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EVALUATION OF THE USE OF ESTERIFIED ACID OILS FOR CANINE DIETS

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SUMMARY

Chemical esterification of free fatty acids (FA) and glycerol, by-products of the refinery and the bio-diesel industries respectively, generates new technological fats with different physico-chemical and nutritional characteristics. Moreover, re-introducing these by-products in commercial canine diets minimizes the amount of residues and contributes to sustainable food manufacturing systems. Thus, this thesis aims to evaluate the use of different esterified acid oils (EAO) for canine diets (**Chapter 3** and **Chapter 4**).

Chapter 3 explores the effect of palm EAO with different mono- (MAG) and diacylglycerides (DAG) content on food preferences (Experiment 1), digestibility, and post-prandial and fasting plasma lipids (Experiment 2) compared to palm native oil in adult dogs. It is hypothesized that esterification of palm acid oil improves the digestibility of certain FA, such as saturated FA (SFA), due to a predicted increase of SFA at the *sn*-2 position within the glycerol molecule and also due to their increased MAG and DAG content, which have fat emulsifying properties. Our results indicate that dogs preferred diets with palm EAO in their composition compared to those with palm native oil. Additionally, the inclusion of palm EAO in a canine basal diet did not result in any differences on lipid digestibility, and fasting and post-prandial lipaemia compared to palm native oil. We did not observe any of the hypothesized expected benefits of the inclusion of palm EAO, which might have been counteracted by negative effects of EAO associated to their melting point on digestion and absorption.

The aim of **Chapter 4** is to investigate the use of EAO with different medium chain fatty acid (MCFA) content from coconut oil on food preferences (Experiment 1), digestibility (Experiment 2), and weight management (Experiment 3) compared to longer chain FA from canola and soybean oils in overweight adult dogs. Several studies have confirmed the potential of MCFA to reduce fat mass (FM) in humans and rodents. However, to our knowledge, there are no published studies in dogs assessing this effect. Our results indicate that the incorporation of FA from coconut oil does not have a negative effect neither on palatability nor on organic matter and energy digestibility compared to unsaturated long chain FA. Moreover, our results also show an improvement in overall fat digestibility in dogs fed diets with MCFA, explained by the better digestibility of saturated MCFA versus longer chain SFA. On the other hand, incorporation of MCFA from coconut oil decreases the percentage of body weight lost and increases body FM after weight loss in comparison to longer unsaturated

FA from canola and soybean oils. However, since the experimental diets differed not only in chain length but also in chain saturation, further research is needed to clearly differentiate these effects.

RESUMEN

La esterificación química de ácidos grasos (AG) libres y glicerol, co-productos de la refinación y de la industria del biodiesel respectivamente, genera nuevas grasas con diferentes características físico-químicas y nutricionales. La introducción de estos co-productos en dietas comerciales para perros, contribuye a reducir la cantidad de residuos y a conseguir un sistema de producción de alimentos para animales de compañía más sostenible. Por todo ello, el objetivo de esta Tesis es evaluar el uso de diferentes aceites ácidos esterificados (AAE) para dietas caninas (**Capítulo 3** y **Capítulo 4**).

El **Capítulo 3** tiene como objetivo explorar el efecto de la inclusión en dietas para perros adultos de AAE de palma con diferente contenido en mono- (MG) y diglicéridos (DG), en comparación con un aceite convencional de palma, sobre las preferencias alimentarias (Experimento 1), la digestibilidad y los niveles de lípidos plasmáticos, tanto postprandiales como en ayuno (Experimento 2). La hipótesis de este capítulo se centra en que la esterificación de aceites ácidos de palma incrementará la digestibilidad de ciertos AG, como la de los AG saturados (AGS), debido a un incremento de los AGS localizados en la posición *sn*-2 de la molécula de glicerol y a un incremento en el contenido de MG y DG, que poseen propiedades emulsificantes de la grasa. Nuestros resultados indicaron que los perros tienen una preferencia por las dietas con AAE de palma en su composición en comparación con las que incorporaban aceite convencional de palma. Además, la inclusión de AAE de palma no produjo ninguna diferencia ni en la digestibilidad de la grasa ni en la lipemia, tanto postprandial como en ayuno, en comparación con el aceite de palma convencional. No observamos ninguno de los beneficios hipotetizados con la inclusión de aceites de palma esterificados, que se podrían haber visto neutralizados por los efectos negativos de los AAE sobre su digestión y absorción relacionados con su punto de fusión.

El objetivo del **Capítulo 4** es investigar el uso de AAE, obtenidos a partir de aceite de coco, con alto contenido en ácidos grasos de cadena media (AGCM) en comparación con AG de cadena más larga, obtenidos a partir de aceite de colza y soja. Para ello, se determinó el efecto sobre las preferencias alimentarias (Experimento 1), la digestibilidad de la dieta (Experimento 2) y el control de peso (Experimento 3) en perros adultos con sobrepeso. Diferentes estudios han confirmado el potencial de los AGCM para reducir la grasa corporal (GC) en humanos y roedores. Sin embargo, no se han publicado estudios que estudien este efecto en perros. Nuestros resultados indicaron que la incorporación de AG de aceite de coco

no tiene ningún efecto negativo ni en la palatabilidad ni en la digestibilidad de la materia orgánica y la energía en comparación con AG insaturados de cadena más larga. Además, nuestros resultados también muestran una mayor digestibilidad total de la grasa en los animales alimentados con AGCM, debido a una mejor digestibilidad de los AGCM respecto a AGS de cadena más larga. Por otro lado, la incorporación de AGCM del aceite de coco disminuye el porcentaje de peso perdido y aumenta la GC después de un tratamiento de pérdida de peso en comparación con AG insaturados de cadena más larga, aportados por aceites de colza y soja. Sin embargo, las dietas experimentales no sólo difieren en longitud de cadena sino que también lo hacen en grado de saturación, por lo cual es necesario más investigaciones enfocadas a diferenciar estos efectos.

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ABBREVIATIONS

AA	arachidonic acid
AAFCO	Association of American Feed Control Officials
ALA	α-linolenic acid
AUC	area under the curve
BCh	butyrylcholinesterase
BCS	body condition score
BW	body weight
CETP	cholesterol ester: transfer protein
Ch	cholesterol
CLA	conjugated linoleic acid
DAG	diacylglyceride
DE	digestible energy
DHA	docosahexaenoic acid
EAO	esterified acid oil
EE	ether extract
EI	energy intake
EPA	eicosapentaenoic acid
EU	European Union
FA	fatty acid
FEDIAF	European Pet Food Industry Federation
FFA	free fatty acid
FLE	fat loss eficiency
FM	fat mass
GE	gross energy
HDL	high-density lipoprotein
IDL	intermediate-density lipoprotein
IL-6	interleukin-6
LA	linoleic acid
LBM	lean body mass
LCAT	lecithin: cholesterol acyltransferase
LCFA	long chain fatty acid
LCT	long chain triacylglycerides
LDL	low-density lipoprotein
LPL	lipoprotein lipase
MAG	monoacylglyceride
MCFA	medium chain fatty acid
MCT	medium chain triacylglyceride
ME	metabolizable energy
MER	maintenance energy requirements
MIU	moisture, impurities, and unsaponifiable matter
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acid
NMR	nuclear magnetic resonance
NRC	National Research Council
OM	organic matter
PUFA	polyunsaturated fatty acid
SCFA	short chain fatty acid
SFA	saturated fatty acid
TAG	triacylglyceride
TBW	total body water
TNF-α	tumour necrosis factor- α
VLDL	very low-density lipoprotein
WL	weight loss

CHAPTER 1:

INTRODUCTION

1.1 Pet food market

The pet food sector is one of the most dynamic in the animal feed world, with similarities to the human food sector. Nowadays, the majority of pet owners in developed countries feed their companion animals with commercially prepared foods rather than homemade diets or table scraps (Laflamme et al., 2008); this trend is growing in parallel with an increase in the variety and quality of the product on offer.

The number of households owning dogs is estimated to have reached 60 million in Western Europe (FEDIAF, 2011), 78 million in United States (according to the National Pet Owners Survey, 2012), and 5 million in Spain (ANFAAC, 2011). Moreover, despite the current recession, the global sales of pet products continued to increase. The worldwide spending reached 56.8 billion Euros in 2010, which represents a growth of 4.4% over 2009 (Taylor, 2011), the European market generated more than 8.5 billion Euros annually (FEDIAF, 2011), and the Spanish market showed a spending of 681,666 Euros in 2011 (ANFAAC, 2011). Additionally, household spending in pet food in Eastern Europe and Latin America, relatively immature markets, has risen tremendously in the last five years (Taylor, 2011).

In recent years, pet owners have become increasingly interested in the quality and safety of the foods that they feed their pets and the pet supply growth has continued to reflect the importance that pets have in our lives (Case et al., 2010). Pet owners are seeking a long and health life for their pets and look to nutrition to provide such support. As a consequence, numerous improvements in companion animal nutrition have resulted in development of a wide array of pet foods that provide complete and balanced nutrition, and are formulated for different life stages, activity levels, and health conditions (Reid and Peterson, 2000). On the other hand, due to the current economic crisis, pet owners tend to buy standard or economic pet foods in grocery stores (65% of volume sales in Spain, according to ANFAAC, 2011) and, as a consequence, pet food manufacturers look for more economic ingredients for their formulations.

1.1.1 Types of pet food

Among the commercially prepared foods, it is important to differentiate between a complete and a complementary pet food. A complete pet food is a food which, by reason of its composition, is sufficient for a daily ration without any other supplementation (EU

Regulation No 767/2009). On the other hand, a complementary pet food is defined as a food which has a high content of certain substances but which, by reason of its composition, is sufficient for a daily ration only if used in combination with other pet foods (EU Regulation No 767/2009). Basically, complementary foods are marketed as treats, to be given occasionally and in small quantities.

Complete and complementary pet foods are presented in many physical forms according to the method of processing used, the ingredients included, and the methods of preservation. They can be separated according to their moisture content: dry, semi-moist, and wet. Dry pet foods are those with a moisture content of less than 14%, semi-moist pet foods have a moisture content of 14% or more and less than 60%, and wet pet foods have a moisture of 60% or more (FEDIAF, 2011). Nowadays, customer demands are increasingly orientated towards complete dry food diets in dogs (Zentek et al., 2004).

In response to the growing interest in quality pet care, pet food can be classified according to the marketing methods and the distribution channels used to sell it. These commercial categories are standard (or economic), premium, and super-premium (Case et al., 2010). Premium and super-premium pet foods are sold mainly through pet supply stores, feed stores, and veterinarians. Premium pet foods include highly digestible ingredients while super-premium foods include highly digestible ingredients along with various types of functional ingredients and/or nutrients that may provide specific health benefits. On the other hand, standard pet foods are usually sold in grocery store chains and through mass-merchant stores, and their formulation can vary. That is, formulas include ingredients that may vary from batch to batch, depending on ingredient availability and cost. It is important to know that there is no legal definition of what premium and super-premium pet foods are and, as a consequence, this classification is of little help to the consumer although it is useful for manufacturers.

During the new millennium, companies have continued to develop foods that are designed for specific stages of life, physiological states, and disease states. Thus, pet foods can also be categorized according to their nutrient content and the purpose for which they are formulated (Case et al., 2010)

The segmentation of the market has increased and has developed products more and more specific as the knowledge and interest about pet nutrition has improved. For example, we can find specific foods for different life stages in dogs (reproduction, growth, adult maintenance, and seniors), different activity levels (high activity or sedentary), size (small, medium, large,

and giant), breed, gender (intact and neutered male, and intact and spayed female), life-style (indoor and outdoor), and also diets for the nutritional management of different disease conditions in dogs and cats (veterinary or therapeutic diets).

1.1.2 Regulation of commercial pet food

A number of agencies and organizations regulate the production, marketing, safety, and sales of commercial pet foods worldwide. Although some regulations are mandatory, others are optional recommendations for the pet food industry. The major agencies and their roles in pet food regulation are presented below.

The *National Research Council* (NRC) is a non-profit scientific organization that collects and evaluates research and makes nutrient recommendations. The NRC includes a standing committee on animal nutrition that identifies problems and needs in animal nutrition, recommends appointments of scientists to subcommittees, and review reports. The latest NRC report was published in 2006 and is an extended version of the 1985 (only for dogs) and 1986 (only for cats) volumes. This newest edition combines both species and offers several different nutrients requirements classifications: minimum requirement, adequate intake, recommended allowance, and safe upper limit. Industry and regulation groups may base their recommendations on the NRC reports, but these are not legally binding.

The *Association of American Feed Control Officials* (AAFCO) is a United States association of state and federal feed control officials that acts in an advisory capacity to provide models for state legislation. These regulations ensure that pet foods are uniformly labelled and nutritionally adequate. Also, AAFCO publishes "AAFCO Official Publications" which include nutrient and energy requirements for dogs, feeding test protocols, and labelling rules for pet food products. Individual states can adopt AAFCO regulations as law, and pet foods that are sold across state lines in the USA must follow AAFCO regulations regarding labelling.

The *European Pet Food Industry Federation* (FEDIAF) represents the national pet food industry associations in the European Union (EU), representing 650 European pet food producing companies across Europe. The FEDIAF reviews are called "Nutritional guidelines for complete and complementary pet food for dogs and cats". The objectives of FEDIAF guidelines are: to provide practical nutrient recommendations for pet food manufacturers and

to assess nutritional values of practical pet foods for healthy animals. These guidelines are based on NRC, EU legislation, and the AAFCO publication.

The FEDIAF guidelines, however, are just recommendations and are not legally binding. The EU has published several regulations that do carry legal standing in the different EU countries (**Table 1.1**) and has endorsed the FEDIAF documentation regarding proper labelling of pet food.

Table 1.1. Relevant European Union (EU) legislation regarding pet food market regulation.

Regulation (EC) No 178/2002	on general food law and food safety
Regulation (EC) No 1829/2003	on genetically modified food and feed
Regulation (EC) No 1831/2003	on additives for use in animal nutrition
Regulation (EC) No 882/2004	on official controls to ensure the verification of compliance with feed and food law, animal health and animal welfare rules
Regulation (EC) No 183/2005	laying down requirements for feed hygiene
Regulation (EC) No 767/2009	on the placing on the market and use of feed
Regulation (EC) No 834/2007	on organic production and labeling of organic products
Council Directive 90/167/EEC	laying down the conditions governing the preparation, placing on the market, and use of medicated feeding stuffs in the Community
Directive 2001/82/EC	on the Community code relating to veterinary medicinal products
Directive 2002/32/EC	on undesirable substances in animal feed
Directive 2006/114/EC	concerning misleading and comparative advertising

1.2 Dietary fat

Lipids are a heterogeneous group of compounds which are classified together because of their solubility in organic solvents and their insolubility in water (NRC, 2006; Christie, 2013; Nelson et al., 2013).

Lipids can be further categorized into glycerol-based and non-glycerol-based lipids. Glycerol-based lipids include both simple lipids; such as hydrophobic triacylglycerides (TAG) that are made up of three fatty acids (FA) linked to one molecule of glycerol (**Figure 1.1**); and compound lipids, that are more polar and are composed of a lipid moiety, such as a FA, linked to a non-lipid molecule. Examples of compound lipids are phospholipids and glycolipids. Lipoproteins, whose function is to carry fat in the bloodstream, are also a type of compound lipid. Cholesterol (Ch) and its FA esters are non-glycerol-based lipids. This category also includes waxes, cerebrosides, terpenes, sphingomyelins, and various sterols.

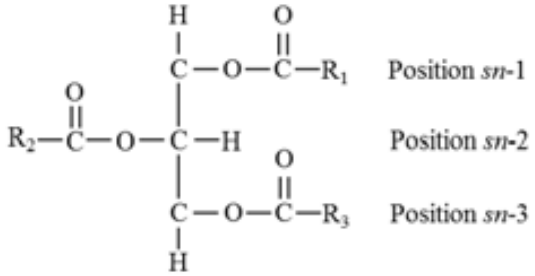

Figure 1.1. The structure of the triacylglyceride molecule.
***sn*, stereospecific numbering position; R, fatty acid.**

Dietary fats and oils used in pet foods are mainly made up of TAG (98%; Christie, 2013). These TAG molecules can be classified according to the types of FA that each TAG contains.

Fatty acids are carboxylic acids with hydro-carbonated chains between 4 and 36 carbon atoms. Fatty acids properties (solubility and melting point) are determined, mainly, by their saturation degree and carbon chain length.

According to FA saturation degree, they can be saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA). Saturated FA do not contain double bonds between carbon atoms, MUFA have one double bond, and PUFA contain two or more double bonds. Double bonds in dietary unsaturated FA can be in either *cis* or *trans* configuration. The location of the double bonds is indicated in the systematic name of the FA according to the delta (where the carboxyl-end of the FA is first) or the omega (where the methyl-end of the FA is first) nomenclature.

Many dietary TAG contain predominantly long chain FA (LCFA) that have a number of carbon atoms ranging between 14 and 26. There are other fat sources which contain appreciable amounts of medium chain FA (MCFA), with carbon chain length between 6 and 12. Short chain FA (SCFA), with a carbon chain length of less than 6, are also called volatile FA and are produced by bacteria in the gut during fermentation of fiber.

1.2.1 Functions and requirements

Dietary fat provides a concentrated source of energy. Dietary fat has more than twice the amount of metabolizable energy (ME) per unit of weight than dietary protein or carbohydrates. Consequently, alteration in the fat content of a diet can significantly affect its caloric density (NRC, 2006). There are equations predict digestible energy (DE) and ME with reasonable precision in dog diets that only include either gross energy (GE) or ether extract (EE) as independent variables (Hervera et al., 2008; Castrillo et al., 2009).

Although, there is not an absolute requirement for dietary fat, some is needed in the diet to provide essential FA, to enhance diet palatability, and to act as a carrier for fat-soluble vitamins. The NRC recommended allowance for total dietary fat for canine diets is 11.7% of ME for adult maintenance and 18% of ME for growth and reproduction (NRC, 2006). These values are also similar to FEDIAF nutrient profile minimum fat recommendations for canine diets.

1.2.1.1 Essential fatty acids

Fat is necessary in the diet of dogs as a source of essential FA. The body has a physiological requirement for two distinct families of essential FA: the omega-6 (n-6) and the omega-3 (n-3), both PUFA. The parental forms of the n-6 and n-3 families are linoleic acid (LA) and α-linolenic acid (ALA), respectively. Both LA and ALA should be provided in the diet, as they are not synthesized by mammals, unable to place a double bond further than the omega-9 position. However, these FA can be converted in the body to other LCFA through elongation and desaturation reactions. The LCFA of greatest physiological importance are arachidonic acid (AA), synthesized from LA, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), produced from ALA.

The rate of conversion of LA to AA provides dogs with adequate quantities of this long chain PUFA (Dunbar and Bauer, 2002). However, although adult dogs are capable of converting ALA to both EPA and to a precursor of DHA, docosapentaenoic acid, low rates of

conversion are found (Bauer et al., 1998). Desaturation reactions for ALA are at least half the velocity as that of LA in dogs (Dunbar and Bauer, 2002). Therefore, while dietary EPA and DHA are probably not required by dogs during adult maintenance, increased demands occurring during early development, growth, and gestation make these long chain PUFA conditionally essential (Bauer et al., 2006). There are some medical conditions where the direct inclusion of EPA and DHA in the diet may be beneficial as well (Bauer, 2011). Dietary requirements for n-6 and n-3, and their respective derivative long chain PUFA must be addressed distinctly because there is no interconversion between the two families of FA (**Figure 1.2**).

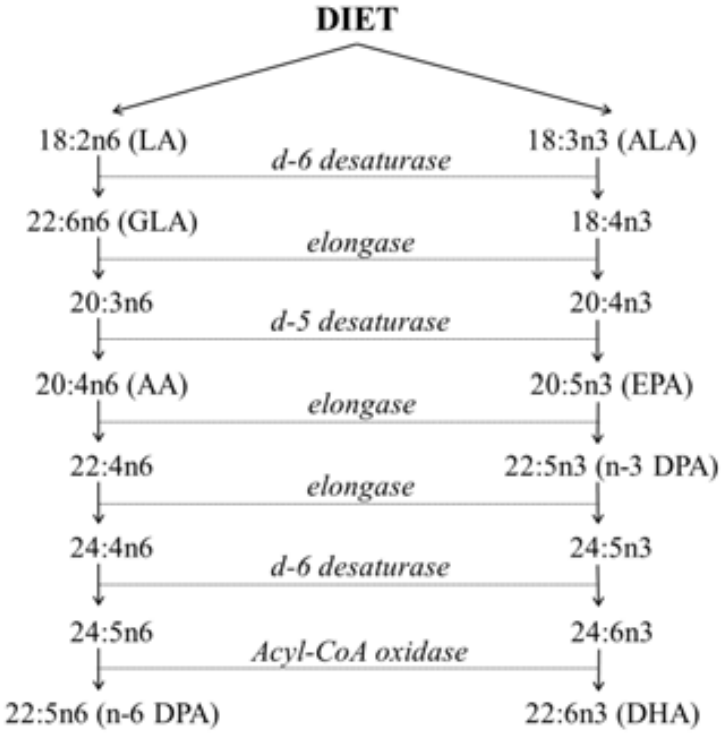

Figure 1.2. Predominant pathways of essential fatty acid metabolism in mammals. LA, linoleic acid; GLA, γ-linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. (from NRC, 2006)

1.2.1.2 Fat and palatability

Fat in the diets of companion animals plays an important role in contributing to the palatability and texture of food. It is well known that fat adds an acceptable texture to food and it increases palatability of pet food (Bauer, 2006). Assessment of palatability of canine food is important for the industry: a greater palatability results in a more enjoyable feeding time for both owner and pet. Since dogs are unable to declare their tastes directly, palatability

assessment must be based on an objective measure in which two or more foods can be ranked on the basis of preference.

The most common method of assessing palatability in dogs is with the two-pan test, which involves comparing the consumption of two different foods (Ferrell, 1984; Griffin et al., 1984). This procedure allows palatability to be determined rapidly but does not control for satiety effects or food interactions in which the presence of one food can alter the palatability of the other. Other approaches circumvent many of the limitations associated with the two-pan test, but may be less robust (Chao, 1984; Rashotte and Smith, 1984; Rashotte et al., 1984). Moreover, in recent years, new palatability assessment methods have been developed that utilize procedures originally developed for assessment of canine cognitive function (Araujo and Milgram, 2004).

1.2.2 Digestion and absorption

Digestion of lipids can be divided into four main phases: emulsification of the bulk fat droplets, hydrolysis of FA ester bonds by lipases, aqueous dispersion of lipolytic products in bile acid mixed micelles, and uptake by the enterocyte (**Figure 1.3**).

The digestion phase of lipids begins with a pre-duodenal emulsification of the ingested fat through gastric and lingual lipases, and also due to mechanical forces. Only traces of lingual lipase activity have been found in adult dogs (Iverson et al., 1991). However, gastric lipase hydrolyzes TAG to a certain extent in the stomach in dogs (Carriere et al., 1992; Vaganay et al., 1998). These enzymes are active at gastric pH, do not require bile salts for activity, and are active mostly breaking the primary (*sn*-1 and *sn*-3) ester links within TAG (Redgrave et al., 1988; Ransac et al., 1990; Rogalska et al., 1990). Different studies in humans and rodents showed that the pre-duodenal hydrolysis of ingested fat results in a more efficient fat digestion due to the emulsification of lipids (Hamosh et al., 1975; Linthorst et al., 1977). The resulting lipid droplets from emulsion are reduced in size by the mechanical strength of peristalsis, and this process is accelerated greatly as the chime enters the small intestine and is mixed with bile and pancreatic secretion. This emulsion or mechanical digestion is important because it increases the ratio surface-to-volume of the droplet, facilitating the contact between lipids and pancreatic lipase, the main enzyme responsible for TAG hydrolysis (Carey et al., 1983).

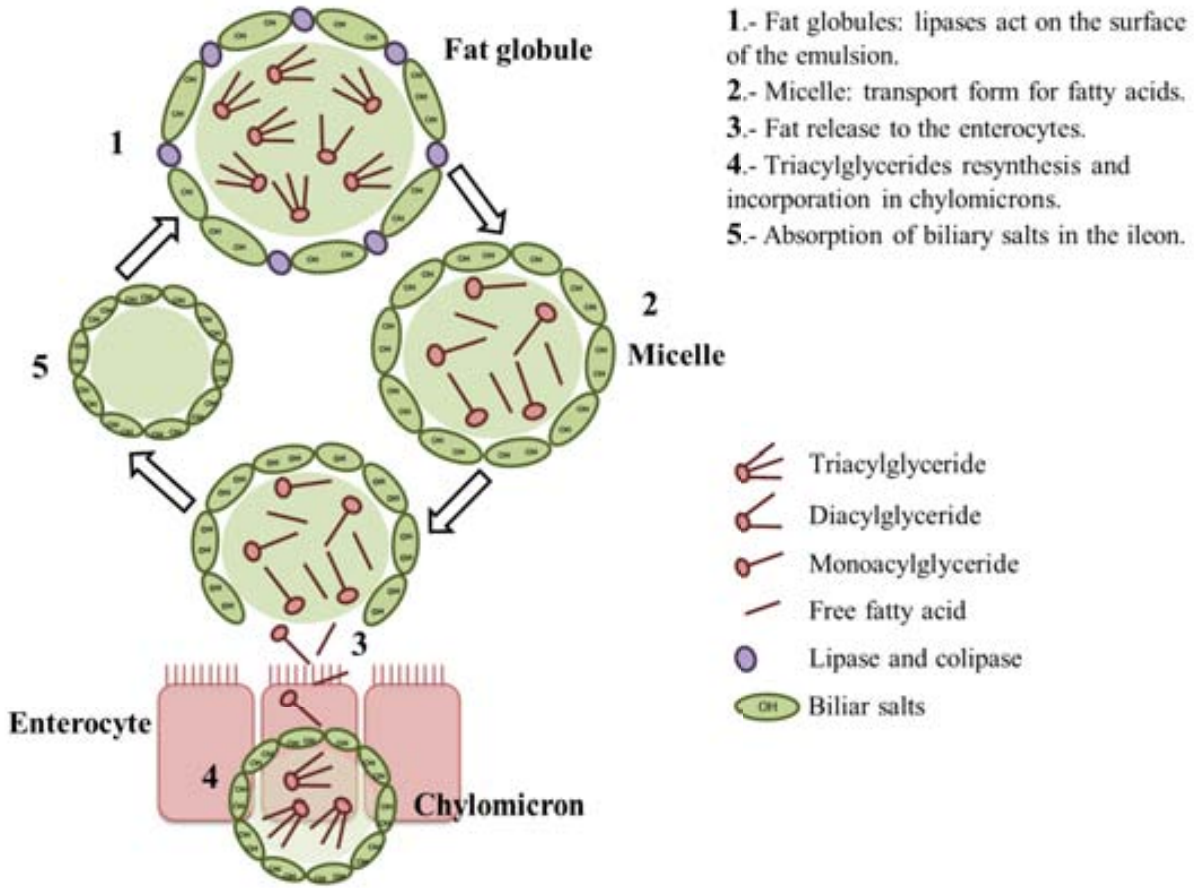

Figure 1.3. Digestion and absorption of lipids.
(from Schenck, 2006)

When lipid droplets exit the stomach and enter the duodenum, the presence of free FA (FFA) helps stimulate cholecystokinin release by the duodenal mucosa. This hormonal response stimulates pancreatic and bile secretions, delivering pancreatic lipase and colipase from the pancreas into the duodenum. Pancreatic lipase has to bind to the surface of colipase before it can act. Colipase is a low molecular protein secreted by the pancreas as pro-colipase which is converted into its active form by trypsin (Borgström, 1975). It protects lipase from denaturation and anchors it to the lipid-water interphase of the droplet, permitting access to the lipase to the inner core of the TAG.

The degradation process is stereospecific and mostly results in the formation of 2-monoacylglycerides (MAG) and FFA. Pancreatic lipase is specific for the primary (*sn*-1 and *sn*-3) ester bond of TAG, although it prefers FA in the *sn*-1 position over FA in the *sn*-3 position (Rogalska et al., 1990) and hydrolysis of diacylglycerides (DAG) over TAG (Lykidis et al., 1995). At the end of the process, all FA in the *sn*-1 and *sn*-3 positions of TAG, but only 22% of FA in the *sn*-2, are hydrolyzed during digestion (Karupaiah and Sundram, 2007).

Moreover, pancreatic lipase selectively hydrolyzes TAG according to the chain length, saturation degree (Bottino et al., 1967), and positional isomerism (Heimermann et al., 1973) of its FA. This could result in a lowering of the hydrolysis rate of some FA. However, Raclot et al. (2001) suggest that the release of FA by pancreatic lipase is only slightly affected by their degree of saturation.

The aqueous solubility of the products from fat hydrolysis is relatively low. Thus, the products of lipolysis are removed from the lipid-water interphase and incorporated into mixed micelles with bile salts secreted by the liver. Unsaturated FFA, 2-MAG and phospholipids are relatively polar and are spontaneously incorporated in mixed micelles (Da Costa, 2003). The entry of these compounds expands the mixed micelle and it is then possible to solubilize other more water-insoluble products, such as long chain SFA, DAG, fat-soluble vitamins, and Ch esters (Carey et al., 1983).

The absorption of the hydrolyzed products of fat digestion occurs in the enterocytes of the small intestine (**Figure 1.3**). Lipid absorption takes place at the apical part of the plasma membrane of the epithelial cells or enterocytes lining the gut. There, micelles dissociate and the lipolytic products are incorporated in the external half of the lipid bilayer of the brush border membrane.

Absorption process occurs either by passive diffusion or protein-mediated mechanisms, depending on the aqueous solubility of the lipolytic products. On the one hand, 2-MAG, SCFA (C4:0 – C6:0), and MCFA (C6:0 – C12:0) are more hydrophilic and are mainly absorbed by passive diffusion, as they are able to cross the unstirred water layer, independently of micellar solubilization. On the other hand, LCFA (> C12:0) and Ch are transported inside the enterocyte via a process mediated by carrier and binding proteins (Yli-Jokipii et al., 2002; Linderborg and Kallio, 2005). These FA-binding proteins facilitate the transport of LCFA into the cytosol of the enterocyte and reduce the potential cytotoxic effect of FA calcium soaps (Da Costa, 2003).

It should be noted that fat hydrolysis proceeds at a faster pace than absorption, which results in FA accumulation in the digestion mixture (Ahrens and Borgström, 1956).

1.2.3 Metabolism

Following absorption across the apical surface of the enterocyte, FA are bound to FA-binding proteins and are transferred to the endoplasmatic reticulum. There, FA are re-esterified to TAG by the glycerol-3-phosphate and the 2-MAG pathways.

The 2-MAG pathway is the major route for new TAG formation, accounting for 80% of synthesized TAG (Lehner and Kuksis, 1996; Linderborg and Kallio, 2005). The enzyme MAG acyltransferase catalyzes the acylation of 2-MAG to DAG. In this reaction, the formation of the 1,2-DAG enantiomer seems to be favored in detriment to 2,3-DAG (Lehner and Kuksis, 1996). The formed DAG are acylated to TAG by DAG acyltransferase (Kuksis et al., 1979). If 2-MAG from the digestion and absorption of dietary TAG are not available, TAG are synthesized via the glycerol-3-phosphate or phosphatidic acid pathway, responsible for 20% of the TAG synthesis (Lehner and Kuksis, 1996; Mu and Hoy, 2004). The FA composition of *sn*-1 and *sn*-3 positions of the new TAG has been found to be similar, regardless of the TAG re-esterification pathway, and it has been suggested that TAG formed by the glycerol-3-phosphate pathway could be then hydrolyzed to 2-MAG and FFA, and re-esterified to TAG via the 2-MAG pathway (Yang and Kuksis, 1991; Linderborg and Kallio, 2005).

In addition to TAG formation, TAG hydrolysis also has been claimed to occur in intestinal cells. In fact, it has been suggested that TAG in all tissues would be constantly hydrolyzed, circulated either intra- or extracellularly, and re-esterified (Reshef et al., 2003; Linderborg and Kallio, 2005).

Finally, TAG formed at the endoplasmic reticulum are transported to the Golgi apparatus where chylomicrons are formed. Chylomicrons are large lipoproteins in charge of blood transport of absorbed dietary fat. Their diameter may be dependent on the saturation degree (Redgrave et al., 1988; Pavero et al., 1992; Sakr et al., 1997) and on the FA positional distribution in TAG (Redgrave et al., 1988). Once formed, chylomicrons particles are released into the circulation via the lymphatic.

1.2.3.1 Lipoprotein metabolism

Lipids, being hydrophobic molecules, have to be transported in plasma as macromolecular complexes known as lipoproteins (Ginsberg, 1998; Bauer, 2004; Johnson, 2005). It is important to highlight that FFA are transported bound to albumin and do not require incorporation into lipoproteins for transport (Ginsberg, 1998; Bauer, 2004; Johnson, 2005). Lipoproteins are composed of a variable lipid fraction and a protein fraction. Proteins used for lipoprotein synthesis are called apoproteins.

Four main classes of lipoproteins, based on their hydrated density, are recognized in dogs: chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and

high-density lipoproteins (HDL; Maldonado et al., 2001). High-density lipoproteins can be subdivided into HDL_1 (unique to dogs), HDL_2, and HDL_3 (Bauer, 2004; Johnson, 2005). In humans, there also are intermediate-density lipoproteins (IDL), with a hydrated density between VLDL and LDL, but their existence has not been verified in dogs (Bauer, 2004; Johnson, 2005).

Chylomicrons are involved in the transport of exogenous (dietary) lipids from the small intestine to other tissues after absorption (**Figure 1.4**). These chylomicrons are formed in the enterocyte with TAG (85-90%), phospholipids, Ch esters, Ch, and apoprotein B_{48}. Once formed, these TAG-rich particles are secreted into the lymphatic system and enter the general circulation, where they acquire apoprotein C and apoprotein E peptides from HDL. One of the apoprotein C peptides (CII) activates the enzyme lipoprotein lipase (LPL) that hydrolyzes TAG in the capillary endothelium of adipose tissue; where FA and glycerol are re-esterified into TAG for energy storage; and in skeletal muscle, where the FFA are used for energy production (Bauer, 2004).

Like the lipases in the digestive tract, LPL is *sn*-1 and *sn*-3 specific, although some authors believe that the *sn*-1 position is favored (Yli-Jokipii et al., 2002; Linderborg and Kallio, 2005). It has been stated that the only lipid molecules of lipoproteins to be transferred from blood into adipose tissue are FFA (Mayes and Botham, 2000). Therefore, FA attached to the *sn*-2 position (that escape hydrolysis by LPL) may be preferentially transported to the liver instead of staying in extrahepatic organs (Berry and Sanders, 2005). Moreover, LPL seems to preferentially hydrolyze unsaturated TAG compared with saturated TAG, and this selective hydrolysis could result in a selective crystallization of the most saturated TAG in the remnant core of the chylomicrons, which would therefore be further inaccessible to lipolytic enzymes (Parks et al., 1981; Clark et al., 1982).

Hydrolisis of core TAG results in a reduction in size of chylomicrons. The Ch-rich remaining particles (chylomicrons remnants), transfer back their apoprotein C to HDL and can be rapidly removed from the circulation by endocytosis when they are recognized by specific hepatic apoprotein E receptors (Bauer, 2004; Johnson, 2005). The residual TAG remaining in the chylomicrons remnants are an important source of hepatic FA and are estimated to account for 73% of the newly synthesized VLDL in mice (Jung et al., 1999).

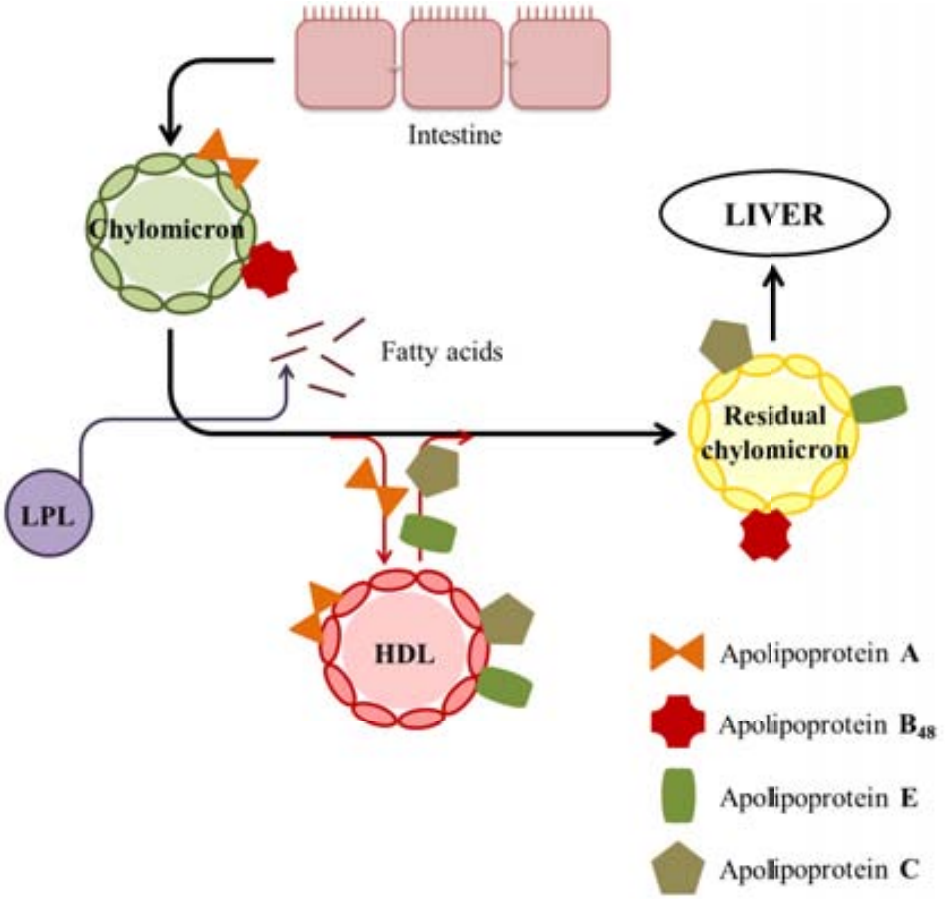

Figure 1.4. Chylomicron metabolism.
HDL, high-density lipoproteins; LPL, lipoprotein lipase.
(from Schenck, 2006)

The lipoproteins VLDL, LDL, and HDL are related particles that transport endogenous (synthesized in the liver) TAG and Ch to peripheral tissues (**Figure 1.5**). As the lipoproteins are delipidated, the diameter of the lipoprotein is reduced and its density increased. Endogenously synthesized TAG combine with Ch, Ch esters, apoproteins (B_{100} and B_{48}), and phospholipids to form VLDL in the liver (Bauer, 2004; Xenoulis and Steiner, 2010). The function of VLDL is similar to that of chylomicrons, but the TAG they transport are from liver origin instead of dietary (Bauer, 2004; Xenoulis and Steiner, 2010). Once secreted, the VLDL acquire apoprotein C and E, release FA and glycerol by the action of LPL, and undergo either hepatic uptake, similarly to chylomicrons, or further delipidation to form LDL. The lipoprotein LDL, which contains mainly Ch esters and phospholipids, circulates in the blood in order to deliver Ch to tissues, which can be used for the synthesis of steroid hormones, incorporation into cell membranes, as well as for hepatic metabolism.

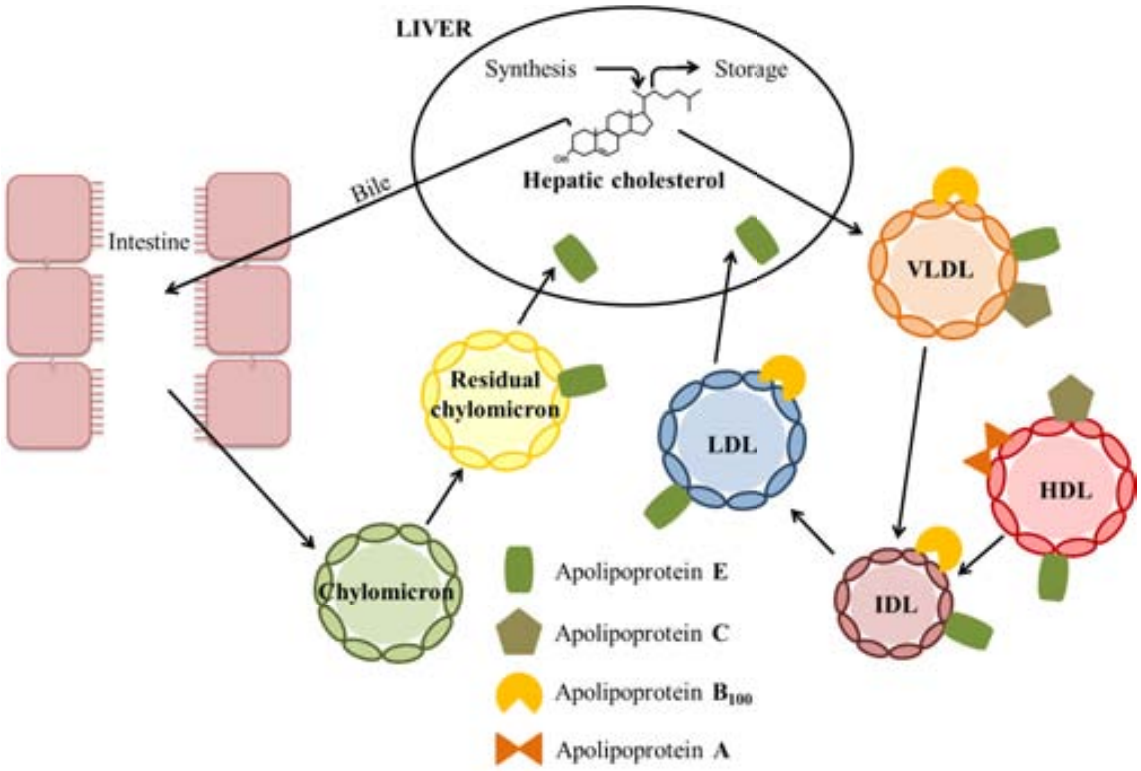

Figure 1.5. Chylomicron, lipoproteins, and liver cholesterol metabolism.
VLDL, very low-density lipoproteins; LDL, low-density lipoproteins; IDL, intermediate-density lipoproteins; HDL, high-density lipoproteins.
(from Schenck, 2006)

Circulating HDL capture plasma non-esterified Ch from cell degradation and convert it to Ch esters by the action of lecithin: Ch acyltransferase (LCAT). Via this process HDL particles are transformed from discoidal nascent HDL to HDL_3. Continued uptake of free Ch and subsequent esterification by LCAT leads to the formation of the Ch ester-rich HDL_2. In humans, a further lipid exchange process takes place between the HDL and apoprotein B-containing lipoproteins (chylomicrons, VLDL, and LDL) by Ch ester: transfer protein (CETP) and results in exchange of TAG from the chylomicrons, VLDL, and LDL with Ch ester from the HDL (**Figure 1.6**). However, in dogs, due to the absence of CETP, HDL_2 molecules continuously acquire Ch esters resulting in the formation of HDL_1, unique in dogs (Bauer, 2004; Johnson, 2005). Thus, with HDL_1, Ch esters are transferred from tissues to the liver and not to apoprotein B-containing lipoproteins which would transfer Ch to peripheral tissues. Therefore, there is little or no transfer of Ch esters to VLDL or LDL and this could be related to the lower incidence of atherosclerotic disorders in dogs compared to humans (Johnson, 2005).

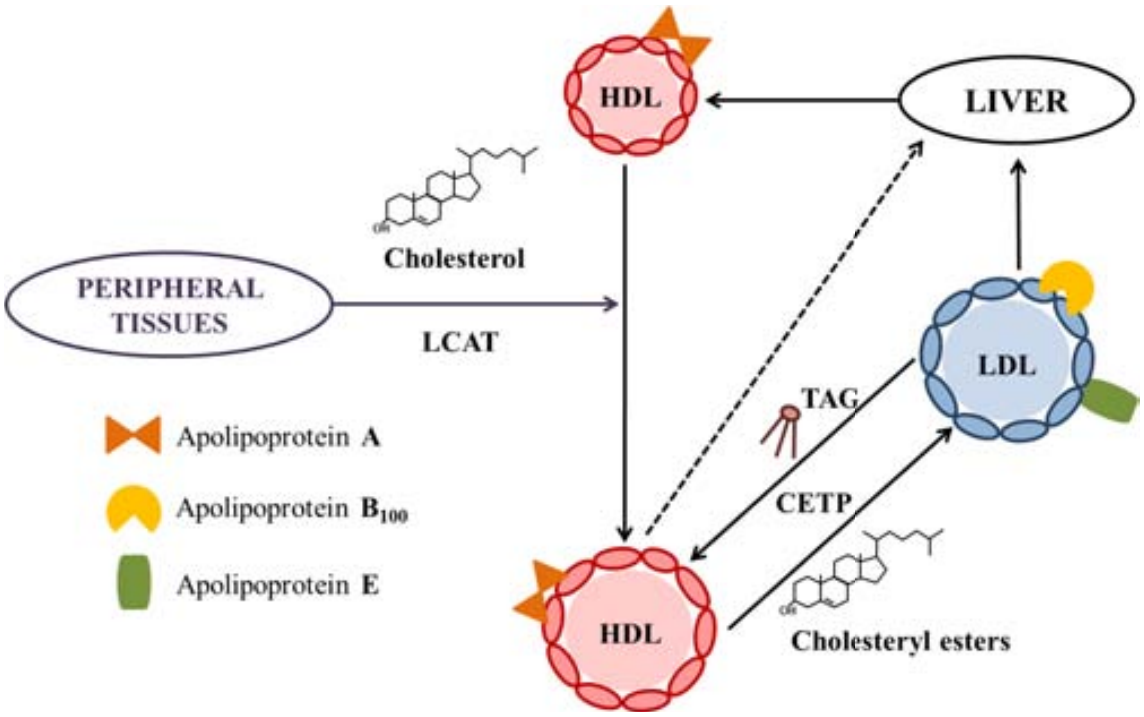

Figure 1.6. Reverse cholesterol transport.
CETP, cholesterol ester: transfer protein; LCAT, lecithin: cholesterol acyltransferase; LDL, low-density lipoproteins; HDL, high-density lipoproteins.
(from Schenck, 2006)

1.2.3.2 Distribution and utilization of body lipids

Adipose tissue is the center of a number of bio-reactions and has numerous functions in the body. Major depots of fat accumulation are present under the skin (as subcutaneous fat), around the vital organs (visceral fat), and in the membranes surrounding the intestines (mesenteric fat). The TAG stored in adipose tissue are constantly undergoing lipolysis and re-esterification, and FA are continuously re-transferred into plasma and eventually used in beta-oxidation.

In general, body fat accumulation may be considered the net result of the balance among dietary absorbed fat, endogenous fat synthesis (lipogenesis), and fat catabolism via beta-oxidation (lipolysis; Leray et al., 2008). However, all these processes are poorly studied in dogs. As a general rule, FA are readily used as an oxidative energy source by many tissues of the body. Different factors as feeding status, dietary fat level, and FA composition influence FA metabolism and their fate between deposition and oxidation in humans and rodents (Poumes-Ballihaut et al., 2001; Arner, 2005; Iritani et al., 2005).

The liver and adipocytes can also synthesize FA via *de novo* lipogenesis in dogs (Bergen and Mersmann, 2005) from non-lipid substrates, mainly carbohydrates. These FA synthesized

de novo undergo esterification to TAG, which are delivered to peripheral tissues by apoprotein B-containing lipoproteins.

1.2.4 Factors affecting fat digestibility, absorption, and metabolism

It is well documented that fat inclusion in canine diets result in an increased total tract organic matter (OM) digestibility in dogs (Hervera et al., 2008; Castrillo et al., 2009), although some differences exist depending on fat type, and amount and the presence of other dietary components (Ballesta et al., 1991; Kienzle et al., 2001). It has been suggested that fat may improve protein digestibility, possibly due to the delaying of gastric emptying (Ballesta et al., 1991).

Several studies indicate a range for crude fat of commercial dog food digestibility from 70 to 95% (Kendall and Holme, 1982; Kendall et al., 1982; Huber et al., 1986; Meyer et al., 1992; Ohshima et al., 1993, 1995; Castrillo et al., 2001). Digestibility trials carried out in our kennels using commercially available canine dry food show a crude fat digestibility of approximately 94% (unpublished data).

The following sub-sections show a whole set of factors that play an important role on digestion, absorption, and metabolism of dietary fat. However, while all these processes have been largely studied in farmed animals, rodents and/or humans, published research in dogs is scarce.

1.2.4.1 Fatty acids saturation degree

It is well established that fat digestibility increases with FA unsaturation degree. On the one hand, considering individual FA digestibilities with the same chain length but different number of double bounds, Duran-Montgé et al. (2007) found that ileal digestibility of stearic (C18:0), oleic (C18:1), and LA (C18:2n6) were 81, 90 and 96%, respectively, in growing pigs fed sunflower oil. Similar results were found by Blanch et al. (1996) in broiler chickens fed soybean oil (69, 87 and 93%, respectively). The low digestibility of long chain SFA may be due to their melting points above body temperature and to their ability to form calcium soaps (Carnielli et al., 1995). Moreover, unsaturation increases the polarity and facilitates micellar solubilization of all FA.

On the other hand, the saturation degree of a fat also has influence on the digestibility of FA that composes it. Thus, Meyer et al. (1992) showed that the digestibility of beef tallow was dependent on the unsaturated FA concentration of the diet in dogs. In this study, the

digestibility of dietary fat improved from 86 to 95% when more 50% of total was present as unsaturated FA. In poultry, palmitic (C16:0) acid digestibility was 83 or 69% depending on whether the fat source was soybean or palm oil (Blanch et al., 1996).

Some studies in broiler chickens and rats indicate that fat deposition seems to be greatly dependent of the unsaturation degree of dietary fat. It has been observed that PUFA inclusion in the diets reduces abdominal fat and total body fat compared to SFA-rich diets in rats and chickens (Mercer and Trayhurn, 1987; Shimomura et al., 1990; Takeuchi et al., 1995; Sanz et al., 1999, 2000; Crespo and Esteve-Garcia, 2001, 2002 a; Ferrini et al., 2008; Wongsuthavas et al., 2008; González-Ortiz et al., 2013). Nevertheless, diets rich in PUFA did not reduce fat deposition with respect to MUFA and SFA in pigs (Realini et al., 2010). Different studies have determined the oxidation rates using different FA in rodents and humans. These studies agree that SFA are catabolized to a lesser extent than unsaturated FA (Jones et al., 1985; Leyton et al., 1987; DeLany et al., 2000), which could explain why their inclusion increases fat deposition in some species.

Clinical and animal studies have shown that saturated fats increase circulating concentrations of serum Ch and TAG, and their replacement by PUFA and MUFA have the opposite effects in human and rodents (Keys et al., 1965; Grande et al., 1970; Denke and Grundy, 1992). Some studies have shown the beneficial effects of long chain PUFA n-3 in lowering levels of Ch and TAG in dogs (Bauer, 1995; Wright et al., 2004; Jeusette et al., 2005 a; LeBlanc et al., 2005; Pasquini et al., 2008).

1.2.4.2 Fatty acid chain length

In general, it has been observed that the digestibility of fat decreases with increasing SFA chain length. Long chain SFA, such as palmitic (C16:0) or stearic (C18:0) acids, are not well absorbed from the intestinal lumen as FFA because their melting point is above body temperatures (70 ºC for C16:0 and 77 ºC for C18:0). These fats are crystalline solids at body temperature and form micelles less readily, and the rate of micelle formation is a critical step in determining the rate of lipolysis (Berry and Sanders, 2005). On the other hand, long chain SFA that are not been incorporated in the micelles have a strong tendency to form non-absorbable insoluble soaps with divalent cations, such as calcium or magnesium, at the alkaline pH of the small intestine lumen and, again, decrease the overall fat digestibility of the diet in several animal models (Renaud et al., 1995; Innis et al., 1997; Lien et al., 1997).

Conversely, higher fat digestibilities are found in dogs fed with high proportions of MCFA (Dongen et al., 2000; Beynen et al., 2002) and this might be partly explained by a different absorption process for MCFA (C6:0 – C12:0). These FA are taken up mainly by passive diffusion across the membrane along a concentration gradient, as they are able to cross unstirred water layer.

As a general rule, oxidation of FA for energy increases as the chain length of the FA decreases. Medium chain FA are a preferred source of energy (beta-oxidation; Babayan, 1987). For that reason (shunting FA towards oxidation rather than storage), recent studies confirmed the potential of MCFA to reduce body weight (BW) and particularly body fat in humans and rodents (Geliebter et al., 1983; Noguchi et al., 2002; St-Onge et al., 2003; St-Onge and Bosarge, 2008).

Numerous studies have shown that SFA increase plasma Ch levels in rodents and humans (Keys et al., 1965; Grande et al., 1970; Denke and Grundy, 1992). However, not all SFA have equivalent effects. Lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids raise plasma Ch levels. By contrast, stearic acid (C18:0) does not appear to have such an effect and has been described as “neutral”.

1.2.4.3 Position of fatty acids in the triacylglyceride molecule

There are numerous studies and literature reviews focused on analyzing the effect of the positional distribution of FA in digestion, absorption, and metabolism processes, mainly in rodents and humans (Bergen and Mersmann, 2005; Berry and Sanders, 2005; Linderborg and Kallio, 2005; Mu and Porsgaard, 2005; Karupaiah and Sundram, 2007; Berry, 2009). As already mentioned, pancreatic lipase hydrolyzes FA in the *sn*-1 and *sn*-3 positions of TAG. The positional specificity of pancreatic lipase may be advantageous for the absorption of SFA located in the *sn*-2 position due to a better absorption of SFA as a 2-MAG compared to free SFA, as shown in rat (Tomarelli et al., 1968; Mattson et al., 1979) and human infants studies (Filer et al., 1969; Carnielli et al., 1995). The 2-MAG are more polar molecules than FFA, have a lower melting point in comparison with its corresponding FA (Schulthess et al., 1994; Ho and Storch, 2001), and are easily incorporated into the micelles.

Mattson and Lutton (1958) reported a specific distribution of FA in the TAG of animal and vegetable fats and oils. In plants, it has been demonstrated that palmitic and stearic acids are esterified predominately at the *sn*-1 and *sn*-3 position of the TAG molecule. However, the

sn-2 positioned FA is almost exclusively for unsaturated FA like oleic, LA and ALA. Conversely, in animal fats, the *sn*-2 position of the TAG contains a high proportion of SFA (**Table 1.2**).

Table 1.2. Positional distribution of fatty acids (mol %) in TAG of some common fats and oils.
(from Berry and Sanders, 2005; Berry, 2009)

Fat or oil[1]	TAG or *sn* position	Fatty acid			
		C16:0	C18:0	C18:1n9	C18:2n6
Cocoa butter (POS, SOS, POP)	TAG	24	35	36	3
	sn-1	34	50	12	1
	sn-2	2	2.0	87	9
	sn-3	37	53	9	Trace
Palm oil (POP, POO, POL)	TAG	45	4	38	10
	sn-1	60	3	27	9
	sn-2	13	Trace	68	18
	sn-3	72	8	14	3
Lard (SPO, OPL, OPO)	TAG	26	15	40	10
	sn-1	22	7	50	11
	sn-2	58	1	15	8
	sn-3	15	5	52	12
Beef tallow (POO, POP, PSO)	TAG	26	20	38	4
	sn-1	41	17	20	4
	sn-2	17	9	41	5
	sn-3	22	24	37	5
Human milk (OPO, OPL, PPO)	TAG	27	7	36	11
	sn-1	16	15	46	11
	sn-2	65	3	13	7
	sn-3	6	2	50	15

TAG, triacylglyceride; P, palmitic acid; O, oleic acid; S, stearic acid; L, linoleic acid.
[1] Major molecular species are in parentheses.

Mattson et al. (1979) showed that rats fed TAG with oleic acid instead of stearic acid in the *sn*-2 position decreased stearic acid digestibility. Similar results have been observed for palmitic acid in rats (Tomarelli et al., 1968; Aoe et al., 1997), poultry (Smink et al., 2008), and human infants (Filer et al., 1969; Carnielli et al., 1995; Lucas et al., 1997). In these studies, when animals and humans were fed ingredients with high proportion of palmitic FA

in *sn*-2 position (human breast milk and lard) instead of being in *sn*-1 and *sn*-3 positions (beef tallow, cocoa butter, and palm oil) total fat digestibility and palmitic FA deposition were increased.

Conversely, other authors also reported that stearic and palmitic FA were well digested when in the outer position of the TAG in human studies (Dougherty et al., 1995; Shahkhalili et al., 2000; Baer et al., 2003; Berry et al., 2007). Thus, the effect on the FA position within the TAG is not clear and can vary depending on the type of fat, characteristics of the FA, and species.

Despite the fact that SFA in *sn*-2 positions appear to be better digested, it has been shown that palmitic and stearic FA situated at this position of the TAG slow down TAG lipolysis in the body by LPL in rat studies (Mortimer et al., 1988; Redgrave et al., 1988) and, as a consequence, chylomicrons and chylomicrons remnants are removed from the circulation more slowly than TAG with SFA in *sn*-1 and *sn*-3 positions. The increased amount of SFA in the *sn*-2 position of the TAG could increase the surface rigidity of the chylomicron and hinder LPL action (Yli-Jokipii et al., 2002).

Delayed post-prandial TAG clearance is an independent risk factor for cardiovascular disease in some species. Animal studies, mainly in rabbit models, have shown that palmitic and stearic acids in the *sn*-2 position of TAG may be more atherogenic because of their higher exposure to the aorta even when lipid levels in the blood did not differ (Kritchevsky et al., 1982, 2000 a; b).

Regarding fasting lipid concentrations, animal fats (saturated) resulted in higher Ch level compared to vegetable saturated fats, such as palm oil, in human studies (Zhang et al., 1997). These results agree with those presented by Innis et al. (1993) in piglets. These differences may be explained by the lowest quantity of SFA in the *sn*-2 position in the vegetable oils (10% of total palmitic FA in palm oil). On the other hand, there are studies in rats and rabbits that have reported no significant effects of the positional distribution of palmitic FA on plasma Ch concentrations (Kritchevsky et al., 1982; de Fouw et al., 1994; Renaud et al., 1995), thus, the role of FA position within the TAG on Ch blood levels is still unclear.

1.2.4.4 Monoacylglyceride and diacylglyceride content

Monoacylglycerides and DAG are emulsifying agents widely used in food industry and are obtained through enzymatic (Yang et al., 2005) or chemical (Noureddini and

Medikonduru, 1997) processes. However, very few studies have focused in their possible effect on fat digestibility. A study with broiler chickens concluded that the presence of mono-olein (2-MAG of oleic acid) can increase palmitic acid absorption of FFA present in the gut as a matter of their emulsifying effect (Garrett and Young, 1975). The absorption of palmitic acid increased with oleic acid and mono-olein. Mono-olein was between twice and five times more effective than oleic acid (**Figure 1.7**).

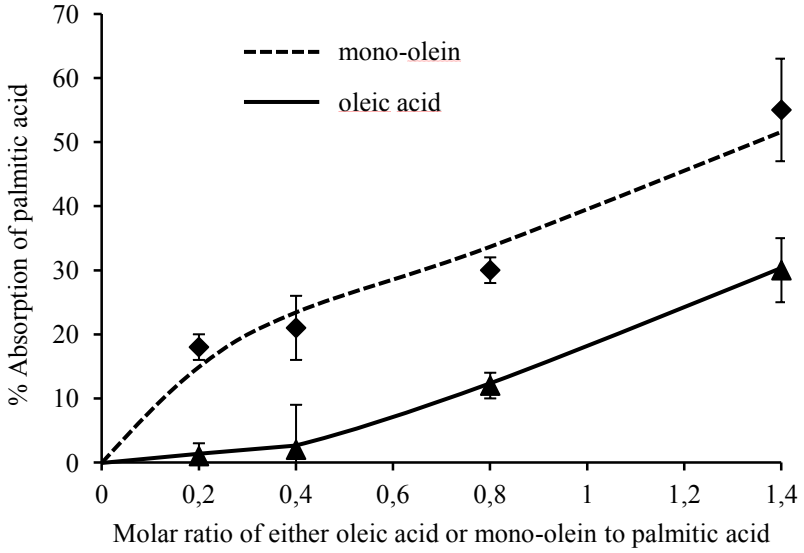

Figure 1.7. Effects of various amounts of oleic acid or mono-olein on the absorption of palmitic acid.
Values are derived from 3 broiler chickens with ligated pancreatic ducts.
(from Garrett and Young, 1975)

Regarding DAG content, recent studies show that DAG can increase beta-oxidation and promote BW loss, depending to the positional distribution of FA in the glycerol molecule (mainly 1,3-DAG) in rodents, humans and dogs (Nagao et al., 2000; Meng et al., 2004; Umeda et al., 2006). After pancreatic lipase action, the main end products of 1,3-DAG are glycerol and FFA, which may be less readily re-synthesized to TAG within the enterocyte compared to the end products of TAG digestion, 2-MAG and FFA. As a consequence, larger amounts of FFA from digested DAG may be released into the portal circulation and lead directly to the liver where they are oxidized for energy.

There are no studies assessing the effect of dietary MAG oils on blood lipids. However, some studies showed decreased fasting and post-prandial TAG concentrations after DAG consumption in humans and rodents (Hara et al., 1993; Yamamoto et al., 2001; Kondo et al., 2003), whereas in others, TAG concentrations remained unchanged (Nagao et al., 2000; Soni

et al., 2001; Maki et al., 2002; Sugimoto et al., 2003). In addition, DAG seems to have no or little effect on serum Ch concentrations in humans and rodents (Yamamoto et al., 2001; Maki et al., 2002; Meguro et al., 2003).

1.2.4.5 Free fatty acid content

It has been established in chickens and pigs that the higher FFA content of a fat, the lower nutritive value, and more so with the more saturated fat and especially in young animals (Wiseman and Salvador, 1991; Blanch et al., 1996; Jørgensen and Fernández, 2000). For example, apparent ME of a fat declines linearly with increasing FFA content and chain length, and increases exponentially with increasing unsaturated/saturated ratio in broiler chickens (Wiseman and Blanch, 1994; Blanch et al., 1996). One possible explanation is that feeding FFA may result in non-absorbable soap formation and the lack of MAG may hinder emulsification, both resulting in incomplete micellar solubilization of FA.

1.3 Dietary fat sources in pet food

Sources of fats in commercial dry diets are usually a combination of animal fat, vegetable oils, and fish oils.

Animal fats are usually rich in SFA and MUFA because animals used in food production are generally fed low fat diets and, as a consequence, their storage fats are the result of *de novo* FA synthesis from carbohydrates. This is always the case in ruminants, where almost all fat is saturated in the rumen. When monogastric animals used in food production are fed high levels of LA (soybean, corn, sunflower, and safflower oils) or ALA (linseed oil) higher percentages of these FA will be found in their fat, like it happens in pigs (Bee et al., 2002; Nuernberg et al., 2005) and poultry (Marion and Woodroof, 1963). Therefore, poultry and pork fat can also contain appreciable amounts of LA and other PUFA, while beef tallow and butter fats contain very little.

Vegetable oils are found in both the seeds (soybean and corn) and, in some cases, the fruit (olive and palm) of plants. Most plant oils; with the exception of palm and coconut oils, contain between 80% and 90% unsaturated FA; animal fats contain between 50% and 60% unsaturated fat. These vegetable oils are mostly rich in LA (corn, safflower, sunflower oils), and some have appreciable amounts of ALA (linseed, but also soybean and canola oils). These 18-carbon essential FA (LA and ALA) are synthesized by terrestrial plants. Marine plants are capable of inserting further double bonds and elongating these FA to produce the longer chain (20 carbons or more) n-3 PUFA. However, plants are not capable of creating very long chain n-6 PUFA from LA. In fact, AA is normally found in fats of animal origin, however commercial sources of AA have been derived from fungus (Gandhi and Weete, 1991; Zhu et al., 2002) or seaweeds (Kumari et al., 2010); whereas n-3 long-chain PUFA are found exclusively in marine sources (fish oils; **Figure 1.8**).

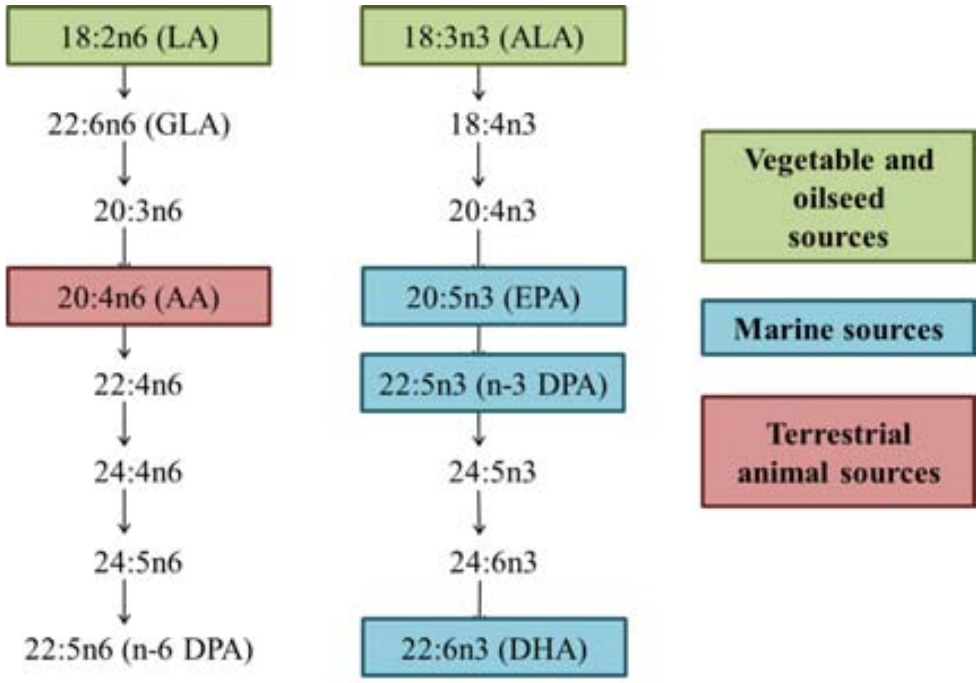

Figure 1.8. Main sources of essential fatty acids.
LA, linoleic acid; GLA, γ-linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. (from NRC, 2006)

1.3.1 Other sources of fat in pet food

The overall FA composition of a finished pet food will ultimately depend on the FA provided by all the ingredients (NRC, 2006). For that reason, it is important to consider fatty protein sources such as eggs, muscle, offal, and meals, typical ingredients used in pet food. In addition, other sources of fat such as conjugated LA (CLA) and medium chain TAG (MCT) have shown several potential benefits in dogs.

1.3.1.1 Conjugated linoleic acid

Conjugated LA is produced in ruminant animals as normal isomerization products of LA or through food processing. Potentially beneficial effects have been associated with one particular CLA isomer. This isomer has been shown to have antiatherogenic properties in rabbit and hamster (Lee et al., 1994; Nicolosi et al., 1997). Moreover, CLA has been reported to provide benefits on body composition in experimental dogs (Schoenherr and Jewell, 1999). However, further research into the use of CLA in pet food diets is needed.

1.3.1.2 Medium chain triacylglycerides

The term MCT refers to mixed TAG of SFA with a chain length between 6 and 12 carbons. The different MCFA found in MCT are hexanoic acid (C6:0; common name capronic acid), octanoic acid (C8:0; common name caprylic acid), decanoic acid (C10:0; common name capric acid), and dodecanoic acid (C12:0; common name lauric acid). There are high amounts of MCFA in some natural sources: coconut and palm kernel oil have more than 50% in weight of FA, and bovine milk have between 6 and 17% in weight of FA (Jensen, 2002).

Distinct chemical properties of MCT compared to long chain TAG (LCT) affect the way MCFA are absorbed and metabolized. There are a high number of studies in this field, as well as a number of reviews in humans and rodents (Bach and Babayan, 1982; Papamandjaris et al., 1998; St-Onge and Jones, 2002; St-Onge, 2005; Marten et al., 2006; Ferreira et al., 2014); however, MCT digestion, absorption, and metabolism are poorly studied in dogs.

After hydrolysis by gastric and pancreatic lipase, the FA from the MCT can be solubilized in the aqueous phase of the intestinal contents after hydrolysis by gastric and pancreatic lipase. Medium chain FA by-pass the re-esterification in the enterocyte, are absorbed bound to albumin, and are transported directly to the liver via the portal vein (**Figure 1.9**). Fat digestibility in dogs fed MCT averaged 95.3% (Beynen et al., 2002). Nevertheless, there is one study that examined the digestion and absorption of MCT in dogs and found no evidence of direct transport via portal venous blood, suggesting that these fats were absorbed in the same manner as LCT (Newton et al., 2000).

If MCT are indeed absorbed by a different route, this may provide benefits in dogs (Remillard and Thatcher, 1989). For that reason, MCT were introduced in veterinary clinical nutrition as a special energy source for dogs with high energy demands or digestive disorders that affect normal fat digestion and absorption, including pancreatic insufficiency and fat malabsorption. Moreover, lipid emulsions containing MCT are available for clinical use to provide total or partial parenteral nutrition to humans and dogs (Ball, 1993; Simoens et al., 2004). In addition, supplementation with MCT has shown long-lasting cognition-enhancing effects in aged dogs (Pan et al., 2010). In spite of those potential benefits, MCT in dogs diets can negatively affect diet palatability (Remillard and Thatcher, 1989) and cause reduced food intake at 22% of dietary ME in the form of MCT (Dongen et al., 2000). However, no food

refusal and no influence on rate of food ingestion in dogs were found at a lower rate of inclusion (11% of ME; Beynen et al., 2002).

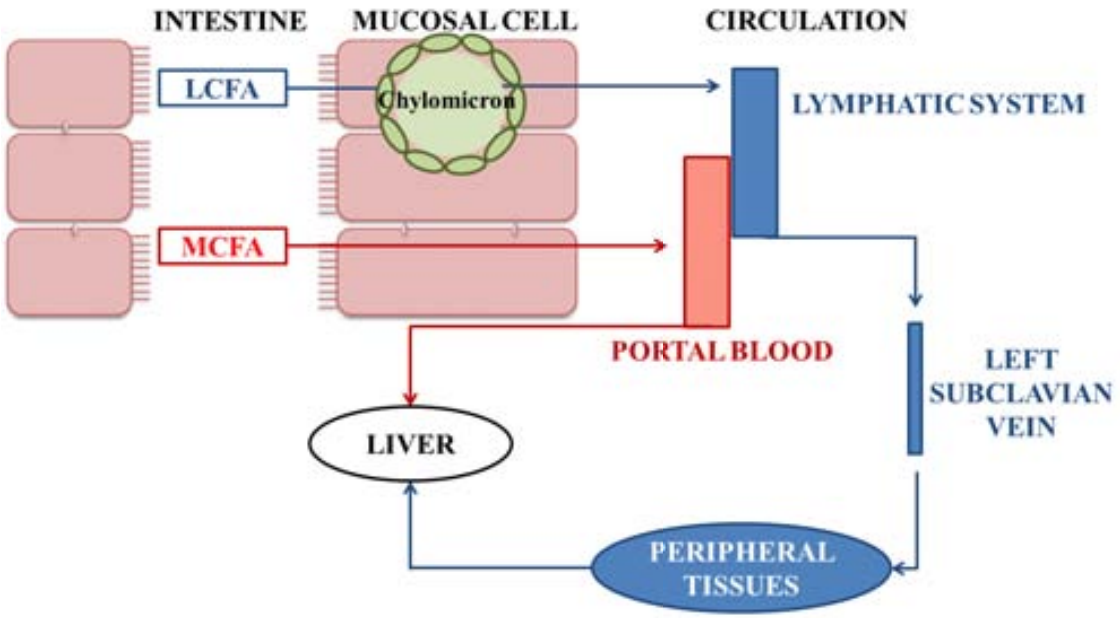

Figure 1.9. Differential medium chain (MCFA) and long chain fatty acids (LCFA) transport.
(from Papamandjaris et al., 1998)

Additionally, MCFA have a high propensity for beta-oxidation because MCFA do not require carnitine palmitoyl transferase for their transport into the mitochondria in hepatocytes. Consequently, oxidation of MCFA is higher than that of LCFA in rodents (Noguchi et al., 2002) and humans (St-Onge et al., 2003). In parallel they also result in increased ketone body production (Tsuji et al., 2001) and hepatic lipogenesis due to the increase in *de novo* FA synthesis (Hill et al., 1990). The minor fraction of MCFA which by-passes the liver is distributed to peripheral tissue via the general circulation.

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In recent years, several studies have focused on the potential role of MCFA for weight management in rodents and humans (Geliebter et al., 1983; Noguchi et al., 2002; St-Onge et al., 2003; St-Onge and Bosarge, 2008). It is generally believed that MCT induce weight loss (WL) secondary to hepatic oxidation of MCFA compared to LCT, which lead to increase energy expenditure. There is no research in this area in companion animals as yet.

1.4 Technical lipids

Technical lipids are defined as fatty products with special characteristics for specific nutritional purposes which, in certain situations, can neither be fulfilled by crude or refined oils nor by non-transformed by-products (Parini and Cantini, 2009; Dijkstra, 2011). Two examples of technical lipids are interesterified fats and esterified acid oils.

1.4.1 Interesterified fats and oils

Nowadays, the possibility of manipulating the TAG structure of native fats has become commercially important in the human food industry (Dijkstra, 2011). Interesterification of fats and oils has become an alternative to the partial hydrogenation of fats, because it increases the melting point of fats without leading to the generation of *trans* FA. Therefore, a fat with new physico-chemical properties is obtained, without changing its unsaturation degree or its isomeric state (Berry and Sanders, 2005; Berry, 2009).

There are two types of interesterification reactions: chemical interesterification and enzyme-directed interesterification. In the process of chemical interesterification, the fat is heated (180 – 240 °C) under a residual pressure of 100 – 300 mbar for a short time, in the presence of a catalyst (usually sodium methoxide 0.05 – 0.15%). The FA are hydrolysed from the glycerol molecule and re-arranged again (Berry, 2009; Dijkstra, 2011). In contrast, enzyme-directed interesterification is used to create TAG with specific positional composition using enzymes with regio-specificity to direct the fatty acid to a specific position within the TAG (Berry, 2009; Dijkstra, 2011).

Interesterification of fats and oils provides suitable functionality of the food industry. On the one hand, this leads to the generation of TAG with different FA distribution. For example, esterification of palm oil can increase the proportion of palmitic acid in the *sn*-2 position. However, in lard, this esterification would decrease the proportion of palmitic acid in the middle position (Berry, 2009).

On the other hand, changes in the positional distribution of FA in TAG may influence the physical properties of fats due to differences in the individual melting point of TAG. Saturated FA-rich TAG can exist in more than one crystalline form in nature. The three basic polymorphic forms are α, β and β'. The α form is the least stable with the lowest melting point, whereas the β has the highest melting point. The β' polymorphic form is commonly generated during interesterification, thus improve the stability and granularity of the fat. For

example, the melting point of α, β' and β of POP (pamitic – oleic – palmitic) TAG molecular species in palm oil are 26.5, 33.5 and 37.2 ºC, respectively (Small, 1991; Berry and Sanders, 2005). In addition, while FA in nature occupy specific positions on the TAG molecule, fat interesterification generates a wide variety of monosaturated, disaturated, and trisaturated each with different melting properties (Berry and Sanders, 2005). The overall effect is the generation of a harder fat. Therefore, the positional distribution of FA in the glycerol molecule could potentially result in a lower digestibility of the interesterified fat, due to the increased proportion of solid fat content at body temperature that could interfere with micelle formation. **Table 1.3** shows the changes in TAG structure and physical characteristics of interesterified fats using cocoa butter as an example.

Table 1.3. Triacylglyceride structure and physical characteristics of native cocoa butter and interesterified cocoa butter.
(from Sanders et al., 2003; Berry, 2009)

	Native cocoa butter	Interesterified cocoa butter
FA in sn-2 position (mol %)		
Palmitic acid	2.90	26.3
Stearic acid	3.70	36.7
Oleic acid	85.6	30.2
Proportion of TAG (% TAG)		
POO	2.20	8.90
SOO	3.10	12.3
POS	44.0	21.0
POP	15.4	15.1
SOS	27.5	12.2
SSP	0.10	8.80
SPP	0.30	7.60
PPP	0.00	2.10
SSS	0.10	3.20
Solid fat content (% solids)[1]		
32ºC	21.1	46.2
37ºC	1.0	37.4
42ºC	<1.0	26.8
47ºC	<1.0	15.8
52ºC	<1.0	7.20

FA, fatty acids; TAG, triacylglycerides; P, palmitic acid; O, oleic acid; S, stearic acid.
[1] Measured using low-resolution nuclear magnetic resonance.

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Salatrim™ and Betapol™ are two examples of interesterified fats that are currently being used in human food industry. Salatrim™ is an interesterified mixture used as a lower-energy fat, due to the presence of MCFA and the position of stearic acid in the outer positions (*sn*-1 and *sn*-3). In contrast, Betapol™ is used in infant feeding to mimic human breast milk fat composition (45% palmitic acid in the *sn*-2 position) and facilitate a greater fat absorption.

1.4.2 Esterified acid oils

Esterified acid oils (EAO) are another type of technical lipids and are manufactured using fatty by-products: acid oils and glycerol.

Fatty by-products are defined as fatty substances which directly derive from primary industrial transformation processes of vegetable oils. Vegetable acid oils, obtained from the chemical and physical refining of the conventional oils (**Figure 1.10**), are economically interesting alternatives for feed and meat producers due to the bio-fuel sector development (Parini and Cantini, 2009). These by-products have a high FFA content and can be added to the diets as is. Nevertheless, as stated before, their energy value is lower than that of the TAG in pigs and broiler chickens (Wiseman and Salvador, 1991; Blanch et al., 1996).

As a consequence of long term strategies for energy production, huge amounts of fatty by-products and waste materials could be available for oleo-chemistry and feed industry to competitive prices. Thus, Parini and Cantini (2009) reported that a quite high and useful amount of acid oils have being produced in the EU and, moreover, their prices (350 – 375 Euros/ton) were slightly lower than the corresponding oil (540 – 600 Euros/ton). The important point that will decide whether the by-product is profitable is the price differential between the conventional oil and the acid oil. According to the Malaysian Palm Oil Board, before October 2009, the discount typically exceeded $200/ton between palm oil and palm acid oil. However, since November 2009, the price differential has narrowed (Cheah et al., 2010).

On the other hand, the glycerol molecule is another by-product from the bio-diesel industry, whose availability has greatly increased in the last few years. The current world glycerol production is about 2.9 million tons, which refers to 10% of total bio-diesel production. Due to the abundant supply of glycerol in the market, in 2007, the price of crude glycerol was about 50 – 80 €/ton (Johnson and Taconi, 2007).

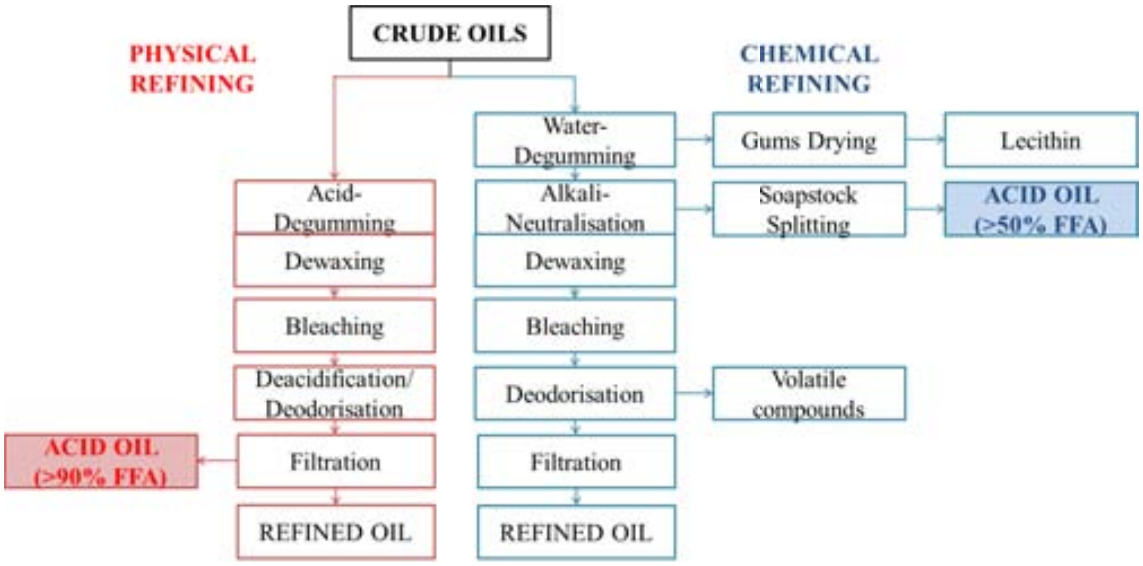

Figure 1.10. Chemical and physical refining of crude oils.
FFA, free fatty acids.
(from Zeldenrust, 2012)

Thus, it is possible to neutralize the FFA of the acid oils by their esterification with glycerol (as a chemical interesterification without previous TAG hydrolysis). This process is called glycerination (Parini and Cantini, 2009). These EAO, similarly to hydrogenated fats, may be used to alter the physical characteristics of fats and are regarded as better alternative to hydrogenated fats because of their low content of *trans* FA.

Furthermore, the amount of MAG, DAG, and TAG of the resulting fat can be fixed during the reaction design, by setting the stoichiometric ratio FA/glycerol (Parini and Cantini, 2009; **Figure 1.11**).

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The EAO used in this PhD dissertation can provide some of the physiological advantages proposed in this literature review, due to their FA position and composition within the glycerol molecule, and their MAG and DAG content. Moreover, re-introducing these by-products in pet food industry would result in a reduction of the amount of residues and would contribute to get sustainable friendly food manufacturing systems.

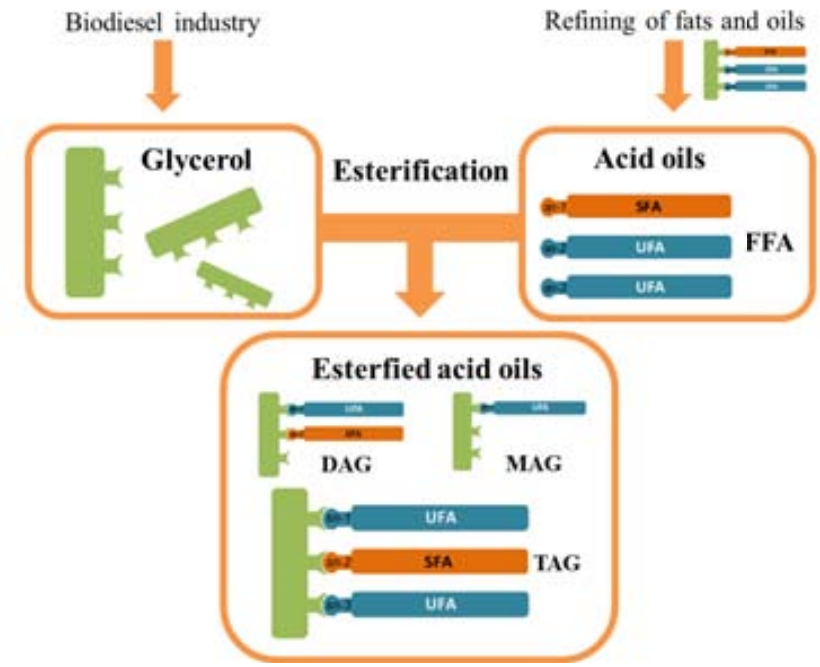

Figure 1.11. Scheme of esterification process between acid oils and glycerol. TAG, triacylglycerides; DAG, diacylglycerides; MAG, monoacylglycerides; FFA, free fatty acids; SFA, saturated fatty acids; UFA, unsaturated fatty acids.

CHAPTER 2:

HYPOTHESES AND OBJECTIVES

The inclusion of fats in pet foods is an effective means to increase energy and palatability and to supply essential fatty acid (FA) in canine foods. Additionally, digestibility, absorption, and metabolism of dietary fat not only depend on their FA saturation degree or chain length, but is also affected by their FA position within the glycerol molecule, and their monoacylglyceride (MAG), diacylglyceride (DAG), and free FA (FFA) content.

On the other hand, the current economic crisis and the imminent re-authorization of processed animal meals to large farmed animals among others have caused that pet food manufacturers look for cheaper and new quality ingredients in formulations of pet foods.

Chemical esterification of acid oils with glycerol generates new technological fats (esterified acid oils; EAO) with different physico-chemical and nutritional properties and potential valuable characteristics for pet food industry. Moreover, introducing these by-products in commercial canine diets can contribute to recycle these residual products in order to avoid environmental contamination. High proportions of FFA are generated from refining oils process, which have a lower cost, but less nutritional value than native oils. Also, large amounts of glycerol are obtained from bio-diesel production, which is another by-product with potential energy value. The resulting fat product could potentially have some benefits that can be used in canine diets as a result of their different physico-chemical characteristics.

The global objective of this PhD dissertation is to **evaluate the use of EAO in adult canine diets**:

- Evaluation of the use of **palm EAO with different proportions of MAG and DAG** for adult canine maintenance diets (**Chapter 3**). It is hypothesized that the inclusion of these EAO will improve digestibility of less soluble fat digestion products, such as SFA. The objectives of this study were:

  1. To compare the palatability of diets containing palm EAO with different proportions of MAG and DAG to diets containing their corresponding palm native oil in adult dogs. *Experiment 1*.

  2. To assess whether the positional distribution of FA within the glycerol molecule and different proportions of MAG and DAG in EAO induce significant changes in the dietary energy value, fat and FA digestibility, and composition of major lipid fractions (triacylglycerides (TAG), DAG, MAG, and FFA) in feces in adult dogs. *Experiment 2*.

3. To test the effects of palm EAO with different proportions of MAG and DAG on post-prandial lipaemia and fasting plasma lipid profile in adult dogs. *Experiment 2*.

- Evaluation of the use of **EAO with different proportions of medium chain FA (MCFA)** for weight loss (WL) adult canine diets (**Chapter 4**). It is hypothesized that the inclusion of MCFA within the glycerol molecule will help reduce fat mass (FM) in adult dogs compared to diets rich in LCFA. The objectives of this study were:

1. To compare the palatability of EAO with different MCFA content from coconut oil with longer unsaturated EAO in adult dogs. *Experiment 1*.

2. To assess whether EAO with different MCFA content from coconut oil induce significant changes in the dietary energy value, fat, and FA digestibility in adult dogs. *Experiment 2*.

3. To assess whether EAO with different MCFA content from coconut oil reduce fat mass compared to longer unsaturated EAO in adult experimentally obese dogs. *Experiment 3*.

4. To test the effects of EAO with different proportions of MCFA on fasting plasma lipid profile in adult experimentally obese dogs. *Experiment 3*.

CHAPTER 3:

EVALUATION OF THE USE OF PALM ESTERIFIED ACID OILS WITH DIFFERENT MONO- AND DIACYLGLYCERIDE CONTENT FOR CANINE DIETS

Summary

Esterified acid oils (EAO) are obtained from the chemical esterification of free fatty acids (FA) and glycerol, by-products of the refinery and the bio-diesel industries, respectively. These EAO have the same FA composition than their native oil, but new physiological characteristics. It is hypothesized that EAO improve the digestibility of certain FA, such as saturated FA (SFA), due to an increase in the presence of SFA at the *sn*-2 position within the glycerol molecule and also due to their increased monoacylglyceride (MAG) and diacylglyceride (DAG) content compared to the native oil. We assessed the effect of including EAO with different MAG and DAG content in dog food on food preferences, digestibility, and plasma lipids. A basal diet was supplemented with 10% as is with 3 different fat sources: PN: palm native oil, PEL: palm EAO low in MAG and DAG, and PEH: palm EAO high in MAG and DAG. The chemical esterification process resulted in a higher fraction of palmitic acid at the *sn*-2 position from 8.0% in PN to 20.4% in PEL and 15.3% in PEH in the experimental oils/diets. Esterification also resulted in the creation of MAG and DAG molecules where the FA were mainly located at *sn*-1,3 positions. Experiment 1 tested food preferences of these EAO enriched diets using a two-pan test procedure. Dogs showed a higher intake score of PEL and PEH compared to PN (PEL: 1.94 ± 0.176 vs. PN: 1.14 ± 0.196, $p = 0.008$; PEH: 2.28 ± 0.168 vs. PN: 1.03 ± 0.178, $p < 0.001$). In Experiment 2, the effects of palm EAO enriched diets on digestibility and blood lipid profile were determined during 4 weeks. No differences were observed among treatments regarding lipid digestibility or post-prandial and fasting blood lipid profile. However, fecal DAG concentrations were higher in both EAO compared to PN.

3.1 Introduction

Due to the current economic crisis and the imminent re-authorization of processed animal meals to large farmed animals (Regulation EU No 56/2013) among others, the search of cheap and new quality ingredients has become an important objective in pet food companies (FEDIAF, 2011). Among the ingredients used in pet food diets, fats are the most concentrated source of energy and contribute both to supply essential fatty acids (FA) and to modify the palatability and texture of food.

Due to reasons of availability and competitive price (Cheah et al., 2010), some fatty by-products obtained from the refining oil processes, such as palm acid oils in the form of free FA (FFA), are interesting ingredients to consider. However, their high saturated FA (SFA) content can affect negatively their energy value, as it has been reported in broiler chickens and pigs (Wiseman et al., 1998). It is possible to give added value to these palm FFA by their chemical esterification with glycerol (a by-product from the bio-diesel industry), obtaining esterified acid oils (EAO). These technical lipids have the same FA composition than their native oil but potentially different physico-chemical properties. In fact, re-esterification processes have actually become an alternative to unsaturated fat hydrogenation in the human food industry, because these processes alter the physical characteristics of fats without leading to the generation of *trans* FA (Berry and Sanders, 2005; Berry, 2009).

On the one hand, it has been reported that chemical esterification process of palm oil increases the proportion of palmitic acid in the triacylglyceride (TAG) *sn*-2 position (Scheeder et al., 2003; Smink et al., 2008). Free long chain SFA have high melting points above body temperature (Small, 1991) and have a high ability to form insoluble soaps with divalent cations in the gut (Carnielli et al., 1995), which result in lower digestibility of these FA. The TAG digestion process results in the formation of FFA and 2-monoacylglycerides (MAG), due to the specificity of pancreatic lipase to act upon *sn*-1 and *sn*-3 positions of the TAG molecule. This, in turn, results in a better absorption of long chain SFA, such as palmitic or stearic acids, as 2-MAG (Renaud et al., 1995; Yli-Jokipii et al., 2001) compared to their FFA counterparts, likely due to their polar nature and the unavailability of these FA to form calcium soaps. Indeed, the human food industry has utilized this process method to mimic human breast milk fat in milk replacers and facilitate a greater absorption of palmitic acid (a SFA) in human infants (Zampelas et al., 1994; Zock et al., 1995; Nelson and Innis, 1999). The effect of the positional distribution of FA within the TAG molecules has been extensively

reviewed by several authors (Bracco, 1994; Hunter, 2001; Mu, 2006; Karupaiah and Sundram, 2007; Berry, 2009).

Additionally, chemical esterification processes generate fats with complex blends of MAG, diacylglycerides (DAG), and TAG. Both MAG and DAG are emulsifying agents widely used in the human food industry and are obtained through chemical (Noureddini and Medikonduru, 1997) and enzymatic (Yang et al., 2005) glycerolysis of fats. Moreover, since MAG, especially 2-MAG, are well absorbed regardless of their constituent FA, these lipid fractions, because their amphiphilic properties, are able to improve fat digestibility and, consequently, the overall digestibility of the diet (Hofmann, 1963; Garrett and Young, 1975). Thus, the use of EAO in canine diets could result in an increased fat digestibility.

In spite of those potential benefits, these EAO could have a negative influence on the post-prandial TAG response. Several studies have suggested that feeding diets with high amounts of SFA in *sn*-2 position slow down TAG lipolysis in the body in rodents and humans (Mortimer et al., 1988; Redgrave et al., 1988; Yli-Jokipii et al., 2002), and, as a consequence, TAG are removed from the circulation more slowly than TAG with SFA predominantly in *sn*-1 and *sn*-3 positions. Thus, it is possible that feeding dogs with EAO enriched diets will show a more sustained post-prandial lipaemia than those fed palm native oil.

Determining the effects of palm EAO on food preferences, digestion, and absorption when included in a canine diet will help define whether EAO are an appropriate ingredient for inclusion in commercial pet foods. The objectives of this research were to examine food preferences of palm EAO compared to palm native oil using two-pan test procedure (Experiment 1) and to determine the effects of EAO on nutrient digestibility, post-prandial and fasting lipaemia, body weight (BW), and body condition score (BCS) (Experiment 2).

3.2 Material and Methods

Experiments. Two experiments were performed in the kennels at the Veterinary School (Servei de Nutrició i Benestar Animal (SNiBA), Universitat Autònoma de Barcelona (UAB), Cerdanyola, Spain), after being approved by the Internal Animal Care and Use Committee of the UAB, to determine the effects of the inclusion of palm EAO in a canine diet on food preferences (Experiment 1), digestion, and absorption (Experiment 2).

Experimental oils and diets. Three different fat sources (**Figure 3.1**; SILO S.p.a., Firenze, Italy) were incorporated into a basal diet via coating of the kibble at the inclusion level of 10% as is. The three dietary treatments were: basal diet with palm native oil (PN), basal diet with palm EAO low in MAG and DAG (PEL), and basal diet with palm EAO high in MAG and DAG (PEH). The characterization of experimental oils and diets is presented in **Table 3.1** and **Table 3.2**.

Figure 3.1. Experimental oils.
PN, Palm native oil; PEL, palm esterified acid oil low in mono- and diacylglycerides; PEH, palm esterified acid oil high in mono- and diacylglycerides.

The lipid fraction composition of experimental oils (% TAG, % DAG, % MAG, and % FFA) was analyzed according to the ISO 18395/2005 method using Agilent 1100 high-performance size-exclusion chromatography with a refractive index detector (Agilent Technologies; Santa Clara, CA, USA), equipped with two Styragel columns (Styragel HR 1 and Styragel HR 0.5) of 30 cm x 0.78 cm i.d. Prior to analysis, the oils were melted and a solution of approximately 10 mg of oil/ml of tetrahydrofurane was prepared. The solution was filtered through a Nylon filter of 0.45 µm and injected to the chromatograph. The mobile phase consisted of tetrahyrofurane at 1 mL/min. The lipid fraction composition of the experimental diets was similarly analyzed and, prior to analysis, the fat was extracted from the experimental foods following the AOAC (2005) Official Method (2003.05).

Additionally, we also analyzed the experimental oils by high-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Bruker, Billerica, MA, USA) as described by Sacchi et al. (1997), in order to distinguish 2-MAG from 1(3)-MAG and 1(3),2-DAG from 1,3-DAG species. These positional isomers can be detected in the area covering 5.3-3.8 ppm, differentiating clearly the H2 protons belonging to 1(3),2-DAG (5.05 ppm), 1,3-DAG (4.03 ppm), 2-MAG (4.88 ppm) and 1(3)-MAG (3.89 ppm) derivatives. The oil samples were dissolved in deuterated chloroform and placed into a 5 mm diameter NMR tubes. Conventional one-dimensional ^{1}H NMR spectra was collected under routine conditions on a 600 MHz spectrometer. All measurements were recorded at 298 K, using a recycle delay of 3 s and 4 scans per sample. After Fourier transformation without any window function and further base line correction, the areas of the selected H2 proton signals of the spectra were quantified by area integration.

The total FA composition of experimental oils was determined by gas chromatography, according to the methylation method described by Guardiola et al. (1994). Briefly, 50 mg of oil were methylated with sodium methoxide (0.5 N), followed by boron trifluoride (20% in methanol), and FA methyl esters were analyzed using an Agilent 4890D gas chromatograph (Agilent Technologies; Santa Clara, CA, USA), equipped with a flame ionization detector and a polar capillary column (SP-2380, 60 m x 0.25 mm i.d., 0.2 μm from Supelco; Bellefonte, PA, USA). Helium was used as the carrier gas. The FA methyl esters were identified by matching their retention times those of their relative standards (Supelco 37 component FAME Mix, Sigma-Aldrich Co.; St. Louis, MO, USA) and quantified by internal normalization. The FA composition and nutrient composition determinations of experimental diets are described below (Experiment 2).

The composition of the FA located at the *sn*-2 position of the experimental oils was determined via the reaction of the oils with pancreatic lipase (from porcine pancreas type II, Sigma-Aldrich Co.; St. Louis, MO, USA) as described in the EU official method (Commission Regulation (EEC) No 2568/91). Afterwards the 2-MAG were separated from FFA and other o-glyceride forms (such as 1(3)-MAG) by thin layer chromatography using 20 × 20 cm Silica gel 60 plates (Merck, Darmstad, Germany) and chloroform: acetone 90:10 as mobile phase. The zone spot of 2-MAG was visualized under UV. Following this step, 2-MAG were scrapped. The FA composition of 2-MAG was determined after obtaining the

corresponding methyl esters by gas chromatography as described above for the global FA oil composition.

The proportion of a particular FA located at the *sn*-2 position (% *sn*-2) was calculated as follows:

% *sn*-2 = (2-MAG, % / Total, %) × *a* × 100,

where 2-MAG is the specific FA composition (% molar) in the *sn*-2 position, Total is the total FA composition (% molar), and *a* is the ratio between the moles of FA located at the *sn*-2 position and the moles of total FA. Thus, an oil composed entirely of TAG has an *a* of 0.33, but our experimental oils had a mixture of TAG, DAG, MAG, and FFA. For this reason, *a* was calculated using the lipid fraction composition of the oil, the average molecular weight (according to the FA composition of the oil), and the glycerol to FA ratio for each molecular species.

Finally, the gross energy (GE) of the experimental oils was measured with an adiabatic bomb calorimeter (IKA-Kalorimeter system C4000, Jankel-Kunkel, Staufen, Germany). Moisture (933.08; AOAC, 2005), impurities (ISO 663/2007), and unsaponifiable matter (926.12; AOAC, 2005) of the experimental oils were also measured.

EAO were obtained from chemical esterification of glycerol and FFA from palm acid oil. According to the stoichometric glycerol to FA ratio, the time, the temperature, and the pressure applied to the reaction, oils with the same FA profile but different FA positional distribution and different TAG, DAG, and MAG proportions were obtained. Thus, while PN experimental oil was mainly comprised of TAG, PEL and PEH had decreasing amounts of TAG and increasing amounts of DAG and MAG (MAG plus DAG were 10.7%, 39.4%, and 76.8% for PN, PEL, and PEH, respectively). In addition, by using ^{1}H NMR, we found that most FA from the MAG and DAG molecules in our experimental oils were located at the *sn*-1,3 positions. Thus, in PEL, the isomeric ratio between 1(3)-MAG and 2-MAG was 86:14, and between 1,3-DAG and 1(3),2-DAG was 67:33. The isomeric ratio in PEH between 1(3)-MAG and 2-MAG was 93:7, and between 1,3-DAG and 1(3),2-DAG was 73:27.

Experimental oils had similar levels of moisture, impurities, and unsaponifiable matter. Regarding the total FA composition of experimental oils, all experimental treatments had a similar FA profile. Palmitic and oleic acids comprised more than 75% of the total FA (44.7 ± 0.52% and 38.0 ± 1.05, respectively). Regarding FA positional distribution, although

chemical esterification did not result in a random distribution of FA over the three positions of the glycerol molecule, this process increased the fraction of palmitic acid located in the *sn*-2 position from 8.0% in PN oil to 20.4% in PEL and 15.3% in PEH. The FA profile of the experimental diets was the same as the experimental oils.

The glycerol: FA ratio was also different among the experimental EAO (0.34, 0.41, and 0.58 for PN, PEL, and PEH, respectively), and this resulted in a different GE content of the experimental oils (9,458 kcal/kg, 9,300 kcal/kg, and 8,955 kcal/kg for PN, PEL, and PEH, respectively). However, the GE of the diets was not as different among treatments (4,552 kcal/kg, 4,538 kcal/kg, and 4,490 kcal/kg for PN, PEL, and PEH, respectively).

Table 3.1. Fatty acid composition, level of *sn*-2 fatty acids, lipid fractions, gross energy, moisture, impurities, and unsaponifiable matter in experimental oils.

	Experimental oils					
	PN		PEL		PEH	
FA profile (%)	*Total*	*% sn-2*[1]	*Total*	*% sn-2*[1]	*Total*	*% sn-2*[1]
C16:0	41.3	7.99	39.7	20.4	39.8	15.3
C18:0	4.57	9.47	8.37	24.4	8.48	18.2
C18:1n9	35.9	50.3	39.0	31.9	39.1	16.8
C18:2n6	11.3	65.3	7.97	34.7	7.66	16.7
Others	6.93	-	4.96	-	4.96	-
SFA	48.1	8.35	51.4	20.5	51.7	15.3
MUFA	38.0	48.4	40.4	31.8	40.5	16.8
PUFA	13.9	61.9	8.20	34.7	7.80	16.8
Lipid fractions (%)						
TAG	84.5		58.8		22.0	
DAG	10.3		33.9		48.9	
1,3-DAG	7.35		22.9		35.8	
1(3),2-DAG	2.95		11.0		13.1	
MAG	0.42		5.55		27.9	
1(3)-MAG	0.28		4.76		25.9	
2-MAG	0.14		0.79		2.04	
FFA	4.78		1.75		1.20	
Glycerol: FA ratio *(mol/mol)*	0,34		0,41		0,58	
Other analytical measurements						
Gross energy *(kcal/kg)*	9,458		9,300		8,955	
Moisture *(%)*	<0.50		<0.50		<0.50	
Impurities *(%)*	<0.50		<0.50		<0.50	
Unsaponifiable matter *(%)*	0.30		1.80		1.55	

FA, fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TAG, triacylglycerides; DAG, diacylglycerides; MAG, monoacylglycerides; FFA, free fatty acids; PN, palm native oil; PEL, palm esterified acid oil (EAO) low in MAG and DAG; PEH, palm EAO high in MAG and DAG.

[1] The proportion of a particular FA located at the *sn*-2 position was calculated as follows % *sn*-2 = (% 2-MAG / Total) × *a* × 100, where % 2-MAG is the FA composition in the *sn*-2 position (mol %, according method by Commission Regulation (EEC) No 2568/91), Total is the total FA composition in the original fat (mol %) and *a* is the ratio between the moles of FA located at the *sn*-2 position and the moles of total FA.

Table 3.2. Fatty acid composition, lipid fractions, and nutrient composition in experimental diets.

	Experimental diets[1]		
	PN	PEL	PEH
FA profile (%)			
C16:0	34.7	32.1	31.9
C18:0	5.34	7.66	7.74
C18:1n9	34.9	35.6	35.4
C18:2n6	18.4	17.2	17.5
Others	6.66	7.44	7.46
SFA	42.0	42.7	42.5
MUFA	37.8	38.7	38.6
PUFA	20.2	18.6	18.9
TFA *(mg/g of food)*	132	127	126
Lipid fractions (%)			
TAG	83.6	69.1	45.3
DAG	9.89	24.4	35.1
MAG	0.36	3.30	17.3
FFA	6.15	3.20	2.30
Nutrient composition (%)			
Dry matter	93.6	94.6	94.7
Ash	9.44	9.76	9.82
Crude protein	22.3	22.9	23.2
Ether extract	13.6	13.3	13.9
Crude fibre	2.16	2.15	1.85
Gross energy *(kcal/kg of food)*	4,582	4,571	4,691

FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TAG, triacylglycerides; DAG, diacylglycerides; MAG, monoacylglycerides; FFA, free fatty acids; PN, basal diet with 10% palm native oil; PEL, basal diet with 10% palm esterified acid oil (EAO) low in MAG and DAG; PEH, basal diet with 10% palm EAO high in MAG and DAG.

[1] Ingredients: corn, poultry meal, whole barley, wheat flour, broken rice, canola meal, hydrolyzed protein, calcium carbonate, sodium chloride, dicalcium phosphate, choline chloride, and vitamin and mineral premix.

3.2.1 Experiment 1

Animals and experimental design. Eighteen Beagle dogs, between 1 and 7 years of age (9 males, 9 females; BW 13.5 ± 0.66 kg and BCS 4.9 ± 0.19) were used in each of three preference tests.

The experiment was designed as a modified two-pan, free choice test (Griffin, 1996), where dogs were able to distribute their intake behavior across two available diets. At the beginning of the assay an adaptation of 4 days was performed as training in order to teach the dogs how to perform the test appropriately. In this training period a commercial maintenance dry pet food was used as a positive dry control (Eukanuba Medium Breed, Coevorden, Netherlands) and the uncoated experimental basal diet was used as a negative control. After the training period, three tests were conducted to determine the preferences of the experimental diets compared one to one (PN vs. PEL, PN vs. PEH and PEL vs. PEH) during 6 days. Dogs were offered 300 g of each test diet for 3 min at the same time each day. At the end of 3 min, any refused or unconsumed food was weighed to determine the amount of each diet consumed. Animals were divided in three groups (n = 6) and rotated among tests in a crossover design. The presentation of the bowls was alternated (left/right) across days and among tests.

Calculations. The intake of each diet (g/3 min) was calculated by subtracting food refusals from the amount of food originally offered and was recorded using an intake score of 0 to 3, namely 0 = 0 g/3 min, 1 = 1-149 g/3 min, 2 = 150-299 g/3 min, 3 = 300 g/3 min. The percentage of preference was calculated by dividing the grams consumed of each diet by the total grams consumed of both diets.

3.2.2 Experiment 2

Animals and experimental design. A total of eighteen Beagle dogs, between 1 and 7 years of age, were used in this experiment. Dogs were separated into three groups as follows: group PN (3 males and 3 females; BW 13.1 ±1.27 kg and BCS 5.0 ± 0.26), group PEL (3 males and 3 females; BW 13.0 ± 1.16 kg and BCS 4.8 ± 0.54) and group PEH (3 males and 3 females; BW 13.1 ± 1.21 and BCS 4.7 ± 0.33). The experimental period was 4 weeks long (30 days). Dogs were fed the experimental diets for 2 and 3 weeks before carrying out the TAG kinetics (day 17) and the digestibility balance (from day 22 to 29), respectively. A fasting plasma lipid profile was determined on day 0 and on day 30.

Animals were fed once a day. Their individual maintenance energy requirements (MER; kcal per day) were calculated following the National Research Council recommendations (NRC, 2006; 132 x (BW in kg)$^{0.75}$). The daily ration was calculated by dividing the MER by the estimated metabolizable energy (ME) density of the diets. The goal was to maintain a stable BW (allowing a BW change of 0 to 1% per week) during all the experimental period. Food intake was controlled daily and food allowance was adjusted weekly by +/- 5% if the dogs had lost or gained weight.

Triacylglyceride kinetics. After the dogs were fed the experimental diets for 2 weeks they were fasted for 18 h. Afterwards, the dogs were fed a single meal accordingly readjusted MER and blood samples (1 mL) were obtained by jugular venipuncture at 0, 1, 2, 3, 4, 5, 9 and 12 h after feeding (n = 6) and collected in tubes lined with heparin (Tubes Lithium Heparin, Aquisel S.L., Spain). The sampling times were decided based on the literature available (Elliott et al., 2011). Plasma was obtained after centrifugation of the blood at 2000 *g* for 10 min and was stored at -20ºC until analysis. Plasma TAG concentrations were measured in each sample using a clinical chemistry autoanalyser (Olympus AU400, Hamburg, Germany).

Digestibility balance. A digestibility balance of each of the experimental diets was performed after the dogs had been fed the experimental diets for 3 weeks, via the total collection method (AAFCO, 2009). Briefly, a marker, iron oxide (Iron (III) oxide purified ≥ 95%, ref. N12342, Sigma-Aldrich Chemical Co., St. Louis, MO) was added to the food the day the balance started (day 22). Fecal collection started with the passage of the first marked (red colored) feces. After 5 days, the marker was added again and fecal collection was finished with the appearance of colored feces. Food samples were taken at the beginning and throughout the digestibility balance and were homogenized, milled and stored at 5ºC until analysis. Fecal samples were frozen, weighed, and homogenized, and a representative sample was then freeze-dried (Kinetic Thermal System: condenser Dura-Dry Model FD2055D0TOO, US), ground, and kept at 5ºC until further analysis. Total tract apparent digestibilities of organic matter (OM), ether extract (EE), FA, and GE was calculated as follows:

Digestibility of X, % = [(X intake, g – X excretion, g) / X intake, g] × 100

Proximate analysis and FA composition of diet and feces. The chemical composition of the diets (**Table 3.2**) was determined according to the following methods of the AOAC

(2005): dry matter (934.01), ash (942.05), crude protein (988.05), and EE (920.39). Hydrolyzed EE and OM were analyzed in the feces. Gross energy was determined in food and feces using an adiabatic bomb calorimeter (IKA-Kalorimeter system C4000, Janke-Kunkel, Staufen, Germany). To calculate digestible energy (DE), the GE digestibility percentage was multiplied by the GE content of each test food. The ME of the experimental diets was calculated from the DE and the crude protein content of the diet according to the NRC (2006) proposed equation:

ME, kcal/kg = DE, kcal/kg – (1.04 × crude protein, g/kg)

The FA content of the experimental diets (**Table 3.2**) and feces was determined by gas chromatography, following the methodology described by Sukhija and Palmquist (1988), using nonadecanoic acid (C:19, ref. N5252, Sigma-Aldrich Chemical Co., St Louis, MO) as internal standard. Briefly, these techniques consist of a direct trans-esterification in which lipid extraction and FA methylation is achieved in only one step. Samples are incubated at 70ºC with methanol chloride and the organic layer is extracted with toluene. Fatty acid methyl esters were analyzed using an Agilent HP 6890 gas chromatograph (Agilent Technologies; Santa Clara, CA, USA), equipped with a flame ionization detector and a polar capillary column (DB23, 60 m x 0.32 mm i.d., 0.25 μm from Agilent Technologies, Santa Clara, CA, USA). Peaks areas are integrated and converted to concentrations (mg/g) by comparison with both internal standards peak areas. Fatty acids were identified by comparing retention times to known standards (Supelco® 37 component FAME Mix, Sigma-Aldrich Chemical Co., St. Louis, MO).

Lipid fraction composition of feces. Lipid fractions in feces were determined according to the ISO 18295/2005 as described above for experimental oils and diets. Prior to analysis, the fat was extracted from the experimental foods. Briefly, about 1 g of lyophilized feces was weighed on a screwed capped glass tube. Then HCl 1N was added and the mixture was stirred for 5 minutes. After this, diethyl ether was added and stirred for 20 minutes. The mixture was centrifuged at 540 *g* for 5 min. The ether phase was transferred to a round bottomed flask. This process was repeated two times. Ether was evaporated by using a vacuum rotator evaporator.

Plasma lipid profile. Blood samples (2 mL) were obtained by jugular venopuncture after an 18-hour fast and collected in tubes with heparin (Tubes Lithium Heparin, Aquisel S.L.,

Spain). Plasma was obtained after centrifugation at 2000 *g* for 10 min and samples were kept frozen at -20ºC until analysis. Plasma total cholesterol (Ch), TAG, high-density lipoproteins (HDL), and low-density lipoproteins (LDL) were determined using a clinical chemistry autoanalyzer (Olympus AU400, Germany).

3.3 Statistical analysis.

Unless otherwise indicated, data in the text are presented as means ± SEM. All analyses were carried out using SAS version 9.2 (SAS Institute INC, Cary, NC, USA). The analysis of intake scores of food preferences (0 to 3) was performed by the General Lineal Mixed Model using a multinomial distribution of GLIMMIX procedure. The model included group (day effect), side of the bowl (left or right), and experimental diet as main effects. Digestibility coefficients, lipid fractions on feces, and plasma lipid profile were analyzed by one-way ANOVA including the treatment as a fixed effect. Data of plasma lipid profile on week 0 was included as a covariate in its analysis on week 4. Both BW and BCS were analyzed with the General Lineal Mixed Model using the MIXED procedure. The model included week and experimental diet as fixed effects and their two-way interaction. Analysis of plasma TAG concentrations was also done with the General Lineal Mixed Model using the MIXED procedure. The model included time of extraction and experimental diets as fixed effects and their two-way interaction. The area under the curve (AUC) of plasma TAG was also calculated and the effect of treatment was tested by ANOVA. Tukey's correction was used for multiple mean comparisons. Alpha level for determination of significance was 0.05. Trends were discussed for an alpha level of 0.10.

3.4 Results

3.4.1 Experiment 1

The intake score average and the percentage of preference for each experimental diet are presented in **Figure 3.2**. The dogs showed a higher intake score of PEL and PEH compared to PN (1.9 ± 0.18 vs. 1.1 ± 0.20, p = 0.008; 2.3 ± 0.17 vs. 1.0 ± 0.18, p < 0.001). There were no differences in intake score when dogs were given the opportunity to choose between PEL and PEH (1.5 ± 0.15 vs. 1.5 ± 0.16, p = 0.874).

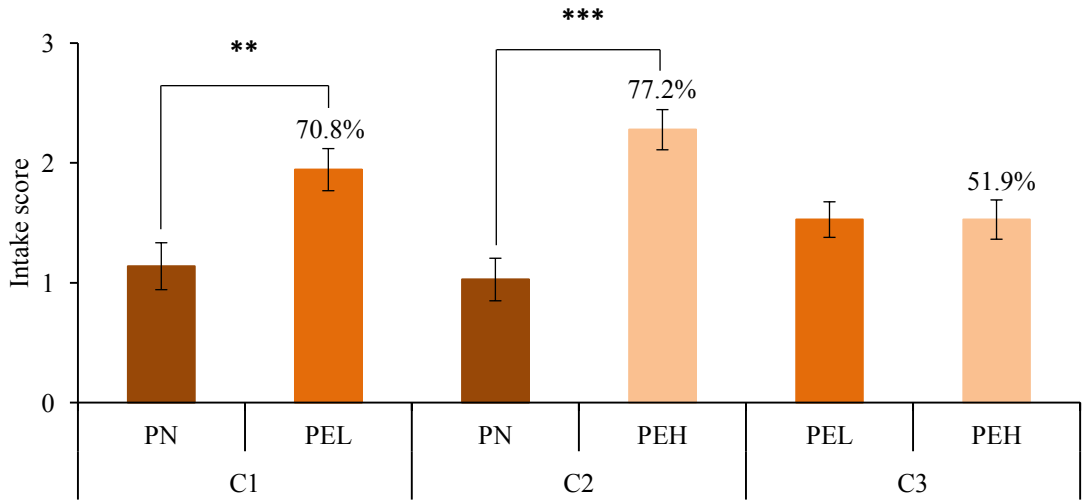

Figure 3.2. Intake score of three diets enriched with native and esterified acid palm oils (PN, PEL and PEH) during a 3 min two-pan test (comparisons 1, 2 and 3).
Numbers in the top of bars indicate the average percent preference for the more preferred diet in each comparison. Asterisks indicate that intakes were significantly different between diets within each comparison (p < 0.01; ***p < 0.001).**

3.4.2 Experiment 2

Body weight and BCS. There were no differences in the weekly weight change rate (0.7 ± 0.16% BW, 0.6 ± 0.26% BW, and 0.8 ± 0.57% BW for PN, PEL, and PEH, respectively, p = 0.978). Subsequently, there were no differences among treatments in BW and BCS throughout the experimental period (p > 0.05). The BW and BCS were 13.0 ± 1.29 kg and 4.8 ± 0.31 for PN, 12.9 ± 1.15 kg and 4.7 ± 0.49 for PEL, and 13.1 ± 25 kg and 4.8 ± 0.17 for PEH at the end of experiment 2.

Post-prandial lipaemia. Dietary treatment had no effect on average TAG concentrations (p = 0.768) and no interaction was found between treatment and time. There were no differences in the TAG AUC among treatments, and the mean values were 63.6 ± 6.12 g/dL/min for PN, 56.2 ± 5.59 g/dL/min for PEL, and 60.5 ± 5.59 g/dL/min for PEH (p = 0.669). There was a time effect (p < 0.001), presented in **Figure 3.3**. Fasting plasma TAG values (47 ± 2.4 mg/dL) increased rapidly after feeding. Peak concentrations of TAG were 102 ± 8.7 mg/dL at 5 h for PN, 97 ± 12.8 mg/dL at 4 h for PEL and 106 ± 17.0 mg/dL at 3 h for PEH. The concentration of plasma TAG was still higher than baseline (p = 0.046) 12 hours post-prandially.

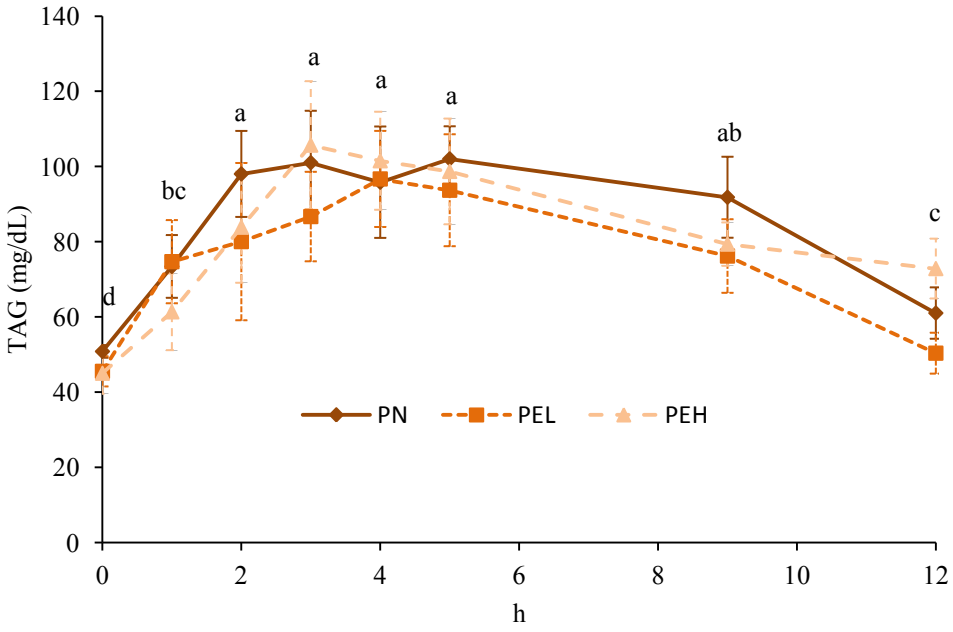

Figure 3.3. Post-prandial plasma triacylglyceride (TAG) concentrations (mg/dL) after the consumption of diets with native and esterified acid palm oils in dogs.
Values are derived from 6 dogs. Time points with no common superscript are significantly different (p < 0.05).

***Digestibility balance*. Table 3.3** shows the nutrient digestibility coefficients obtained from the digestibility balances. There were no differences among treatments for OM, EE and ME digestibility coefficients ($p > 0.05$). There were no differences on FA digestibility among treatments ($p > 0.05$). However, palmitic acid tended to have a higher digestibility coefficient in the PEH group than in the PN group ($p = 0.088$) and polyunsaturated FA (PUFA) digestibility tended to be higher in the PN treatment compared to the PEH treatment ($p = 0.081$).

Table 3.3. Macronutrient and fatty acid digestibility (%) in dogs fed palm oil and palm esterified acid oils.

	Treatments			Statistics
	PN	PEL	PEH	p-value
	(digestibility, %)[1]			
Energy	86.2 ± 0.40	86.0 ± 0.54	85.2 ± 0.52	NS
DE (*kcal/kg*)[2]	4,222 ± 19.8	4,155 ± 26.1	4,110 ± 25.2	NS
ME (*kcal/kg*)[3]	3,974 ± 19.8	3,904 ± 26.1	3,862 ± 25.2	NS
Organic matter	86.1 ± 0.45	86.3 ± 0.51	85.2 ± 0.56	NS
Ether extract	94.6 ± 0.36	93.9 ± 0.27	94.0 ± 0.32	NS
Fatty acids				
Total FA	95.6 ± 0.24	95.9 ± 0.23	95.9 ± 0.33	NS
SFA	94.5 ± 0.38	95.4 ± 0.33	95.5 ± 0.47	NS
C16:0	95.1 ± 0.31	96.0 ± 0.26	96.1 ± 0.41	0,085
C18:0	92.2 ± 0.80	93.7 ± 0.64	93.8 ± 0.75	NS
MUFA	96.8 ± 0.16	96.8 ± 0.15	96.8 ± 0.23	NS
C18:1n9	96.9 ± 0.14	97.0 ± 0.14	96.9 ± 0.21	NS
PUFA	95.6 ± 0.14	95.0 ± 0.20	95.0 ± 0.24	0,070
C18:2n6	95.8 ± 0.15	95.3 ± 0.14	95.3 ± 0.22	NS

DE, digestible energy; ME, metabolizable energy; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PN, basal diet with 10% palm native oil; PEL, basal diet with 10% palm esterified acid oil (EAO) low in mono- (MAG) and diacylglycerides (DAG); PEH, basal diet with 10% palm EAO high in MAG and DAG.

Values are expressed as mean ± SEM of 6 observations per treatment.

[1] Digestibility, % = [(amount ingested, g – amount excreted, g) / amount ingested, g] × 100

[2] DE, kcal/kg = [(GE, kcal/kg × energy digestibility, %) / 100]

[3] ME, kcal/kg = DE, kcal/kg – (1.04 × crude protein, g/kg)

Lipid fraction content on feces. The TAG, MAG and FFA content on feces was not affected by treatments. However, PEL and PEH had a higher fecal DAG content ($p < 0.001$) than PN (**Table 3.4**). In all cases, the fecal concentration of FFA was at least 5 times higher than that of TAG, DAG and MAG.

Table 3.4. Feces lipid fraction (g/kg food intake) in dogs fed native and esterified acid palm oils.

	Treatments			Statistics
	PN	PEL	PEH	p-value
Lipid fractions	*(g lipid fraction/kg food intake)*			
TAG	0.78 ± 0.046	0.81 ± 0.035	0.85 ± 0.044	NS
DAG	0.85 ± 0.075[b]	1.32 ± 0.045[a]	1.31 ± 0.059[a]	<0.001
MAG	0.64 ± 0.056	0.67 ± 0.054	0.67 ± 0.079	NS
FFA	5.61 ± 0.385	5.94 ± 0.266	5.82 ± 0.320	NS

TAG, triacylglycerides; DAG, diacylglycerides; MAG, monoacylglycerides; FFA, free fatty acids; PN, basal diet with 10% palm native oil; PEL, basal diet with 10% palm esterified acid oil (EAO) low in MAG and DAG; PEH, basal diet with 10% palm EAO high in MAG and DAG.
Values are expressed as mean ± SEM of 6 observations per treatment.
Values within the same row no common superscript are significantly different, $p < 0.01$.

Plasma lipid profile. There were no differences ($p > 0.05$) regarding plasma lipid profile among experimental treatments (**Table 3.5**).

Table 3.5. Plasma lipid profile taken of dogs after feeding native palm oil and palm esterified acid oils during 4 weeks.

	Treatments			Statistics
	PN	PEL	PEH	p-value
Cholesterol (*mg/dL*)[1]	222 ± 22.0	202 ± 23.6	245 ± 23.1	NS
TAG (*mg/dL*)[1]	52.3 ± 7.64	45.8 ± 6.07	46.3 ± 6.53	NS
HDL (*mmol/L*)[1]	4.05 ± 0.341	3.80 ± 0.345	4.65 ± 0.224	NS
LDL (*mmol/L*)[1]	1.31 ± 0.190	1.18 ± 0.224	1.50 ± 0.218	NS

TAG, triacylglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PN, basal diet with 10% palm native oil; PEL, basal diet with 10% palm esterified acid oil (EAO) low in mono- (MAG) and diacylglycerides (DAG); PEH, basal diet with 10% palm EAO high in MAG and DAG.
[1] Baseline measurements on week 0 are included as a covariate.
Values are expressed as mean ± SEM of 6 observations per treatment.

3.5 Discussion

To our knowledge, there are no published studies assessing food preferences of acid oils or EAO in dogs. In our study (**Figure 3.2**), we did not find a negative effect of esterification of palm acid oil on palatability and, moreover, EAO enriched diets were consumed in higher amounts compared to native palm oil. However, further studies using a negative control group (diets enriched with acid oil, high in FFA), the use of other vegetable acid oils and animal fats will be necessary to evaluate whether there is a clear benefit on palatability of the use of esterified fats in dogs.

There were no differences for ME, OM, EE, and total FA digestibility among treatments (**Table 3.3**). These results are in agreement with some authors (Smink et al., 2008) in broiler chickens using esterified palm oils at 8% inclusion level, but do not agree with our initial hypothesis and with other studies in broiler chickens (Lin and Chiang, 2010), human infants (Filer et al., 1969), and rats (Tomarelli et al., 1968; Renaud et al., 1995; Lien et al., 1997) using esterified palm oils, lard or human milk replacers. Most of these studies use fats with a higher palmitic acid content at the *sn*-2 position (more than 33%) than the ones used in our study (20.4% for PEL and 15.3% for PEH). Despite not find differences on digestibility among experimental diets, some tendencies were found regarding FA digestibilities. Palmitic acid tended to have a higher digestibility in PEH than in PN, which is in agreement with our initial hypothesis. The positional specificity of pancreatic lipase may be advantageous for the absorption of palmitic acid in the *sn*-2 position due to a better absorption of SFA as a 2-MAG compared to free SFA that have a strong tendency to form non-absorbable insoluble soaps with divalent cations, as shown in rat (Tomarelli et al., 1968; Mattson et al., 1979; Renaud et al., 1995) and human infant studies (Filer et al., 1969; Carnielli et al., 1995). However, this trend was not observed in the PEL treatment, which had a higher amount of palmitic acid in *sn*-2 position compared to PEH, which suggests that the higher amounts of MAG and DAG in PEH might be contributing to this trend, potentially due to their emulsifying effect, able to enhance fat digestion and absorption in broiler chickens (Garrett and Young, 1975). However, it is not possible with our results to clearly differentiate these effects or to know the exact mechanism of action that is in play. Additionally, PUFA tended to be more digestible in PN than in PEH. One explanation for the lower PUFA digestibility could be the loss of *sn*-2 position in PEH relative to the native oil, as shown in broiler chickens (Blanch et al., 1996),

which means that a better SFA digestibility could penalize PUFA digestibility. In any case, the effect, if present, is numerically very low.

Thus, we hypothesize that the percentage of SFA in the *sn*-2 position of dietary glycerides in the EAO enriched diets, were not high enough in magnitude to result in a better fat and FA digestibility, although it is also possible that these EAO do not have the same positive effects on fat digestibility in dogs compared to other species.

On the other hand, the differences in the physical characteristics of the test oils used could also be partly responsible for the lack of effect of EAO in fat digestibility in the current study. In particular, we hypothesize that the experimental oils in PEL and PEH could have a different melting point and thus a different proportion of solid fat at body temperature compared to native palm oil.

One study carried out in our group (Vilarrasa et al., 2013) determined the melting profile by differential scanning calorimetry of palm EAO with different proportions of MAG and DAG, produced in the same manner as palm EAO used in our study. The authors showed that increasing the MAG and DAG content of oils resulted in a larger melting range (they started to melt earlier but finished melting later). Moreover, according this study, the percentage of solid fat of EAO and native palm oil at canine body temperature (39 ºC) was 0.10% for palm native oil (with 13.8% of MAG and DAG), 10.0% for EAO (with 53.7% of MAG and DAG), and 19.0% for EAO (with 83.9% of MAG and DAG). Fats that are crystalline solids at body temperature form micelles less readily, and the rate of micelles formation is a critical step in determining the rate of lipolysis (Berry and Sanders, 2005). Thus, the increase of the solid fat content of the diets could negatively affect fat digestion.

On the other hand, while native palm oil has a fixed FA positional distribution and mainly consists of disaturated TAG (POP with a melting point of 35.2ºC) and diunsaturated TAG (POO with a melting point of 18.2ºC), where P and O represent palmitic and oleic acids respectively (Small, 1991; Lida et al., 2002); the EAO used in this study likely have a higher solid fat percentage at body temperature due to a high content of trisaturated TAG (mainly PPP with a described melting point of 66.4ºC; Small, 1991) and a lower content of the above mentioned disaturated and diunsaturated molecular species (Berry, 2009).

Moreover, some authors have reported that the melting point of TAG with palmitic acid in all its positions is lower (66.4ºC) than the melting points of saturated MAG (2-P and 1(3)-P have melting points of 68.5ºC and 70.5ºC, respectively; Small, 1991; Bracco, 1994) and saturated DAG (1(3),2-PP and 1,3-PP have melting points of 70.1ºC and 74.9ºC, respectively;

Small, 1991; Bracco, 1994). This further suggests that the EAO used in this study might have had a higher solid fat percentage compared to native palm oil.

In addition, the melting points of saturated 1,3-DAG and 1(3)-MAG (with the *sn*-2 position unoccupied) are higher than their respective 1(3),2-DAG and 2-MAG isomers. The EAO used in this study have a large proportion of 1,3-DAG and 1(3)-MAG, which may have contributed to further increase the solid fat percentage of PEL and PEH. The higher fecal DAG concentrations of the EAO treatments compared to PN (**Table 3.4**) are consistent with this hypothesis: it is possible that digestion of DAG has been affected by a relatively higher melting point of the DAG species in PEL and PEH. On the other hand, it is important to note that the FFA content in feces is at least five times higher than the other lipid fractions, suggesting a proper performance of pancreatic lipase to act upon *sn*-1 and *sn*-3 position of TAG, DAG and MAG molecules.

Therefore, the different physico-chemical properties of oils obtained by chemical esterification process, which we hypothesized to be positive, may in fact have negative aspects associated to their melting point upon digestion and absorption that may counteract any expected benefits of the increase of SFA in the *sn*-2 position and the presence of MAG and DAG as potentially emulsifying agents.

To our knowledge, no data were available on the effect of palm EAO on post-prandial lipaemia in dogs. In this study, we hypothesized that dogs fed EAO would show a more sustained post-prandial lipaemia than those fed a palm native oil supplemented diet, as a result of the combination of enhanced absorption and persistence in the circulation of fats with long chain SFA in the *sn*-2 position. The increased proportion of saturated 2-MAG may alter the physical properties of the surface layer of chylomicron or lipoprotein particles, limiting further hydrolysis, and slowing their removal by the liver (Redgrave et al., 1988; Linderborg and Kallio, 2005). However, we did not observe any effect of dietary treatment on post-prandial or fasting TAG concentrations. The results found in the literature in human studies (with fat sources different from palm oil) are contradictory. Similarly to our results, several authors have not found differences in plasma post-prandial lipids of adult men and women after consuming TAG with different FA positional distribution (Zampelas et al., 1994; Summers et al., 1998, 1999). On the other hand, consumption of interesterified fats has resulted in a reduced post-prandial lipaemia in other human studies, and these authors hypothesized that this was a result of a slower absorption rate, in turn due to a high proportion of solid fat content at body temperature (Yli-Jokipii et al., 2001; Tuomasjukka et al., 2009). In

contrast, some studies in humans have shown that feeding interesterified vegetable TAG results in a more pronounced post-prandial lipaemia. In this case, the authors hypothesized that it might be the result of an increased proportion of SFA in the *sn*-2 position, which may be absorbed more efficiently but cleared from circulation at a slower rate than TAG with SFA in the *sn*-1 and *sn*-3 positions (Kubow, 1996; Yli-Jokipii et al., 2002).

Finally, it is important to note that TAG concentrations were still higher than the baseline 12 h after feeding. These results agree with those presented by Elliott et al. (2011), who showed that post-prandial TAG concentrations at 12 h after feeding were at least 1.4 times higher than the baseline concentrations using a diet containing animal fat at an inclusion level of 16% as is. This is very important in the clinical setting, since in order to obtain fasted bloodowork is common to request a 12-hour fast, which might be insufficient if we want to evaluate fasted TAG concentrations in dogs.

Regarding the fasting lipid profile (**Table 3.5**), we did not see any differences among treatments, which might reflect the relatively small percentage of SFA located at the *sn*-2 position in the EAO treatments compared to PN. Studies in rodents have reported no significant effects of the positional distribution of palmitic acid on plasma Ch concentrations (de Fouw et al., 1994; Renaud et al., 1995), whilst piglets fed formula with a 70% of palmitic acid at the *sn*-2 position showed higher plasma total Ch and HDL concentrations than piglets fed similar amounts of palmitic acid, but with <5% at the *sn*-2 position (Innis et al., 1993). Moreover, the effect of interesterified fats on plasma Ch concentrations in humans remains controversial. A study in human infants (Nelson and Innis, 1999) observed higher Ch concentrations in infants fed breast milk with 57% of palmitic acid at the *sn*-2 position than those fed a standard formula with 5% of palmitic acid at the sn2 position. However, Forsythe et al. (2007) reported lower total Ch following palmitic acid-rich diets with 65% of palmitic acid at the *sn*-2 position compared with a diet with 23% of palmitic acid at the *sn*-2 position in adult subjects. Finally, different studies with palmitic acid percentages between 20% and 70% found no differences in total plasma Ch and lipoprotein concentration in adult humans (Nestel et al., 1995; Zock et al., 1995; Meijer and Weststrate, 1997).

Studies in dogs (Umeda et al., 2006), rodents (Meng et al., 2004), and humans (Nagao et al., 2000) suggest an anti-obesity effect by DAG, especially 1,3-DAG, as a result of beta-oxidation and thermogenesis in the small intestine and an increased activity of enzymes involved in the beta-oxidation pathway in the liver. Overweight dogs (Umeda et al., 2006) fed

a diet containing 7% DAG from canola, soy, and safflower oils lost 2.3% of their starting BW over 6 weeks, whereas the control dogs (fed a diet containing 7% TAG from the same fat sources), consuming the same amount of calories, maintained their BW. In our study, there were no differences either on BW or BCS throughout the experimental period. The difference with these findings can be due to the fact that all these authors use unsaturated fat sources, rich in oleic and linoleic acids, while our experimental fat was palm oil, rich in SFA. On the other hand, assessing the effect of DAG in weight management was not an aim of our study, thus, the food allowance of the dogs was readjusted weekly to maintain stable BW and this might have precluded identifying any possible effect of our treatments on BW.

In conclusion, palm EAO coated in kibble at 10% inclusion level is more preferred than palm native oil in adult dogs. The inclusion of 10% EAO in a canine kibble basal diet did not result in any differences on lipid digestibility and post-prandial plasma concentration compared to palm native oil in adult dogs.

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CHAPTER 4:

EVALUATION OF THE USE OF ESTERIFIED ACID OILS WITH DIFFERENT MEDIUM CHAIN FATTY ACID CONTENT FOR WEIGHT LOSS CANINE DIETS

Summary

Esterified acid oils (EAO) are obtained from esterification of vegetable acid oils with glycerol. These fat sources have the same fatty acid (FA) composition than their respective native oil but new physico-chemical properties. Several studies have confirmed the potential of medium chain FA (MCFA) to reduce fat mass (FM) in humans and rodents. This study investigates the use of EAO with different MCFA proportions on food preferences, digestibility, and weight management in dogs. A basal diet was supplemented with 8% of 3 different fat sources: C0: soybean-canola EAO, C20: soybean-canola (80%) coconut (20%) EAO, C40: soybean-canola (60%) coconut (40%) EAO. Medium chain FA provided 2.35% of the metabolizable energy (ME) in the C20 and 3.65% ME in the C40. Experiment 1 tested food preferences of these EAO using a two-pan test procedure. Dogs presented a higher intake of C20 and C40 compared to C0 (C20: 155 ± 18.6 g vs. C0: 17 ± 7.0 g, $p < 0.001$; C40: 117 ± 13.9 g vs. C0: 28 ± 10.5 g, $p < 0.001$). In Experiment 2, digestibility of diets was studied. C20 and C40 showed higher ether extract ($p = 0.009$), total FA ($p = 0.037$), and saturated FA ($p = 0.002$) digestibility than C0 diet. In Experiment 3, 14-weeks weight loss period after induced obesity was investigated. Body weight (BW) reduction was lower (C0: 20.1 ± 2.32%, C20: 14.6 ± 1.43%, and C40: 15.7 ± 1.23%, $p < 0.05$) for diets C20 and C40 than for C0, and FM was higher (18.7 ± 3.42%, 27.9 ± 3.90%, and 28.2 ± 2.88% for C0, C20, and C40, respectively, $p = 0.048$) for diet C40 than for C0. Feeding diets with MCFA at these inclusion levels to experimentally obese dogs during 14 weeks does not result in an improved weight loss compared to unsaturated long chain FA.

4.1 Introduction

It is estimated that the prevalence of obesity and excess body weight (BW) ranges between 22 and 40% of the dog population all over the world (Edney and Smith, 1986; McGreevy et al., 2005; Lund et al., 2006). It is well known that obesity predisposes dogs to a large number of disorders such as: metabolic abnormalities, endocrinopathies, orthopedic disorders, cardio-respiratory disease, functional disorders, and even decrease in lifespan (Kealy et al., 2002; German, 2006). Many nutritional strategies have been developed to promote weight loss (WL) and prevent obesity in dogs. Ultimately, successful WL depends, above all, on inducing a negative energy balance (Laflamme, 2012).

Recently, several studies in rodents and humans have focused in the use of medium chain fatty acids (MCFA), mainly caprylic (C8:0) and capric (C10:0) acids, in weight management (Geliebter et al., 1983; Noguchi et al., 2002; St-Onge et al., 2003; St-Onge and Bosarge, 2008). These MCFA comprise saturated fatty acids (SFA) with carbon chain length ranging from 6 to 12 carbons; and are obtained from natural sources, like coconut or palm kernel oil. Intraluminal absorption of MCFA is theoretically faster and more efficient than that of long chain fatty acids (LCFA); for that reason, MCFA were proposed as a special energy source in various diseases in dogs, including pancreatic insufficiency, fat malabsorption, or even long-lasting cognitive dysfunction (Nelson and Couto, 1992; Pan et al., 2010), although evidence of their efficacy in gastrointestinal disease is lacking. However, the poor palatability of MCFA at high inclusion levels in dogs is considered as a negative point (Remillard and Thatcher, 1989; Dongen et al., 2000).

Furthermore, it has been suggested that MCFA are absorbed via the portal venous system rather than via the lymphatic system and, in contrast to LCFA, may not be incorporated into chylomicrons. Besides, MCFA are a preferred source of energy for beta-oxidation in rodents (Noguchi et al., 2002) and humans (St-Onge et al., 2003), and reduce body fat mass (FM) through down-regulation of adipogenic genes in murine adipocytes (Han et al., 2003). Nevertheless, the potential of MCFA to reduce BW and, particularly, FM has not been studied in dogs.

Esterification of vegetable acid oils with glycerol, both fatty by-products from chemical refining of conventional oils and the bio-diesel industry respectively, generates technical fats with special physico-chemical properties. On the one hand, higher content of diacylglycerides (DAG) and monoacylglycerides (MAG) can be achieved by reacting vegetable acid oils with glycerol whereas native oils are mainly composed of triacylglycerides (TAG). On the other

hand, these technical lipids contain the same fatty acid (FA) composition, but different positional distribution within the glycerol molecule than their native oils.

The aim of this study was to evaluate the use of vegetable EAO with different proportions of MCFA from coconut oil and LCFA from soybean and canola oil on food preferences, digestibility, and weight lost after induced obesity in adult dogs.

4.2 Materials and methods

Experiments. Three experiments were performed in the kennels at the Veterinary School (Servei de Nutricio i Benestar Animal (SNiBA), Universitat Autònoma de Barcelona (UAB), Cerdanyola, Spain), after being approved by the Internal Animal Care and Use Committee of the UAB: food preferences (experiment 1), digestibility trials (experiment 2), and WL after induced obesity (experiment 3).

Experimental oils and diets. Three different fat sources (**Figure 4.1**; SILO S.p.a., Firenze, Italy) were incorporated to a basal diet via coating of the kibble (**Figure 4.2**), at the inclusion level of 8% as is. The three dietary treatments according to their experimental oils were: basal diet with soybean-canola EAO (C0; control diet), basal diet with soybean-canola (80%) and coconut (20%) EAO (C20), and basal diet with soybean-canola (60%) and coconut (40%) EAO (C40).

Figure 4.1. Experimental oils.
C0, soybean-canola esterified acid oil (EAO); C20, soybean-canola (80%) and coconut (20%) EAO; C40, soybean-canola (60%) coconut (40%) EAO.

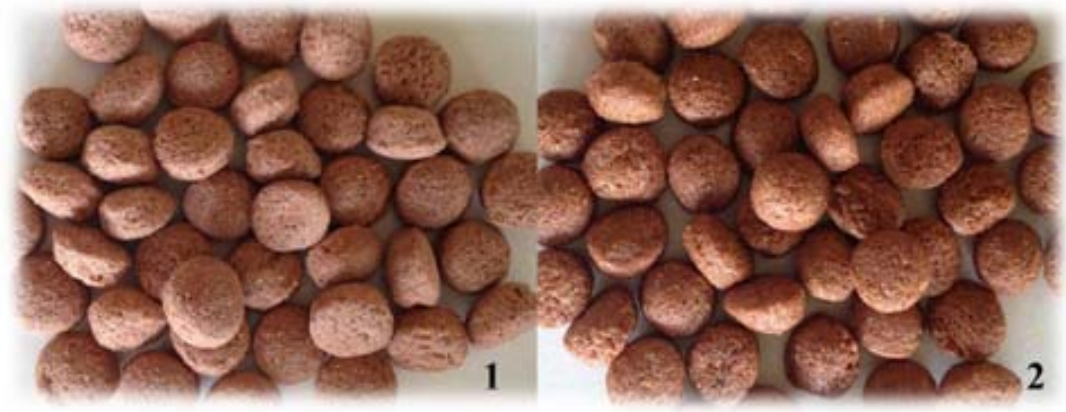

Figure 4.2. Basal diet without additional oil (1) and basal diet coated with added oil (2).

The characterization of experimental oils and diets are presented in **Table 4.1** and **Table 4.2**. The lipid fraction composition (% TAG, % DAG, % MAG, and % FFA) of experimental oils was analyzed according to the ISO 18395/2005 method by size-exclusion chromatography. Moreover, we also analyzed the experimental oils by high-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy as described by Sacchi et al. (1997), in order to distinguish 2-MAG from 1(3)-MAG and 1(3),2-DAG from 1,3-DAG species. The FA profile of the experimental oils was determined by gas chromatography as described by Guardiola et al. (1994) and the *sn*-2 FA composition was analyzed as described in the EU official method (Commission Regulation (EEC) No 2568/91). Moisture was determined according 933.08 method by AOAC (2005), impurities were analyzed according ISO 663/2007, and unsaponifiable matter was determined according 926.12 method by AOAC (2005).

The lipid fraction composition of the experimental diets was analyzed according to the ISO 189395/2005 as exposed above; prior to analysis, fat was extracted from food following the AOAC (2005) Official Method (2003.05). The FA profile and nutrient composition determinations of experimental diets are described below (Experiment 2).

Esterified acid oils were produced by setting the stoichiometric ratio between FA, from vegetable acid oils, and glycerol under fixed pressure, temperature, and time conditions. Due to this esterification process, established amounts of TAG, DAG, and MAG were found in experimental diets. However, these lipid fraction profiles were fixed constant to avoid any variation factor among experimental diets. Thus, lipid fraction profiles and positional isomers were similar among oils and diets (see **Table 4.1** and **Table 4.2**).

Experimental oils had similar levels of moisture, impurities, and unsaponifiable matter. Regarding total FA composition of experimental oils and diets, C0 had higher polyunsaturated fatty acids (PUFA; mainly linoleic acid) and monounsaturated FA (MUFA; mainly oleic acid) content than C40. On the other hand, C40 had higher SFA than C0, due to its content in lauric (C12:0) and miristic (C14:0) acids. C20 presented an intermediate profile. Moreover, the proportion of MCFA located at *sn*-2 position during chemical esterification process was low in both experimental oils from coconut oil (4.52% for C20 and 7.45% for C40).

Table 4.1. Fatty acid composition, level of *sn*-2 fatty acids, lipid fractions, gross energy, moisture, impurities, and unsaponifiable matter in experimental oils.

	Experimental oils					
	C0		C20		C40	
FA profile (%)	*Total*	*sn-2*[1]	*Total*	*sn-2*[1]	*Total*	*sn-2*[1]
C12:0	0.00	0.00	8.94	0.54	17.1	1.64
C14:0	0.15	0.01	3.97	0.50	7.62	1.03
C16:0	9.88	1.17	9.83	1.97	9.81	1.77
C18:0	3.91	0.67	3.54	0.90	3.18	0.74
C18:1n9	36.3	7.43	30.3	6.61	24.9	5.78
C18:2n6	43.9	9.52	36.0	8.08	28.7	7.04
C18:3n3	1.66	0.36	1.45	0.30	1.22	0.28
Others	4.20	-	5.97	-	7.47	-
SFA	15.9	2.28	30.6	3.94	43.8	5.22
MCFA	0.00	0.00	11.7	0.53	21.9	6.19
MUFA	38.4	7.84	31.8	7.09	26.2	7.33
PUFA	45.7	9.91	37.6	8.40	30.0	1.63
Lipid fractions (%)						
TAG	36.6		30.5		25.0	
DAG	46.5		51.1		56.0	
1,3-DAG	33.8		36.5		40.4	
1(3),2-DAG	12.7		14.6		15.6	
MAG	14.6		14.9		13.7	
1(3)-MAG	13.2		13.5		12.5	
2-MAG	1.39		1.43		1.15	
FFA	2.30		3.50		5.30	
Other analitical measurements						
Gross energy *(kcal/kg)*	8,983		8,932		8,831	
Moisture *(%)*	1.07		0.86		0.69	
Impurities *(%)*	<0.50		<0.50		<0.50	
Unsaponifiable matter *(%)*	3.41		3.15		3.30	

FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; MCFA, medium chain fatty acids; TAG, triacylglycerides; DAG, diacylglycerides; MAG, monoacylglycerides; FFA, free fatty acids; C0, soybean-canola esterified acid oil (EAO); C20, soybean-canola (80%) coconut (20%) EAO; C40, soybean-canola (60%) coconut (40%) EAO.

[1] Total profile was multiplied by the proportion of a particular FA located at the *sn*-2 position of the acylglycerol molecule was calculated as follows: % *sn*-2 = (*2-MAG* / Total) × *a* × 100, where *2-MAG* is the FA composition at the *sn*-2 position (mol %), Total is the total FA composition in the original fat (mol %), and *a* is the ratio between the moles of FA located at the *sn*-2 position and the moles of total FA.

Table 4.2. Fatty acid composition, lipid fractions, and nutrient composition in experimental diets.

	Experimental diets[1]		
	C0	C20	C40
FA profile (%)			
C12:0	0.00	6.59	9.76
C14:0	0.59	6.86	8.66
C16:0	14.1	14.0	13.7
C18:0	6.10	6.00	5.80
C18:1n9	34.7	31.3	29.8
C18:2n6	37.9	27.9	25.3
C18:3n3	1.56	1.55	1.33
Others	5.05	5.80	5.65
SFA	22.0	35.4	39.9
MCFA	0.00	7.36	11.1
MUFA	38.0	34.6	32.9
PUFA	40.0	30.0	27.2
Lipid fractions (%)			
TAG	54.7	53.1	56.9
DAG	32.1	33.7	32.1
MAG	9.62	9.09	6.74
FFA	3.58	4.11	4.26
Nutrient composition (%)			
Dry matter	93.4	93.4	92.9
Ash	12.3	12.0	12.1
Crude protein	23.7	23.5	23.7
Ether extract	15.6	15.9	15.6
Crude fibre	2.43	2.66	2.50
Gross energy *(kcal/kg of food)*	4,552	4,538	4,490

FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; MCFA, médium chain fatty acids; TFA, total fatty acids; TAG, triacylglycerides; DAG, diacylglycerides; MAG, monoacylglycerides; FFA, free fatty acids; C0, basal diet with 8% soybean-canola esterified acid oil (EAO); C20, basal diet with 8% soybean-canola (80%) coconut (20%) EAO; C40, basal diet with 8% soybean-canola (60%) coconut (40%) EAO.

[1] Ingredients of basal diet: corn, wheat, poultry meal, poultry fat, hydrolyzed protein, beet pulp, soybean oil, beer yeast, methionine, lysine, salt, and vitamin and mineral premix.

4.2.1 Experiment 1

Animals and experimental design. Eighteen Beagle dogs, between 1 and 8 years of age (10 castrated males, 8 castrated females; BW, 16.0 ± 0.92 kg) were used in each of three preference tests.

The experiment was designed as a modified two-pan, free choice test, where dogs were able to distribute their intake behavior across two available diets. At the beginning of the assay an adaptation of 4 days was performed as training, so the dogs learned to appropriately perform the test. In this training period, a commercial maintenance dry pet food was used as a positive dry control (Eukanuba Medium Breed, Coevorden, Netherlands) and the uncoated basal diet was used as a negative control. After the training period, three tests were conducted to determine preferences of the experimental diets (C0 vs. C20, C0 vs. C40, and C20 vs. C40) during 6 days. Dogs were offered the test foods in excess (500 g of each test diet) for 5 min. At the end of the 5 min, any refused or unconsumed food was weighed to determine the amount of each diet consumed. Animals were divided in three groups (n = 6) and were rotated among tests in a crossover design. Presentation of the bowls was alternated (left/right) across days and among tests.

Calculations. The intake of each diet (g/5 min) was calculated by subtracting food refusals from the amount of food originally offered. The percentage of preference was calculated by dividing the grams consumed of each diet by the total grams consumed of both diets.

4.2.2 Experiment 2

Animals and experiment design. A total of 18 Beagle dogs, between 1 and 8 years of age, were used in three digestibility trials as follows: group C0 (3 castrated males and 3 castrated females; BW, 15.2 ± 1.49 kg), group C20 (4 castrated males and 2 castrated females; BW, 15.5 ± 1.51 kg), and group C40 (3 castrated males and 3 castrated females; BW, 14.4 ± 1.62 kg). The digestibility balances were performed using the total collection method (AAFCO, 2009), where digestibility of organic matter (OM), ether extract (EE), FA, and gross energy (GE) was calculated as explained in Chapter 3.

Controls and sampling. Animals were fed once a day according to their calculated maintenance energy requirements (MER) according to their respective BW (NRC, 2006; 132 x (BW in kg)$^{0.75}$). Food samples were taken at the beginning and throughout the experimental

period, and were homogenized, milled, and stored at 5ºC until analysis. Excreta samples were frozen, weighed, and homogenized, where a representative sample was freeze-dried (Kinetic Thermal System: condenser Dura-Dry Model FD2055D0TOO, US), ground, and kept at 5ºC until further analysis.

Proximate analyses and FA composition of diet and feces. The chemical composition of the diets (**Table 4.2**) was determined according to the following methods of the AOAC (2005): dry matter (934.01), ash (942.05), crude protein (988.05), crude fibre (962.09), and EE (920.39). Hydrolyzed EE and OM were analyzed in the feces. GE was determined in food and feces using an adiabatic bomb calorimeter (IKA-Kalorimeter system C4000, Janke-Kunkel, Staufen, Germany). The FA content of the experimental diets (**Table 4.2**) and the feces was determined by gas chromatography (HP 6890 Agilent, Waldbronn, Germany) following the methodology described by Sukhija and Palmquist (1988), using nonadecanoic acid (C19, ref. N5252, Sigma-Aldrich Chemical Co., St Louis, MO) and nonanoic acid (C9, ref. 73982, Sigma-Aldrich Chemical Co., St Louis, MO) as internal standards.

To calculate digestible energy (DE), the GE digestibility percentage was multiplied by the GE of each test food. The metabolizable energy (ME) of the experimental diets was calculated from the DE and the crude protein content of the diet according to the NRC (2006) proposed equation exposed in Chapter 3.

4.2.3 Experiment 3

Animals. Seventeen Beagle dogs, between 1 and 8 years of age, were used in this experiment. At the start of the experiment mean BW and body condition score (BCS) were 13.9 ± 0.81 kg and 5.7 ± 0.16, respectively. In order to determine the body condition of the dogs, a 9-point BCS system was used (Laflamme, 1997), where a score of 4 or 5 is considered ideal, a score below 4 underweight and a score above 5 overweight.

Experiment design. The experiment was divided in 2 phases. In the first phase (obese), obesity was induced in all dogs by consuming a high-energy diet (Royal Canin Professional Energy 4800, Aimargues, France) *ad libitum* for 16 weeks, at which time their BW and food intakes were stable.

During the second phase (WL), the dogs were randomly divided in three groups where they were fed the experimental diets in sufficient amounts to induce WL. Initially, dogs were fed 80% of their previously measured energy intake (EI) to achieve WL during 14 weeks. The

EI at the end of the obese state was calculated by multiplying the average daily food intake (grams per day) times the ME (kcal/g) provided by the manufacturer. The amount of experimental diet initially offered (80% of initial EI) was calculated with the ME of the experimental diets obtained *in vivo* in Experiment 2.

The BW and BCS were measured weekly. Food intake was determined daily by weighing the food bowls prior to and after feeding. The WL of the dogs was monitored according to an individual caloric restriction protocol (Fundació Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Bellaterra, Spain), where food (and thus, energy) intake was adjusted every two weeks according to the weekly WL rate for each individual dog. This percentage was calculated each week as follows:

WL rate, % = [(Current weight, kg – Previous weight, kg) / Previous weight, kg] × 100

The overall aim was to achieve a WL of between 1 and 2% BW per week. Every two weeks, changes were made to the WL plan, as follows: if the dog had gained or maintained weight, the amount fed was reduced by 10%. If the dog had lost less than 1% BW per week, the amount fed was reduced in 5%. If WL was higher than 2% BW per week, the amount of the experimental diet was increased in 5%. If WL was between 1 and 2% BW weekly, no changes were made to the plan.

Body composition. Fat mass and lean body mass (LBM) were measured prior to (day 0) and after WL (day 98) using a stable isotope dilution method, using a single dose of deuterium oxide (D_2O; 275 mg/kg) as previously described (Burkholder and Thatcher, 1998; Son et al., 1998). Briefly, animals were fasted for 18 h and deprived of water for 2 h before and during the D_2O equilibration period. Blood (5 mL) was obtained before (basal) and after 2 h of D_2O administration (post-injection) by jugular venopuncture, and collected in tubes with heparin (Tubes Lithium Heparin, Aquisel S.L., Spain). Plasma was obtained after centrifugation at 2000 *g* for 10 min and samples were kept frozen at -20ºC until analyzed for D_2O by use of NMR (Bruker Avance 400 MHz). Fat mass and LBM were calculated from the equations proposed by Burkholder and Thatcher (1998), and expressed as % of BW and kg per dog.

Calculations. Total body water (TBW) content was calculated from the equation:

$$TBW, g = [[(D_2O \text{ injected, g}) - [(m_0 - m_1) \times (D_1 - D_0 / 100)]] \times (0.985) \times (18/20)] / (D_1 - D_0) / 100,$$

where m_0 is dog BW (g) immediately before D_2O delivery; m_1 (g) is dog BW at the time of sample collection; D_1 is atom % D_2O in the plasma sample obtained after D_2O administration and equilibrium of D_2O; D_0 is atom % D_2O in the plasma sample before the dose was administered (D_1 and D_0 are obtained from NMR measurements); 0.985 is a correlation for incorporation of D_2O into organic constituent (d_9 t-butyl alcohol), and 18/20 is the correction factor for the difference in molecular weight between D_2O and water.

Percentage of LBM and FM were calculated from measured TBW according to the following formulas, assuming fat-free mass to contain 74.4% of moisture (Ferrier et al., 2002):

$$LBM, \% = (TBW, \% / 0.744)$$

$$FM, \% = (100 - LBM, \%)$$

Blood samples determinations. Basal bloods samples (5 mL) extracted prior to D_2O administration were used to determine hormones, enzymes, pro-inflammatory cytokines, and plasma lipid profile before (day 0) and after WL (day 98).

Plasma butyrylcholinesterase (BCh) was measured using a reported method by Tecles et al. (2000), using butyrylthiocholine iodide as substrate and adapted for an automated analyzer (Olympus AU2700, Germany). Plasma total cholesterol (Ch), TAG, glucose, high-density lipoproteins (HDL), low-density lipoproteins (LDL), and non-esterified fatty acids (NEFA) were determined using a clinical chemistry autoanalyzer (Olympus AU400, Germany). Finally, insulin (Insulin, Canine ELISA, Mercodia AB, Uppsala, Sweden), adiponectin (Human Adiponectin ELISA, High Sensitivity Kit, BioVendor, Laboratorni Medicine), leptin (Multi-Species Leptin RIA kit, Linco Research, Inc., Saint Charles, Missouri), and tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6; Research & Diagnostics Systems, Abingdon, UK) were also measured.

4.3 Statistical analysis

Unless otherwise indicated, data in the text are presented as means ± SEM. All analyses were carried out using SAS version 9.2 (SAS Institute INC, Cary, NC, USA). The analysis of

intake of food preferences (g/5 min) was performed by the General Lineal Mixed Model using the MIXED procedure of SAS. The model included group (day effect), side (left or right), and experimental diet as main effects. Digestibility values and data prior to and after WL obtained for body composition, BW, BCS, plasma lipid profile, BCh, and hormones were analyzed by one-way ANOVA including experimental diet as fixed effect. Data prior to WL was included in the model as covariate in the analysis after WL. A paired t test procedure of SAS was used to compare changes in BCh and hormones prior to and after WL. Percentage of BW lost and EI were analyzed by General Lineal Mixed Model using the MIXED procedure of SAS. The model included week of WL and experimental diet as fixed effects and the two-way interaction. The Fisher exact test was used to compare the proportions of samples positive for TNF-α and IL-6 prior to and after WL. Correlation between FM and BCS was analyzed through the regression procedure. Tukey's correction was used for multiple mean comparisons. Alpha level for determination of significance was 0.05.

4.4 Results

4.4.1 Experiment 1

The average of food intake and the percentage of preference for each treatment are presented in **Figure 4.3**. The two-pan tests showed that dogs presented a higher intake of C20 and C40 compared to C0 (C20: 155 ± 18.6 g vs. C0: 17 ± 7.0 g, $p < 0.001$; C40: 117 ± 13.9 g vs. C0: 28 ± 10.5 g, $p < 0.001$). When dogs were given the opportunity to choose between diets with MCFA in their composition (C20 vs. C40), there were no differences between them (67 ± 14.3 g vs. 85 ± 15.0 g, $p = 0.218$).

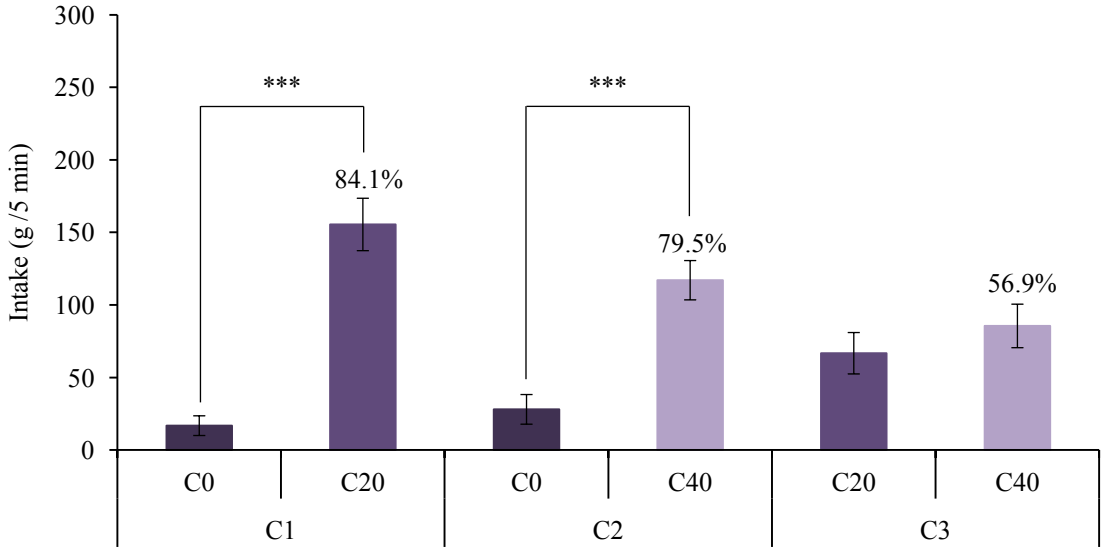

Figure 4.3. Intake (g/5 min) of three diets enriched with different esterified acid oils (C0, C20 and C40) during 5 min two-pan test (comparisons 1, 2 and 3).
Numbers in the top of bars indicate the average percent preference for the more preferred diet in each comparison. Asterisks indicate that intakes were significantly different in each comparison (*p < 0.001).**

4.4.2 Experiment 2

There was no effect of the treatments on OM and GE digestibility ($p > 0.05$). However, C20 and C40 showed higher EE digestibility ($p = 0.009$) than the C0 diet. Concerning the obtained FA digestibility values, the diets with MCFA resulted in higher SFA ($p = 0.002$) and total FA ($p = 0.039$) digestibility compared to the C0 diet (**Table 4.3**). As expected, digestibility values of SFA decrease with increasing SFA chain length. Thus, the highest FA digestibility values were found in MCFA (99.0 ± 0.31% and 98.9 ± 0.28% for C20 and C40, respectively).

Table 4.3. Macronutrient and fatty acid digestibility (%) in dogs fed three diets enriched with different esterified acid oils varying in medium chain fatty acid content.

	Treatments			Statistics
	C0	C20	C40	p-value
		(digestibility, %)[1]		
Energy	80.3 ± 0.46	82.3 ± 0.95	81.4 ± 0.53	NS
DE (*kcal/kg*)[2]	3,912 ± 22.5	4,000 ± 46.4	3,937 ± 22.7	NS
ME (*kcal/kg*)[3]	3,648 ± 22.5	3,738 ± 46.4	3,672 ± 22.7	NS
Organic matter	79.9 ± 0.43	81.5 ± 0.93	80.5 ± 0.61	NS
Ether extract	87.7 ± 0.73[b]	90.6 ± 0.86[a]	90.9 ± 0.39[a]	0.009
Fatty acids				
Total FA	92.8 ± 0.28[b]	94.4 ± 0.61[a]	94.3 ± 0.26[a]	0.039
SFA	91.4 ± 0.34[b]	94.2 ± 0.83[a]	94.6 ± 0.26[a]	0.002
MCFA	-	99.0 ± 0.13	98.9 ± 0.11	NS
C12:0	-	98.9 ± 0.12	98.6 ± 0.10	NS
C14:0	96.9 ± 1.07	96.9 ± 0.12	96.9 ± 0.16	NS
C16:0	91.7 ± 0.31	92.9 ± 0.84	92.6 ± 0.34	NS
C18:0	88.5 ± 0.55	89.8 ± 1.92	89.4 ± 0.65	NS
MUFA	94.2 ± 0.49	95.3 ± 0.58	95.2 ± 0.23	NS
PUFA	92.9 ± 0.16	93.8 ± 0.40	93.0 ± 0.29	NS

DE, digestible energy; ME, metabolizable energy; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; MCFA, medium chain fatty acids.C0, basal diet with 8% soybean-canola esterified acid oil (EAO); C20, basal diet with 8% soybean-canola (80%) coconut (20%) EAO; C40, basal diet with 8% soybean-canola (60%) coconut (40%) EAO.

Values are expressed as mean ± SEM of 6 observations per treatment.

[1] Digestibility, % = [(amount ingested, g – amount excreted, g) / amount ingested, g] × 100

[2] DE, kcal/kg = [(GE, kcal/kg × energy digestibility, %) / 100]

[3] ME, kcal/kg = DE, kcal/kg – (1.04 × crude protein, g/kg)

Values within the same row no common superscript are significantly different, $P < 0.05$.

4.4.3 Experiment 3

Energy intake, BW, and body composition. The BW, body composition via stable isotope, and BCS of the dogs were assessed prior to and after WL. The BCS and percent FM were positively correlated ($p < 0.001$), with a correlation coefficient of 0.91 and a CV of 12.5%. There were no differences in the basal measurements among the dogs assigned to each diet group after obese induction (**Table 4.4**). There was a decrease in FM, LBM, BW, and BCS ($p < 0.05$ for all) after WL in all treatment groups.

At the end of the WL phase (**Table 4.5**), the C0 group had lower kg of FM per dog than the C40 ($p = 0.046$), and there were no differences regarding the kg of LBM ($p = 0.507$). When FM and LBM were expressed as percentage of BW, these differences in FM loss resulted in the C0 group showing a higher % of LBM than the dogs fed C40 ($p = 0.048$) due to the higher loss of FM relative to LBM.

As expected, there were no differences in the weekly WL rate among treatments since those were adjusted bi-weekly (-1.73 ± 0.337% BW, -1.23 ± 0.232% BW, and -1.43 ± 0.297% BW for C0, C20, and C40, respectively, $p = 0.377$). However, the percentage of BW lost was higher in dogs fed C0 than in dogs fed C20 and C40 from week 6 to week 14 as shown in **Figure 4.4**. On week 14 of WL phase, the dogs lost 20.1 ± 2.32% of their initial BW for group C0, 14.6 ± 1.43% for group C20 and 15.7 ± 1.23% for group C40.

Although dogs were initially offered 80% of their previously measured EI to achieve WL, the dogs did not initially ate all the food offered and EI was actually the equivalent to 69.9 ± 6.26% of the calculated *in vivo* ME for group C0, 72.3 ± 3.58% ME for C20, and 71.1 ± 4.44% ME for C40 at the beginning of the WL phase. Afterwards, there were no differences among treatments regarding their EI during the WL phase ($p = 0.687$; **Figure 4.5**).

Table 4.4. Baseline measurements of body composition, body weight, and body condition score in three groups of obese dogs prior to feeding three diets enriched with different esterified acid oils varying in medium chain fatty acid content.

	Treatments			Statistics
	C0	C20	C40	p-value
Body composition				
FM (*%*)	33.4 ± 2.78	35.6 ± 1.77	34.4 ± 3.56	NS
LBM (*%*)	66.6 ± 2.78	64.4 ± 1.77	65.6 ± 3.56	NS
FM (*kg*)	6.03 ± 1.124	6.22 ± 0.793	6.09 ± 1.175	NS
LBM (*kg*)	11.6 ± 1.41	10.9 ± 0.85	11.0 ± 1.05	NS
Characteristics				
BW (*kg*)	17.7 ± 2.35	17.2 ± 1.58	17.1 ± 2.03	NS
BCS	7.10 ± 0.400	7.25 ± 0.423	7.50 ± 0.408	NS

FM, fat mass; LBM, lean body mass; BW, body weight; BCS, body condition score; C0, basal diet with 8% soybean-canola esterified acid oil (EAO); C20, basal diet with 8% soybean-canola (80%) coconut (20%) EAO; C40, basal diet with 8% soybean-canola (60%) coconut (40%) EAO.
Values are expressed as mean ± SEM, where n = 5 dogs for the C0 diet and n = 6 for C20 and C40 diets.

Table 4.5. Measurements of body composition, body weight, and body condition score taken from three groups of dogs after feeding three diets enriched with different esterified acid oils varying in medium chain fatty acid content during 14 weeks.

	Treatments			Statistics
	C0	C20	C40	p-value
Bodycomposition				
FM (*%*)[1]	18.7 ± 3.42^{b}	27.9 ± 3.90^{ab}	28.2 ± 2.88^{a}	0.048
LBM (*%*)[1]	81.3 ± 3.42^{a}	72.1 ± 3.90^{ab}	71.8 ± 2.88^{b}	0.048
FM (*kg*)[1]	2.71 ± 0.744^{b}	4.31 ± 0.860^{ab}	4.32 ± 0.988^{a}	0.046
LBM (*kg*)[1]	11.2 ± 1.47	10.5 ± 0.79	10.2 ± 0.95	NS
Characteristics				
BW (*kg*)[1]	13.9 ± 1.94	14.8 ± 1.34	14.5 ± 1.88	NS
BCS[1]	5.50 ± 0.447	6.25 ± 0.461	6.33 ± 0.357	NS

FM, fat mass; LBM, lean body mass; BW, body weight; BCS, body condition score; C0, basal diet with 8% soybean-canola esterified acid oil (EAO); C20, basal diet with 8% soybean-canola (80%) coconut (20%) EAO; C40, basal diet with 8% soybean-canola (60%) coconut (40%) EAO.
Values are expressed as mean ± SEM, where n = 5 dogs for the C0 diet and n = 6 for C20 and C40 diets
[1] Baseline measurements prior to weight lost (Table 4.5) are included as a covariate.
Values within the same row no common superscript are significantly different, $P < 0.05$.

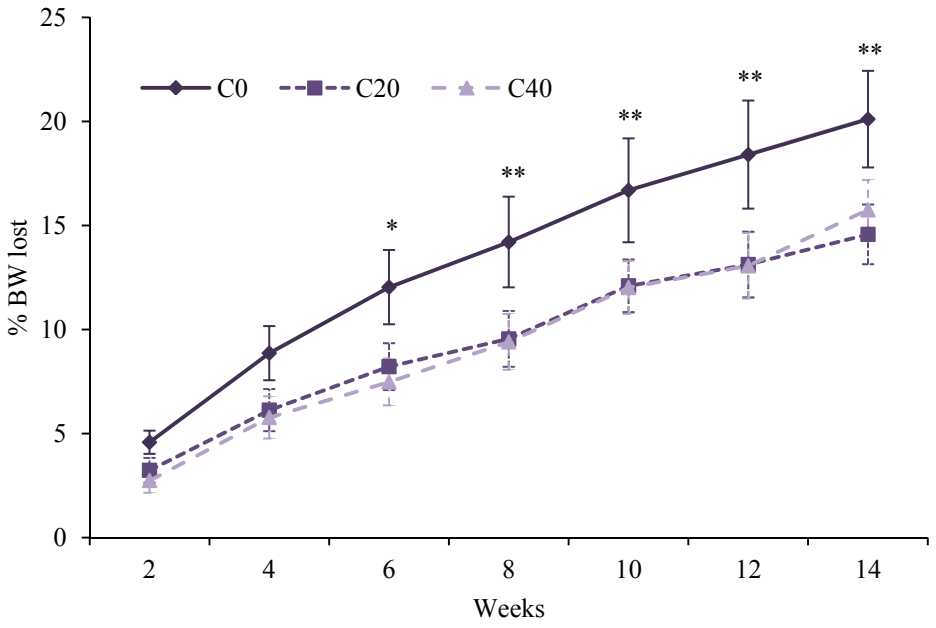

Figure 4.4. Average weekly body weight lost (%) for dogs eating three diets enriched with different esterified acid oils varying in medium chain fatty acid content.
Values are derived from 5 dogs for the C0 diet and 6 dogs for C20 and C40 diets. Asterisks over the top of the points indicate differences on percentage of weight lost between C0 and C40 (*) or between C0 and both diets with medium chain fatty acids (C20 and C40) (**).

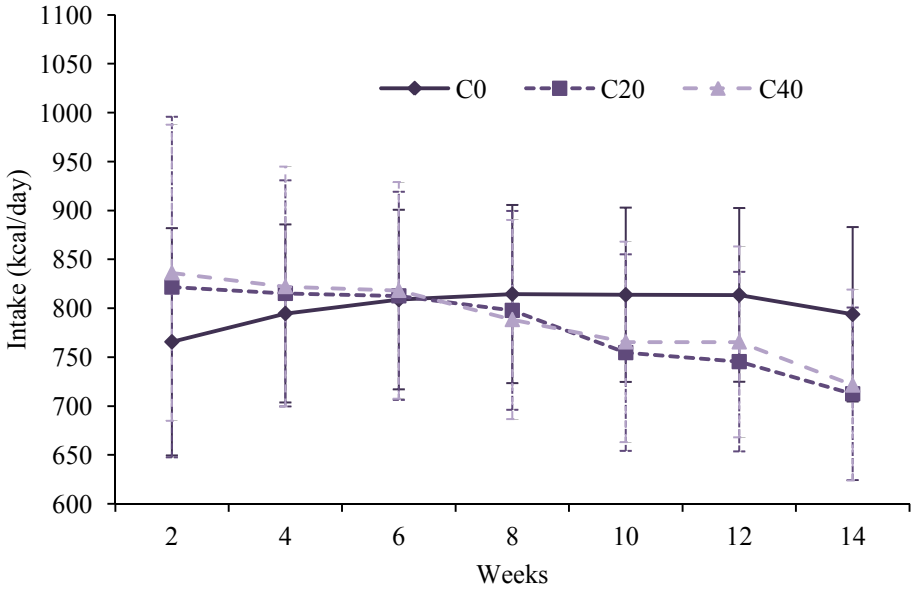

Figure 4.5. Average weekly food intake (kcal/day) for dogs eating three diets enriched with different esterified acid oils varying in medium chain fatty acid content.
Values are derived from 5 dogs for the C0 diet and 6 dogs for C20 and C40 diets.

Plasma analysis of lipid profile, BCh, hormones, and pro-inflammatory cytokines. There were no differences regarding the analyzed plasma lipid profile neither among treatments (**Table 4.6**) nor between days ($p > 0.05$ for all).

Plasma adiponectin, leptin, insulin, and BCh concentrations did not differ among treatments ($p > 0.5$ for all). However, there were some changes in these values after WL. Plasma adiponectin concentration was higher post-WL ($p = 0.032$; **Figure 4.4A**). Plasma leptin was lower post-WL compared to basal values ($p = 0.004$; **Figure 4.4B**). Similarly, plasma insulin concentration was lower post-WL compared to basal values ($p = 0.043$; **Figure 4.4C**). No changes in plasma concentration of BCh occurred with WL ($p = 0.159$). Prior to and after WL plasma BCh concentrations were 4.61 ± 0.273 μmol/ml and 5.29 ± 0.332 μmol/ml, respectively.

Regarding plasma TNF-α and IL-6 concentrations, most of the samples were below the detectable limit of the assay (2 pg/mL and 11.8 pg/mL for TNF-α and IL-6, respectively). Plasma TNF-α was above the detectable limit of the assay in 3 dogs (18%) prior to WL and in only 1 dog (8%) after WL ($p = 0.601$). However, plasma IL-6 concentrations were above the detectable limit of the assay in 3 dogs (18%) both prior to and after WL ($p = 1.000$).

Table 4.6. Plasma lipid profile taken from three groups of dogs after feeding three diets enriched with different esterified acid oils varying in medium chain fatty acid content during 14 weeks.

	Treatments			Statistics
	C0	C20	C40	p-value
Cholesterol (mg/dL)[1]	227 ± 25.0	248 ± 11.1	226 ± 24.8	NS
TAG (mg/dL)[1]	44.1 ± 2.61	46.8 ± 2.48	44.4 ± 4.24	NS
Glucosa (mg/dL)[1]	68.5 ± 5.63	67.8 ± 3.03	58.3 ± 4.16	NS
HDL (mmol/L)[1]	4.31 ± 0.484	4.66 ± 0.251	4.25 ± 0.398	NS
LDL (mmol/L)[1]	1.42 ± 0.201	1.55 ± 0.123	1.41 ± 0.234	NS
NEFA (mmol/L)[1]	0.98 ± 0.189	0.98 ± 0.102	1.08 ± 0.093	NS

TAG, triacylglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NEFA, non-esterified fatty acids. C0, basal diet with 8% soybean-canola esterified acid oil (EAO); C20, basal diet with 8% soybean-canola (80%) coconut (20%) EAO; C40, basal diet with 8% soybean-canola (60%) coconut (40%) EAO.

[1] Baseline measurements prior to weight loss are included as a covariate

Values are expressed as mean ± SEM, where n = 5 dogs for the C0 diet and n = 6 for C20 and C40 diets.

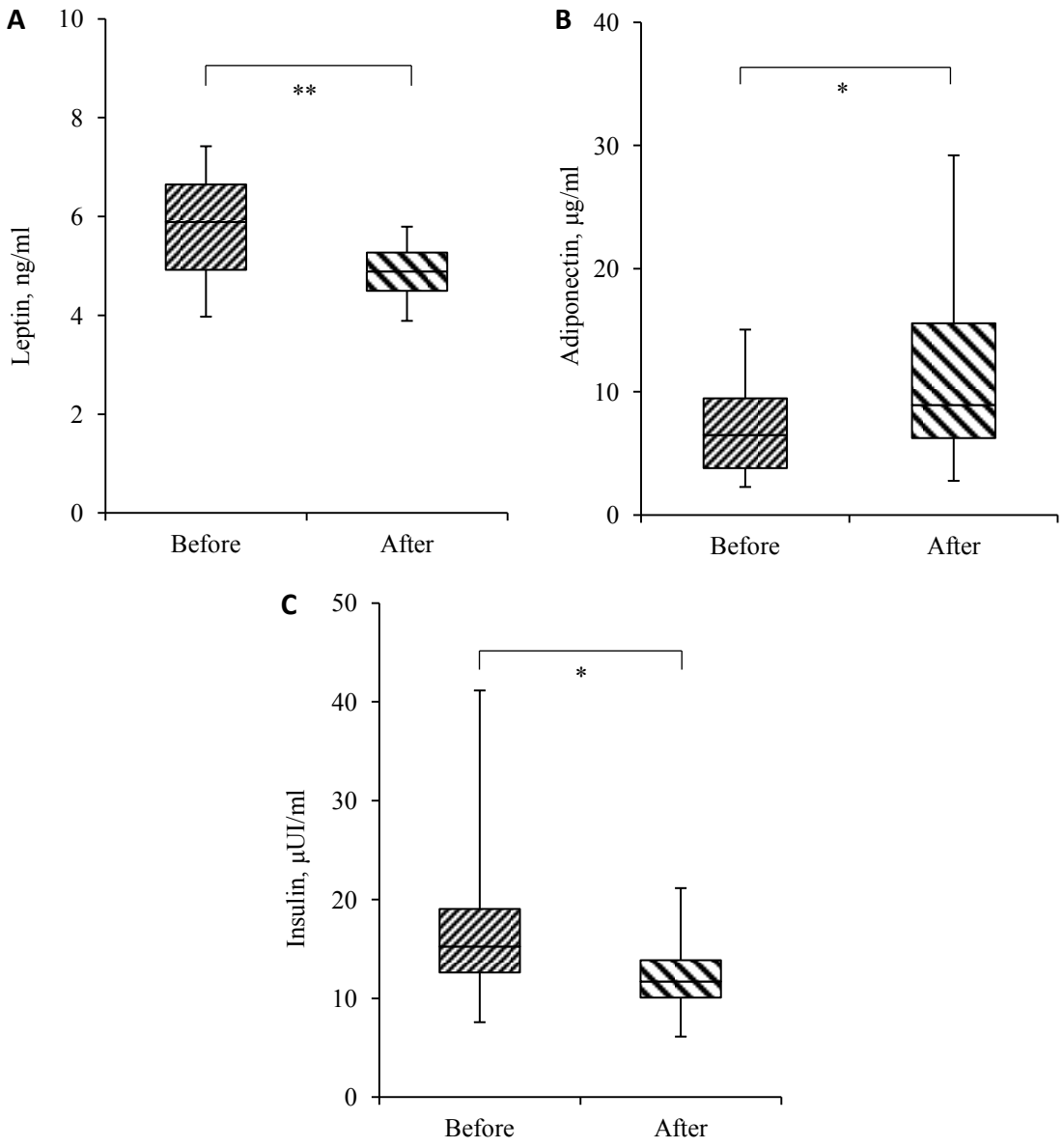

Figure 4.6. Boxplots demonstrating pre- and post-weight loss plasma leptin (A), adiponectin (B), and insulin (C) concentrations in 17 dogs.
Asterisks indicate that intakes were significantly different prior to and after weight loss (*p < 0.05; **p < 0.01).

4.5 Discussion

In previous studies, MCFA have shown decreased food palatability for dogs (Remillard and Thatcher, 1989; Dongen et al., 2000). In the study by Dongen et al. (2000), a reduction in food intake was found feeding dogs with a diet containing 22% of ME in the form of MCFA. However, Beynen et al. (2002) did not find differences in food intake at much lower doses (11% of dietary ME as MCFA). In the present study, dogs fed diets with MCFA from coconut oil showed a preference for vegetable sources from canola and soybean acid oils with longer chain unsaturated FA in its composition (**Figure 4.1**). The amount of MCFA used in our study (2.35% in C20 diet and 3.65% in C40 diet of dietary ME as MCFA) was even lower than the one used in the study by Beynen et al. (2002). Our results suggest that there is no negative effect on palatability of low inclusion levels of MCFA in canine diets, compared to vegetable LCFA. However, we did not compare it to more palatable sources of fat (like chicken or beef fat), and that comparison may have probably yielded different preference results.

A digestibility balance was carried out with the three experimental diets for two purposes. Firstly, it was performed in order to obtain a more accurate ME value of the experimental diets, rather than estimating ME using the chemical composition. Thus, a better caloric intake control was possible during the WL process. Secondly, the digestibility balance was performed to check whether fat digestibility was improved in dogs fed MCFA.

Digestibility of total FA was increased by 1.7% units for C20 and 1.6% units for C40 in comparison with dogs fed the diet containing LCFA. These results agree with those presented by Beynen et al. (2002) in dogs where apparent digestibility of crude fat was increased by 0.9% units when the dogs received the diet containing MCFA. In our study, the improvement in overall fat digestibility in dogs fed MCFA is explained by the improvement in SFA digestibility, explained by the better digestibility of MCFA, mainly C12:0 (98.9% for C20 and 98.6% for C40), versus longer chain SFA, mainly palmitic (C16:0) and stearic (C18:0) acids (**Table 4.3**). Similar improvements in fat digestibility were found in weanling pigs (Cera et al., 1990), broiler chickens (Wiseman and Blanch, 1994), and rats (Vissia and Beynen, 2000) fed with MCFA-rich sources.

Regarding the effect of MCFA in WL, the dogs fed EAO with MCFA in their composition (C20 and C40) lost less BW from week 6 to week 14 compared to C0. Moreover, the animals fed the experimental diet with higher MCFA content (C40) showed higher FM (both in

percentage and in total kg) without changes in kg of LBM at the end of WL. The worse performance of dogs fed MCFA versus the control group (**Figure 4.2**) occurred despite there being no differences in the EI of the different groups during the WL phase (**Figure 4.3**). These results were contrary to our initial hypothesis and are different from results found in other species.

First of all, it is important to mention that most studies analyzing the use of MCFA in dogs (Remillard and Thatcher, 1989; Nelson and Couto, 1992; Dongen et al., 2000; Beynen et al., 2002; Pan et al., 2010) and its use in weight management in rodents and humans (Geliebter et al., 1983; Noguchi et al., 2002; St-Onge et al., 2003; St-Onge and Bosarge, 2008) actually use purified medium chain TAG (MCT). These compounds contain almost exclusively C8:0 and C10:0 from coconut or palm kernel oils. In our study, almost 90% of MCFA content was composed by C12:0. This fact may have a direct impact on subsequent metabolism of experimental diets used in this study. For example, higher gains were observed in weanling pigs fed with coconut oil (>32% C12:0) compared with those fed diets contained roasted soybean (>98% LCFA, 80% as unsaturated FA) or MCT (45% C8:0 and 21% C10:0 acid) at 7.75% added fat inclusion level (Cera et al., 1990), suggesting that C12:0 from coconut oil might enter the peripheral blood circulation largely in esterified form in the lymph following absorption rather than via direct portal absorption (Cera et al., 1990). Moreover, there is one study that suggests there is no direct transport via portal venous blood using MCT (60.2% C8:0 and 27.5% C10:0) at 5% inclusion level in dogs (Newton et al., 2000).

Since the experimental diets had the same ME content, the same inclusion level of fat, and the same lipid fraction composition, another explanation for the lower fat deposition in the C0 treatment could be the substitution of unsaturated (mainly PUFA) for SFA among experimental diets. The ratio of unsaturated FA to SFA was higher than 3 in C0 (3.53) and lower than 2 in C20 (1.82) and C40 (1.50). Some studies in broiler chickens (Sanz et al., 1999, 2000; Crespo and Esteve-Garcia, 2001, 2002 a; Ferrini et al., 2008; Wongsuthavas et al., 2008; González-Ortiz et al., 2013) and in rats (Mercer and Trayhurn, 1987; Shimomura et al., 1990; Takeuchi et al., 1995) have observed a lower fat content in animals fed diets rich in PUFA using sunflower oil (Sanz et al., 1999, 2000; Ferrini et al., 2008), linseed oil (Crespo and Esteve-Garcia, 2001, 2002 a; González-Ortiz et al., 2013), soybean oil (Wongsuthavas et al., 2008), and safflower oil (Shimomura et al., 1990) compared to SFA sources such tallow or lard. There are some hypotheses regarding this fat lowering effect of PUFA. Some of these studies suggest that PUFA cause a higher beta-oxidation of fats (Sanz et al., 2000; Crespo and Esteve-Garcia, 2002 b), others suggest that PUFA decrease hepatic lipogenesis (Sanz et al.,

2000), and others propose a higher diet-induced thermogenesis by unsaturated oils (Mercer and Trayhurn, 1987; Takeuchi et al., 1995). In spite of these results in broiler chickens and rats, Realini et al. (2010) did not find differences in fat deposition in pigs fed diets rich in PUFA (sunflower oil or linseed oil) with respect to SFA (tallow).

On the other hand, since the C0 differs from the C20 and the C40 diets not only in chain length but also in chain saturation, our results might have been different if we had used a source of saturated LCFA (such as palm oil) as control.

Numerous studies have shown that SFA increase plasma Ch and TAG levels in rodents and humans (Keys et al., 1965; Grande et al., 1970; Denke and Grundy, 1992). In these studies, C12:0, C14:0, and C16:0 increased plasma lipids in a greater magnitude compared to C18:0. Coconut oil (>32% C12:0) resulted in elevated serum TAG concentrations compared to corn oil (57% linoleic acid) or MCT (45% C8:0 and 21% C10:0) in weanling pigs (Cera et al., 1990). Moreover, the feeding of MCFA at 22% of ME has been showed to raise plasma TAG in dogs (Dongen et al., 2000), but no hypertriglyceridaemic effect was found at much lower doses (11% of ME) compared to corn oil (Beynen et al., 2002). Although the FA present in the C20 and C40 treatments could potentially have higher hyperlipidemic effects compared to C0, according to the studies in other species, this was not observed in this study (**Table 4.6**), likely due to the low doses of MCFA used.

A number of hormones, cytokines, and enzymes were also analyzed in the current study as markers of adiposity. This study did not show any differences among experimental treatments after WL. However, we were able to document alterations prior to and after WL in some of these substances, showing that they are indeed affected by changes in adiposity (**Figure 4.6**).

Adiponectin concentration increased after WL as reported by other authors in dogs (Ishioka et al., 2006; Tvarijonaviciute et al., 2012 a; b). The plasma concentrations observed in the current study agree with those found in other studies (Ishioka et al., 2006; German et al., 2009; Tvarijonaviciute et al., 2012 a; b), although other authors have reported much lower serum concentrations (0.85 to 1.5 μg/ml). Unlike in our study, other authors did not find an effect of obesity on adiponectin concentrations in dogs (German et al., 2009; Verkest et al., 2011 a; Wakshlag et al., 2011). These differences among studies may be related with the involvement of high and low adiponectin molecular weight multimers. Unfortunately, these protein complexes were not measured in the current study and they may differ in importance in development of obesity associated consequences (Verkest et al., 2011 b).

In the current study, obese dogs had higher plasma leptin concentrations compared with lean dogs. Higher leptin concentrations observed in obese dogs reflect a higher body FM (Ishioka et al., 2002; Jeusette et al., 2005 a; Grant et al., 2011; Verkest et al., 2011 a; Wakshlag et al., 2011). Plasma leptin concentrations obtained were of a magnitude equivalent to those found with experimentally obese dogs (Ishioka et al., 2002, 2006). Chronically obese dogs also present higher leptin concentrations compared to lean dogs (Ishioka et al., 2002; Jeusette et al., 2005 a; Grant et al., 2011; Verkest et al., 2011 a; Wakshlag et al., 2011). Regarding plasma insulin concentrations, our findings agree with some (Jeusette et al., 2005 b; German et al., 2009), but not all (Diez et al., 2004), previous.

Studies in humans (Calderon-Margalit et al., 2006; Iwasaki et al., 2007) and dogs (Tvarijonaviciute et al., 2010) have shown associations between BCh and adiposity. Obese animals have an incremental flux of NEFA from adipose tissue. This discharge stimulates the hepatic synthesis of plasma BCh in humans (Cuauianu et al., 2002). However, mobilization of NEFA from adipocyte following energy restriction may explain the lack of difference on plasma BCh prior to and after WL.

There are several cytokines released from adipose tissue in obese subjects that contribute to chronic inflammation in obesity, such as TNF-α and IL-6 (Trayhurn and Wood, 2005). German et al. (2009) have shown that naturally occurring obesity is associated with increases of circulating level of TNF-α. In the present study, we found TNF-α and IL-6 concentrations below the detection limit of the assay in obese dogs. In our study, dogs were obese induced through feeding a high calorie diet *ad libitum* during 16 weeks. It is probable that short time obesity, as opposed to natural chronic obesity, does not result in an inflammatory state due to excess of adipose tissue. However, there is one study that demonstrates increase in TNF-α in dogs made experimentally obese through overfeeding (Gayet et al., 2004).

As mentioned before, successful WL depends, above all, on creating a negative energy balance in the patient. Therefore, a low calorie food should be considered. Furthermore, an important goal for WL is to promote fat loss while minimizing loss of LBM, which may be influenced by dietary composition. Thus, some nutritional strategies have been considered in dog food formulated for WL to achieve the described goals: increase dietary protein to minimize LBM loss (Hannah and Laflamme, 1998; Diez et al., 2002; Bierer and Bui, 2004), increase dietary fibre to reduce energy density and potentially promote satiety (Jackson et al., 1997; Jewell et al., 2000; Bosch et al., 2009), and include other compounds such as DAG (Umeda et al., 2006) that have shown beneficial effects on WL in dogs. In our study, DAG

and its positional isomers (1(3),2-DAG and 1,3-DAG) were maintained constant among diets to avoid any effect on WL.

To our knowledge, there are no published studies in dogs assessing the effect of dietary FA profile on body composition after WL. In addition, our results indicate that incorporation of FA from coconut into a dry dog food at our inclusion levels increases fat digestibility, does not have a negative effect on palatability, negatively affects the percentage of BW lost during a WL process compared to unsaturated LCFA, and increases body FM after WL in comparison to longer FA from canola and soybean oils. However, as chain length and degree of saturation might affect WL in dogs, further research is needed to clearly differentiate these effects.

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CHAPTER 5:

GENERAL DISCUSSION

5.1 Food preferences

Chapter 3, Experiment 1; Chapter 4, Experiment 1

According to the food preferences analyzed on this Thesis, palm esterified acid oils (EAO) are more preferred than native palm oil (**Chapter 3; Experiment 1**) and EAO with medium chain fatty acid (MCFA) from coconut oil on their composition are more preferred than longer unsaturated fatty acids (FA) from canola and soybean oil (**Chapter 4; Experiment 1**). It is unknown at this time why palm EAO would be more palatable than native palm oil, or why MCFA from coconut are preferred over LCFA from vegetable oils. However, we did not compare EAO to a negative control (acid oils) or to an animal fat, which is the most common form of added fat in pet food and, arguably, will be more palatable to dogs than vegetable oils such as palm, canola or soybean oils. This would give us more data regarding the usefulness of esterification compared to animal fats currently used in pet food industry.

In a preliminary study to validate the two-pan test, the experimental diets used in **Chapter 3** were compared to a maintenance commercial pet food with poultry fat in its composition (Brekkies Excel Complet Chicken and Rice, Barcelona, Spain). As expected, dogs showed a very clear preference for the commercial pet food (**Figure 5.1**). However, the pet foods compared not only differed in their fat source, they also differed in their composition, chemical analysis, and processing. For this reason, we cannot draw any conclusions, but these results warrant further comparisons between EAO and animal fats.

The two-pan tests used to assess food preferences in this Thesis allowed us to estimate palatability but does not account for satiety effects or interactions among foods (Griffin et al., 1984; Araujo and Milgram, 2004). Additionally, Beagle dogs are not an optimal breed for the preference analysis in comparison to another breeds, due to their voracious feeding behavior (Smith et al., 1983).

It is also important to note that we used a different feeding protocol in each chapter which resulted in a different statistical analysis. Concerning food preferences of palm oils (**Chapter 3; Experiment 1**), the animals were fed only 300 g of food and, as a consequence, most of the dogs ate all the food in the allotted time. Therefore, the data did not follow a normal distribution because of censorship of the data occurred in one of the tails of the Gaussian distribution. For this reason, the intake of each diet was recorded using an intake score and was analyzed using a multinomial distribution of GLIMMIX procedure of SAS in this study.

To correct for this, the tested foods during the preference studies in **Chapter 4** were offered in excess (500 g), thus allowing us to obtain normally distributed data.

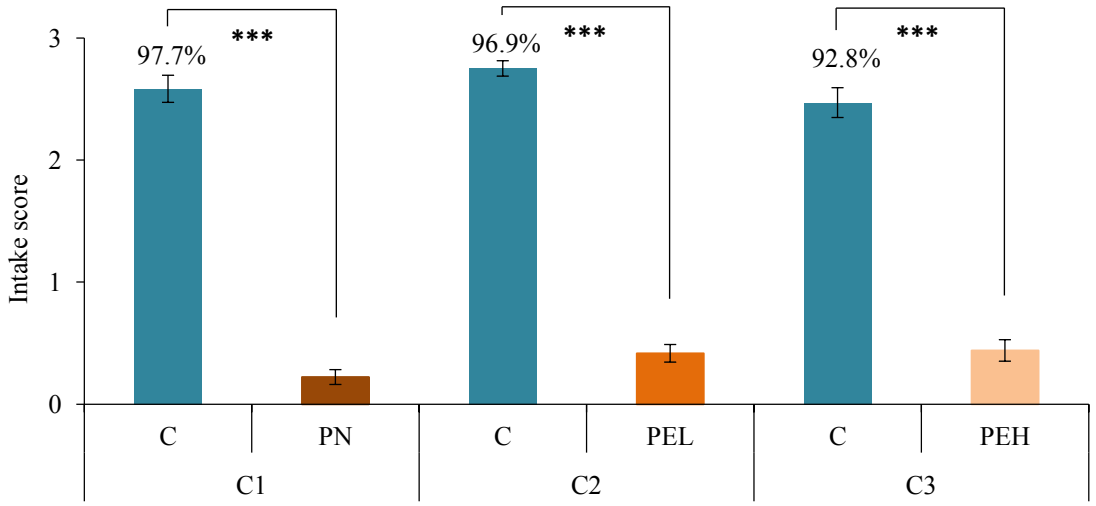

Figure 5.1. Intake score of three diets enriched with native and esterified acid palm oils (PN, PEL and PEH) versus a control diet with poultry fat (blue bars) during 3 min two-pan test (comparisons 1, 2 and 3).
Numbers in the top of bars indicate the average percent preference for more preferred diet in each comparison. Asterisks indicate that intakes were significantly different in each comparison (*p < 0.001).**

5.2 Digestibility

Chapter 3, Experiment 2; Chapter 4, Experiment 2

The apparent fecal digestibility obtained for organic matter (OM), energy, and crude fat in both experiments appears to be comparable to those usually found in dry extruded canine foods. As expected, unsaturated FA were better digested than saturated FA (SFA) and the digestibility of SFA increased with decreasing chain length, as it has been reported in poultry and pigs (Blanch et al., 1996; Duran-Montgé et al., 2007).

However, fat digestibility of EAO from soybean, canola, and coconut oil (unsaturated and short-SFA) was not higher than digestibility from palm oils, both native and esterified. This was unexpected, since palm oil has a higher amount of long chain SFA, which are overall less digestible. A possible explanation lies on the moisture, impurities, and unsaponifiable matter (MIU) values found in the experimental oils from **Chapter 4**, which were above the recommended 2% for oils and fats used as ingredients in farmed animal diets (FEDNA, 2010), and at least twice higher than MIU values of experimental oils from palm oil (**Chapter 3**). These high MIU values may have impaired their digestibility, as it has been found in

weanling pigs (DeRouchey et al., 2004). However, given that experiments were conducted at different times, another factors such as the time of the year when they were performed, could also explain these differences.

5.3 Weight loss protocol

Chapter 4; Experiment 3

The weight loss (WL) protocol used in the present Thesis was chosen to be as close as possible to field conditions in veterinary practices. Firstly, we chose to use the previously measured energy intake (EI) during stable obese state to calculate the energy allowance necessary to achieve WL. Since animals can differ greatly in their maintenance energy requirements (MER), estimation of MER from formulas (NRC, 2006) may be misleading (Laflamme, 2006). Thus, the amount of energy required to maintain stable (obese) body weight was considered to be the "true" MER of each individual dog.

On the other hand, regular adjustments in calorie allowances is also a common practice in WL feeding protocols, since a gradual WL is more likely to allow long-term maintenance of the reduced body weight (BW; Laflamme and Kuhlman, 1995) and a very slow rate of WL might result in owners dropping from the WL plan. In our study, our goal was to achieve a weekly WL rate between 1 and 2% of BW. Since the rate of WL was fixed, we could not use this value to assess the different diets. However, differences both on BW lost and on body composition were found. Since diets had the same metabolizable energy (ME) and the same inclusion of experimental oils, differences are likely due to differences in the efficiency of ME utilization, likely explained by the better fat deposition of SFA versus unsaturated FA.

Unfortunately, unlike the majority of overweight dogs attending veterinary practices, our experimental dogs were not chronically obese. This might explain why we did not observe any differences in the pro-inflammatory cytokines concentration studied before and after WL. To our knowledge, there are no published studies comparing experimentally and naturally obese dogs. However, in our study, we included a chronically obese dog (had been obese for at least 2 years prior to the beginning of the study), with a body condition score (BCS) of 8 (in a 9-point scale; Laflamme, 1997) . This dog was included in C0, but, at the end of the experiment, we decided to discard the data obtained from him, due to its statistical outlier behavior. His chronic obesity might be responsible for the lower percentage of BW lost in this individual dog (8.05% of his initial BW) compared to the rest of the animals, who became

obese after overfeeding them with a high-energy diet (C0: 20.1 ± 2.32%, C20: 14.6 ± 1.43%, C40: 15.7 ± 1.23%, $p < 0.05$; **Figure 5.2**).

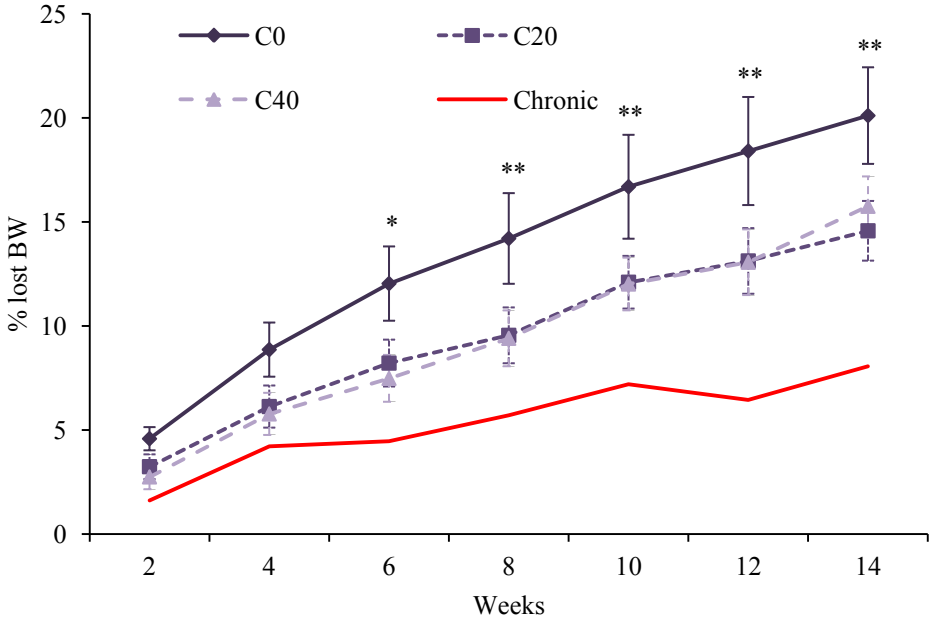

Figure 5.2. Average weekly body weight lost for dogs eating three diets enriched with different esterified acid oils varying in medium chain fatty acid content during 14-weeks weight loss. Red line shows weekly body weight lost for 1 chronically obese dog during 14-weeks weight loss.
Values are derived from 5 dogs for the C0 diet and 6 dogs for C20 and C40 diets. Asterisks over the top of the points indicate differences on percentage of weight lost between C0 and C40 (*) or between C0 and both diets with medium chain fatty acids (C20 and C40) (**).

We defined fat loss efficiency (FLE) as the amount of kcal of fat mass (FM) lost per kcal consumed. This percentage can be calculated by dividing the kcal of FM lost by the kcal consumed during the 14-weeks of WL period, assuming that each gram of FM has 9 kcal of ME. The FLE follows the same trend as body composition and percentage of BW lost. Thus, animals fed experimental diets from soybean and canola oils in their composition presented higher (and, therefore, better) FLE percentages than animals fed C40 with 40% of coconut oil in its composition (C0: 32.6 ± 6.03%, C20: 19.5 ± 3.48%, C40: 16.1 ± 1.97%, $p = 0.042$; **Figure 5.3**). Additionally, **Figure 5.3** shows that the chronically obese dog (indicated as a red point) presented the worst FLE percentage (8.9%) compared to the non-chronic obese animals included in the trial, showing again a different behavior.

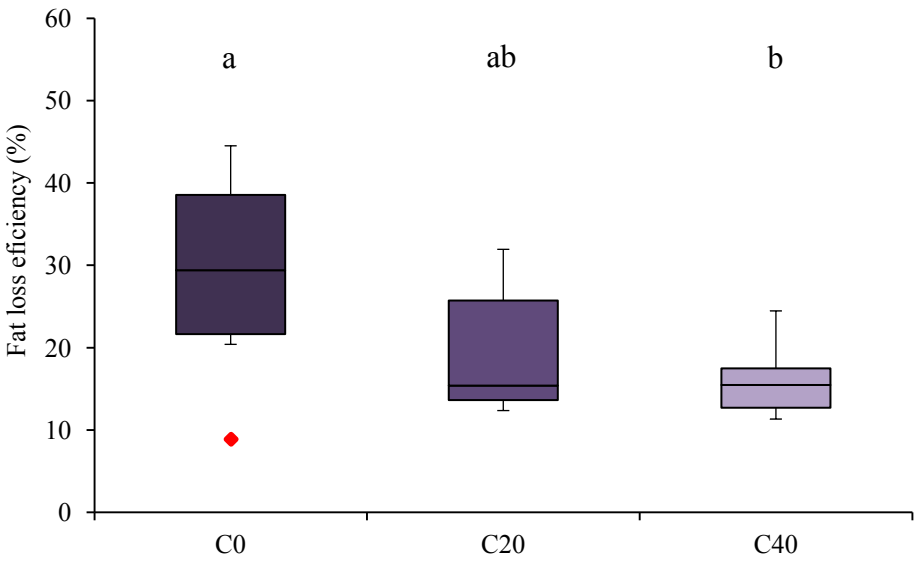

Figure 5.3. Boxplots demonstrating fat loss efficiency (%) for dogs eating three diets enriched with different esterified acid oils varying in medium chain fatty acid content during 14-weeks weight loss.
Outliers are shown as separate red points.

5.4 Other considerations

The present Thesis is part of a larger project whose main goal is to study the use of EAO in different monogastric species (poultry, swine, fish, and dogs). Therefore, this affected the choices made in order to choose the EAO to study, since maintaining the comparative aspect of the project line was a priority (mainly in experiments derived from **Chapter 3** with palm EAO). Additionally, the composition of EAO was chosen from a practical point of view in order to evaluate their potential use as an ingredient for canine diets. As consequence, several limitations were found on the experiments.

Referring to **Chapter 3**, the experimental design does not completely allow differentiating between the effect of the FA positional distribution within the glycerol moiety and the effect of monoacylglyceride (MAG) and diacylglyceride (DAG) inclusion. We can say that, at the two levels of MAG/DAG inclusion, we found no differences among treatments, so MAG/DAG inclusion between 40% and 80% of the added oil does not seem to provide any benefit on fat digestibility, ME content, and palatability. It would be very interesting to repeat our experiment using EAO composed mainly of triacylglycerides (TAG). On the other hand, as it was mentioned above, the inclusion of a control negative group (basal diet supplemented with acid oil) will help us to define whether there is a clear benefit on palatability,

digestibility, and plasma lipid profile of the use of EAO over the acid oils, which are a cheaper alternative.

Another limitation is that the experimental oils on **Chapter 4** differ not only in chain length but also in chain saturation. For this reason, further research should include longer SFA from vegetable (palm oil) or animal (lard or beef tallow) sources in comparison to shorter SFA (coconut oil or medium chain TAG; MCT) to evaluate the effect of chain length on body composition, independently of the saturation effect of the oils. We hypothesize that one explanation for the worse performance of the MCFA rich EAO in the weight loss plan could be a result of the substitution of unsaturated for SFA among experimental oils. A lower fat deposition in animals eating long chain unsaturated fats compared to long chain saturated fats has been reported by several authors in broiler chickens and rodents (Mercer and Trayhurn, 1987; Shimomura et al., 1990; Takeuchi et al., 1995; Sanz et al., 1999, 2000; Crespo and Esteve-Garcia, 2001, 2002 a; Ferrini et al., 2008; Wongsuthavas et al., 2008; González-Ortiz et al., 2013). Further studies should confirmed this effect in dogs fed diets rich in polyunsaturated fatty acids (PUFA) both omega-6 (n-6; corn, safflower, sunflower, soybean oils) and omega-3 (n-3; linseed or marine oils) compared to diets rich in longer SFA (palm oil or animal fats).

CHAPTER 6:

CONCLUSIONS

According to the results presented in this dissertation, the following conclusions can be drawn:

1. Diets supplemented with palm esterified acid oils at 10% inclusion level are more preferred than those supplemented with palm native oil in adult dogs.

2. The inclusion of 10% palm esterified acid oils in a canine kibble basal diet did not result in any differences on digestibility levels of organic matter, energy, fat, and fatty acids (FA) compared to palm native oil in adult dogs.

3. The fecal concentration of free fatty acids is at least 5 times higher than that of triacylglycerides, diacylglycerides, and monoacylglycerides in adult dogs fed either palm esterified acid oils or palm native oil at 10% inclusion level. However, diets supplemented with palm esterified acid oils leads to higher fecal diacylglycerides excretion than diets supplemented with palm native oil.

4. The use of palm esterified acid oils at 10% inclusion level in adult dogs has no impact on post-prandial lipaemia compared to palm native oil.

5. The use of palm esterified acid oils at 10% inclusion level did not change the fasting plasma lipid profile in adult dogs after 1 month of diet consumption.

6. Diets supplemented with esterified acid oils at 8% inclusion level with at least 2.35% of dietary metabolizable energy as medium chain fatty acids from coconut oil are more preferred than diets with longer unsaturated fatty acids from canola and soybean oils in adult dogs.

7. Dogs fed diets supplemented with esterified acid oils at 8% inclusion level with at least 2.35% of dietary metabolizable energy as medium chain fatty acids from coconut oil showed a better fat digestibility, mainly the saturated fraction, compared to those fed diets supplemented with longer unsaturated fatty acids from canola and soybean oils in adult dogs.

8. The incorporation of fatty acids from coconut oil into a dry dog food negatively affects the percentage of body weight lost during a weight loss process and results in a lower decrease in fat mass after 14-weeks weight loss compared to longer unsaturated fatty acids from canola and soybean oils.

9. The incorporation of fatty acids from coconut oil into a dry dog food has no effect on plasma lipid profile, butyrylcholinesterase, adiponectin, leptin, insulin, interleukin-6, and tumour necrosis factor-α compared to fatty acids from canola and soybean oils in adult experimentally obese dogs.

10. Weight loss increases plasma adiponectin concentration, decreases insulin and leptin concentrations, and has no effect in butyrylcholinesterase, tumour necrosis factor-α, and interleukin-6 concentrations after 14-weeks weight loss in adult experimentally obese dogs.

CHAPTER 7:

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CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Fragua Fernández, Víctor
Nationality: Spain
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Birth date: 16/10/1984

ACADEMIC TRAINING

PhD student, Animal Science 2011 - Present
(Universitat Autónoma de Barcelona; UAB)

Master of Science, Veterinary Research (UAB) 2010

Bachelor in Veterinary Science (UAB) 2008

PROFESSIONAL EXPERIENCE

PhD student (UAB, Barcelona, Spain) 2011 - Present
- Thesis topic: Use of random esterified acid oils in dog diets.
- Conducted research for different companies and in national public projects.

Research technician (UAB, Barcelona, Spain) 2008 - 2011
- Conducted research for different companies and in national public projects.

Veterinary clincian (Clínica Veterinària Animàlia, Barcelona, Spain) 2008 - 2011
- Conducted emergencies, internal medicine, and surgeries.

Veterinary clincian (Gortlands Veterinary Clinic, Belfast, United Kingdom) 2009
- Conducted emergencies, internal medicine, and surgeries.

Collaborator student (UAB, Barcelona, Spain) 2007 - 2008
- Trainee in clinical nutrition. Fundació Hospital Clínic UAB.
- Conducted research for different companies and in national public projects.

ATTENDING POST-GRADUATE COURSES

An Introduction to Marketing (Wharton School, on-line) 2014

Biological agents: risks and preventive measures (UAB, Barcelona, Spain) 2013

Official European training course for researcher on laboratory animal management. Category C (UAB, Barcelona, Spain). 2012

Initial training course on occupational risk prevention (UAB, Barcelona, Spain). 2009

SCIENTIFIC PUBLICATIONS

Fragua et al. (2013). Comparison of postprandial lipaemia between native and palm random esterified acid oils in two different monogastric species (dogs and broiler chickens). J. Anim. Physiol. Anim. Nutr. *97,* 74-79.

Fragua et al. (2011). Preliminary study: voluntary food intake in dogs during tryptophan supplementation. Br. J. Nutr. *106,* 162-165.

Fragua et al. Evaluation of esterified acid oils with different medium chain fatty acid content in weight loss dog diets. J. Anim. Physiol. Anim. Nutr. (Submitted)

Fragua et al. Evaluation of esterified acid oils with diferent proportions of mono- and diacylglycerides in dog diets. J. Anim Sci. (In preparation).

Molina et al. Preliminary study: fibre content in pet rabbit diets: crude fibre vs. Total dietary fibre. J. Anim. Physiol. Anim. Nutr. (Submitted)

CONGRESSES CONTRIBUTION

Fragua et al. (2014). Evaluation of esterified acid oils with different medium chain fatty acid content for weight loss canine diets. 14th Annual American Academy of Veterinary Nutrition Symposium. Nashville, Tennessee. Poster.

Fragua et al. (2013). Effects of the use of vegetable esterified acid oils with different medium chain fatty acid content on food preferences and digestibility in adult dogs. 17th Congress of the European Society of Veterinary and Comparative Nutrition. Ghent, Belgium. Poster.

Fragua et al. (2012). Effects of the use of esterified acid oils with different monoacylglicerides content on food digestibility in adult dogs. 16th Congress of the European Society of Veterinary and Comparative Nutrition. Bydgoszcz, Poland. Poster.

Fragua et al. (2012). Food preferences between esterified palm acid oil and native palm oil in adult dogs. 12th Annual American Academy of Veterinary Nutrition Symposium. New Orleans, Louisiana. Oral communication.

Fragua et al. (2011). Comparision of postprandial lipemia between native and randomized palm oils in two different monogastric species (dogs and broiler chickens). 15th Congress of the European Society of Veterinary and Comparative Nutrition. Zaragoza, Spain. Poster.

Fragua et al. (2010). Tryptophan supplementation increases voluntary food intake in dogs. Waltham International Sciences Symposium. Cambridge. Poster.

Molina et al. (2013). Fibre content in pet rabbit diets: crude fibre vs. total dietary fibre. 17th Congress of the European Society of Veterinary and Comparative Nutrition. Ghent, Belgium. Poster.

Vilarrasa et al. (2013). Use of palm esterified acid oils in monogastric animal nutrition. 17th Congress of the European Society of Veterinary and Comparative Nutrition. Ghent, Belgium. Poster.

Vilarrasa et al. (2012). Effects of the use of esterified acid oils with different saturation degree and different monoglyceride content in broiler chicken diets. XXIV World's Poultry Congress. Salvador, Bahia, Brasil. Poster.

Vilarrasa et al. (2012). Influence of dietary fat saturation degree on postprandial lipaemia in broiler chickens. XXIV World's Poultry Congress. Salvador, Bahia, Brasil. Poster.

Vilarrasa et al. (2011). Efectos del uso de aceites esterificados con diferente contenido en monoglicéridos en la ración del pollo de carne. XLVIII Simposio Científico de Avicultura. Santiago de Compostela. Spain. Poster.

Villaverde et al. (2009). Nutritional Management of chronic kidney disease: how to choose an appropriate diet. 13th Meeting of the European Society of Veterinary and Comparative Nutrition. Oristano, Italy. Oral communication.

NATIONAL PUBLICATIONS

Fragua et al. (2009). Tratamiento de la obesidad. ARGOS informativo veterinario. *105*, 36-38.

Villaverde et al. (2011). Importancia del manejo dietético de enfermedad renal en perros y gatos. ARGOS informativo veterinario. *128*, 50-53.

CONTRIBUTION IN RESEARCH DEVELOPMENT AND INNOVATION PROJECTS, AND CLINICAL TRIALS

Project title: USE OF ESTERIFIED ACID OILS IN MONOGASTRIC ANIMAL DIETS. COMPARATIVE NUTRITION AND IMPACT ON MEAT LIPID QUALITY.

Project title: THE EFFECTS OF A TRYPTOPHAN ENRICHED DIET AND PHYSICAL ACTIVITY ON BEHAVIOUR AND BIOMARKERS IN DOGS.

Project title: DIGESTIBILITY OF DRY-EXTRUDED DOG DIETS AND FECAL CONSISTENCY EVALUATION IN DOGS.

Project title: FOOD PREFERENCES OF DRY-EXTRUDED DOG DIETS.

Project title: INNOVATIVE DOG FOOD DEVELOPMENTS.

Project title: EFFECT OF A FIBER SOURCE ON FECAL EXCRETION OF HAIR IN CATS.

Project title: CANINE LEISHMANIASIS AND XANTHINE UROLITHS.

Project title: PARENTERAL NUTRITION USES IN ICU PATIENTS.

TEACHING EXPERIENCE

Undergraduate (Veterinary Degree)

	Year	
Subject: **Animal Production and Management**		
Activity: practical sessions	2011-2012	12 hours
	2012-2013	12 hours
	2013-2014	12 hours
Subject: **Nutrition**		
Activity: practical sessions	2012-2013	2 hours
Subject: **General Surgery**		
Activity: theory lessons on parenteral nutrition	2012-2013	2 hours
Subject: **Clinical Nutrition**		
Activity: theory lessons on nutritional management of gastrointestinal diseases	2010-2011	1 hour
	2011-2012	1 hour
	2012-2013	1 hour
Subject: **ICU training period**		
Activity: workshop on nutrition support in ICU patients	2008-2009	4 hours
	2009-2010	4 hours

Invited speaker

Course on Clinical Nutrition – IVSA		
Topic: Use of omega-3 fatty acids on inflammatory disease in dogs and cats	March, 2012	1 hour

PERSONAL SKILLS AND COMPETENCES

Native languages: Spanish and Catalan

Other languages:

- English (reading, writing and verbal skills (excellent)
- German (reading, writing and verbal skills (basic, A2 level)
- French (reading, writing and verbal skills (basic, A2 level)

Technical skills:

- Window MS Office® and related programs user.
- Experience in statistical analysis using SAS® system.
- Experience in a large number of laboratory techniques.

Driving license: B1