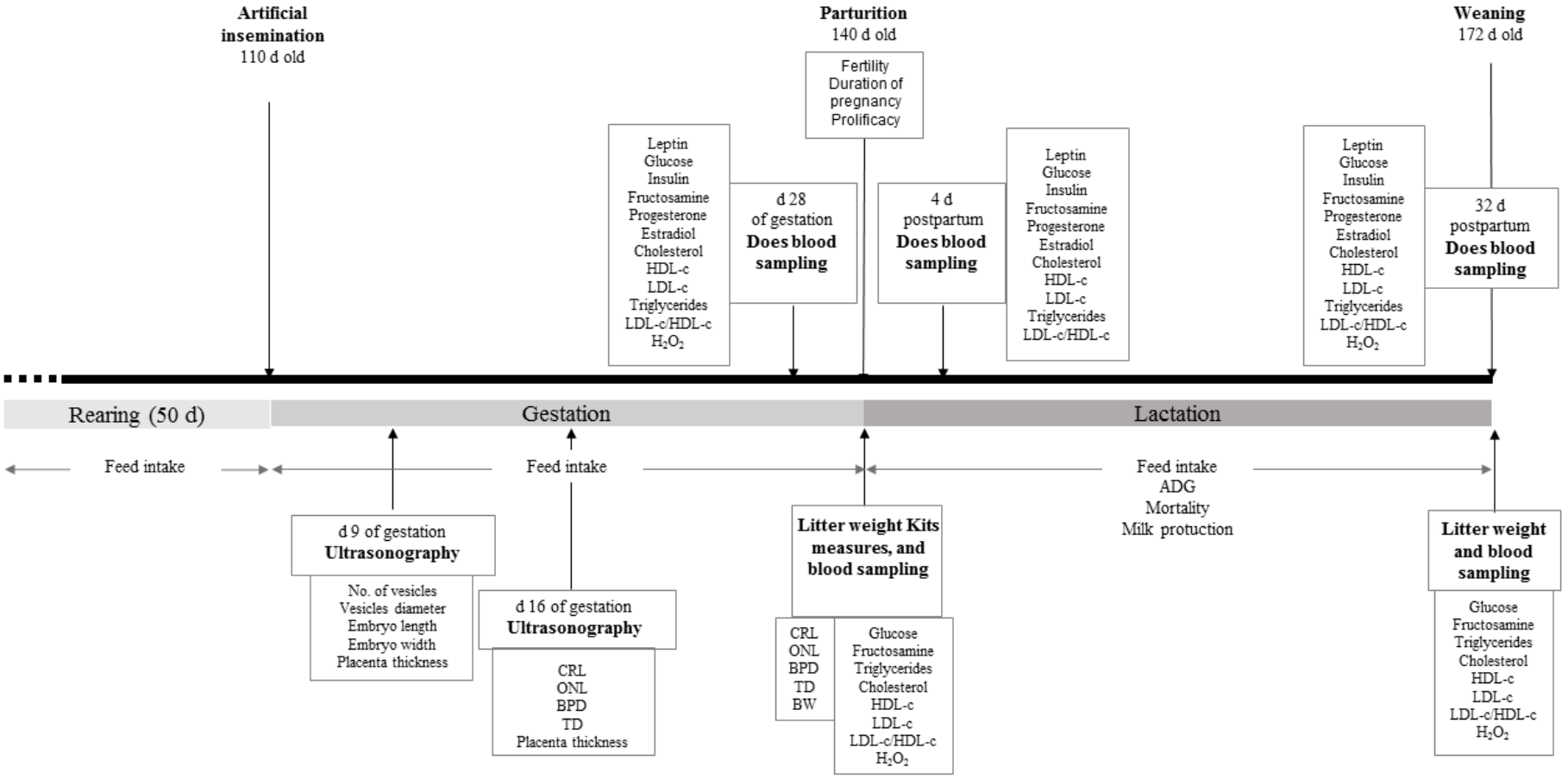

Figure 3.1. Sampling timeline of different variables in mothers and their offspring. CRL = crown-rump length; ONL = occipito-nasal length; BPD = biparietal diameter; TD = thoracic diameter; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; H_2O_2 = Hydrogen peroxide; Fertility = (number of does giving parturition/total number of does inseminated)×100; Prolificacy (number of newborn, live born, and stillborn kits); BW = body weight.

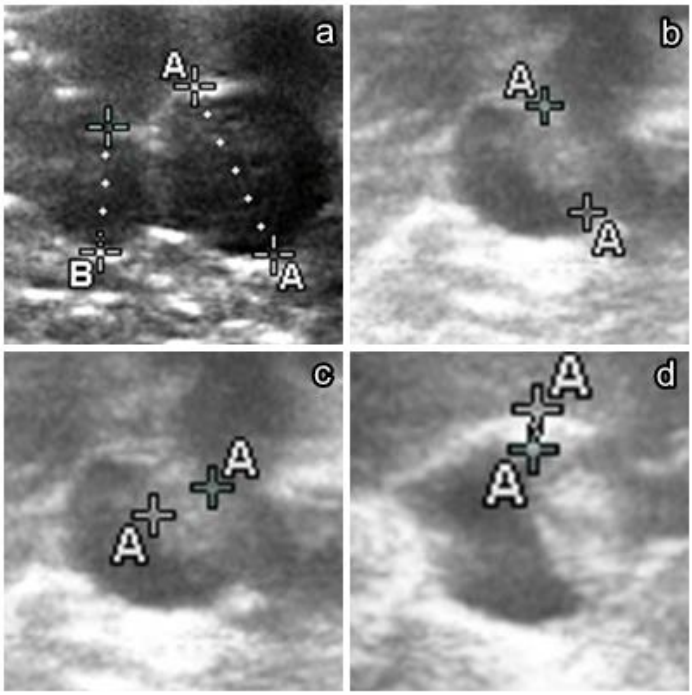

Image 3.1. (a) Diameter of all chorionic vesicles, (b) embryo length, and (c) width and (d) placental thickness measured by ultrasonography assay in pregnant rabbit does on d 9 of gestation.

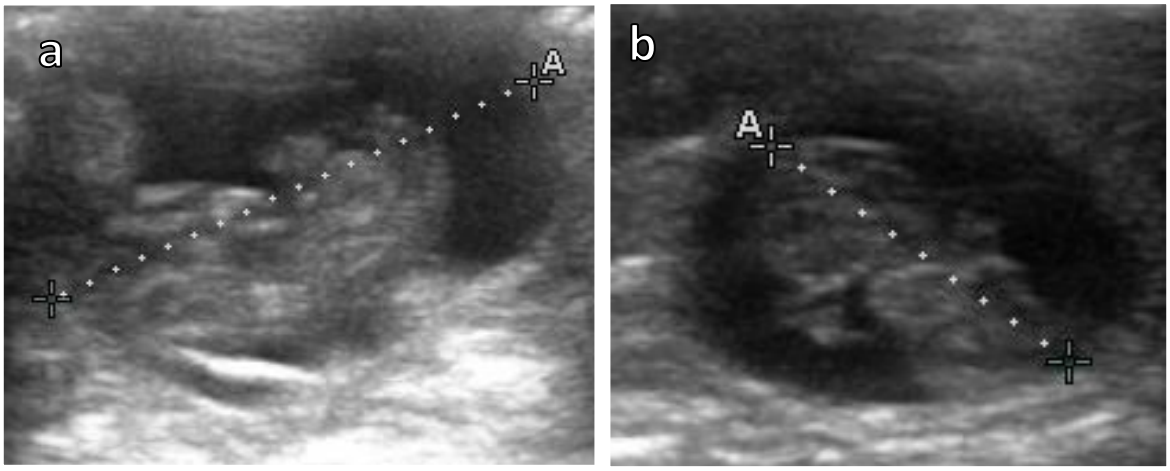

Image 3.2. (a) Crown-rump length and (b) biparietal diameter measured by ultrasonography assay in pregnant rabbit does on d 16 of gestation.

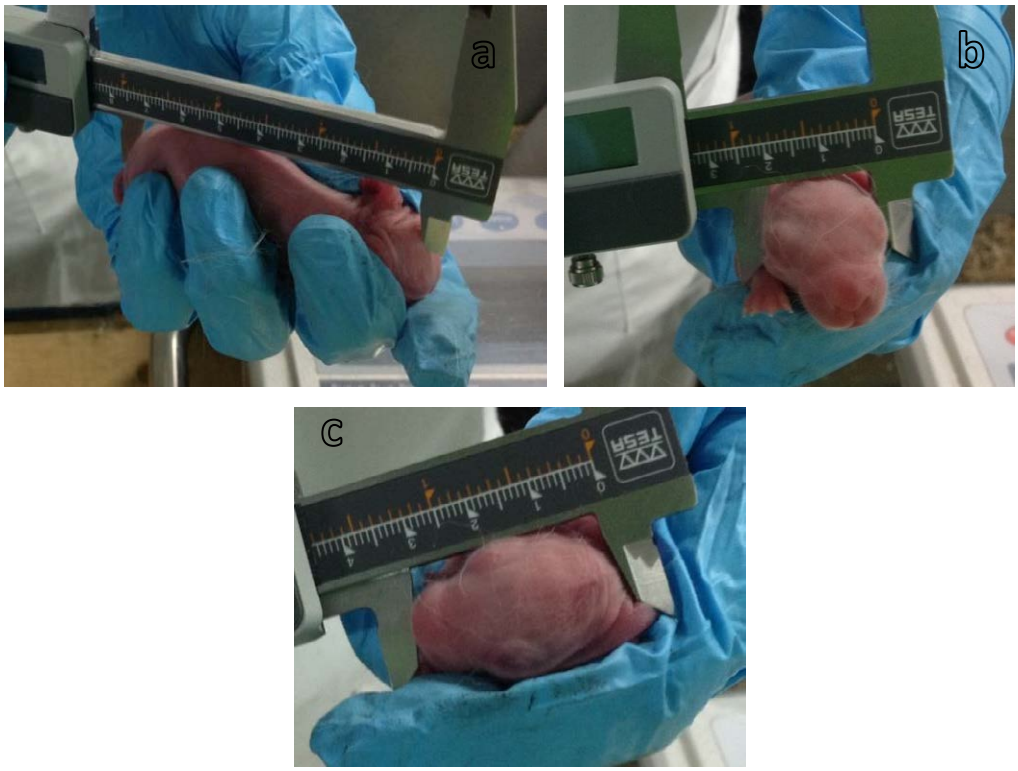

Image 3.3. (a) Crown-rump length and, (b) biparietal diameter and (c) occipito-nasal length measured in 1 day old kits.

IIn a random subsample of 30 pregnant females (15 control and 15 enriched does), plasma concentrations of reproductive and metabolic hormones (progesterone, estradiol, leptin, and insulin) as well as glycemic (glucose and fructosamine) and lipid (TG, total cholesterol, HDL-c, and LDL-c) levels were measured in blood samples collected from the central ear artery (2.5 mL) and placed in tubes containing EDTA at 3 times: d 28 of pregnancy, 4 dpp, and 32 dpp (early lactation and weaning, respectively) (Image 3.4). Oxidative stress, using hydrogen peroxide ($\mathbf{H_2O_2}$) as marker, was also measured in blood samples on d 28 of pregnancy and at weaning in dams. Plasma was obtained after centrifugation at 1,200 *g* for 10 min at 4ºC and stored at -20ºC until analyzed. Data for oxidative stress at 4 dpp were not considered due to a fail in the storage conditions of the collected samples that may affect reliability of the results.

Image 3.4. Blood sampling from the central ear artery in tubes containing EDTA.

In addition, at early lactation (1 dpp) and at weaning two kits per doe were selected from a random subsample of 16 litters from previous 30 rabbit does (8 control and 8 enriched litters) in order to have data from individuals representing heterogeneity within the litter (i.e.: normal birthweight and low birthweight) (Image 3.5). With that purpose, every kit of each dam was weighed, and once all the individual weights were collected, the smallest and the largest kit of each litter were taken. The average of BW of the smallest kits was 35.1 ± 29.4 g and 532 ± 32.2 g, at early lactation and weaning respectively. For the largest kits, the average of BW was 59.7 ± 25.1 g and 728 ± 28.3 g, at early lactation and weaning respectively. After slaughter by cervical dislocation, immediately intracardiac blood samples were obtained and plasma was processed as described above in order to analyze glycemic and lipid compounds (glucose, fructosamine, triglycerides, total cholesterol, HDL-c, and LDL-c) as well as H_2O_2 for oxidative stress at both times. There was no replacement of slaughtered kits.

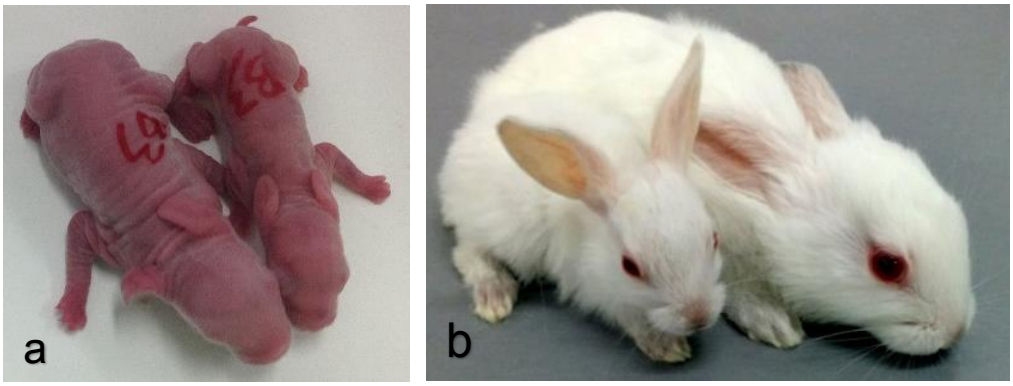

Image 3.5. Normal weight and low weight kit (a) at early lactation (1 dpp) and (b) at weaning.

2.2. Analytical methods

Diets. Chemical analysis of experimental diets followed the official methods of the AOAC (2000) for dry matter (**DM**) (oven drying method: 934.01), ash (muffle furnace incineration: 923.03), ether extract (solvent extraction: 920.39), and crude protein (**CP**) (Dumas method: 968.06; FP-528 LECO, St. Joseph, MI, USA) determinations. Gross energy was determined by combustion in an adiabatic calorimetric pump (model 1356, Parr Instrument Company, Moline, IL, USA). The neutron detergent fiber (**NDF**), acid detergent fiber (**ADF**) and acid detergent lignin (**ADL**) content was determined according to the sequential method of Van Soest *et al*. (1991) using an ANKOM220 Fiber Analyzer unit (ANKOMTechnology Corporation, Fairport, NY, USA). Sodium sulphite and heat-stable amylase were used in the sequential analysis of NDF, ADF and ADL, and they were expressed exclusive of residual ash.

The FA profiles were analyzed according to Sukhija and Palmquist (1988) and the identification and quantification was made by chromatography using a Hewlett Packard HP-5890 gas chromatograph (Avondale, PA, USA) equipped with a flame ionization detector (capillary column HP-Innowax, 30 m x 0.32 mm internal diameter and 0.25 µm film thickness) (Agilent Technologies Gmbh, Ratingen, Germany). A split ratio of 50:1 was used and C15:0 was included as the internal standard.

Endocrine, metabolic and oxidative status variables. Plasma progesterone and estradiol concentrations were measured in a single assay using EIA kits (Demeditec Diagnostics GmbH, Kiel, Germany). Previously, plasma samples were extracted with petroleum ether at a 5:1 (vol/vol) ether:sample ratio (extraction efficiency was around 85%). The sensitivity of the assay was 0.045 ng/mL and 1.4 pg/mL for progesterone and estradiol, respectively. The intra-assay coefficients of variation were 5.5 and 5.7% for progesterone and estradiol, respectively.

Glucose and fructosamine were measured with a clinical chemistry analyzer (Saturno 300 Plus; Crony Instruments s.r.l., Rome, Italy). Insulin was determined with an Insulin ELISA kit (Mercodia AB, Uppsala, Sweden), with a sensitivity of 0.26 IU/L and intra-assay variation coefficient of 3.5%.

Concentrations of leptin were determined in a single analysis using the Multi-species Leptin RIA kit (Demeditec Diagnostics GmbH, Kiel, Germany). The assay sensitivity was 1.0 ng/mL and the intra-assay variation coefficient was 3.1%.

Triglycerides, total cholesterol, HDL-c, and LDL-c were measured with the same analyzer (Saturno 300 Plus; Crony Instruments s.r.l., Rome, Italy). Plasma LDL-c/HDL-c ratio was obtained by dividing LDL-c levels by HDL-c concentrations. Systemic oxidative stress was assessed using H_2O_2 as a marker. Plasma hydrogen peroxide concentrations were determined by an EIA (Abcam, Cambridge, United Kingdom) with an assay sensitivity of 0.04 µ*M*.

2.3. Statistical analysis

Statistical analysis was performed with SAS software (SAS Inst. Inc., Cary, NC). The experimental unit was the rabbit doe. Duration of pregnancy, total number of newborn, born alive, and stillborn kits, and litter weight at parturition and at weaning (considering the total number of newborn per litter as covariable), as well as ultrasonography measures, individual kit development, and plasma biochemical variables of kits at parturition and at weaning were analyzed as a completely randomized design with feeding regime as the main source of variation, using the GLM procedure. Feed intake, plasma levels of endocrine and biochemical variables of does were studied by repeated measure analysis using the MIXED procedure with feeding regime, time, and their interaction as main effects. Doe was considered a random effect nested in the treatment. The effect of dietary supplementation on fertility rate was analyzed by means a χ^2 test (proc CATMOD). All means were compared using a protected *t*-test, and differences were considered significant at $P < 0.05$ and a trend when $P < 0.10$. Results are presented as least squares mean.

3. RESULTS

3.1. Maternal trial

The FA composition of experimental diets confirmed that the supplement added to the enriched diet increased EPA and DHA concentrations (Table 3.3). Control and enriched does had a similar feed intake during rearing (186 ± 4.14 and 187.4 ± 18 g/d), pregnancy (135 ± 4.64 and 142 ± 4.70 g/d), and lactation (386 ± 4.68 and 385 ± 4.70 g/d) ($P > 0.05$). Ultrasonographic determinations of the number and diameter of embryo vesicles and embryo dimensions on d 9 of pregnancy, and the size of placenta and fetuses on d 16 were similar in both dietary treatments (Table 3.4).

Fertility, evaluated in all inseminated does, was similar among control and enriched group (84.6 ± 5.2 and 83.0 ± 5.1%; $P > 0.05$), as well as pregnancy duration (30.8 ± 0.07 and 30.9 ± 0.07 d), kits born alive (10.1 ± 0.38 and 9.7 ± 0.38 kits), and stillborn (0.1 ± 0.11 and 0.3 ± 0.11 kits). Regarding individual growth of kits born alive from a random subsample of litters (Table 3.4), head and thoracic diameters were similar but CRL was larger in enriched kits, which may be related to a tendency toward a 19.53% greater BW in that treatment ($P = 0.07$). Nevertheless, the number and the litter-weight of kits at weaning were not different between diets (9.3 ± 0.25 and 9.1 ± 0.24 weaned kits; 5,676 ± 144 and 5,624 ± 140 g in control and enriched does, respectively; $P > 0.05$). Rabbit does had similar milk production and consequently the ADG of the offspring was also similar during lactation period (6,058 ± 184 and 5,769 ± 181 g; 17.9 ± 0.48 and 17.9 ± 0.48 g/d in control and enriched group, respectively; $P > 0.05$). In addition, there were no differences between experimental groups for mortality values (5.14 ± 1.43 and 2.17 ± 1.39% in control and enriched kits, respectively; $P > 0.05$).

Table 3.4. Measurements of placenta and fetuses by ultrasonography at d 9 and 16 and of newborn kits from rabbit does fed a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 15 g/kg. All values are least squares means

	Diet			
Item	Control	Enriched	RMSE[1]	*P*-value
Pregnancy				
No. of does	8	8		
d 9				
No. of vesicles	12.8	13.4	3.47	0.7440
Vesicles diameter, mm	12.1	11.7	2.07	0.2251
Embryo length, mm	8.29	8.08	1.61	0.6435
Embryo width, mm	4.00	3.76	0.684	0.1958
Placenta thickness, mm	2.39	2.02	1.51	0.2028
d 16				
CRL,[2] mm	14.8	15.6	2.55	0.4070
ONL,[3] mm	8.14	9.13	1.84	0.1591
BPD,[4] mm	5.76	5.88	0.501	0.3754
TD,[5] mm	6.61	6.57	0.528	0.7608
Placenta thickness, mm	4.48	4.22	0.525	0.0654
Parturition				
Born alive	9.71	10.89	3.45	0.5099
Stillborn	0.14	0.00	0.247	0.2711
Individual development				
No. of does[6]	15	15		
BW[7], g	42.5	50.8	7.07	0.0744
CRL, mm	71.6	79.5	6.37	0.0306
ONL, mm	28.4	30.3	4.22	0.3950
BPD, mm	18.3	18.7	1.93	0.7327
TD,[5]mm	18.3	18.8	2.09	0.6629

[1]RMSE = root mean square error (n = 8 rabbit does per diet for determinations on d 9, d 16 and parturition; 15 rabbit does per diet for individual development). [2]CRL = crown-rump length. [3]ONL = occipito-nasal length. [4]BPD = biparietal diameter. [5]TD = thoracic diameter. [6]Does with litters of 10-11 kits. [7]BW = body weight.

Regarding plasma variables in mothers, diet affected plasma leptin and glucose concentrations. The supplementation with PUFA always induced a greater leptinaemia at all times when it was determined (Figure 3.2a), whilst glycaemia in control does was greater only at weaning (Figure 3.2b). Diet did not affect the mean plasma concentrations of insulin nor fructosamine in does (Figures 3.2c and 3.2d). All cited variables showed the same changes overtime; they significantly increased from d 28 of pregnancy to d 4 postpartum (except for leptin) and then decreased again at weaning, except fructosamine, which increased. The interaction between diet and time was not significant.

The assessment of variables related to the lipid metabolism of does showed a similar pattern in both diets through time, with lower values of total and HDL-c at 4 dpp in the enriched group than in the control one ($P < 0.05$; Figures 3.2e and 3.2f). At weaning, HDL-c concentrations were still lower in enriched does than in controls ($P < 0.05$), while total cholesterol levels were similar in both ($P > 0.05$). Both total and HDL-c increased from the end of pregnancy overtime until 4 dpp and remained high until weaning. Diet did not affect LDL-c and TG concentrations (Figures 3.2g and 3.7h), but both increased at 4 dpp ($P < 0.05$), in relation to the end of pregnancy and weaning. The LDL-c/HDL-c ratio was high on d 28 of pregnancy, decreasing at 4 dpp and remained low until weaning (Figure 3.2i). Finally, assessment of systemic oxidative stress showed significantly greater values of H_2O_2 in enriched does on d 28 of pregnancy ($P < 0.05$), but there were no differences at weaning (Figure 3.2j).

Regarding reproductive hormones (Figures 3.2k and 3.2l), the enriched group had lower concentrations of progesterone on d 28 of pregnancy ($P < 0.05$), decreasing to basal levels in both groups after parturition. Nevertheless, time did not affect plasma estradiol, but greater concentrations were observed in enriched does at 32 dpp ($P < 0.05$).

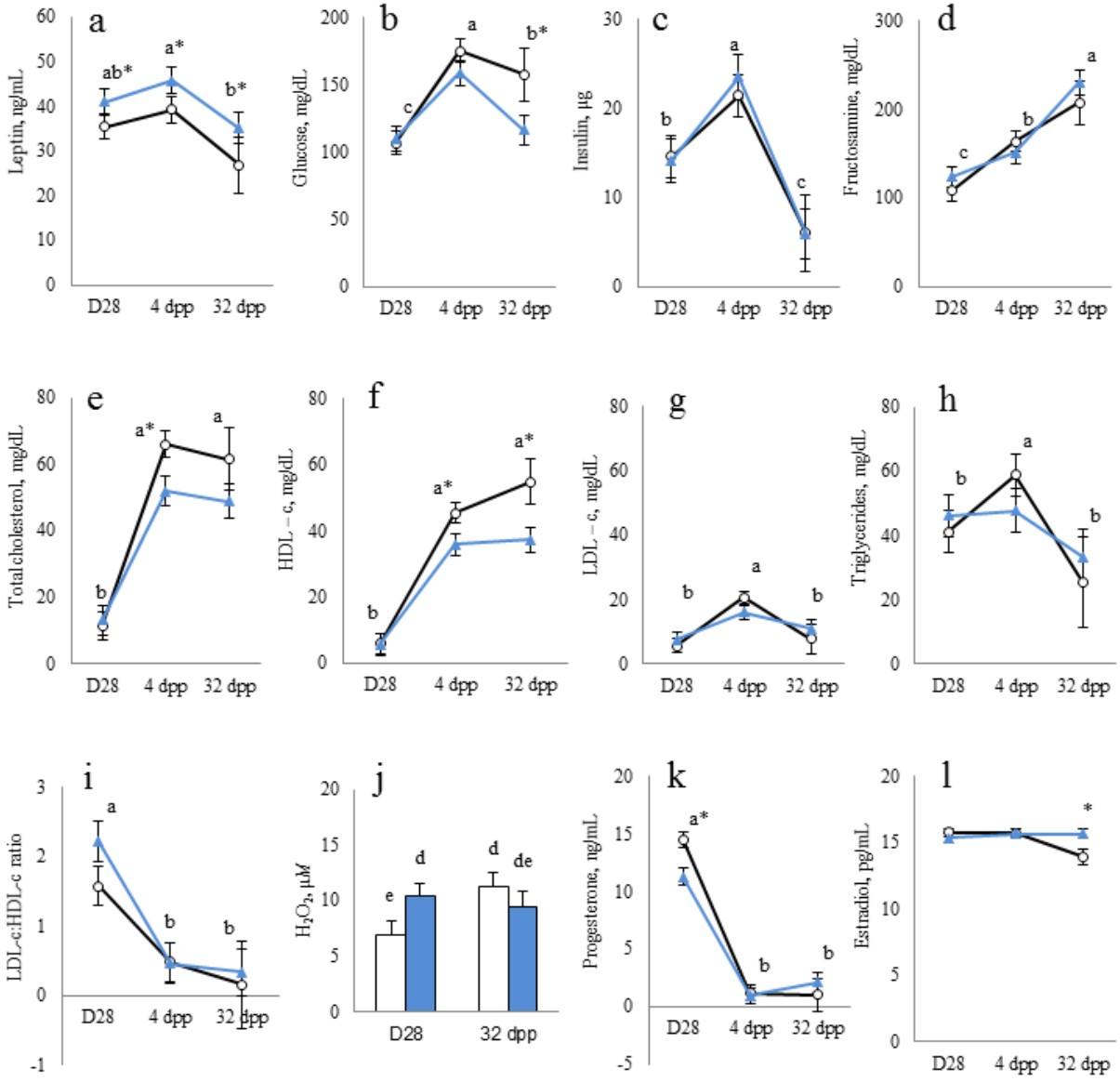

Figure 3.2. Plasma variables of rabbit does fed a control diet (○; n = 15) and an enriched diet with a supplement based on n-3 PUFA (Enriched; ▲; n = 15) measured on d 28 of gestation (D28), at 4 d postpartum (**dpp**) and at 32 dpp. All values are least squares means. *: significant differences between diets ($P < 0.05$); a, b, c: time significant differences ($P < 0.05$); d,e: significant differences of interaction between diet and time ($P < 0.05$). HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol. H_2O_2: hydrogen peroxide (white bars = control, darkened bars = enriched).

3.2. Offspring trial

Variables related to glucose metabolism (glucose and fructosamine) were similar in the offspring of either treatments, without significant effects of age (1 dpp/32 dpp) or size (the largest/the smallest kits) (Figures 3.3a and 3.3b; 3.3c and 3.3d). By contrast, there were significant effects of PUFA supplementation on TG, total, and HDL-c in newborns, with greater plasma concentrations in the enriched than in the control group ($P < 0.05$), in both the largest (Figures 3.3e, 3.3g and 3.3i) and the smallest kits (Figures 3.3f, 3.3h and 3.3j). There were no differences between groups in plasma LDL-c in newborns regardless of their size (Figures 3.3k and 3.3l). At weaning, all those variables were significantly lower than at early lactation ($P < 0.05$), without significant differences between groups, except for LDL-c/HDL-c ratio. It was low at early lactation, increasing significantly at weaning, with a greater increase in the enriched treatment, considering the largest kits (Figure 3.8m). However, for the smallest ones, newborns from both diets had low LDL-c/HDL-c ratio at early lactation, increasing significantly in enriched group at weaning, but keeping intermediate in control kits (Figure 3.3n).

Assessment of systemic oxidative stress showed greater plasma H_2O_2 values in the kits from the enriched group at early lactation ($P < 0.05$), irrespective of their size, but no significant differences were observed at weaning (Figures 3.3o and 3.3p).

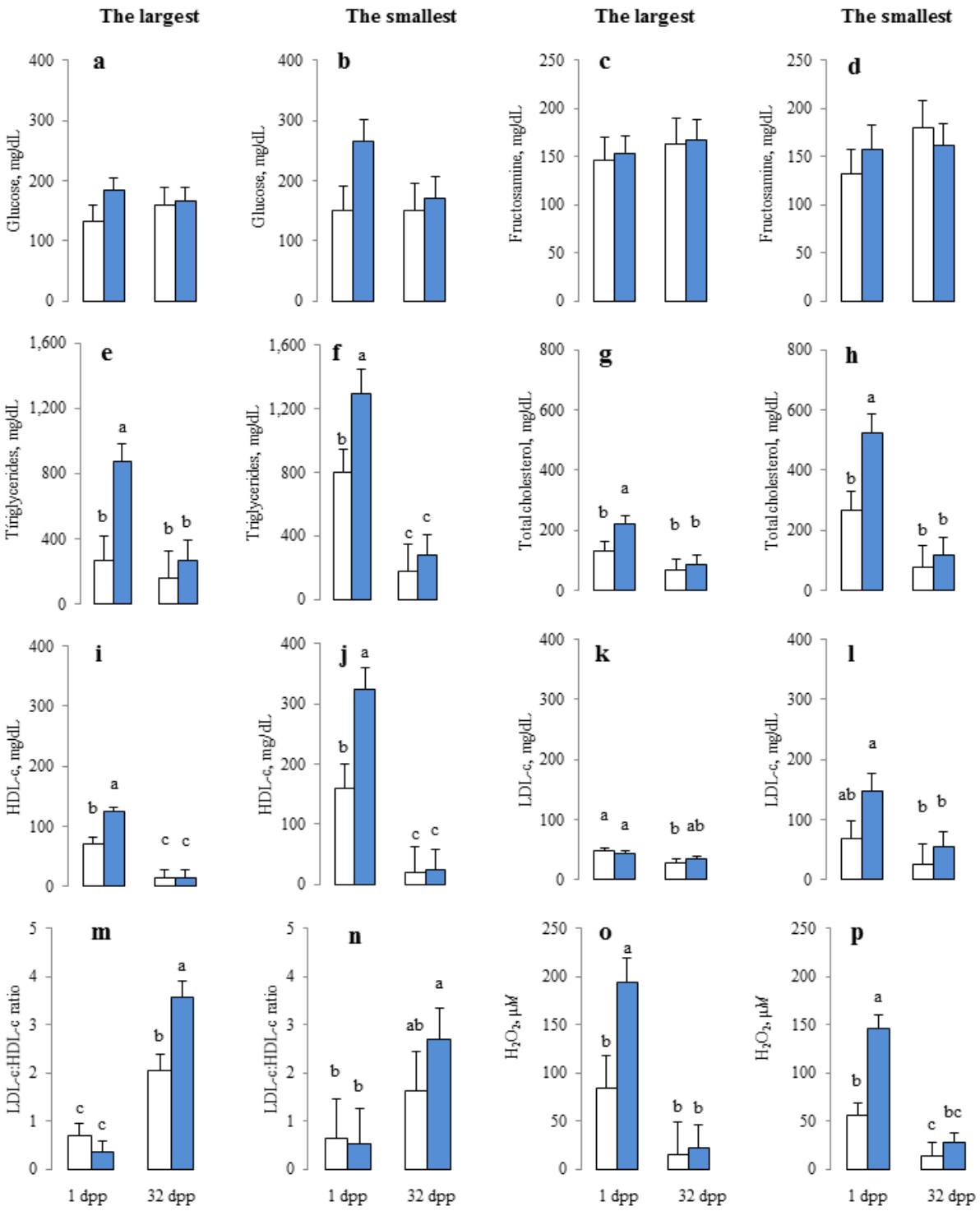

Figure 3.3. Plasma variables of the largest and the smallest kit of the litter born from rabbit does fed a control diet (□; n = 8) and an enriched diet with a supplement based on n-3 PUFA (Enriched; ■; n = 8) at 1 d postpartum (dpp) and at 32 dpp. All values are least squares means. a, b, c: significant differences of interaction between diet and time ($P < 0.05$). HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol. H2O2: hydrogen peroxide.

4. DISCUSSION

Although the use of a supplement based on n-3 PUFA during pregnancy and lactation did not improve the reproductive performance of rabbit females, it modified some plasma variables of both, rabbit females and their offspring.

Both diets covered maternal nutritional requirements because feed intake was normal and similar among treatments during rearing, pregnancy, and lactation (Rebollar *et al.,* 2011). As a result, there were no differences in morphometric features of the placenta, embryo, and fetus throughout pregnancy, nor in fertility and prolificacy at first parturition. As in previous trials in sows supplemented with fish-oil (Tanghe *et al.,* 2013), the enrichment of rabbit diets with n-3 PUFA had no effects on prolificacy. However, at birth, offspring from PUFA supplemented females were heavier and longer than controls, as previously reported with similar supplementation in rabbits (Rebollar *et al.,* 2014), in humans (Imhoff-Kunsch *et al.,* 2012), and in rats (Olsen *et al.*, 1990). It has been suggested that the beneficial effects on neonatal growth of n-3 PUFA could be determined by a low production of $PGF_{2\alpha}$ in uterine and placental tissues, which leads to a less susceptibility to preterm parturition (Elmes *et al.*, 2004, 2005), prolonging gestation and increasing the size and weight of newborn. Nonetheless, in the current study pregnancy duration was similar in both groups.

Most plasma values of metabolic hormones suggested that the glycemic and lipid metabolism of mothers were similar between treatments. That is probably because the feed intake, and thus the nutritional and metabolic status, were similar. In both groups, lipid and glucose values were low at the end of pregnancy, increasing at 4 dpp and remaining constant or decreasing again at weaning. That is related to the well-known feed intake behavior of pregnant does, which decreases at the end of pregnancy due to the limited space available in the gastrointestinal tract and the lack of available carbohydrates (Wang *et al.,* 2001; Mizoguchi *et al.,* 2010). Furthermore, there is an increased energy demand for fetal growth at the end of pregnancy (Mizoguchi *et al.,* 2010; Rebollar *et al.,* 2011). Both feed intake and energy demands would explain the lower levels of plasma leptin, glucose, fructosamine, insulin, total cholesterol, LDL-c, HDL-c, and TG concentrations observed at the end of pregnancy.

At early lactation there was a significant increase in glucose, fructosamine, and insulin concentrations in both groups, as well as lipid metabolites, coinciding with the dramatic increase in feed intake at that time (Gidenne *et al.,* 2010), which could improve the metabolic status of lactating females. Conversely, plasma concentrations of leptin, glucose, and insulin decreased at the end of lactation and weaning, as in previous studies (Rebollar *et al.,* 2011), presumably due to a negative energy balance caused by the high demand for milk components (Lebas, 1975). Despite reduce leptin concentration in supplemented does during the experiment, the observed values are within the physiological range (Rebollar *et al.*, 2011).

By contrast, PUFA supplementation influenced plasma levels of lipids in rabbits does during lactation, with significant decreases (although within physiological values; Palinski *et al.,* 2001) in plasma total and HDL-c concentrations, whereas LDL-c and TG remained the same between diets throughout the experiment. In previous studies on diet-induced hyperlipidemic rabbit models (Cayli *et al.,* 2010), supplementation with 300 mg · kg^{-1} · d^{-1} EPA significantly decreased plasma concentrations of total cholesterol, LDL-c, HDL-c, and TG. Concomitantly, some studies have corroborated the positive effects of long-term n-3 PUFA supplementation on postprandial lipidemia in humans (Sanders *et al.,* 1997). All together, these results suggest that even though plasma values of lipid observed in control females do not suppose health risk, PUFA supplementation could promote a healthier lipid profile in lactating rabbit does.

Regarding steroid hormones, estradiol was similar between groups on d 28 of pregnancy and on d 4 postpartum, increasing in enriched does at the end of lactation. This possibly could help to improve sexual receptivity and productive results later on, if animals were artificially inseminated at 32 dpp, as observed in additional works using the same commercial supplement as used in the current study (Rodríguez *et al.,* 2014). Progesterone values at the end of pregnancy were lower in enriched group, however this difference had no effect on pregnancy length, supporting previous data in rabbits (Rebollar *et al.,* 2014) and sows (Tanghe *et al.,* 2013) supplemented with n-3 PUFA.

In kits, the glycemic index was not altered by PUFA supplementation at any time. However, the lipid profiles of newborns from enriched does changed at early lactation, in spite of similar maternal lipidemia in both groups at the end of pregnancy. All the kits born from does supplemented with PUFA, regardless of their

size, had greater plasma concentrations of total cholesterol, TG, and HDL-c than control kits; the smallest kits also showed a trend for greater LDL-c plasma concentrations. During advanced pregnancies, most cholesterol is synthesized *de novo* by the fetus and, to a lesser extent, by cells on the fetal side of the placenta (Palinski, 2009). The results of the present study also indicate an inverse relation of cholesterol between mothers and kits (elevated in control does on d 4 postpartum but lower in their offspring), that has also been reported in earlier (sows; Torres-Rovira *et al.,* 2014) and in advanced stages of pregnancy (primates; Cox *et al.,* 2009).

Being conscious that we are speculating, this situation in newborn lipid metabolism may have important implications for their survival. Thermal stress can be particularly important in small sized kits, because they have a high skin surface area to body mass ratio. As altricial animals, thermoregulation is even more important in the first hours of life when lipids are used as the substrate for thermogenesis via BAT (which is more abundant in neonates than adults; reviewed by Mutinati *et al.*, 2014). The hyperlipidaemia and the high oxidative stress observed in newborn (the largest and the smallest) from enriched females may be related to the increased FA oxidative capacity of their tissues (Clarke, 2001; Oster *et al.,* 2010) for a heat production. According to Thompson and Danesh (2006), a lower LDL-c/HDL-c ratio will indicate less vascular aggression by plasma cholesterol and more effective reverse transport of cholesterol. As a result of the dynamic of both lipid profiles of mother and offspring (despite the low lipid profile of does at d 28 of pregnancy), all does had a greater LDL-c/HDL-c ratio probably due to the use of lipid stores accumulated during the anabolic phase of pregnancy. Later, at early lactation, lipid metabolism of females normalised, decreasing the LDL-c/HDL-c ratio, and implying adequate reverse transport to the liver. Nevertheless in kits, the results were opposite to does; at 1 dpp they had a low LDL-c/HDL-c ratio because their metabolism was rapid and efficient to generate heat using lipids (Mutinati *et al.*, 2014). Later, at weaning, these lipids are accumulating in tissues for development, growth and energy storage, causing an increase of this ratio (Rommers *et al*., 1999).

Moreover, the greater concentration of lipids in newborn kits from does fed the enriched diet was concomitant with a greater oxidative stress, in parallel with a greater oxidative stress in does of this group on d 28 of pregnancy. These results support previous data from Gladine *et al.* (2012), who demonstrated that the

incorporation of n-3 PUFA is positively correlated with lipid peroxidation, twice as high in the supplemented group, and Van Kuijk *et al.* (1990), who found that this lipid peroxidation increases in a dose-dependent manner in the liver of rabbits receiving tuna-oil supplements.

Newborns are more prone to develop oxidative stress than adults due to the exposure to high oxygen concentrations (Mutinati *et al.*, 2014). To counteract this situation of the newborn, antioxidant vitamins are administrated to the mother and transferred to the fetus (Capper *et al.*, 2005). In the current study, in spite of the inclusion of higher vitamin E concentrations in the enriched diet, it seems that it was not enough. Possibly, it was due to vitamin E and other major antioxidants show reduced activity in newborns (Gitto el at., 2009).

Afterwards, during lactation, the females of each group continue feeding on the same diet as during pregnancy. Usually milk composition is closely related to the FA composition of the corresponding diet (Tanghe *et al.*, 2013). However, in the present study, diet did not affect lipid plasma profile or the oxidative status of kits at weaning, except the LDL-c/HDL-c ratio, as discussed previously.

In conclusion, the present data suggest that a supplementation based on n-3 PUFA in rabbits: 1) does not induce major effects on reproductive performances, 2) increases leptin and estradiol in lactating does without relevant changes in progesterone, and 3) at early lactation, favors the hyperlipidemic status of neonates, reduces cholesterol concentration of does during lactation, nonetheless increases the oxidative stress in both does and kits. This study provides information on the benefits and consequences of a supplementation based on n-3 PUFA in nulliparous rabbit does and their first progeny until the weaning age that could be applied to rabbit farms.

CHAPTER 4

A diet supplemented with n-3 polyunsaturated fatty acids influences performance, meat quality and cecal fermentation of growing rabbits

1. INTRODUCTION

Increasing the quality of animal products in production systems that also enhance animal welfare is an important goal of animal nutritionists. Human dietary recommendations often focus on the need of increasing the intake of n-3 LCPUFA (18, 20, and 22 carbon atoms) due to their beneficial effects on health (Gil and Gil, 2015). Dietary supplementation of n-3 LCPUFA has been shown to increase their content in rabbits meat (Xiccato, 1999; Peiretti, 2012; Bianchi *et al*., 2009), and meat content in EPA (C20:5n-3), DPA (C22:5n-3), and DHA (C22:6n-3) was reported to be higher by enriching with fish than with vegetable oils (Benatmane *et al*., 2011; Tres *et al*., 2014). These n-3 LCPUFA are implicated in the development of the immune response (Fortun-Lamothe and Boullier, 2007), and their dietary supplementation can increase both kits BW at birth (Rebollar *et al*., 2014) and postweaning viability (Maertens *et al*., 2005). Moreover, dietary supplementation with vegetable fats can modify feed efficiency and productive performance in rabbits (Peiretti *et al*., 2007; Casado *et al*., 2013), and it has been reported that medium-chain FA (caprilyc and capric) acids can alter the intestinal microbiota, thus affecting cecal fermentation (Marounek *et al*., 2002) and the development of pathogenic strains (Skřivanová *et al*., 2009). Nonetheless, studies with PUFA of marine origin are scarce (Castellini *et al*., 2004; Kowalska and Bielanski, 2009) and have focused on meat characteristics. Our hypothesis was that fish oil (rich in E and C22:6n-3) supplementation to rabbits would influence meat FA profile, but might also modify cecal fermentation and affect positively animal performance by reducing morbidity and/or mortality. The aim of this study was to evaluate the influence of a supplement derived from fish oil on performance, meat quality, ileal morphology, and cecal fermentation of growing rabbits.

2. MATERIALS AND METHODS

2.1. Experimental diets

Diets used in this trial were the same used in the experiment of the chapter 3 of the present PhD Thesis work. As has been already commented, two isoenergetic, isoproteic, and isofibrous diets were formulated following the nutritional recommendations of De Blas and Mateos (2010). Both diets had the same basal mixture of ingredients and only varied in the type of fat added: either 7.5 g/kg of lard (control group) or 15 g/kg of a commercial supplement (Optomega-50; Optivite International Ltd., Barcelona, Spain) consisting in a concentrated mixture of n-3 PUFA derived from salmon oil (enriched group). According to the manufacturer, Optomega-50 contained 50% of ether extract (35% of n-3 and 8% of n-6) and vitamin E (2,500 mg/kg) on a mineral based carrier. In order to equal the carrier of Optomega-50, 7.5 g/kg of sepiolite were included in the control diet.

Image 4.1. Greowing rabbits fed a control diet and an enriched diet with a supplement based on n-3 polyunsaturated fatty acids from 30 to 60 d of age.

The ingredient and chemical composition of diets were given in Table 3.1 and Table 3.2, respectively, and FA profiles were shown in Table 3.3 of the chapter 3 of the current Thesis. Only one batch of each diet was used during the study. Diets were prepared at the beginning of the trial, vacuum-packed and stored at 5ºC to protect against lipid oxidation and other degradative processes. Samples of each diet were collected weekly, maintained at 5ºC and composited at the end of the trial for chemical analysis.

2.2. Animals and experimental design

All experimental procedures used were approved by the Animal Ethics Committee of the Community of Madrid (Reference Procedimiento experimental 302/15), and were in compliance with the Spanish Guidelines for Care and Use of Animals in Research (Boletín Oficial del Estado, 2013). Temperature (19-22°C), air circulation (15 renovations per h), humidity (50 ± 5%), and light program (16 h of light and 8 h of darkness) of the building were maintained through the trial.

The litters from 24 primiparous rabbit does that had been fed the experimental diets (12 control and 12 enriched does) during pregnancy and lactation were used in the study. Results on the effects of diet supplementation with n-3 PUFA on endocrine, reproductive and productive responses of rabbit does have been reported by Rebollar *et al*. (2014), and those on plasma metabolites in their offspring in the chapter 3 of the present work. All kits were weaned after 30 d of lactation. They were weighed and subsequently standardized to 8 kits per litter (kits with a BW similar to the average litter BW removing the outliers). Each litter was housed in a flat-deck cage (60 x 50 x 33.5 cm). The 24 litters (192 kits in total) were divided into 4 homogeneous groups (6 litters each; 2 groups/diet) according average BW. The initial average BW of kits was 711 ± 25.9 and 710 ±28.3 g for control and enriched group, respectively. Rabbits continued to be fed their *ad libitum* maternal diet through the growing period and viability of rabbits was monitored every day. The experimental design is shown in Figure 4.1.

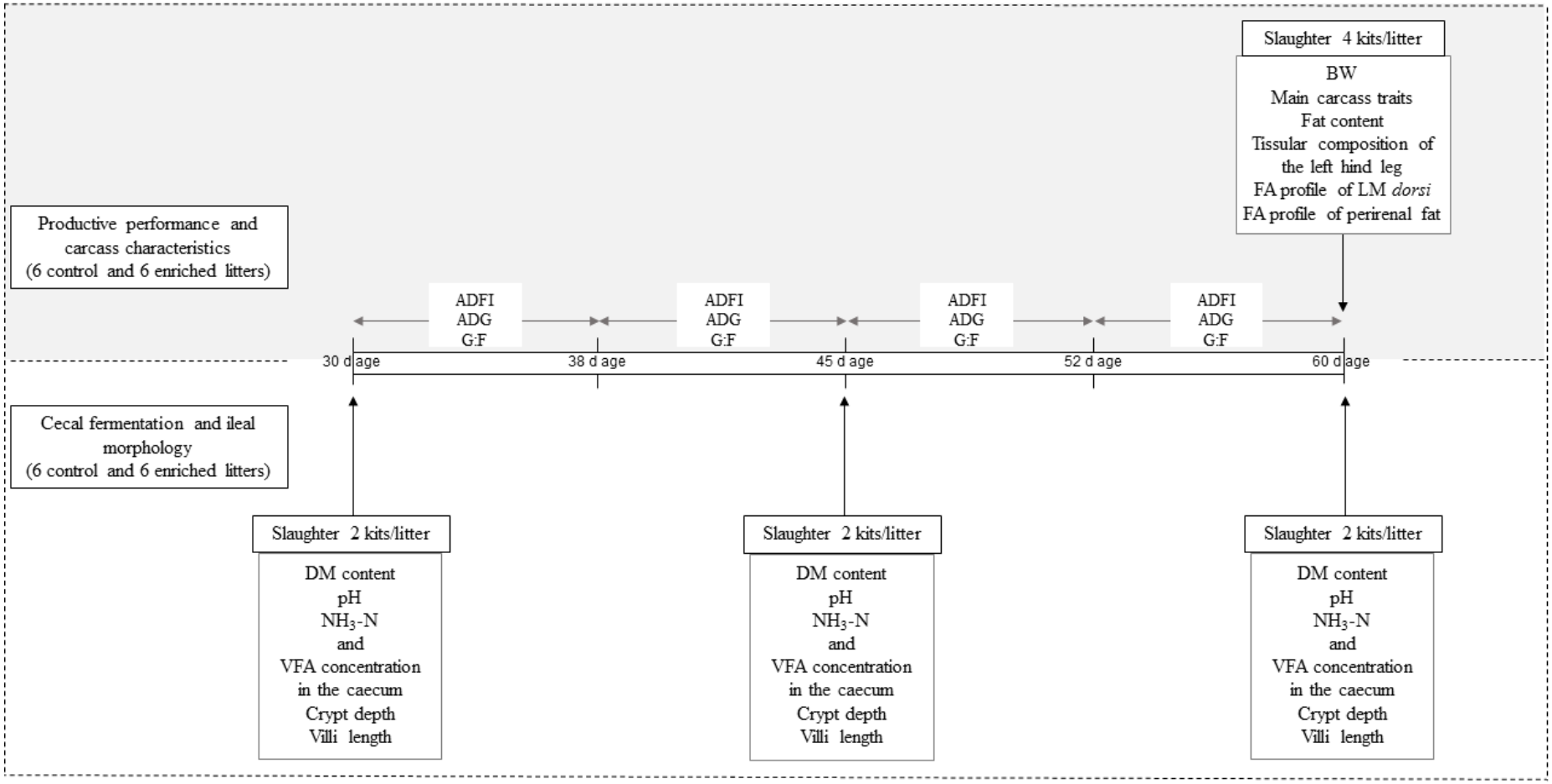

Figure 4.1. Sampling timeline of different variables related to productive performance and carcass characteristics, as well as, cecal fermentation and ileal morphology of kits during their growing period. DM = dry matter. VFA = volatile fatty acids. ADFI = average daily feed intake. ADG = average daily gain. G:F = gain/feed ratio. BW = body weight. FA = fatty acids. LM = *Longissimus dorsi* muscle.

2.3. Productive performance and carcass characteristics

Feed intake, productive performance and carcass quality were measured in 6 litters per diet (48 rabbits per diet). Live BW and feed consumption were determined weekly in each cage to calculate average daily feed intake (**ADFI**), ADG and gain/feed ratio (**G:F**). At d 60, all rabbits (48 rabbits per diet) were weighed and 4 animals from each litter, having a BW similar to the average litter BW, were stunned at low voltage (90 V; 5 s). Animals were then bled by cutting the carotid arteries and jugular veins. All further procedures followed the recommendations of the World Rabbit Science Association described by Blasco and Ouhayoun (2010). The full gastrointestinal tract, skin, distal legs and tail, and urogenital tract were removed. Carcasses (with the head, heart, lungs, liver, kidneys, perirenal fat and scapular fat; Image 4.2) were weighed, and then the perirenal, suprascapular, and abdominal fat were removed and weighed individually. Samples (2-3 g) of perirenal fat and *Longissimus dorsi* muscle (**LM**) (dorsal portion) were taken and immediately frozen (-20ºC) until lyophilization and analysis of FA profile (Image 4.3). Muscle pH was measured on the 10th rib face of the LM using a portable Crisson 25 pHmeter with a penetration electrode 5053 T (Crisson Instruments, Barcelona, Spain) at 0, 30 min and 24 h post mortem. Meat color was assessed 30 min after slaugther in the LM according to the Commission International de l'Eclairage (2004) system, and was reported as L* (lightness), a* (redness), and b* (yellowness). Color was determined using a Konica Minolta CM-700d colorimeter (Azuchi-Machi Higashi-Ku, Osaka 541, Japan) under daylight illumination and a 45/0 viewing angle (Image 4.4). The left hind leg was separated, weighed and vacuum packaged until dissection and determination of the amount of muscle, bone and fat by weight (Precisa 125A precision balance, Precisa Gravimetrics AG, Dietikon, Switzerland) (Image 4.5). Finally, carcasses were chilled to 5°C for 24 h in a ventilated room, and weighed to calculate drip loss percentage and carcass yield.

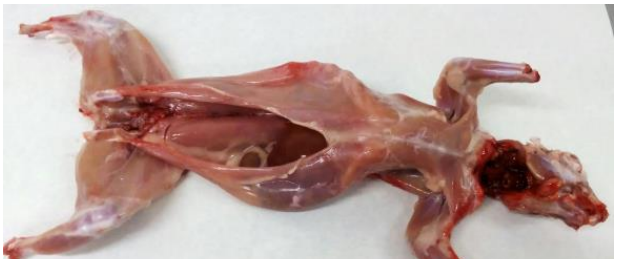

Image 4.2. Rabbit carcass weighing 2 kg after slaughtering at 60 days old.

Image 4.3.. Samples of (A) perirenal fat and (B) *Longissimus dorsi* muscle

Image 4.4. (A) pHmeter and colorimeter used to determine pH and color carcass. (B) Carcass pH determination.

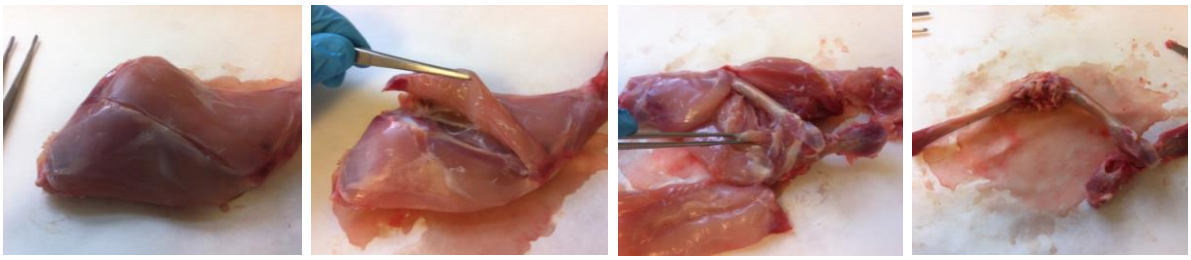

Image 4.5. Left hind leg before and after dissection of the amount of muscle, bone and fat by weight

2.4. Cecal fermentation and ileal morphology

The other 6 litters per diet (48 rabbits per diet) were used to assess the influence of the experimental diets on cecal fermentation and ileal morphology. Two

rabbits per litter (12 kits per diet), with a BW similar to the average litter BW, were slaughtered by cervical dislocation at 30 (weaning), 45 and 60 d of age. The cecum was removed and its full weight was recorded. The cecal content was then extracted, weighed, homogenized, and the pH immediately measured with a Crison Basic 20 pHmeter (Crison Instruments, Barcelona, Spain). About 2 g of cecal content were weighed, mixed with 2 mL of 0.5 *N* HCl, and immediately frozen (-20°C) until analysis of NH_3-N and VFA concentrations. The remaining cecal content was used to determine DM content (Image 4.6).

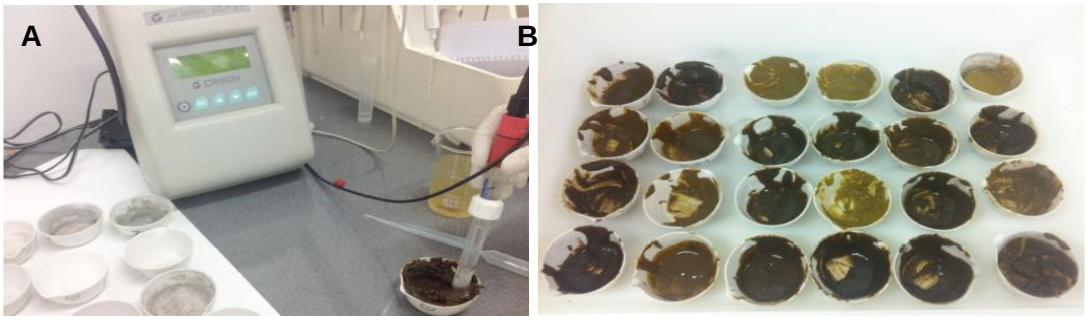

Image 4.6. (A) pH and (B) dry matter determination of cecal content.

In addition, a sample from the ileum (4 cm) was taken next to the last Peyer's patch from each animal to study mucosa morphology. Samples were preserved in a formaldehyde solution (10%; vol/vol), and were later gradually dehydrated with increasing concentrations of ethyl alcohol (50 to 100%). The dehydrated samples were embedded in paraffin and stained with hematoxylin-eosin before cutting histological sections of 5 µm. The sections were analyzed by light microscopy (Olympus BX40; Olympus Optical Co., Hamburg, Germany) using an image analyzer (Soft Imaging System, Olympus, GmbH, Hamburg, Germany) (Image 4.7). Villi length (from the top of the villi to the villi crypt junction) and depth of corresponding crypts were measured according to Hampson (1986) in four cross sections from the mean value of 30 vertically-oriented villi per animal. Due to a problem during the storage of the samples taken at weaning (30 d of age), they could not be analyzed and ileal morphology values for this sampling are not reported.

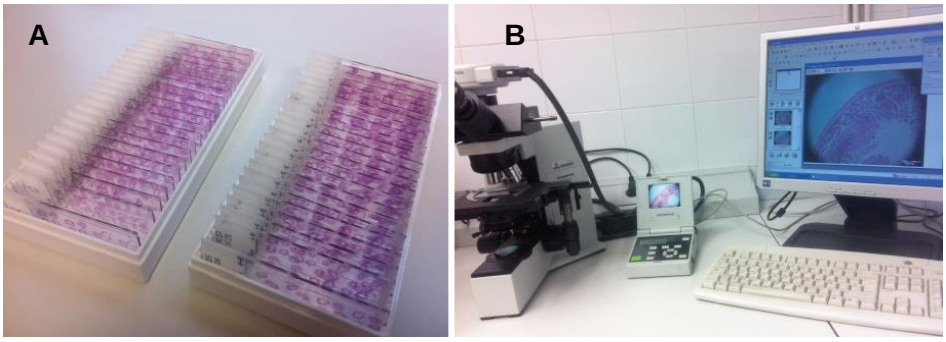

Image 4.7. (A) Processed ileum samples and (B) subsequent microscopy analysis in the laboratory

2.5. Chemical analyses

Chemical analysis of experimental diets followed the official methods of the AOAC (2000) for DM (oven drying method: 934.01), ash (muffle furnace incineration: 923.03), ether extract (solvent extraction: 920.39), and CP (Dumas method: 968.06; FP-528 LECO, St. Joseph, MI, USA) determinations. Gross energy was determined by combustion in an adiabatic calorimetric pump (model 1356, Parr Instrument Company, Moline, IL, USA). The NDF, ADF and ADL content was determined according to the sequential method of Van Soest *et al*. (1991) using an ANKOM220 Fiber Analyzer unit (ANKOMTechnology Corporation, Fairport, NY, USA). Sodium sulphite and heat-stable amylase were used in the sequential analysis of NDF, ADF and ADL, and they were expressed exclusive of residual ash.

The extraction of lipids from dietary samples followed the procedure of Sukhija and Palmquist (1988). Briefly, ground samples (1 mm screen; 200 mg) were mixed with 1 mL of toluene containing 1 mL of internal standard (pentadecanoic acid (C15:0), Sigma–Aldrich, Madrid, Spain) of 10 mg/mL concentration, 1 mL of toluene and 3 mL of freshly made 5% methanolic HCl in a culture tube. Tubes were capped, vortexed (1 min) and heated for 2 h in a shaking water-bath at 70°C. After tempering, 5 mL of 5% potassium carbonate solution were slowly added and the mixture was vigorously vortexed and centrifuged (5 min, 3000 rpm). The upper phase (3 phases are formed) was dried with anhydrous sodium sulphate and collected for FA analysis as described below. Lipids from muscle samples were extracted using the procedure described by Segura and López-Bote (2014). Briefly, lyophilized samples (200 mg; in triplicate) were homogenized in dichloromethane:methanol (8:2; vol/vol) using a mixer mill (MM400; Retsch technology, Stuttgart, Germany). The final

biphasic system was separated by centrifugation. Solvent was evaporated under nitrogen stream, lipids were dried by vacuum desiccation and total lipid content was determined gravimetrically. The FA methyl esters were prepared from total lipids by transesterification using a mixture of sodium methylate–methanol and methylated in the presence of sulphuric acid as described by Segura and López-Bote (2014). The FA methyl esters were separated using a gas chromatograph (HP 6890 Series GC System; Hewlett Packard Co., Avondale, PA, USA) equipped with a flame ionization detector and a HP-Innowax polyethylene glycol column (30 m x 0.316 mm x 0.25 µm; J&W Scientific/Agilent Technologies, Santa Clara, CA, USA). Nitrogen was used as a carrier gas. The FA profiles from adipose tissue samples were extracted using a mixture of chloroform/methanol (2:1, vol/vol), methylated in the presence of sodium methoxide and quantified as previously described (Cordero *et al*., 2011). A Hewlett Packard HP-5890 (Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector was used (capillary column HP-Innowax, 30 m × 0.32 mm internal diameter and 0.25 m film thickness) (Agilent Technologies Gmbh, Germany). A split ratio of 50:1 was used and C15:0 was included as internal standard.

Cecal samples were thawed at 4ºC, homogenized, centrifuged (14,000 rpm, 15 min, 4 ºC) and the supernatant was used for NH_3-N and volatile fatty acid (**VFA**) analyses. The analysis of the ammonia concentration was performed by the colorimetric technique described by Weatherburn (1967). Preparation of samples and analysis of VFA concentration followed the procedures described by García-Martínez *et al*. (2005). Briefly, 1.0 mL of the supernatant was mixed with 0.5 mL of a deproteinizing solution (10% metaphosphoric acid and 0.06% crotonic acid) and the obtained mixture was allowed to stand for 24 h at 4°C. Samples were centrifuged (14,000 rpm, 15 min, 4 ºC) and the supernatant was transferred to chromatography vials. The VFA concentration was determined by gas chromatography using a Pelkin Elmer Autosystem XL gas chromatograph (PerkinElmer Inc., Shelton, CT, USA) equipped with an automatic injector, detector flame ionization and a semi-capillary column TR-FFAP 30 m x 0.53 mm x 1 µm (Supelco, Barcelona, Spain).

2.6. Calculations and Statistical Analyses

Unsaturation index of diets, muscle and fat were calculated as the sum of the unsaturated FA, each multiplied by the number of double bonds in their chain, and

divided by 100. Atherogenic and thrombogenic index of muscle and fat were calculated according to the equations proposed by Ulbricht and Southgate (1991), where MUFA represent the content of monounsaturated fatty acids.

Atherogenic index = (C12:0 + 4 x C14:0 + C16:0) / (ΣMUFA + Σn-6 + Σn-3)

Thrombogenic index = (C14:0 + C16:0 + C18:0) / [0.5 x ΣMUFA + 0.5 x Σn-6 + 3 x Σn-3 + (Σn-6/Σn-6)]

The experimental unit for ADFI, ADG, G:F, cecal fermentation parameters, and ileal morphology values measurements was the cage, whereas the individual rabbit was the experimental unit for carcass and meat characteristics. Data on ADFI, ADG, G:F, cecal fermentation, and ileal morphology were analyzed as a repeated measures ANOVA using the MIXED procedure of SAS (version 9.2; SAS Inst. Inc., Cary, NC). The statistical model included diet, time (sampling day), and the interaction between diet and time as fixed effects. The rest of the data were analyzed by the same model excluding the effects of time. All means were compared using a protected *t*-test, and significance was declared at $P < 0.05$, and $0.05 < P < 0.10$ values were considered to be a trend. Results are presented as least squares means.

3. RESULTS AND DISCUSSION

Experimental diets were formulated with the same ingredients, and were isoenergetic and isoproteic in order to avoid confounding effects of different ingredients and/or different energy levels due to fat supplementation. As shown in Table 3.3, total PUFA concentration and n-6/n-3 ratio were 1.3-fold higher and 3.3-fold lower, respectively, in the enriched diet compared with the control one. No C20:5 n-3, C22:5 n-3 or C22:6 n-3 were detected in the control diet but they were present in the enriched diet, with proportions ranging from 9.2 to 40.0 g/kg of total FA methyl esters.

3.1. Productive performance and carcass characteristics

No mortality or morbidity was registered through the trial. As shown in Table 4.1, there were no differences (P = 0.127 to 0.688) between experimental groups either in ADFI, ADG or G:F, and no interactions between diet and time were observed, with the exception of a trend for ADFI (P = 0.093). These results indicate that the level of inclusion of a supplement derived from fish oil used in our study had no detrimental effect either on feed intake or on animal growth. In agreement with our results, Kowalska and Pielanski (2009) reported no effects of dietary fish oil supplementation (3% as-fed basis) on growth performance of rabbits and feed conversion efficiency. Using other PUFA-rich fats (palm fat (99% SFA) or linseed oil (>70% PUFA)), Trebušak *et al*. (2015) also observed no differences in *g*rowth performance. Nonetheless, others (Bianchi *et al*., 2009; Casado *et al*., 2013) have reported lower growing performance when linseed or linseed oil was included in the diet. Both FA composition of the supplements and level of fat inclusion in the diet can be involved in the variable response of rabbits to fat supplementation observed in the different studies. Values of ADFI, ADG, and G:F during the first 3 wk of the study were similar to those reported in the literature for similar production conditions (Fernández *et al*., 1994; Kouba *et al*, 2008; Kowalska and Bielanski, 2009), but a lack of increase in feed intake and a reduced growth were observed at wk 4 in both groups, which was attributed to the high density of rabbits in the cage at the end of the growing period. Muguerza *et al*. (2008) reported that a density of 0.044 m^2 per animal was optimal to reach a slaughter BW of 2.0 – 2.1 kg in 56-60 d (typical values for rabbit production in Spain), but in our study the density was higher (0.038 m^2 per

animal) and the animals occupied most of the floor space in the cage the last wk of the study. This probably made difficult the access to the feeder and consequently reduced feed intake, which was lower than that reported by others in similar studies (> 155 g/d; Corrent *et al*., 2007; Casado *et al*., 2013). However, the lack of morbidity and mortality registered in our study indicates that the reduced feed intake did not negatively affect the health of the rabbits in any group. As observed by others (Gutiérrez *et al*., 2002; Read *et al*., 2016), G:F was decreasing over time, as feed conversion rate is more favorable in younger animals than in those reaching slaughter BW (Maertens, 2009).

Table 4.1. Average daily feed intake, ADG, and G:F of fattening rabbits fed a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 15 g/kg from 30 to 60 d of age[1]. All values are least squares means

	Week of growing period						*P*-value		
Item	1	2	3	4	SEM_D[2]	SEM_T[2]	Diet	Time	Diet
ADFI[3], g/d									
Control	84.5	131	146	145	0.52	0.74	0.688	<0.001	0.093
Enriched	86.0	138	143	137					
ADG[4], g/d									
Control	48.6	52.2	46.4	32.7	1.12	1.58	0.144	<0.001	0.433
Enriched	49.0	52.0	43.6	30.9					
G:F[5], g/g									
Control	0.575	0.401	0.318	0.226	0.0048	0.0067	0.127	<0.001	0.626
Enriched	0.574	0.378	0.302	0.222					

[1]Six litters of 8 rabbits each were fed each diet. [2]SEM_D and SEM_T = standard error of the mean for diet and time effects, respectively. [3]ADFI = average daily feed intake. [4]ADG = average daily gain. [5]G:F = gain/feed ratio.

No differences (*P* = 0.160 to 0.842) between diets were observed either in BW at slaughter, hot and chilled carcass weights, carcass yield, drip loss percentage, and full gastrointestinal tract weight (Table 4.2). Values were in the range of those reported in the literature for similar rabbit production systems (Chamorro *et al*., 2007; Casado *et al*, 2011; El Abed *et al*, 2012). The type of fat in the diet had no effect (*P* = 0.144 to 0.935) either in meat color parameters measured

30 min after slaughter or in pH values at 0, 30 min and 24 h after slaughter. Nonetheless, the weight of the skin tended to be lower (P = 0.055) in the enriched group than in control one. This trend might be due to a higher deposition of subcutaneous fat with a more saturated profile in the control group, which would result in higher fat densification and therefore subcutaneous fat would be more easily removed at skinning. This hypothesis is also supported by the higher amount of perirenal fat (P = 0.020) and the trend (P = 0.063) to higher abdominal fat observed in the control group than in the enriched one, although there were no differences (P = 0.851) between groups in the amount of scapular fat.

A decrease in carcass fat content after PUFA-rich fat supplementation have been previously observed by others in rabbits (Kowalska and Bielanski, 2009; Volek and Marounek, 2011), as fat deposition is affected by the saturation degree of FA (Lin *et al*., 1993). It has been consistently reported (Piers *et al*., 2002; Ukropec *et al*., 2003; Jans *et al*., 2012) that PUFA are more readily oxidized than SFA, mainly in the liver, but also in the muscle, which can contribute to the reduction on lipogenesis by n-3 PUFA supplementation that has been repeatedly shown in different animal species (Wood *et al*., 2008).

No differences (P = 0.253 to 0.946) between groups were detected in total weight and tissue composition (bone, muscle and fat) of the left hind leg (Table 4.2). Similar results have been reported by others in rabbits when the diet was supplemented with fish oil (Kowalska and Bielanski, 2009) or other PUFA-rich fats (Peiretti *et al*., 2007; Volek and Marounek, 2011).

Table 4.2. Body weight, main traits and fat content of carcass, and tissue (bone, muscle and fat) composition of the left hind leg from growing rabbits fed a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 15 g/kg and slaughtered at 60 d of age[1]. All values are least squares means

	Diet			
Item	Control	Enriched	SEM	*P*-value
BW, g	1999	1971	13.4	0.205
Carcass traits				
Hot carcass weight, g	1197	1177	9.2	0.221
Hot carcass yield, %	59.9	59.8	0.24	0.823
Chilled carcass weight, g	940	925	7.4	0.160
Color				
L*	54.9	54.4	0.52	0.497
a*	3.11	3.51	0.348	0.414
b*	1.25	1.19	0.342	0.297
pH				
Slaughter time	7.40	7.48	0.039	0.144
30 min	7.03	7.11	0.036	0.166
24 h	6.05	6.05	0.029	0.935
Drip loss percentage, %	7.30	7.42	0.165	0.431
Skin weight, g	387	374	4.685	0.055
Full gastrointestinal tract weig	325	325	5.9	0.842
Fat, g				
Abdominal	26.2	24.4	0.65	0.063
Scapular	7.11	7.19	0.272	0.851
Perirenal	14.7	12.8	0.57	0.020
Left hind leg, g				
Total weight	170	169	1.467	0.403
Bone	26.7	26.2	0.27	0.253
Muscle	131	129	1.5	0.299
Fat	5.22	5.20	0.183	0.946

[1] 24 rabbits per group. [2]BW = body weight.

The FA profile and indexes related to human health of LM and perirenal fat are presented in Tables 4.3 and 4.4, respectively. There was no difference (P = 0.476) between groups in total fat content of LM. In contrast, dietary supplementation with fish oil resulted in greater PUFA ($P \leq 0.021$) and lower ($P \leq 0.002$) MUFA content in both LM and perirenal fat. The C20:5n-3, C22:5n-3 and C22:6n-3 content of LM was 3.2, 2.1 and 4.4 times greater ($P < 0.001$) in the enriched rabbits compared with control ones. Although these FA were not supplied by the control diet (Table 3.3), the three FA were detected in all samples of LM from control animals, thus reflecting the ability of rabbits to synthesise endogenous n-3 FA (Bernardini *et al*., 1999). The amount of endogenous n-3 FA synthesized seems to depend on dietary n-6/n-3 PUFA ratio, and Bernardini *et al*. (1999) reported an efficient conversion of ALA (C18:3n-3) into n-3 LCPUFA in rabbits fed diets with low n-6/n-3 ratio. An increase in the C20:5n-3, C22:5n-3 and C22:6n-3 content in rabbit carcass has also been observed by dietary C18:3n-3 supplementation (Bernardini *et al*., 1999; Kouba, 2006), although the level of C18:3n-3 that can be converted into C20:5n-3 and C22:6n-3 appears to be variable in different body tissues, with the heart and the liver having greater capacity than the muscle (Ander *et al*., 2010). In our study there were no differences (P = 0.768) between diets in C18:3n-3 concentrations in LM. Total SFA content in perirenal fat was not affected by the type of fat in the diet, but SFA content in LM was greater (P = 0.012) in the enriched group than in control one; this was due to the greater (P = 0.003) C20:0 concentrations observed in enriched rabbits, as there were no differences between diets ($P \geq 0.158$) in the rest of analyzed SFA. In agreement with our results, previous studies have shown the influence of dietary FA profile on FA deposition in rabbit carcass (Xiccato, 1999; Benatmane *et al*., 2011; Tres *et al*., 2014). In addition, good relationships between dietary FA and their content in LM and perirenal fat have been reported by Peiretti (2012) in a review of studies involving 27 diets varying in FA profile. However, individual FA differed in their incorporation in muscle and fat, and regression equations of individual FA in LM and perirenal fat on the same FA in the diet were different for the two tissues (Peiretti, 2012). A different response to dietary FA in LM and perirenal fat was also observed in our study, as n-3 LCPUFA concentrations and n-6/n-3 ratio in PUFA rabbits were greater and lower, respectively, in LM than in perirenal fat. In addition, no C20:5n-3 and C22:6n-3 were

detected in the perirenal fat of control rabbits, but both were detected in the LM samples of the same animals. The FA profile observed in our study is consistent with previous work (Bernardini *et al*., 1999; Kouba *et al*., 2008; Peiretti, 2012), showing greater SFA and MUFA deposition in fat compared with muscle, but greater PUFA deposition in muscle than in fat. It has to be noticed that the major lipid class in adipose tissue is triacylglycerol or neutral lipid, whereas in muscle a significant proportion is phospholipid, which has a much higher PUFA content in order to perform its function as a constituent of celular membranes (Wood *et al*., 2008). The FA profile in neutral lipids and phospholipids differ considerably, which can help to explain the the different individual FA incorporation observed in LM and perirenal fat.

The atherogenic and thrombogenic indexes can be considered a measure of the atherogenic and thrombogenic potential for human consumption, respectively, of the fat deposited in the carcass (Ulbricht and Southgate, 1991). The LM and perirenal fat from enriched rabbits showed lower ($P < 0.001$) atherogenic and thrombogenic indexes, respectively, than those from unsupplemented rabbits. Values of both indexes showed more individual variability in LM than in perirenal fat, but they indicate that carcass from enriched rabbits had a higher nutritional quality than that from control rabbits. Moreover, the n-6/n-3 ratio in the muscle of PUFA enriched rabbits was in the range recommended for healthy eating (< 4.0; Simopoulos, 2002; Wood *et al*., 2003), whereas meat from control rabbits exceeded this value.

Table 4.3. Total lipids content, fatty acid (FA) profile (g/100 g FA acid methyl esters), and indexes related to human health in LM of growing rabbits fed a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 15 g/kg and slaughtered at 60 d of age[1]. All values are least squares means

	Diet			
Item	Control	Enriched	SEM	*P*-value
Total lipids, g/100 g	1.63	1.52	0.078	0.476
Fatty acid profile				
C12:0	0.39	0.36	0.031	0.591
C14:0	2.43	2.49	0.200	0.831
C16:0	23.4	22.1	0.729	0.209
C18:0	6.43	6.14	0.141	0.158
C20:0	0.13	0.29	0.033	0.003
Total SFA[2]	38.6	39.5	0.227	0.012
C16:1n-7	2.64	2.64	0.198	0.993
C18:1n-9	21.6	17.5	0.647	<0.001
C18:1n-7	1.34	1.23	0.039	0.072
C20:1n-9	0.38	0.27	0.038	0.002
Total MUFA[3]	27.8	23.9	0.797	0.002
C18:2n-6	19.1	15.0	0.578	<0.001
C18:3n-3	0.93	0.95	0.062	0.768
C20:4n-6	4.63	4.37	0.222	0.424
C20:5n-3	0.80	2.59	0.095	<0.001
C22:5n-3	2.52	5.28	0.520	<0.001
C22:6n-3	0.99	4.34	0.268	<0.001
Total PUFA[4]	33.6	36.6	0.860	0.021
n-6	23.8	19.4	0.517	<0.001
n-3	5.24	13.2	0.788	<0.001
n-6/n-3 ratio	5.80	1.61	0.643	<0.001
Unsaturation index[5]	1.24	1.52	0.013	<0.001
Atherogenic index[6]	0.79	0.51	0.043	<0.001
Thrombogenic index[7]	0.53	0.51	0.017	0.456

[1] 12 rabbits per group. [2]SFA = saturated fatty acids. [3]MUFA = monounsaturated fatty acids. [4]PUFA = polyunsaturated fatty acids. [5]Calculated as the sum of the unsaturated fatty acids, each multiplied by the number of double bonds in their chain, and divided by 100. [6] Atherogenic index=(C12:0 + 4 x C14:0 + C16:0) / (ΣMUFA + Σn-6 + Σn-3). [7] Thrombogenic index=(C14:0 + C16:0 + C18:0) / [0.5 x ΣMUFA + 0.5 x Σn-6 + 3 x Σn-3 + (Σn-6/Σn-6)].

Table 4.4. Fatty acid (FA) profiles (g/100 g total FA methyl esters) and indexes of relevance for human health of perirenal fat of growing rabbits fed a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) *in a level of inclusion of 15 g/kg* and slaughtered at 60 d of age[1]. All values are least squares means

	Diet			
Fatty acid	Control	Enriched	SEM	*P*-value
C12:0	1.32	1.24	0.043	0.172
C14:0	4.34	4.68	0.050	<0.001
C16:0	29.3	29.5	0.230	0.541
C18:0	5.74	5.47	0.104	0.079
C20:0	0.10	0.10	0.003	0.633
Total SFA[2]	42.5	42.2	0.263	0.546
C16:1n-7	3.43	4.41	0.134	<0.001
C18:1n-9	28.0	23.0	0.177	<0.001
C18:1n-7	1.54	1.60	0.031	0.174
C20:1n-9	0.53	0.61	0.010	<0.001
Total MUFA[3]	34.2	30.4	0.260	<0.001
C18:2n-6	20.9	21.8	0.234	0.008
C18:3n-3	1.69	1.99	0.024	<0.001
C20:4n-6	0.18	0.26	0.006	<0.001
C20:5n-3	nd[1]	0.10	0.019	<0.001
C22:5n-3	0.08	0.84	0.014	<0.001
C22:6n-3	nd[1]	0.93	0.013	<0.001
Total PUFA[4]	23.1	27.2	0.277	<0.001
n-6	21.3	22.4	0.240	0.004
n-3	1.77	4.76	0.048	<0.001
n-6/n-3 ratio	12.04	4.71	0.064	<0.001
Unsaturation index[5]	0.83	0.97	0.006	<0.001
Atherogenic index[6]	0.84	0.86	0.010	0.138
Thrombogenic index[7]	1.20	0.97	0.012	<0.001

[1] 12 rabbits per group. [2]SFA = saturated fatty acids. [3]MUFA = monounsaturated fatty acids. [4]PUFA = polyunsaturated fatty acids. [5]Calculated as the sum of the unsaturated fatty acids, each multiplied by the number of double bonds in their chain, and divided by 100. [6] Atherogenic index=(C12:0 + 4 x C14:0 + C16:0) / (ΣMUFA + Σn-6 + Σn-3). [7] Thrombogenic index=(C14:0 + C16:0 + C18:0) / [0.5 x ΣMUFA + 0.5 x Σn-6 + 3 x Σn-3 + (Σn-6/Σn-6)].

3.2. Cecal Fermentation and Ileal Morphology

There were no differences (P = 0.126 to 0.924) between groups either in full cecum or cecal content weight at any sampling time (data not shown; averaged values across sampling times: 56.1 and 34.7 g for control diet, and 58.0 and 37.0 g for enriched diet, respectively). As shown in Table 4.5, control rabbits showed greater DM content (P = 0.005) and lower (P < 0.001) total VFA concentrations in the cecum than those fed the enriched diet, but diet did not affect (P > 0.05) cecal pH or NH_3-N concentrations. The reason for the greater total VFA concentration in the cecum of enriched rabbits is unclear, but it might reflect greater fermentation activity, although VFA concentrations do not necessarily reflect changes in VFA production if there are concomitant changes in VFA absorption and/or digesta passage rate. Some studies have shown that n-3 PUFA supplementation can modify the intestinal microbiota in poultry (Knarreborg *et al*., 2002) and pigs (Andersen *et al*., 2011), and it has been suggested that n-3 PUFA can protect against dysbiosis (Ghosh *et al*., 2013). Some studies in rabbits have reported that dietary supplementation with caprylic and capric acids decreased bacterial shedding in rabbits infected with enteropathogenic *E. coli* (Skrivanova and Marounek, 2006; Skrivanova *et al*., 2009) and other FA had antimicrobial activity on selected microorganisms (Marounek *et al*., 2002), but to our best knowledge there is no information on the effects of n-3 PUFA on rabbits gut microbiota. There were no differences between groups in VFA profile, with the exception of the proportion of minor VFA (calculated as the sum of isobutyrate, isovalerate, and valerate), which was greater (P = 0.013) in control than in supplemented rabbits. The lack of differences between diets in the proportions of the main VFA and NH_3-N concentrations observed in our study might indicate that the activity of the microbiota rather than the microbiota itself was affected by PUFA supplementation. Total VFA concentrations were 1.43, 1.35, and 1.10 times greater in enriched rabbits than in control ones at 30, 45, and 60 d of age, respectively, indicating that differences became less marked with age. Rabbit does received the same experimental diet than their litters during gestation and lactation, and therefore differences in cecal VFA concentrations at weaning (30 d) might be partly due to differences in milk FA profile (data will be showed in the chapter 5). In agreement with previous observations (Gidenne and Bellier, 2000), total VFA concentrations increased (P <

0.001) and molar proportions of acetate and butyrate decreased ($P < 0.001$) and increased ($P < 0.001$), respectively, from 30 to 60 d of age. Cecal pH decreased ($P < 0.001$) with age, which is in accordance with the increased VFA concentrations observed over time.

Diet did not affect ileal crypt depth (P = 0.319) or villi length (P = 0.255), indicating no effect of fish oil supplementation on the development of the ileal mucosa. Whereas ileal crypt depth increased ($P = 0.012$) from 45 to 60 d of age, no changes ($P = 0.619$) in villi length were detected. There were no diet x time interactions ($P = 0.115$ to 0.979) in any cecal and ileal parameter analyzed, with the exception of cecal pH ($P = 0.036$).

Table 4.5. Dry matter content, pH values, and NH_3-N and VFA concentrations in the cecum and ileal morphology of growing rabbits fed control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) and slaughtered at 30 (weaning), 45, and 60 d of age[1]. All values are least squares means

	Age (d)					*P*-value		
Item	30	45	60	SEM_D[2]	SEM_T[2]	Diet	Time	Diet x Time
Caecum								
DM[3], %								
Control	22.8	23.2	22.4	0.24	0.29	0.005	0.512	0.627
Enriched	21.5	22.0	21.9					
pH								
Control	5.78	5.92	5.72	0.029	0.036	0.749	<0.001	0.036
Enriched	5.93	5.94	5.59					
NH_3-N, mg/L								
Control	101	123	92	7.9	9.6	0.926	0.121	0.631
Enriched	89	122	107					
Total VFA[4],								
Control	43.8	64.2	72.8	2.02	2.47	<0.001	<0.001	0.109
Enriched	62.5	86.8	80.4					
Molar proportions (mol/100 mol)								
Acetate	85.4	78.3	73.9	0.47	0.58	0.332	<0.001	0.819
Control	86.1	78.4	75.1					
Enriched								
Propionate								
Control	3.59	4.44	5.01	0.182	0.223	0.688	0.039	0.115
Enriched	4.22	4.79	4.34					
Butyrate								
Control	10.1	15.4	19.1	0.38	0.47	0.431	<0.001	0.744
Enriched	9.1	15.2	19.0					
Minor VFA[5]								
Control	0.95	1.88	2.00	0.090	0.110	0.013	<0.001	0.912
Enriched	0.65	1.58	1.58					
Ileal morphology								
Crypt depth, µm								
Control	-	131	138	2.1	2.6	0.319	0.012	0.979
Enriched	-	131	146					
Villi length, µm								
Control	-	501	471	10.5	12.8	0.255	0.619	0.376
Enriched	-	450	480					

[1] Six litters (8 rabbits each) were fed each diet, and 2 rabbits per cage were sampled at each time (n=6). [2] SEM_D and SEM_T: standard error of the mean for diet and time effects, respectively. [3]DM = dry matter. [4]VFA = volatile fatty acids. [5]Calculated as the sum of isobutyrate, isovalerate, and valerate.

Finally, it is worth to mention that right hind legs were cooked (fried in olive oil without any condiment) and assessed by an untrained panel of 12 people, who did not find any abnormal odor or flavor in the enriched group (data not shown).

In conclusion, replacing lard by a supplement derived from fish oil in the diet of rabbits changed the FA profile of meat and fat depots, resulting in a more favorable profile for human nutrition, reduced the perirenal and abdominal fat content and had no adverse effects on growth performance, carcass characteristics and ileal morphology. The reason for the increased total VFA concentrations in the cecum of rabbits fed a supplement derived from fish oil should be further investigated.

CHAPTER 5

Improvements in the conception rate, milk composition and embryo quality of rabbit does after dietary enrichment with n-3 polyunsaturated fatty acids

1. INTRODUCTION

In previous studies, Rebollar *et al*. (2014) established that long-term dietary enrichment with n-3 PUFA (half of the fat of diet was substituted for a fish oil supplement rich in EPA (C20:5n-3) and DHA (C22:6n-3) in a concentration of 15 g/kg) did not affect feed intake and tended to improve endocrine function of corpora lutea around the implantation period (day 5-7 post AI), increasing weight and size of kits, and reducing mortality at the second parturition. In this line of investigation, using the same commercial supplement that in the chapter 3 of the present Thesis, we observed in primiparous rabbit does an increase of plasma leptin during lactation and plasma estradiol concentrations at 32 dpp. This indicates better energy condition and enhanced sexual receptivity that could improve the fertility rate in followings inseminations and could help to mitigate the principal problematic of these animals.

Moreover, diet consumed by females around the fertilization period and first days of pregnancy conditions the oviductal ambient (Ashworth *et al*., 2009), which can affect the first phases of embryo development. Thus, a continuous and higher level of n-3 PUFA supplementation during some critical periods as AI and the first phase of pregnancy, could have unknown effects on fertilization and early embryo development processes.

Similarly, dietary FA are incorporated into diverse tissues of pregnant females (Rebollar *et al*., 2014), which can be transferred to the offspring, increasing into the muscle and fat of carcass, as it has been corroborated in the chapter 4 of the present Thesis work. This transfer is due, at least in part, to the appearance of FA in milk as it has been demonstrated in sows (Mateo *et al*., 2009). Therefore, the diet used for the mother can improve the meat quality of the progeny. Nonetheless, to our knowledge there are no studies that analyse the milk FA profile of rabbits after consumption of a PUFA enriched diet from fish oil origin.

Considering the positive effects observed in previous studies, this work intends to determine the extent to which a long period of supplementation with n-3 PUFA in a 4-fold higher amount from rearing and during two reproductive cycles could affect: (1) daily feed intake of does, (2) performance parameters of does and viability of their litters, (3) progesterone response during gestation, (4) milk FA profile and (5) early embryo development and quality in terms of apoptosis rate.

2. MATERIAL AND METHODS

2.1. Animals and housing

The study was performed according to the Spanish Policy for Animal Protection RD53/2013, assessed and approved by the Animal Ethics Committee of the Community of Madrid (Ref. PROEX 302/15). A total of 127 New Zealand x California white rabbit does were fed the experimental diets from rearing and during two reproductive cycles. All animals were always fed *ad libitum* and were housed individually in flat-deck cages (700 x 500 x 330 mm) with a 16 h of light and 8 h of darkness light program. Temperature (18-23ºC), air circulation (15 renovations per hour) and humidity (50 ± 5%) of the building were maintained throughout the trial.

2.2. Experimental diets and fatty acid composition

Two isofibrous, isoenergetic, and isoproteic diets were formulated following the nutritional recommendations for breeding does issued by De Blas and Mateos (2010). Both diets had the same basal mixture of ingredients and only varied in the type of added fat: either 30 g/kg mixed fat (Control group, n = 63 does) or 60 g/kg of a commercial supplement (Optomega-50; Optivite International Ltd., Barcelona, Spain) containing a 50% of ether extract and 35% of n-3 PUFA and 2500 mg/kg of vitamin E (Enriched group, n = 64 does). The ingredients and chemical composition of diets are given in Table 5.1 and Table 5.2, respectively, and the FA profiles of experimental diets are shown in Table 5.3. Only one batch of each diet was used during all study. Diets were vacuum-packet and stored at 5ºC protecting them of lipid oxidation and other degradative processes. Samples of both diets were collected weekly and composited for further analysis. Feed intake was determined during rearing, pregnancy and lactation of the first cycle to assess the palatability of the diet.

Chemical analysis of diets followed the AOAC official methods (2000) for DM (oven drying method: 934.01), ash (muffle furnace incineration: 923.03), ether extract (solvent extraction: 920.39) and CP (Dumas method: 968.06; FP-528 LECO, St. Joseph, MI, USA) determinations. Gross energy was determined by combustion in an adiabatic calorimetric pump (model 1356, Parr Instrument Company, Moline, IL, USA). Neutral detergent fibre, ADF and ADL were measured using a filter bag system (Ankom Technology, New York, NY, USA) and following the procedures of

Mertens (2002) for aNDFom and the AOAC official method 973.18 for ADFom and ADL (AOAC, 2000).

Fatty acid profiles were analysed according to Sukhija and Palmquist (1988). A Hewlett Packard HP-5890 (Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector was used (capillary column HP-Innowax, 30 m × 0.32 mm internal diameter and 0.25 m film thickness) (Agilent Technologies Gmbh, Ratingen, Germany). A split ratio of 50:1 was used and C15:0 was included as internal standard. The unsaturation index (UI) was calculated as the addition of the unsaturated FA, each one multiplied by the number of double bonds in their chain and divided by 100.

2.3. Productive trial

Experimental design is shown in Figure 5.1. All females were artificial inseminated at 16 weeks of age and following AI was performed at 32 dpp according to the recommendations for primiparous does (Arias-Álvarez *et al*., 2009). Seminal doses with at least 20 million spermatozoa in 0.5 mL of diluent (Magapor S.L., Zaragoza, Spain) were prepared using a pool of fresh heterospermic semen from selected bucks. To induce ovulation, does were given an intramuscular injection of 20 µg gonadorelin (Inducel-GnRH, Lab. Ovejero, León, Spain).

Fertility [(number of parturitions/number of AI) x100] and duration of pregnancy (days) were determined. Total number of newborn, kits born alive and stillborn per litter was recorded and litters (total born alive) were weighed.

In order to confirm previous work (Rebollar *et al.*, 2014) with lower level of inclusion than the current study, 10 does per experimental group at first parturition with 10-11 kits born alive were sampled to determine individual kits body size. Using a slide calliper, CRL (maximum distance from crown to tail basis), BPD (from one parietal eminence to the other) and TD (were measured when they were one day old. After that, litter size of all does was standardized to 10–12 pups in average by removing or adding kits within each dietary treatment. At weaning (25 dpp), kits were counted, litters were weighed and ADG was determined. Lactation mortality of kits was recorded and expressed as the percentage of rabbits dead at weaning with respect to the number of rabbits after standardizing litter size. Non-pregnant females after AI were excluded from the experiment.

Table 5.1. Ingredient and chemical composition of a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 60 g/kg (g/kg, as-fed basis unless otherwise indicated)

	Diet[1]	
Ingredients	Control	Enriched
Barley grain	109	120
Corn DDGS	10	8.3
Gluten Feed	80	80
Bran	300	286
Sunflower meal 280 (g/kg CP)	221	220
Palmkernel 160 (g/kg CP)	60	60
Lucerne grain 15	73.1	70
Cereal straw	66	66
Mixed fat	30	-
Optomega 50[2]	-	60
Calcium carbonate	15	17.4
Sodium chloride	4	4
Lysine 500 (g)	2	2.3
Organic acids	1	0.7
Choline clorhide	0.2	0.2
Min-vitpremix[3]	3	3
Antioxidants[4]	4	0.1
Zinc bacitracin premix[5]	2	2
Sepiolite	20	-

[1]Only one batch of each diet was used during the study. Samples of both diets were collected weekly and composited for further analysis. [2]Optivite International Ltd. (Barcelona, Spain). Contained salmon fish oil, 100%; ether extract, 50%; n-6, 8%; n-3, 35%; CP, 4%, ME, 5254 kcal/kg;and Vitamin E, 2500 mg/kg. [3]Mineral and vitamin premix supplied per kg of complete diet: Vitamin A 9999.9 IU; Vitamin D 1080 IU; Vitamin E, 200 mg/kg; vitamin K3: 1.7 mg; Tiamine:1.7 mg; Riboflavin: 4.3 mg; Pantithenic acid: 13.6 mg; Pyrodoxine: 1.7 mg; Mn:22.7 mg; Co:595 µg; Se: 140 µg; I: 1.2 mg. [4]Supplied per kg of diet: [E320 Butilhidroxianisol (BHA)+E324 Etoxiquina+E321 Butilhidroxitolueno (BHT) 30000 mg; E562 sepiolite 910000 mg] (Trow Nutrition Spain SA, Madrid, Spain); Luctanox 3000 mg (Lucta, Barcelona, Spain). [5]Contained 100 mg Zinc-bacitracin/kg (Andrés Pintaluba, S.A., Reus, Spain).

Table 5.2. Chemical composition of a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 60 g/kg (g/kg, as-fed basis unless otherwise indicated)

	Diet[1]	
Item	Control	Enriched
Chemical composition analysed		
Gross Energy(MJ/kg)	16.8	16.7
Dry matter	899	900
Ash	87	86
Crude protein	16.3	16.6
Ether extract	56.4	55.9
aNDFom[2]	344	372
ADFom[3]	145	154
ADL[4]	32.3	28.9
Chemical composition calculated		
Digestible Energy (MJ/kg)	9.6	9.5
Lysine	6.7	6.8
Methionine	2.9	2.9
Methionine + Cystine	5.7	5.7
Threonine	5.5	5.4
Tryptophan	2.0	1.9
Isoleucine	5.6	5.6

[1] Only one batch of each diet was used during the study. Samples of both diets were collected weekly and composited for the further analysis. [2]nNDFom = amylase neutral detergent fiber organic matter basis. [3]ADF = acid detergent fiber organic matter basis. [4]ADL = acid detergent lignin.

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Table 5.3. Fatty acid (FA) composition (g/kg total FA methyl esters) of a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 60 g/kg

	Diet[1]	
Item	Control	Enriched
Total SFA[2]	369.5	211.7
C12:0	39.1	40.6
C14:0	52.1	42.0
C16:0	197.7	100.3
C18:0	79.2	26.2
Total MUFA[3]	319.8	210.4
C16:1n-7	14.2	17.7
C18:1n-9	288.7	176.3
C20:1n-9	5.6	11.8
Total PUFA[4]	310.6	577.8
C18:2n-6	277.9	289.1
C18:3n-3	30.3	35.2
C18:4n-3	1.6	5.3
C20:5n-3	0.0	75.1
C22:5n-3	0.0	32.2
C22:6n-3	0.0	139.5
n-9	304.3	189.5
n-6	278.0	289.5
n-3	31.9	287.7
n-6/n-3 ratio	8.72	1.01
IU[5]	105	143

[1] Only one batch of each diet was used during the study. Samples of both diets were collected weekly and composited for further analysis. [2]SFA = Saturated fatty acids. [3]MUFA = Monounsaturated fatty acids. [4]PUFA = Polyunsaturated fatty acids. [5]UI = Unsaturation index

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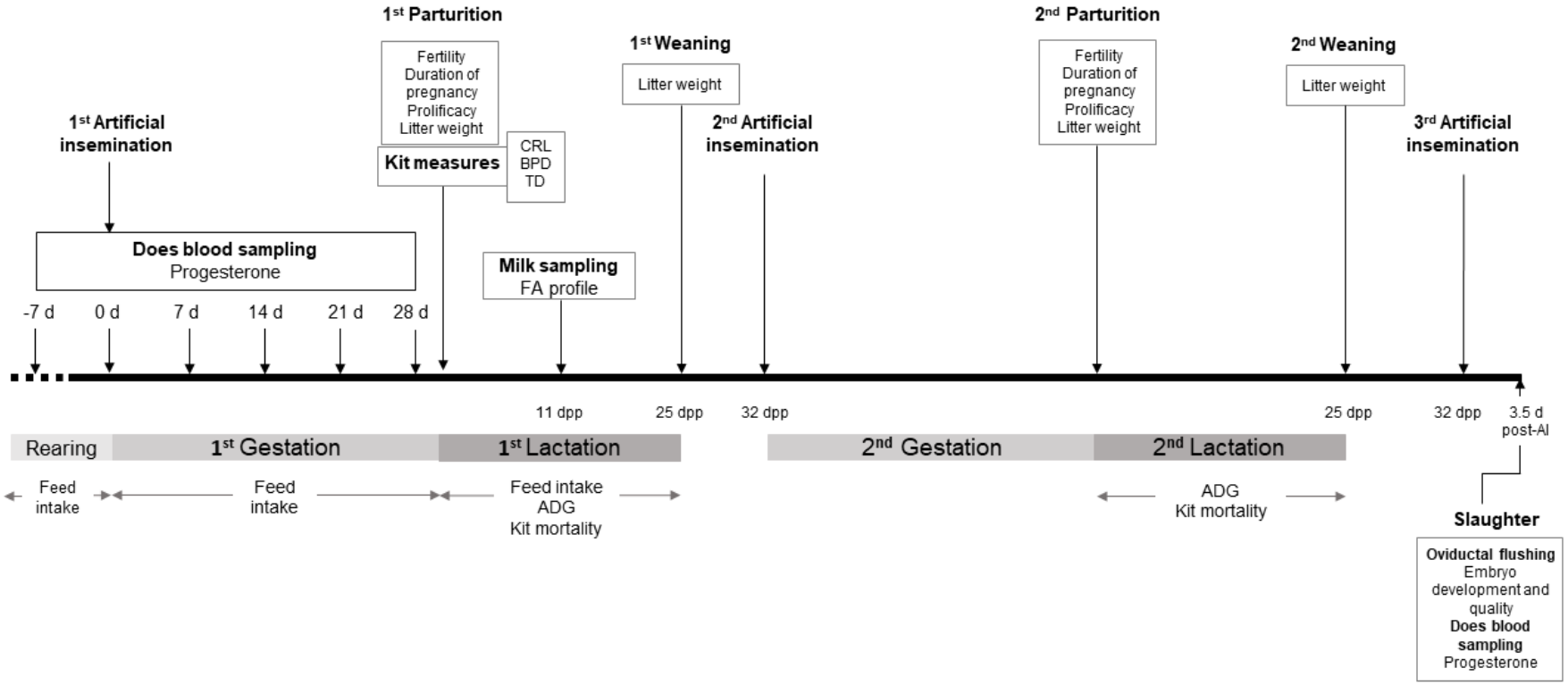


Figure 5.1. Sampling timeline of different variables in mothers and their offspring. CRL = crown-rump length; BPD = biparietal diameter; TD = thoracic diameter; FA = fatty acids; dpp = days postpartum; ADG = average daily gain; AI = artificial insemination

2.4. Plasma progesterone determination

A random subsample of 24 pregnant females (12 control and 12 enriched does) were taken to assess plasma progesterone levels during their first pregnancy. Considering that time 0 was the AI moment, blood samples at Days -7, 0, 7, 14 and 28 of gestation were taken from the marginal ear vein (2.5 mL) between 09:00 h and 10:00 h, avoiding circadian rhythm, by collecting samples in tubes containing EDTA. Plasma was obtained after centrifugation at 1 200 x g for 10 min at 4ºC and stored at −20ºC until further analysed.

Plasma progesterone concentrations were analysed using a commercial kit (Progesterone ELISA, Demeditec Diagnostics GmbH, Kiel, Germany) based on the principle of competitive binding. Previously, plasma samples were extracted with petroleum ether at a 5:1 (vol/vol) ether:sample ratio (extraction efficiency was 85%). Sensitivity was 0.045 ng/mL. The intra- and inter-assay coefficients of variation were 5.5% and 6.9%, respectively. Absorbance was measured in a Bio-Tek automatic plate reader (EpochTM Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, VT, USA) at 450 and 630 nm, and hormone concentrations calculated by means of a software developed for these techniques (Gen5TM ELISA, Bio-Tek Instruments, Winooski, VT, USA)

2.5. Milk sampling and fatty acid composition

During first lactation (at 11 dpp), a random subsample of 10 animals (five control and five enriched does), were used to collect milk. To stimulate the secretion of milk, does were injected intravenously 5 IU of oxytocin (IVEN Laboratories, Madrid, Spain) in the marginal ear vein. We proceeded to shave the chest and abdominal area leaving visible the nipples. Milk was collected in empty clean glass tubes and frozen until further analysed (Image 5.1).

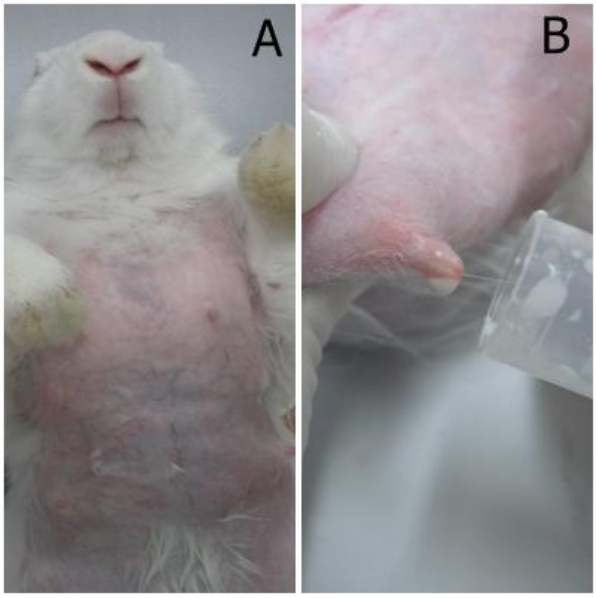


Image 5.1. (A) Rabbit doe with the chest and abdominal area shaved leaving visible the nipples. (B) Sampling milk.

Milk lipids extraction was performed in triplicate as proposed by Segura and López-Bote (2014). The lipid content was determined gravimetrically. Lipids obtained were methylated following the procedure of Sandler and Karo (1992) and FA were identified and quantified by gas chromatography according to conditions previously described. The UI was calculated with the above mentioned formula.

2.6. Embryo development and quality

At the end of trial, a random subsample of 28 multiparous rabbit does with two previous cycles (14 control and 14 enriched does) were chosen. They were inseminated under the same condition above-mentioned. Three days and a half after AI, a punctual blood sample was obtained to determine progesterone concentration of each doe and was stored until analysis as previously mentioned. Immediately, animals were euthanized with an overdose of barbiturate (30 mg/kg of Pentothal; Dolethal, Lab, Vetoquinol, Madrid, Spain). The ovaries and reproductive tract were collected in PBS at 37ºC. Ovulation rate was determined as the percentage of females with corpora lutea respect to the number of females inseminated, and the number of corpora lutea per female was counted. Subsequently, embryos were recovered by flushing the reproductive tract from the infundibulum to uterus with PBS + 0.1% BSA at 37ºC (Image 5.2). The flushing fluid

was deposited in a Petri dish on a heated plate (Minitub, Tiefenbach, Germany) at 37ºC to proceed with the evaluation of the embryos with a stereoscopic microscope (Nikon SMZ-800, Tokyo, Japan).

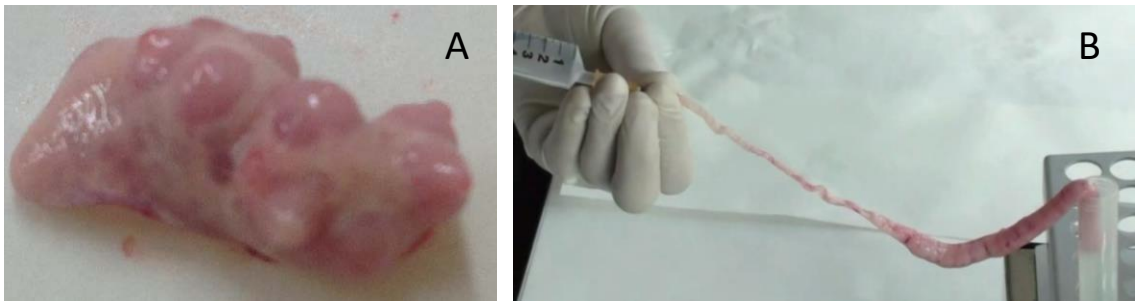


Image 5.2. (A) Ovulated ovary and (B) flushing of the reproductive tract from the infundibulum to uterus.

Embryo recovery rate was calculated as the percentage of embryos recovered respect to the number of corpora lutea counted on ovarian surface. At the time of collection, the different embryonic stages observed were defined on the basis of according to the guidelines of the International Embryo Technology Society and were classified into the following categories: morula, blastocyst and retarded embryos (embryos with delayed development, e.g.: oocyte, 2-cell, 4-cell...). The percentage of these categories was given over total of recovered structures.

Embryo quality was determined analysing the apoptosis rate. A total of 37 embryos (n = 19 for control group and n = 18 for enriched group) were washed in PBS supplemented with 1 mg/mL PVP, fixed in 4% paraformaldehyde solution for 30 min at room temperature, washed and stored in PBS at 4ºC until their use. Protocol was adapted from that previously described by Arias-Álvarez *et al*. (2009). First, embryos were permeabilized by incubation in 0.5% Triton X-100 in PBS containing 1 mg/mL BSA during 1 h and 20 min in humidified chamber, in darker conditions at room temperature. Strand breaks of DNA were detected using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling kit (TUNEL; In Situ Cell Death Detection Kit, POD, Roche Diagnostics S.L., Applied Science, Barcelona, Spain). The positive control sections were treated with DNAse I (Roche Diagnostics S.L., Applied Science) for 1 h at 37ºC, before incubation with the TUNEL reagent. For negative controls, samples were incubated with the label solution of the TUNEL reaction mixture without the enzymatic solution. Embryos were treated

with RNAses before staining. Finally, embryos were counterstained with 10 µg/mL Bisbenzamide (Hoechst 33342, Sigma, Madrid, Spain) and fixed on glass slides in mounting solution (ProLong Gold antifade reagent, Invitrogen, OR, USA). Slides were examined under confocal microscope (Leica TCS SP2, Wetzlar, Germany) using a 488 nm excitation laser to visualize TUNEL-positive cells and 460 nm excitation laser to assess the blue fluorescence. The format, laser, gain and offset were kept constant for all samples. The images were analysed using Image J software (free downloaded from NIH website: http://rsbweb.nih.gov/ij/). The apoptosis rate was calculated as the relation between the number of green cells over total cells x100.

2.7. Statistical analysis

Statistical analysis was performed with SAS software (SAS Inst. Inc., Cary, NC, USA). The experimental unit was the rabbit doe. The daily feed intake, body size of kits, FA profile of milk, as well as the determinations of punctual progesterone, embryo morphology, ovulation and apoptosis rates were analysed as a completely randomized design with feeding regime as the main source of variation by using the GLM procedure. Progesterone concentrations during first pregnancy were analysed by repeated measure analysis using the MIXED procedure considering the diet as the main effect. The effect of dietary supplementation and cycle (1st and 2nd AI) on fertility rate was analysed by means a χ^2 test (proc CATMOD). Prolificacy, litter weight, ADG and mortality data were also analysed by repeated measures (proc MIXED) to study the effect of diet, cycle and their interaction. All means were compared using a protected *t*-test. Differences were considered significant at $P < 0.05$ and a trend when $P < 0.07$. Results are presented as least squared mean (lsmeans).

3. RESULTS

3.1. Productive trial

The ADFI of rabbit does was similar between experimental groups in all periods (240 ± 3.9 g/d) increasing during lactation respect to rearing and pregnancy (351 ± 8.99 *vs.* 185 ± 7.69 and 183 ± 4.78 g/d respectively; $P < 0.05$).

Productive variables of does and viability of their litters during the first two production cycles were unaffected by diet (Table 5.4), except for the individuals measurements of kits born alive, where the CRL, BPD and TD were higher in kits from mothers fed enriched diet than in control ones ($P < 0.05$).

On the other hand, AI order had influence in the reproductive outcome. We found that in the second cycle, fertility rate was lower ($P < 0.05$) compared with the first one in both experimental groups. Nonetheless, the number of born alive kits, weaned kits, litter weight at parturition and at weaning, as well as kits mortality and ADG during the second lactation were greater ($P < 0.05$) compared to the first cycle. However, pregnancy duration and the number of stillborn were similar in both productive cycles ($P > 0.05$). There was a trend in the interaction between dietary treatments and parturition order on fertility rate ($P = 0.067$; Figure 5.2). Control does at second AI had a lower fertility value comparing to the first one ($P < 0.05$), while n-3 PUFA supplemented does did not experiment this decrease. There was a significant interaction on the ADG during lactation period (Figure 5.3A; $P < 0.05$), and on litter weight at weaning time (Figure 5.3B; $P < 0.0001$). The ADG increased in the control group during the second lactation, whilst the enriched group kept the same values in both cycles (slightly higher than control group during the first lactation, but lower during the second). The litter weight increased in the two groups in the second cycle at weaning, although the enriched group did not augment so much as control group, keeping in intermediate values.

Table 5.4. Productive variables of rabbit does fed a control and an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 60 g/kg and artificially inseminated (AI) either at 16 weeks of age (AI 1) or at 32 days postpartum (AI 2). All values are least squares means

	Diet		AI order			*P*-value		
	Control	Enriched	1	2	SE	Diet	AI	Diet x AI
No. of does	114	118	127	105				
Fertility (%)	79.4	86.9	89.7	76.6	3.688	0.1179	0.0090	0.0669
Pregnancy (days)	30.7	30.8	30.8	30.8	0.059	0.6491	0.9429	0.1167
Parturition								
No. births	87	89	111	65				
Born alive (n)	10.8	10.4	9.75	11.4	0.234	0.4727	0.0001	0.8761
Stillborn (n)	0.49	0.42	0.33	0.57	0.106	0.6710	0.1594	0.2693
Litter weight (g)	576	582	499	658	8.898	0.7307	0.0001	0.0734
Individual measurements								
No. of does[1]	10	10						
CRL[2]	95.4	101	-	-	0.891	0.0001	-	-
BPD[3]	18.3	19.6	-	-	0.184	0.0001	-	-
TD[4]	16.1	19.0	-	-	0.372	0.0001	-	-
Lactation[5]								
Weaned (n)	9.68	9.04	8.93	9.79	0.197	0.0954	0.0129	0.3842
Litter weight (g)	6068	5825	5499	6394	99.70	0.0960	0.0001	0.0001
Mortality (%)	8.14	11.2	6.70	12.6	1.430	0.2168	0.0082	0.1422
ADG (g/d)[6]	18.8	18.7	18.0	19.5	0.345	0.8911	0.0079	0.0489

[1] Does with litters of 10-11 kits. [2] CRL = crown-rump length [3] BPD = biparietal diameter [4] TD = thoracic diameter [5] Lactation at 25 day postpartum [6] ADG = average daily gain

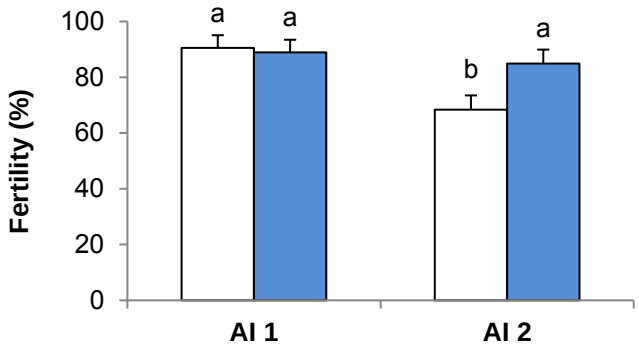


Figure 5.2. Fertility of rabbit does fed a control diet and an enriched diet with a supplement based on n-3 PUFA (Enriched), at first (AI1) and second (AI2) artificial insemination. Each bar represents the least squares means from 63, 51, 64 and 54 rabbit does from left to right; a, b: significant differences of interaction between diet and AI (P< 0.05); (white bars = control group, blue bars = enriched group).

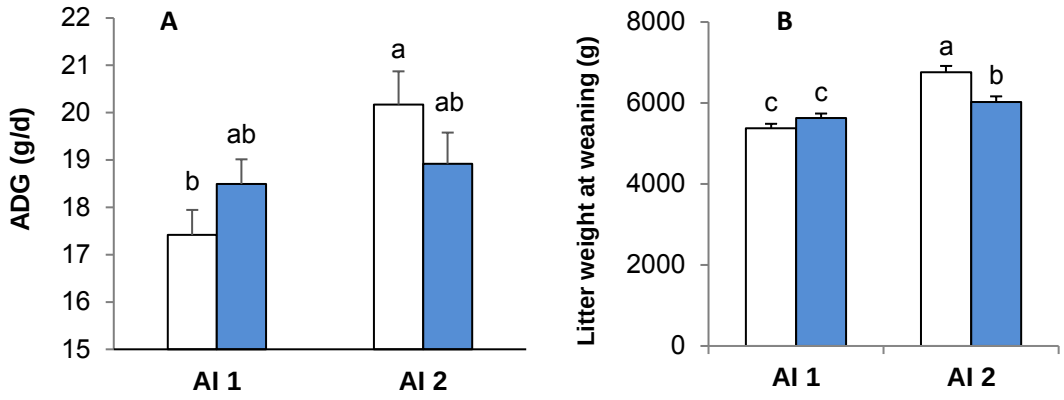


Figure 5.3. (A) Average daily gain (ADG) during lactation and (B) litter weight at weaning of kits from rabbit does fed a control diet and an enriched diet with a supplement based on n-3 PUFA, at first (AI1) and second (AI2) artificial insemination. Each bar represents the least squares means from 53, 54, 30 and 35 litters from left to right;a, b, c: significant differences of interaction between diet and AI (P< 0.05); (white bars = control, blue bars = enriched).

3.2. Plasma progesterone concentration during pregnancy

During the first pregnancy, plasma progesterone concentrations (Figure 5.4) increased at 7 (P < 0.05) and 14 days (P < 0.05) after AI and decreased without reaching baseline levels on Day 21 and 28 of gestation in both experimental groups.

Rabbit does fed with enriched diet had greater progesterone concentrations than those fed with control diet on Day 7 and 14 ($P < 0.05$) after AI.

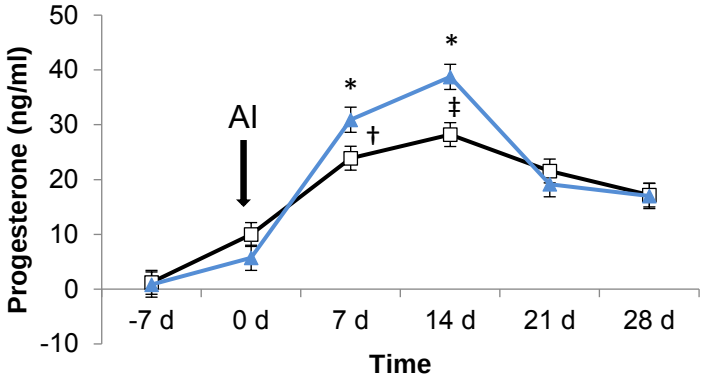


Figure 5.4. Plasma progesterone concentrations in pregnant rabbit does fed a control diet (□; n=12) and an enriched diet with a supplement based on n-3 PUFA (▲; n=12) at -7, 0, 7, 14, 21 and 28 days of gestation respectively, being day 0 when artificial insemination (AI) was performed. All values are least squares means; * Time effect at 7 and 14 days (P< 0.05); Diet effect at 7 d (†: $P = 0.029$) and at 14 d (‡: $P = 0.001$).

3.3. Fatty acid composition of milk

Milk FA profile is shown in Table 5.5. The diet consumed had a significant effect on the FA analysed ($P < 0.05$), except for the palmitoleic acid (C16:1n-7) concentration that was similar between diets. Milk analysis showed 16.76% and 13.71% more of total SFA and MUFA in the control group than in the enriched group ($P < 0.05$), respectively. Moreover, milk of supplemented does had 43.53% more total PUFA than milk of control ones ($P < 0.05$). This resulted in greater milk UI in that group compared to the control one ($P < 0.05$)

Table 5.5. Fatty acids profile (g/100 g total FA methyl esters) of rabbit milk does of females fed control diet or an enriched diet with a supplement based on n-3 PUFA (Enriched) in a level of inclusion of 60 g/kg

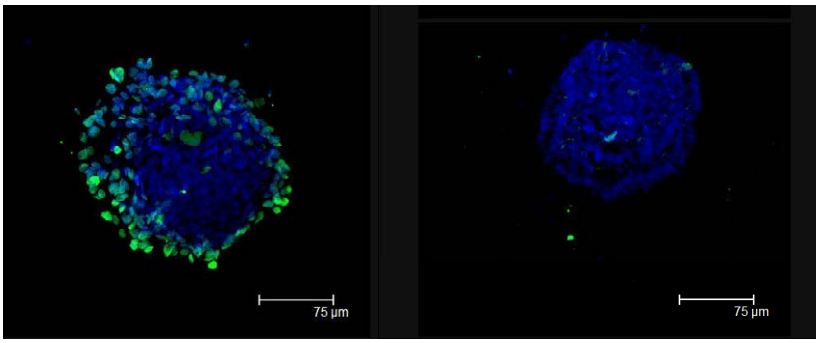
	Diet			
Item	Control	Enriched	SD	*P*-value
N	5	5		
Total SFA[1]	40.4	34.6	0.3	0.0001
C12:0	2.96	3.99	0.21	0.0081
C14:0	3.15	3.52	0.09	0.0178
C16:0	25.1	20.8	0.41	0.0001
C18:0	7.56	4.55	0.17	0.0001
Total MUFA[2]	36.5	32.1	0.3	0.0001
C16:1n-7	0.31	0.22	0.02	0.3448
C18:1n-9	30.1	24.8	0.26	0.0001
C20:1n-9	0.38	1.37	0.02	0.0001
Total PUFA[3]	23.2	33.3	0.02	0.0001
C18:2n-6	20.4	24.3	0.19	0.0001
C18:3n-3	1.56	2.78	0.02	0.0001
C18:4n-3	0.12	0.34	0.00	0.0001
C20:5n-3	0.00	1.25	0.02	0.0001
C22:5n-3	0.09	1.17	0.02	0.0001
C22:6n-3	0.05	2.24	0.05	0.0001
n-9	30.5	25.2	0.26	0.0001
n-6	20.9	24.8	0.20	0.0001
n-3	1.81	6.64	0.08	0.0001
n-6/n-3 ratio	11.6	3.74	0.14	0.0001
IU[4]	0.86	1.20	0.00	0.0001

[1]SFA = Saturated fatty acids. [2]MUFA = Monounsaturated fatty acids. [3]PUFA = Polyunsaturated fatty acids. [4]UI = Unsaturation index

3.4. Embryo development and quality

No differences in plasma progesterone concentration (mean 3.4 ± 0.31 ng/mL; P > 0.05) between experimental groups were observed at 3.5 days after AI. The ovulation rate (100%; P > 0.05) with a mean of 12.5 ± 1.24 corpora lutea/doe and embryo recovery rate (mean 69.9 ± 9.04%; P > 0.05) were also similar between groups.

Similar percentage of morula (mean 75.8 ± 10.69%), blastocyst (mean 10.65 ± 6.32%) and retarded embryos (mean 13.6 ± 10.28%) were observed (P > 0.05) among the experimental groups. Apoptosis rate was almost twice higher in control group than in supplemented one, being 31.1 ± 4.56 and 17.1 ± 3.87%, respectively (P < 0.05) (Figure 5.7).



Control Enriched

Image.5.3. Embryo quality assessed by apoptosis rate in early embryos (3.5 days post artificial insemination) from rabbit does fed a control and an enriched diet with a supplement based on n-3 PUFA. Green immunofluorescence shows apoptotic cells and blue denotes nucleus cell.

4. DISCUSSION

According to the present data, the long-term use of an enriched diet with 60 g/kg of a supplement based on n-3 PUFA from rearing and during two reproductive cycles maintains improved reproductive and productive outcomes, also confirming the endocrine features enhancement that were shown by Rebollar *et al*. (2014) and in the chapter 3 of the present Thesis.

In the current study, there were no differences in feed intake due to the diet. This result could be attributed to several reasons: 1) the long-term administration of the supplement and the adaptation of animals in their different physiological status and 2) the appropriated recommendations in the ingredients and diets formulation avoiding problems of palatability in them. By contrast, noticeable differences between physiological periods were highlighted and as we expected, feed intake of both groups during lactation was higher than during rearing and gestation due to high energy expenditure existent in these periods (Xiccato *et al*., 2004).

At first AI high fertility was observed in both groups, difficult to improve with the enriched diet due to an adequate corporal condition and a proper live BW of does at this time (Rebollar *et al*., 2009). By contrast, at the second AI, after the first lactation and weaning, fertility of control females significantly decreased. However, n-3 PUFA enriched diet maintained the high fertility of the first cycle. It could be related to a possible positive effect of a high plasma estradiol concentrations observed at the end of lactation period and before the second AI in supplemented does described in the chapter 3 of the present work.

For the productive parameters, in accordance to the similar basal chemical composition and digestible energy content of the two diets, there was no effect on litter size and weight at birth. The similar litter size at birth obtained in both experimental groups determined a similar duration of pregnancy because the available space of foetuses in uterus is one of the most important condition that define the pregnancy length (Manchisi *et al*., 1991). Nonetheless, although there were no differences in the weight of neonates, PUFA supplementation had a favourable effect on the individual kits body size. That was evident the day of parturition, probably due to the higher plasma progesterone concentration when placenta formation occurs (Khan *et al*., 2012). The significant increment of size observed in the current study is important from a practical point of view because it

has been described that the size of the newborn is directly related to their vitality around the first days of life (Bautista *et al*., 2008), when the location of nipples to survival is very important.

The lower weight of kits from enriched mothers at 32 dpp could be due to diets rich in PUFA use to generate smaller abdominal fat deposits than diets rich in MUFA and SFA as it was described in chicken (Crespo and Esteve-García, 2002). In humans, Rosqvist *et al*. (2014) also observed that fat deposits in the liver and visceral fat were lower with rich PUFA diets. Furthermore, some rabbit researchers concluded that with PUFA diets less carcass fat (Kowalska and Bielánski, 2009) or abdominal and perirenal fat, as in the chapter 4 of the present Thesis, is obtained. All these findings would support the low weaning weight of kits of enriched does. Nonetheless, this little weight difference could be compensated with a healthier lipid profile in both the muscle and the fat of the carcass, as it is described in the chapter 4 of the current Thesis.

Other positive result obtained in the present study was that pregnant animals fed enriched diet showed increased plasma progesterone concentrations on day 7 and 14 of pregnancy. In this period, embryo implantation and, immediately, placenta formation occur (Khan *et al*., 2012), therefore, the higher the progesterone concentrations, the better implantation and placentation process would take place, with an improved survival post-implantation of foetuses (Froment *et al*., 2006). Similar progesterone results have been obtained in previous work with lower levels of inclusion of the same supplement (Rebollar *et al*., 2014) on day 7 of pregnancy. Regarding hormonal response, MacLaren *et al*. (2006) suggested that the possible beneficial effects of n-3 PUFA on progesterone production could be due to the activation of the nuclear family of PPAR in luteinized cells. According to Zerani *et al*. (2013), PPAR have the function of preserving the rabbit corpora lutea benefiting the fertility found at second AI in enriched group in present work.

Focusing on the milk composition, Lin *et al*. (1991) observed that in rabbits, PUFA represented 34% of total FA, almost all of which were LA and ALA, with only some traces of DHA, likewise to the current study. Moreover, using fish oil to enrich the diet, these authors observed a higher total level of PUFA in the milk, principally by increasing the proportion of long-chain n-3, where DHA represented 3.8% of total FA. We observed that milk of the enriched group had a higher total PUFA concentration, UI and DHA concentrations than the control one. A mechanism

through which n-3 PUFA could influence kits survival is by improving their immune system. The IgG in colostrum is the main source of antibodies that boosts the passive immune system of neonatal pigs, and colostral IgG concentrations were greater in sows fed a n-3 PUFA rich diet (Mateo *et al*., 2009) and FA influenced the expression of immune related genes (Kitajka *et al*., 2004). In this regard, Maertens *et al*. (2005) observed that in a farm affected by epizootic rabbit enteropathy, animals weaned from does fed with a n-3 PUFA diet and that continued consuming the same diet after weaning, reduced their mortality with respect to a control diet. The lack of differences in terms of mortality between groups that could evidence the beneficial effect of n-3 PUFA in the current work could be due to the trials were carried out in an experimental farm under optimal controlled ambient and sanitary conditions, and consequently, very low mortality values throughout the entire experience were found.

Aligned with Rebollar *et al*. (2014), there were no differences between diets in plasma progesterone concentration at 3.5 days after AI. This is probably because in early stages of gestation corpora lutea produce scant but still enough amount of progesterone to sustain early embryo events which also could explain the similar embryo development observed in the current study. On the other hand, embryo rate was similar to that usually obtained on 3.5 days after AI as previously described (Arias-Álvarez *et al*., 2010). In this regard, in cows, Fouladi-Nashta *et al*. (2009) confirmed that despite altering proportions of major FA in plasma and milk, dietary PUFA supplementation had little effect on FA composition of granulosa cells, and consequently, there was no effect of diet on follicle numbers and post-fertilization development of oocytes in vitro.

The current study reports that embryo quality in terms of apoptosis rate was better for enriched group. Previous research showed that adding ALA to bovine and goat oocytes maturation media resulted in better-quality blastocysts in terms of apoptosis rate (Marei *et al*., 2009 and Veshkini *et al*., 2016, respectively).

In conclusion, our data suggest that the use of a supplement based on n-3 PUFA in a higher level of inclusion than previously employed in this PhD Thesis: (1) did not influence feed intake, (2) maintained fertility rate on critical second insemination and improved size of kits at parturition even though the lower ADG observed during lactation period was translated in lower litter weight at weaning time, (3) increased plasma progesterone concentrations on days 7 and 14 of

gestation, (4) modified the milk FA profile and (5) did not improve development of early embryos but their quality, in terms of apoptosis rate, was enhanced.

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSIÓN

In the last few decades, several studies that go into detail about the effect of the dietary supplementation with n-3 FA in rabbits have been done. Many of them have been made with vegetal sources such as seeds or vegetal oils (Maertens *et al.,* 2005; Schmitz and Ecker, 2008; Mordenti *et al.,* 2010; Delgado, 2017). Supplements of animal origin have also been used (Castellini *et al.,* 2004; Tres *et al.,* 2014), nonetheless, the direct inclusion of particular FA, such EPA and DHA, supposes a more efficient supplementation since the necessary elongation and desaturation reactions for its synthesis from its precursors (LA and ALA) are avoided (Wathes *et al.,* 2007). In this regard, Rebollar *et al.* (2014) already used a supplement composed mainly by EPA and DHA, focusing principally in reproductive and productive responses of rabbit does. To the best of our knowledge, this is the first time that a dietary direct inclusion of EPA and DHA is used to study the effects, not only on productive or carcass parameters, but also on metabolic parameters and lipid profile of does and their offspring as well as intestinal health or meat quality.

As it has been commented, all experimental diets were formulated following the recommendations for breeding does described by De Blas and Mateos (2010). It is important to notice that the manufacturing process was appropriate and both experimental diets used in each specific trial of this Thesis had the same chemical composition analysed. In this sense, the enriched diets used in chapters 3 and 4 of the present work had a 25% more of total PUFA, and the n-6/n-3 PUFA ratio was more than 3-fold lower than control ones. In addition, after the increase the level of inclusion, the experimental diet used in chapter 5 of the current Thesis had a 86% more of total PUFA that was translated in a n-6/n-3 PUFA ratio 8-fold lower than in the control diet.

Feed intake and cecal fermentation responses

As previously described by Rebollar *et al.* (2014) using the same supplement than in the current study, the apparent digestibility coefficients of DM, NDF, CP, ether extract and digestible energy content of diets were not affected. As it is known, all animals eat to satisfy their energy needs (Xiccato and Trocino, 2010), therefore, the same apparent digestibility coefficient of digestible content entailed the same feed intake by control and enriched rabbit does of the present study. In the chapter

3 and 5 of the present Thesis, the two experimental diets covered maternal nutritional requirements during all productive phases studied (rearing, pregnancy and lactation) because feed intake was similar to those described in these animals under analogous conditions (Rebollar *et al.,* 2011) and there was not differences among dietary treatments. These results of consumption gave rise to similar milk production of rabbit does and consequently, the ADG of the offspring during lactation was similar when 15 g/kg of the suppelement was included.

Lastly, during growing period, ADFI, ADG and G:F of young rabbits studied in the chapter 4 of this Thesis work were similar between groups, in agreement with Kowalska and Bielanski (2009), who reported no effects after dietary fish oil supplementation on growth performance and feed conversion efficiency of rabbits. Even though, lower growth performance has been observed after vegetal supplementation (linseed or linseed oil) in diet (Bianchi *et al.,* 2009; Casado *et al.,* 2013), recent works of Delgado (2017) observed improves in the growth rate with no effect on feed intake, which accounted for the increase of the feed efficiency. These results has been corroborated in recent works using the same fish oil supplement that in the current Thesis with a level of inclusion of 60 g/kg, obtaining improves in growth rate and feed efficiency (Rodríguez *et al.,* 2017). This variability of the results after dietary n-3 PUFA enrichment could be due to the specific FA composition of the different supplements and the level of fat inclusion in the diet.

Furthermore of the performance during growing period, it has been reported a significant relation between an enriched diet with medium-chain FA (caprilyc and capric) and a possible alteration of intestinal microbiota, affecting cecal fermentation (Marounek *et al.,* 2002) and decreasing the development of pathogenic strains (Skřivanová *et al.,* 2009). A previous study carried out in mice also concluded that a rich dietary source of n-3 PUFA (flaxseed/fish oil) may have a bifidogenic effect on the intestinal microbiota composition of the host by increasing the levels of *Bifidobacterium* (Patterson *et al.,* 2014). Our study was carried out with a LCPUFA supplement and no major effects were found in the most of the items studied regarding cecal fermentation. Only the concentration of total cecal VFA was greater in the enriched rabbits compared with control ones, even though the reason of this result is unclear. Cecal VFA concentrations do not necessarily reflect changes in VFA production if there are concomitant changes in VFA absorption or digest passage rate.

A reduction of the villous/crypt height ratio may indicate an impairment of gut barrier function (Gómez-Conde *et al*., 2007). In this sense, our study about the ileal morphology in young rabbits supplemented or not during the growing period did not show differences between them; both values of crypt depth as well as values of villi length were found within those observed in a normal development of the ileal mucosa in this species (Gallois *et al.,* 2005; Gómez-Conde *et al.,* 2007). Maertens *et al.* (2005) observed that in an experimental farm naturally affected by epizootic rabbit enteropathy, animals weaned from does fed with a n-3 rich diet and fed with the same diet after weaning (n-6/n-3 ratio = 1.0, using extruded linseed), reduced their mortality with respect to a control diet. According some studies developed in human, the anti-inflammatory benefits of n-3 PUFA on gut microbiome composition may be attributed to the products of DHA metabolism (Serhan *et al.,* 2011), which are mediator substances used in experimental models of inflammatory bowel diseases with protective effects (Schwanke *et al.,* 2016). However, supported by recent works in human, the composition of the microbiota is also influenced by multiple environmental factors such as diet, antibiotic therapy, and environmental exposure to varied microorganisms (Vernocchi *et al.,* 2016). In this Thesis work the n-3 PUFA source of the diet had animal origin and n-6/n-3 ratio was 2.20, nevertheless, the lack of improvements in gastrointestinal health of growing rabbits could be due to the fact that the trials were carried out in an experimental farm under optimal controlled ambient and sanitary conditions, and consequently, neither morbidity nor mortality throughout the entire experience were found.

Consequently, from a digestive point of view, enriched diets seem to be healthy, there was no effect on palatability and their digestion process was suitable.

Steroidogenesis

Regarding hypothalamus-hypophysis-ovarian response, and aligned with Rebollar *et al*. (2014) the PUFA enrichment of diet did not suppose differences in plasma progesterone concentration at 3.5 days after AI, as it has been corroborated in the chapter 5 of the current Thesis. This is probably because in early stages of gestation corpora lutea produce scant but still enough amount of progesterone just to sustain early embryo events which also could explain the similar embryo development observed in the chapter 5 of the current study. As gestation progress a positive plasma progesterone response was observed on days 7 and 14 after

artificial insemination, corresponding with the embryo post-implantational period. As it is known, to inhibiting the proliferative effect of oestrogen on uterine epithelial cells, progesterone facilitates uterine receptivity by inducing stromal cell proliferation, decidual growth and the expression of adhesion molecules via its genomic actions (Gellersen *et al.*, 2009), allowing the embryo to implant. Progesterone acts as a negative regulator of trophoblast invasiveness and suppresses the deleterious maternal immune responses. Via these effects, progesterone is a key to the establishment and maintenance of pregnancy. Any disturbances in its actions may contribute to pathological pregnancies, e.g. early foetal loss, habitual abortions or preterm delivery. MacLaren *et al*. (2006) suggested that the possible beneficial effects of n-3 LCPUFA on progesterone production could be due to the activation of the nuclear family of PPAR in luteinized cells. According to research carried out by Zerani *et al.* (2013) in rabbit does, PPAR has the function of preserving the corpora lutea and the progesterone secretion benefiting the gestation. Another hypothesis could be the lower synthesis of 2-series PG, including $PGF_{2\alpha}$, when the enzyme system PGTS use the phospholipids from cell membranes rich on DHA and EPA. A reduction of this luteolytic hormone could improve the health of corpora lutea, their progesterone production and embryo viability. In cows, Elis *et al.* (2016) have observed that feeding with a n-3 PUFA diet (fish oil encapsulated) tended to decrease the non-fertilization and/or early embryo mortality rate. However, in our study, this hypothesis is not so accurate later, because at the end of pregnancy (Day 28), a similar level of this hormone was detected in plasma of the enriched group compared with control one. Nonetheless, it had no effect on pregnancy length, supporting previous data in sows (Tanghe *at al.,* 2013).

Polyunsaturated FA and eicosanoids modulate steroid synthesis in a variety of different steroidogenic tissues such the regulation of adrenal steroidogenesis (Sarel and Widmaier, 1995; Matthys and Widmaier, 1998). In relation to the action of PUFA on the synthesis of sexual steroids as oestradiol, previous works have suggested that AA and its metabolites have long been implicated by means their direct effects on the Steroid Acute Regulator (**STAR**) protein, or by the indirect effects via PG (Stocco *et al*., 2005). Specific inhibition of prostaglandin-endoperoxide synthase 2 by n-3 LCPUFA, as mentioned before, has been also associated with an increased STAR expression and steroid output (Fiedler *et al.,* 1999; Wang *et al.,* 2003). This indirect mechanism mediated by n-3 LCPUFA could

explain the higher oestradiol values observed on day 32 of lactation in enriched rabbits that has been showed in the chapter 3 of the present Thesis work.

In summary, n-3 LCPUFA could improve embryo implantation via progesterone and sexual receptivity at the end of lactation via oestradiol, explaining the better results of fertility in the second cycle that has also been observed in the chapter 5 of this PhD Thesis.

Reproductive/productive response

Foetal metabolism, and consequently foetal growth, directly depends on the nutrients crossing the placenta, and therefore, the mother adapts her metabolism in order to support this continuous draining of substrate. Considering the foetal development and the maternal metabolism, the gestation could be divided into two stages. In early pregnancy, the embryo is practically microscopic and although its energy demands are very low, this moment is a critical time since the formation and development of the cell mass, which later will become the foetus and foetal structures take place. In this regard, better embryo quality, in terms of apoptosis rate, was observed in the present Thesis confirming recent *in vitro* studies carried out in goat by Veshkini *et al.* (2016) after PUFA addition to oocytes maturation media. Nonetheless, although n-3 LCPUFA supplementation implied better embryo quality and an increase in progesterone around the implantation period, no differences were observed in the number or size of embryonic vesicles at day 9, as well as in foetal determinations at day 16 of gestation as it is shown in the chapter 3 of the current study. In relation to the mother, these moments are a preparation period to support the late-pregnancy and can be considered as an anabolic state in the mother (King, 2000). Although we did not observe differences in the ultrasound scans test, we can assume that PUFA supplementation has a positive effect on the management of energy reserves during gestation periods. In recent works (Rodríguez *et al.,* 2016) using the same supplement with the same level of inclusion that in the chapter 5 of the present Thesis (60 g/kg) we have observed some improves in fetoplacental development, obtaining larger foetuses (in terms of CRL, ONL, BPD, and TD) at the end of pregnancy (Day 28). In the current PhD Thesis, the management of energy reserves during gestation periods was evident because a greater availability of the energy deposits and, consequently, a greater foetal growth at the end of pregnancy was observed giving rise to larger kits and

corroborating previous results in rabbits (Rebollar *et al*., 2014), in humans (Imhoff-Kunsch *et al.,* 2012) and in rats (Olsen *et al.,* 1990). These results have special relevance from the point of view of rabbit production. In this area, does are selected for high prolificacy, reducing the uterine space for the foetus and consequently decreasing the body weight of the newborn (Cifre *et al.,* 1998) with decisive consequences on their survival (Bautista *et al.,* 2015), that could be amended with these larger kits.

On the other hand, due to the fact that rabbits are highly selected by their productive performance, at the second AI, when their first lactation is recently finished, fertility of primiparous females significantly decrease because does cannot satisfy the high nutritional demands (Xiccato *et al*., 2004). This situation is usually translated to lower reproductive efficiency (Castellini *et al.,* 2006). Align with Rebollar *et al*. (2014), fertility was similar between diets regardless of the level of inclusion, however, increasing it, the fertility of primiparous enriched does did not decrease as usually happen in the second AI. In both chapter 3 and 5 the size of the newborn kits of the enriched nulliparous females were higher than control ones, nonetheless, after increase the level of inclusion of dietary LCPUFA, the greater values of mortinatality observed in previous works (Rebollar *et al.,* 2014) were lost. Moreover, it should be taken into consideration that the ADG of kits during lactation and consequently, the litter weight at weaning were harmed after increase the LCPUFA added to the diet.

Summing up, dietary LCPUFA enrichment improved neonate sizes and maintained fertility rate on critical second insemination, however, a lower ADG was observed during lactation period, although did not translate in a higher mortality at weaning time.

Metabolic response

With regard to metabolic response, it has been already mentioned, that the role of leptin in hypothalamic-mediated appetite suppression in response to caloric intake is not its only function. This hormone is the responsible to communicate to the hypothalamus the adequacy of peripheral energy stores to sustain reproduction (Zieba *et al.,* 2005). A hyperleptinemic status throughout the lactation observed with the dietary enrichment in chapter 3 could mean an optimal fat depots. In that sense,

previous works (Castellini *et al.,* 2006; Feugier and Fortun-Lamothe, 2006) described that rabbit does with optimal body condition reach higher fertility rates and show a better pituitary response. Results that have been confirmed in the present PhD Thesis, where higher values of oestradiol combined with higher values of leptin at the end of lactation were translated in a higher fertility rate of primiparous does.

In addition, during periods of high energy demand, total serum protein levels are reduced which in turn produces the mobilization of body reserves from fat depot by lipolysis to increase energy supply (Melillo, 2007). High total cholesterol and HDL-c values were detected during lactation due to the increased demands of TG to synthesise milk fat. Moreover, a lower LDL-c/HDL-c ratio observed during lactation period indicates less vascular aggression by plasma cholesterol and increases more effective reverse transport of cholesterol to the liver, as well as other beneficial effects, thereby reducing the risk of cardiovascular disease (Dobiasova and Frohlich, 1998). All together, these results suggest that even though plasma values of lipid observed in control females do not suppose health risk, PUFA supplementation could promote a healthier lipid profile in lactating rabbit does.

On the other hand, the hyperlipidemic situation observed in newborn in the chapter 3 of this PhD Thesis may have important implications for their survival. In this regard, the relationship between lipid metabolism of the mother, the placenta and the foetus is vital for controlling many aspects of normal gestation (Coleman, 1989; Crawford *et al.,* 1989). Maternal TG are not transported intact, since the mechanism in the placenta only allows transfer esterified FA to the foetus, which together with the transport of NEFA from maternal circulation fulfil the requirements of essential FA by the developing foetus. Dietary deviations in maternal FA intake throughout pregnancy may affect the nature of FA crossing the placenta, having consequences to foetal neuronal maturation and postnatal development (for review, see Herrera *et al*., 2002). Consequently, the supply of essential FA and LCPUFA is critical and central to the synthesis of structural lipids and hence, to normal foetal development (Leaf *et al.,* 1992). In addition, a differential behaviour has been proposed, indicating that during early gestation, embryonic and foetal lipids are derived from maternal free FA crossing the placenta, whereas in advanced gestation, there is a gradual shift to de novo synthesis in foetal tissue (Van Aerde *et al*., 1998). However, the degree to which the foetus is capable of FA desaturation and elongation is not clear. Foetal baboons have been shown to effectively

synthetize both DHA and AA from their precursors ALA and LA, respectively (Su *et al.,* 1999, 2001). In human, although human foetal liver desaturase-elongase chain reaction has not been clearly demonstrated in physiological condition, parallel increases in plasma DHA in woman and newborn were found after fish oil supplementation during pregnancy. This demonstrates the importance of a maternal dietary FA to control the availability of LCPUFA by the foetus and newborns (Connor *et al.,* 1996).

Taking into account the discoidal hemochorial structure of the rabbit placenta, this organ optimizes feto-maternal exchanges compared to others species (Fischer *et al.,* 2012). Placental tissue has been shown to express lipoprotein lipase activity (Bonet *et al.,* 1992) as well as phospholipase A2 (Rice *et al.,* 1998) and intracellular lipase activities (Kaminsky *et al.,* 1991). Through this mechanism, maternal plasma TG are hydrolysed and taken up by the placenta, where reesterification and intracellular hydrolysis facilitates diffusion of the released FA to the foetus, and their subsequent transport to foetal liver. Placental released free FA at the foetal side are transported in foetal blood bound to a specific oncofetal protein, the alphafetoprotein (Benassayag *et al*., 1997). Those FA are rapidly taken up by foetal liver, where they are esterified and released back into circulation as TG. These mechanisms of placental transfer could explain the hyperlipidemic status found in the neonates in the chapter 3 of the present Thesis. Moreover, this correlation between maternal and foetal lipids may also have important implications in newborn weight, as it has been demonstrated in human (Kitajima *et al*., 2001) and corroborated in rabbits with the present work.

On the other hand, circulating free FA reaching maternal liver can be used for either esterification in the synthesis of glycerides or oxidation and ketone body synthesis. Moreover, ketone bodies in foetal plasma reach the same level as in the mother, despite that ketogenesis is not active in the foetus since they easily cross the placenta (Shambaugh, 1985). Therefore, the foetus benefits from this product of maternal FA metabolism, since ketone bodies may be used not only as fuels (Shambaugh, 1985) but also as lipogenic substrates (Edmond, 1974), that would contribute to their hyperlipidemic status at birth.

As it has been previously mentioned, the hyperlipidemic situation observed in newborn of the present study may have important implications for their survival. The high skin surface area to body mass ratio that kits have, can cause that thermal

stress to be particularly important, even more in small-size kits. Moreover, as altricial animals, kits are born after a short gestation with an immature HPA axis, maintaining their body temperature by huddling together in their nest. Consequently their brown adipose tissue (BAT) matures postnatally in parallel with the maturation of the HPA. Brown adipose tissue is a thermogenic organ that protects the body from cold environment via dissipating chemical energy (lipids and glucose) as heat. The thermogenesis of BAT is predominately governed by the sympathetic nervous system via the adrenergic receptor signalling pathways. Upon stimulation, sympathetic nerve releases norepinephrine that binds to β3 adrenergic receptors in the membrane of brown adipocytes to activate a cascade of signalling pathways, leading to increases in FA β-oxidation and heat production (Cannon and Nedergaard, 2004). It has been appreciated that BAT uses lipids as substrates for thermogenesis and activation of BAT promotes oxidative metabolism and heat production, leading to a great increase in energy utilization (Cannon and Nedergaard, 2004). In this regard, in mice, Bartelt *et al.* (2011) have reported that cold exposure drastically accelerated plasma clearance of TG as a result of increased uptake into BAT. Thus, the higher plasma concentration of TG observed in the newborns could be due to a greater oxidation by BAT for heat production, even superior in enriched neonates due to the LCPUFA are easily oxidised.

Hence, greater metabolic response, in terms of leptin concentration, was observed in enriched rabbit does during lactation that was translated in reproductive improvements. Moreover, healthier lipidaemic profile was observed in enriched mothers, concomitantly with an hyperlipidemic status of neonates that could mean greater used of TG as substrate of thermogenesis the first hours of life, with important implications in their survival.

Oxidative stress response

The greater concentration of lipids in newborn kits and their mothers fed the enriched diet was concomitant with a greater oxidative stress in both. As it has been mentioned, the degree of unsaturation affects fat stability because double bonds are easily oxidised, thereby the higher content of PUFA in the diet, the more likely will peroxidation occur. Thus, excess intake of PUFA may reduce antioxidant capacity (Chu and Choi, 1994), enhancing susceptibility to oxidative damage (Mazière *et al*., 1998), a condition that has been shown to be responsible for foetal damage during

pregnancy in rats (Viana *et al*., 2000). Nonetheless, diets of the current study were formulated following the recommendations stablised by De Blas and Mateos (2010) and an extra amount of vitamin E was added to the experimental diet. This resulted in that although the mothers of the experimental group had a greater oxidative stress on day 28 of pregnancy, there were no negative consequences or foetal damage throughout this period.

Several investigators studied the relationship between the oxidative state of the mother and the newborn. Auguelles *et al.* (2006) measured oxidative stress markers in human blood and found a good correlation between the oxidative status of the mother and that of the neonate based on measurements in the umbilical cord blood. Results that has been confirmed in enriched mothers and newborns of the chapter 3 of the present Thesis. Furthermore, newborns are more prone to develop oxidative stress than adults due to the exposure to high oxygen concentrations. The first breaths of the neonate imply also an increase in ROS generation (Wiedemann *et al.* 2003; Gaál *et al.* 2006) which leads to oxidative stress (Rizzo *et al.* 2012), as it can be observed in the kits (regardless of the size) of the current Thesis. The transition from foetal to neonatal life at birth induces acute and complex physiological changes. During delivery, in fact, the foetus is transferred from an intrauterine hypoxic environment with 20–25 mmHg oxygen tension (PO2) to an extrauterine normoxic environment with approximately 100 mmHg PO2 (Gitto *et al.* 2009). Furthermore, as it has been previously mentioned, and based upon lamb studies, we know now that the neonate depends on the BAT as a primary source for heat production (Thompson and Jenkinson, 1969; Klein *et al*., 1983). The mobilization of the BAT results in an increase in oxygen consumption and causes, consequently, an increase in free radical generation (Barja De Quiroga, 1992). Hence, newborns are more predisposed to reach an oxidative stress condition than kits or adults do, as can be observed in kits at 32 dpp, where oxidative stress equals the values of their mothers.

Animal and human studies have investigated the protective benefit of antioxidant therapies on free radical-mediated newborn diseases (Davis *et al.,* 1993). In this connection, Vitamin E is an integral component of the lipid membranes and is a natural antioxidant that prevents fat degeneration in animal cells and the consequent free radical formation (Hatfield *et al*., 2000). Therefore, the administration of antioxidant vitamins to the mother is certainly of particular interest

to improve colostrum/milk quality and, consequently, neonatal survival (Capper *et al.,* 2005; De Renobales *et al.,* 2012). It is important to note that in comparison with adults, increased concentrations of some antioxidants such as ascorbate and bilirubin are found in newborns (Gopinathan *et al.,* 1994; Wiedemann *et al.,* 2003). However, other major antioxidants such as vitamin E show reduced activity in them (Gitto *et al.,* 2009). In the chapter 3 of the current work, in spite of the inclusion of higher vitamin E concentration in the enriched diet, it seems that it was not enough for counteract the greater oxidative stress found in enriched neonates, probably due to the reduced activity of this vitamin in them.

In consequence, because newborns are suddenly exposed to high oxygen concentrations and due to the higher content of PUFA in the diet, the more easily will be oxidised, observing high values of oxidative stress indicators in neonates. However, this situation was normalised at weaning and similar values to rabbit does were achieved.

Transfer and deposition in different tissues

As it has been previously mentioned, once absorbed, the FA are metabolized by mammalian cells (Sprecher, 2000). They are components of phospholipids and sphingolipids which are part of biological membranes, of fats (TG) which are used as energy storage devices inside cells (adipose tissue), they are transported in the blood (HDL-c and LDL-c), and through covalent attachment they are used for protein modification. The effect of using in mothers an animal source of n-3 LCPUFA as supplementation has been proved in the chapter 5 of the present Thesis, where the FA profile of diet after n-3 LCPUFA enrichment was directly reflected in the FA profile of rabbit milk. In this regard, Delgado (2017) used linseed oil as PUFA source and observed that milk FA profile were closely correlated to their profile in rabbit females diets. Castellini *et al.* (2004) using flaxseed, observed a similar milk composition in PUFA that was reflected in suckling rabbit plasma profile. In cows supplemented with fish oil encapsulated, milk composition of both EPA and DHA increased more than three-fold (Elis *et al.,* 2016).

It is known that maternal diet FA composition during pregnancy and lactation affects the development and growth of both the foetus and the neonate (through the maternal milk) (Gerfault *et al*., 1999; Lapillonne, 2007). According to Lin *et al.* (1991), rabbit milk composition may be too low in DHA to completely meet the need

of growing rabbit and prenatal dietary treatment is more effective than postnatal. Consequently, deepening the study of the enrichment of diet with supplements composed by fish oil could be a good way to resolve the low milk concentrations of DHA, making that kits start the fattening period with more PUFA in their fat deposits.

The continuous administration of the same diet to mothers and offspring during the fattening period was translated in a higher content of PUFA in muscle and perirenal fat in the enriched group compared with the control one. Even though EPA, DPA and DHA were not detected in control diet, these FA appeared later in muscle of control animals, reflecting the ability of rabbits to synthesise endogenous n-3 FA (Bernardini *et al.,* 1999). In agreement with our results, previous studies have shown the influence of dietary FA profile on FA deposition in rabbit carcass (Bernardini *et al.,* 1999; Xiccato, 1999; Kouba, 2006; Benatmane *et al*., 2011; Tres *et al.,* 2014). The FA profile observed in both tissues in our study is consistent with previous work (Bernardini *et al.,* 1999; Kouba *et al,* 2008; Peiretti, 2012), showing greater SFA and MUFA deposition in fat compared with muscle, but greater PUFA deposition in muscle than in fat. It has to be noticed that the major lipid class in adipose tissue is triacylglycerol or neutral lipid, whereas in muscle a significant proportion is phospholipid, which has a much higher PUFA content in order to perform its function as a constituent of cellular membranes (Wood *et al.,* 2008).

The potential atherogenic and thrombogenic risk of fat deposited in the carcass for human consumption can be measured by atherogenic and thrombogenic indexes, respectively (Ulbricht and Southgate, 1991). According to the results of the chapter 4 in the present study, carcass from enriched rabbits had higher nutritional quality than that from control ones. Other indicator of healthy food for human is the n-6/n-3 ratio, and this parameter in the muscle of PUFA enriched rabbit was <4.0 (Simopoulos, 2002; Wood *et al.,* 2004), whereas meat from control rabbit exceeded this value. In any case, the n-6/n-3 ratio was lower in both muscle and perirenal fat in enriched than in control rabbits, translating in a healthier FA profile for human consumption.

As it has been commented, rabbits fed with both experimental diets had the same productive performance; therefore the body weight of rabbit at slaughter time was similar. All carcass traits evaluated was also similar barring the weight of the skin, which tended to be lower after the dietary enrichment. This trend might be due to a higher deposition of subcutaneous fat with a more saturated profile in the control

group, which would result in higher fat densification and therefore subcutaneous fat would be more easily removed at skinning. Regard to differences observed in the chapter 4 of the current study related to abdominal fat weight, it had been already described in chickens, where diets rich in PUFA generated smaller abdominal fat deposits than those diets rich in MUFA and SFA (Crespo and Esteve-García, 2002). In addition, a reduction in carcass fat content after PUFA enrichment have been previously described in rabbits (Kowalska and Bielanski, 2009; Volek and Marounek, 2011), due to the fat deposition is affected by the saturation degree of FA (Lin *et al.,* 1993). Nevertheless, hot carcass yield and total weight as well as tissue composition (bone, muscle and fat) of the left hind leg were not affected after supplementation as previously described Kowalska and Bielanski (2009) enriching the diet with fish oil.

Consequently, the supplement based on fish oil used in this Thesis to feeding rabbits during their growth period, has allowed to obtain enriched carcass which contain a fat with a FA profile mainly unsaturated that imply healthy properties to human consume.

CHAPTER 7

GENERAL CONCLUSSIONS

Data of the present PhD Thesis work suggest that dietary enrichment with a supplement based on n-3 polyunsaturated fatty acids as fat source in rabbit does and, consequently in their offspring:

1. Did not affect either daily feed intake in any productive stage (rearing, gestation and lactation) or productive variables in the first cycle. Nonetheless, the supplementation determined a healthier lipid profile and greater estradiol concentration in lactating does as well as a hyperlipidemic status in newborn kits, increasing indicators of oxidative stress.

2. Enhanced beneficial long-chain n-3 polyunsaturated fatty acids and decreased n-6/n-3 ratio in rabbit meat and fat, being healthier for human consumption, without having negative effects on growth performance, cecal fermentation, and ileal morphology or carcass characteristics.

3. Confirmed the higher plasma progesterone concentrations during pregnancy, the maintenance of fertility, the healthier milk fatty acid profile, and the larger size of neonates of primiparous does, supporting the accumulative long-term beneficial effect of n-3 PUFA supplementation in rabbit does.

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