Bioactive compounds for functional foods: delivery systems, bioaccessibility and biological activity

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Compuestos bioactivos para alimentos funcionales: vehiculización, bioaccesibilidad y actividad biológica

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Memoria presentada por **Dña. Lucía Gayoso Morandeira** para aspirar al grado de Doctor por la Universidad de Navarra.

El presente trabajo ha sido realizado bajo la dirección de la **Dra. Iciar Astiasarán Anchía** y de la **Dra. Diana Ansorena Artieda** en el Departamento de Ciencias de la Alimentación y Fisiología y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

En Pamplona, octubre de 2018

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La directora del Departamento, Dra. Dña. Diana Ansorena Artieda hace constar que el presente trabajo de investigación ha sido realizado por Dña. Lucía Gayoso Morandeira, en el Departamento de Ciencias de la Alimentación y Fisiología de la Facultad de Farmacia y Nutrición de la Universidad de Navarra.

Dra. Diana Ansorena Artieda

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ABSTRACT

Issues related to bioaccessibility of bioactive compounds provide valued information for designing functional foods. Relevant bioactive compounds, such as phenolic compounds from plant extracts, omega 3 fatty acids and α-lipoic acid have been evaluated as potential functional ingredients for the development of new healthier food formulations. This evaluation has addressed bioaccessibility, biological activity and chemical, methodological and technological aspects.

In vitro gastrointestinal digestion models are a valuable tool for assessing the bioaccessibility of bioactive compounds, being the interpretation of results highly influenced by the methodology applied.

Phenolic compounds from extracts of *Melissa officinalis* L., *Origanum vulgare* L. and *Lavandula latifolia* Medicus were chemically characterized. After an *in vitro* gastrointestinal digestion, compounds responsible for the antioxidant activity of these extracts remained bioaccessible and showed biological activity in the *Caenorhabditis elegans* nematode model.

Emulsion-based delivery systems (O/W emulsions and gelled emulsions) showed to be an effective strategy to increase the bioaccessibility of long chain omega 3 fatty acids and the antioxidant properties of an aqueous extract of *M. officinalis*.

The supplementation of eicosapentaenoic acid and α-lipoic acid in overweight/obese women following a hypocaloric diet had a positive influence in the plasma lipid profile of the volunteers.

Therefore, the bioactive compounds and delivery systems studied in this work are promising ingredients to design bioaccessible functional foods that contribute to promote human health.

RESUMEN

Aspectos relacionados con la bioaccesibilidad de compuestos bioactivos ofrecen una información relevante para el diseño de alimentos funcionales. Algunos de los compuestos bioactivos más destacados, como son compuestos fenólicos de extractos de plantas, ácidos grasos omega 3 y ácido α-lipoico, se han evaluado como potenciales ingredientes funcionales para el desarrollo de nuevas formulaciones de alimentos más saludables. Esta evaluación ha abordado bioaccesibilidad, actividad biológica y aspectos químicos, metodológicos y tecnológicos.

Los modelos de digestión gastrointestinal *in vitro* son una valiosa herramienta para evaluar la bioaccesibilidad de los compuestos bioactivos, si bien la interpretación de resultados pueda estar altamente influida por la metodología aplicada.

Los compuestos fenólicos de extractos *de Melissa officinalis* L., *Origanum vulgare* L. y *Lavandula latifolia* Medicus se caracterizaron químicamente. Después de la digestión gastrointestinal *in vitro*, los compuestos responsables de la actividad antioxidante de estos extractos permanecieron bioaccesibles y mostraron actividad biológica en el modelo de nematodo *Caenorhabditis elegans.*

Los sistemas de vehiculización basados en emulsiones (emulsiones O/W y emulsiones gelificadas) fueron una estrategia efectiva para incrementar la bioaccesibilidad de los ácidos grasos omega 3 de cadena larga y las propiedades antioxidantes de un extracto acuoso de *M. officinalis*.

La suplementación con ácido eicosapentaenoico y ácido α-lipoico en mujeres con sobrepeso/obesidad siguiendo una dieta hipocalórica tuvo una influencia positiva en el perfil lipídico plasmático de las voluntarias.

Por lo tanto, los compuestos bioactivos y los sistemas de vehiculización estudiados en este trabajo son ingredientes prometedores para diseñar alimentos funcionales bioaccesibles que contribuyan a promover la salud humana.

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INTRODUCTION

1. Bioactive compounds

Bioactive compounds are essential and non-essential compounds that occur in nature, are part of the food chain, and can be shown to have an effect on human health (Biesalski et al., 2009). Figure 1 shows a classification of the main classes of bioactive compounds, including vitamins, minerals, fatty acids, dietary fibre, bioactive peptides, polyphenols, glucosynolates, carotenoids, alkaloids and phytosterols. However, given their wide diversity, these compounds can be classified taken into account other factors, such as chemical structure, food source or biological actions. For instance, from a chemical point of view, bioactive compounds include from hydrophilic polyphenols (Alminger et al., 2014) to lipophilic bioactives (polyunsaturated lipids, fat-soluble vitamins, phytosterols, curcuminoids, carotenoids and flavonoids) (McClements, 2015). The majority of these compounds are predominantly derived from the plant kingdom, with some from animal sources (Rein et al., 2013). In particular, plant kingdom produces an overwhelming range of structurally diverse secondary metabolites, being polyphenols the biggest group of phytochemicals, with more than 8000 phenolic structures identified (Tsao, 2010).

Bioactive compounds are mainly found in plant foods such as vegetables, fruits, cereals, legumes, nuts, seeds, fungi, herbs and spices (Kurmukov, 2013). The intake of these foods has been associated with low incidence of cancers and chronic diseases, including cardiovascular disease, type II diabetes, and impaired cognitive function (Del Rio et al., 2013). Their biological effects have been associated with different actions: antioxidant effect, inhibition or induction of enzymes, inhibition of receptors activities, and induction and inhibition of gene expression (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014).

Bioactive compounds are aimed to promote and improve human health. In order to exert a health benefit, bioactive compounds, whether hydrophilic or lipophilic, needs to withstand food processing, be released from food matrix post-ingestion and be bioaccessible in the gastrointestinal tract, undergo metabolism and reach the target tissue of action (Rein et al., 2013). Therefore, it is important

before concluding on any potential health effect, to analyse whether the digestion process affects bioactive compounds and their stability, as this, in turn, will affect their bioavailability and their possible beneficial effects (Carbonell-Capella et al., 2014).

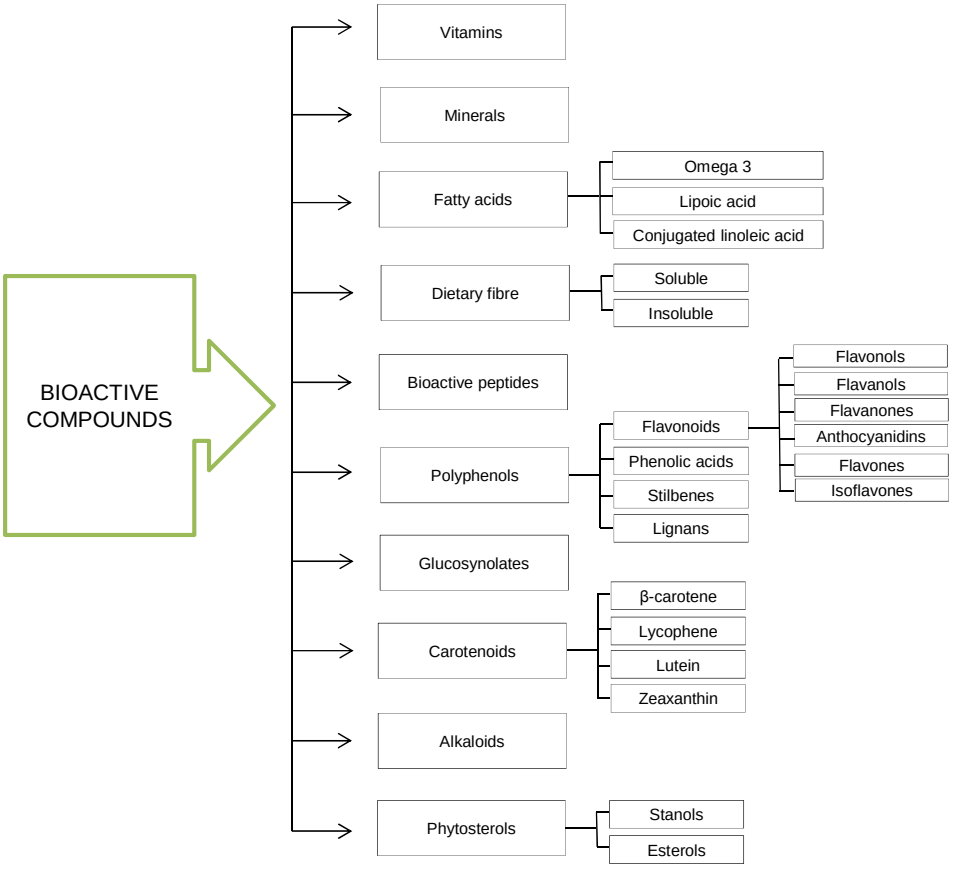

Figure 1. Classification of the main classes of bioactive compounds.

1.1. Polyphenols

The ability of plants to produce bioactive compounds such as polyphenols has been exploited by humans for thousands of years, and plant extracts have been used since ancient times as herbal medicinal products to treat different diseases (Maffei, 2003). Among the medicinal and aromatic herbs, three species belonging to the *Lamiaceae* family, *Melissa officinalis* L., *Origanum vulgare* L. *and Lavandula latifolia* Medicus, have been studied in this work as potential sources of polyphenols (Figure 2).

Figure 2. Images of *Melissa officinalis* (Photo: Hugo Lizama), *Origanum vulgare* (Photo: Laboratorio de Botánica Collection, Universidad de Navarra) and *Lavandula latifolia* (Photo: Laboratorio de Botánica Collection, Universidad de Navarra).

Melissa officinalis, also known as lemon balm, is usually consumed as infusion and it has been studied by its therapeutics properties, such as sedative, carminative, antispasmodic, neuroprotective and antiproliferative (Barros et al., 2013; López et al., 2009; Saraydin et al., 2012; van Wyk & Wink, 2015). *Origanum vulgare* is a widely consumed spice, with antiviral, anti-inflammatory and cell damaging preventive activities (González, Lanzelotti, & Luis, 2017; van Wyk & Wink, 2015, 2017; Zhang et al., 2014). *Lavandula latifolia*, commonly known as spike lavender, it is mainly used due to its aromatic properties and its medical properties, such as antispasmodic, sedative, antihypertensive, antiseptic, healing and anti-inflammatory (Herraiz-Peñalver et al., 2013; van Wyk & Wink, 2015). Most of these health benefits have been attributed to different bioactive compounds and, in particular, to polyphenols, which are molecules associated with antioxidant actions among others (Embuscado, 2015; López et al., 2007; Milevskaya, Temerdashev, Butyl'skaya, & Kiseleva, 2017; Shan, Cai, Sun, & Corke, 2005; van Wyk & Wink, 2015).

Polyphenols are secondary metabolites that plant produce to protect themselves from other organisms and contribute substantially to their colour and other organoleptic properties. They are ubiquitous in all plant organs and therefore, they are widespread constituents of plant-based foods, mainly fruits, vegetables, spices, herbs and beverages such as tea, coffee and wine (Mojzer, Hrnčič, Škerget, Knez, & Bren, 2016). The diversity and wide distribution of polyphenols in plants have led to different ways of categorizing these compounds. According to the number of phenol rings that they contain and of the structural elements that bind these rings to one another, dietary polyphenols can be classified into four classes: flavonoids, phenolic acids, stilbenes, and lignans (Manach, Scalbert, Morand, Remesy, & Jiménez, 2004). Flavonoids, the most common in our diet, can be further categorized as flavonols, flavanols, flavanones, anthocyanidins, flavones and isoflavones (Manach et al., 2004). Some of the most common flavonoids include quercetin, a flavonol abundant in onion, broccoli, and apple; catechin, a flavanol found in tea and several fruits; naringenin, the main flavanone in grapefruit; cyanidin-glycoside, an anthocyanin abundant in berry fruits; and daidzein, genistein and glycitein, the main

isoflavones in soybean (D'Archivio et al., 2007; Dai & Mumper, 2010). Phenolic acids can be divided in two classes: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic, ferulic and sinapic acid. Stilbenes and lignans are less common in the human diet. The main representative of stilbenes is resveratrol, mainly found in red grapes and wine (D'Archivio et al., 2007). The richest dietary source of lignans is linseed, which contains secoisolariciresinol and low quantities of matairesinol (Manach et al., 2004).

Absorption of dietary polyphenols is poor, in a range of 2-20% (Hu, 2007). The chemical structure of polyphenols, more than the concentration, determines the rate and extent of absorption and the nature of the metabolites circulating in the plasma (D'Archivio et al., 2007). Figure 3 shows the compartments involved in their metabolism.

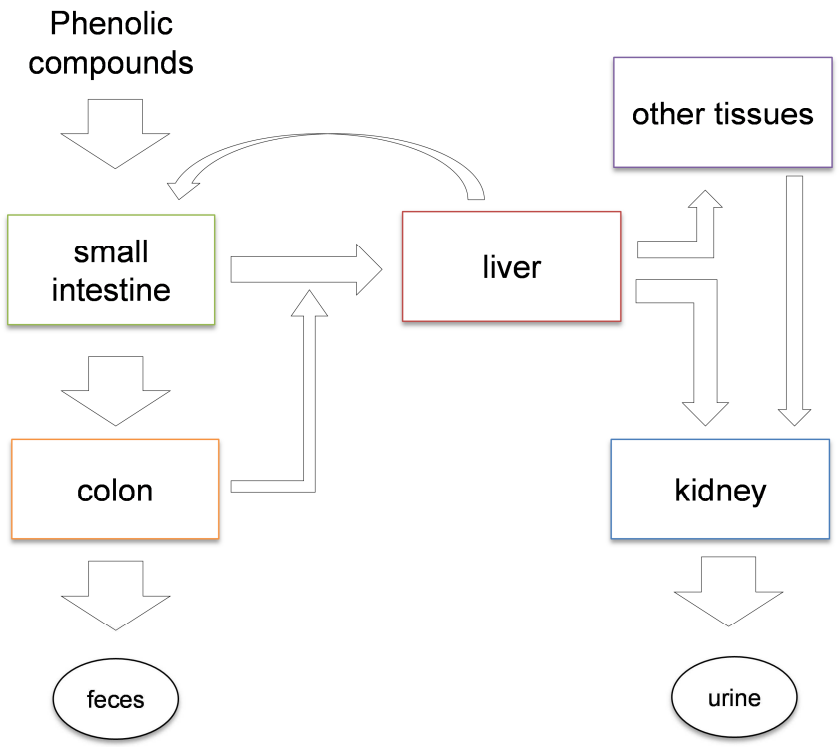

Figure 3. Compartments involved in the metabolism of plant phenols. Adaptation from Hollman (2001).

Most polyphenols are present in food in form of esters, glycosides or polymers that cannot be absorbed in the native form (Manach et al., 2004). These substances must be hydrolysed into respective aglycons by intestinal enzymes (cytosolic β-glucosidase and lactase-phlorizin hydrolase) or by the colonic microflora before they can be absorbed (Németh et al., 2003). Once absorbed, compounds undergo phase II enzymatic metabolism with three main types of conjugation (methylation, sulfation and glucuronidation), that represents a metabolic detoxication process, common to many xenobiotics, that facilitates their biliary and urinary elimination by increasing their hydrophilicity (Manach et al., 2004). Non-absorbed polyphenols or those re-excreted via bile or the pancreas reach the colon where are metabolized via esterase, glucosidase, demethylation, dehydroxylation, and decarboxylation activities of bacteria (Selma, Espín, & Tomás-Barberán, 2009), resulting in smaller metabolites such as phenolic acids and short-chain fatty acids, some of which can be absorbed across the intestinal mucosa (Etxeberria et al., 2013).

Despite the low bioavailability of these molecules, they display activity at low plasma concentrations. Nowadays, there is extensive evidence about their antioxidant, anti-inflammatory and other biological effects that exert in the prevention of various pathologies including cardiovascular diseases, neurodegenerative diseases and cancer (Dai & Mumper, 2010; Del Rio et al., 2013; Mojzer et al., 2016; Rodriguez-Mateos et al., 2014).

1.2. Omega 3 fatty acids

Omega 3 polyunsaturated fatty acids (n-3 PUFAs) have long been studied for their health benefits. The three major n-3 PUFAs are eicosapentaenoic acid (EPA, C20:5 n-3), docosahexaenoic acid (DHA, C22:6 n-3) and α-linolenic acid (ALA, 18:3 n-3). In particular, long chain n-3 PUFAs, EPA and DHA, are among the most studied bioactive compounds of marine origin, and they have been linked to a wide range of beneficial physiological effects. It has been pointed out their protective and beneficial effects on inflammatory diseases, cancer and cardiovascular disease (Saini & Keum, 2018). Since the first time that cardiovascular health was associated to n-3 PUFAs ingestion analysing the

"Inuit diet" (based on high-fish consumption), numerous studies have been conducted, attempting to elucidate the effects of n-3 PUFAs on cardiovascular disease. The combined effect of regulation of cholesterol levels, adipocytes metabolism, lipogenesis, inflammation, thrombosis, and arterial stiffness contribute to this cardiovascular benefit (Colussi, Catena, Novello, Bertin, & Sechi, 2017; Tortosa-Caparrós, Navas-Carrillo, Marín, & Orenes-Piñero, 2017). EPA and DHA have also been reported to counteract obesity-related metabolic changes, including chronic inflammation, insulin resistance and dyslipidaemia, by modulation of lipid metabolism (Albracht-Schulte et al., 2018; Martínez-Fernández, Laiglesia, Huerta, Martínez, & Moreno-Aliaga, 2015). Specifically, DHA plays a key role in maintaining membrane fluidity of the retina and brain, which is essential for proper neurological and cognitive functions (Cardoso, Afonso, & Bandarra, 2017).

Fish, krill and algae are rich sources of EPA and DHA (Astiasarán & Ansorena, 2009; Schuchardt & Hahn, 2013). ALA is abundant in several vegetables oils, such as linseed, chia, hemp, canola and perilla oil (Saini & Keum, 2018). The conversion of ingested ALA to EPA and DHA within the body is not usually considered to be an adequate source of these long chain PUFAs. The elongation and desaturation conversions are highly inefficient as most of the fatty acid precursors are utilized for energy (Deckelbaum & Torrejon, 2012). Furthermore, conversion of ALA to EPA is only 0.3-8% in men and 21% in women (Arterburn, Hall, & Oken, 2006). The conversion of ALA to DHA is <4% in men and 9% in women (Arterburn et al., 2006). This poor production of long chain PUFAs in the body makes necessary a direct intake of EPA and DHA to achieve an optimal consumption. Different organizations have published recommendation for EPA and DHA. European Food Safety Authority (EFSA) recommends that the dietary advice for adults should be 250 mg/day of EPA and DHA (EFSA, 2010). The 2015 Dietary Guidelines for Americans recommend consuming about 8 ounces (227 g) per week of seafood, which would provide about 250 mg/day of EPA and DHA (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). The World Health Organization (WHO) recommended a regular fish consumption (1-2

servings/week; providing 200-500 mg/serving of EPA and DHA) (WHO, 2015). The new recommendation from the American Heart Association supports consume seafood, especially species higher in long chain n-3 fatty acids, 1 to 2 times per week for cardiovascular benefits, including reduced risk of cardiac death, coronary heart disease, and ischemic stroke (Rimm et al., 2018). Current guidelines from cardiac societies recommend the use of n-3 PUFA supplements (1 g/day of EPA and DHA) for the prevention of coronary heart disease and major vascular events in people with prior coronary heart disease (Siscovick et al., 2017). The U.S. Food and Drug Administration has stated that levels up to 3 g/day are generally recognized as safe (Albracht-Schulte et al., 2018), although EFSA have reported no adverse effects at up to 5-6 g/day (EFSA, 2012).

General population use enriched foods and supplements to get and maintain adequate amounts of these fatty acids. In recent years, the number of enriched foods and dietary supplements of long chain n-3 PUFAs in the form of triglycerides (fish oil), free fatty acid, ethyl esters, or phospholipids (krill oil) has markedly increased (Saini & Keum, 2018). However, the incorporation of these fatty acids into food is often challenging due to their low water solubility, poor oxidative stability, undesirable taste and variable bioavailability (Walker, Decker, & McClements, 2014). The analysis of these aspects, especially concerning their bioavailability, requires further exploration.

1.3. α-Lipoic acid

α-lipoic acid (1, 2- dithiolane-3-pentanoic acid) (LIP) has become a common ingredient in multivitamin formulas and anti-aging supplements (Shay, Moreau, Smith, Smith, & Hagen, 2009). This antioxidant is a short-chain fatty acid, and an essential co-factor for mitochondrial function, and thus plays a critical role in mitochondrial energy metabolism (Gao et al., 2018). It is naturally found in several animal and vegetal nutritional sources, including liver and kidney, spinach, broccoli, tomato, Brussels sprouts and rice bran (Rodriguez-Perdigon, Solas, Moreno-Aliaga, & Ramirez, 2016). The human body can synthesize small amounts of LIP through lipoic acid synthase (Padmalayam, Hasham, Saxena, & Pillarisetti, 2009). It is both water and fat soluble and therefore cross biological

membranes easily, reaching all the compartments of the cell (Gorąca et al., 2011).

Most of the biological effects of LIP are usually attributed to its potent antioxidant properties: quenching of reactive oxygen species, regeneration of endogenous antioxidants such as glutathione and vitamins C and E, chelation of metal ions and repair of proteins from oxidative damaged (Vidoviü et al., 2014). On account of these properties, LIP may improve several metabolic disorders that are mediated by oxidative stress, such as cardiovascular, neurodegenerative and autoimmune diseases, diabetes, acquired immune deficiency syndrome, and cancer (Gorąca et al., 2011).

Several studies have suggested important anti-obesity properties for LIP. In animal studies, LIP supplementation can promote body weight and fat mass loss by reduction in food intake and feed efficiency (Prieto-Hontoria et al., 2009) and stimulating energy expenditure (Wang, Li, Guo, Chan, & Guan, 2010). Studies in humans have also suggested that supplementation with LIP could promote body weight loss and beneficial effects on insulin sensitivity, glucose and lipid metabolism (Carbonelli et al., 2010; Huerta, Navas-Carretero, Prieto-Hontoria, Martínez, & Moreno-Aliaga, 2015; Koh et al., 2011). Furthermore, clinical trials have reported that LIP is safe and well-tolerated. Oral doses up to 2400 mg/person/day did not show adverse effects versus placebo (Shay et al., 2009). However, besides the positive effects on weight loss observed in some trials, the anti-obesity properties of LIP in humans have been inconsistent (Huerta et al., 2015). Recent meta-analysis has revealed that supplementation with LIP significantly decrease body weight (Kucukgoncu, Zhou, Lucas, & Tek, 2017; Namazi, Larijani, & Azadbakht, 2018). Further studies are needed to examine the effect of different doses and the long-term benefits of LIP on weight management (Kucukgoncu et al., 2017).

2. Bioaccessibility, bioavailability and bioactivity of bioactive compounds

From the nutritional point of view, bioaccessibility has been defined as the amount of an ingested compound that is released from a food matrix in the gastrointestinal lumen and is available for intestinal absorption (Parada & Aguilera, 2007). This includes digestive transformations of food into material ready for absorption into intestinal epithelium. It is usually evaluated by *in vitro* simulation of human digestion (Carbonell-Capella et al., 2014).

The concept of bioavailability refers to the amount of an ingested compound that is absorbed and available for physiological functions (Etcheverry, Grusak, & Fleige, 2012). This term includes gastrointestinal digestions, absorption, metabolism, tissue distribution, and bioactivity (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009). According to this definition, bioavailability of a compound is determined *in vivo* in animals or humans as the area under the curve (plasma-concentration) of the compound obtained after administration of an isolated compound or a compound-containing food (Carbonell-Capella et al., 2014).

Bioactivity is the specific effect upon exposure to a substance. It includes tissue uptake and the consequent physiological response. It can be evaluated *in vivo*, *ex vivo*, and *in vitro* (Carbonell-Capella et al., 2014).

Knowledge of the physicochemical changes that occur in bioactive compounds during the digestion process and the factors influencing their bioaccessibility and bioavailability would be helpful for designing functional foods that maximize the health benefits of these compounds (Lucas-González, Viuda-Martos, Pérez-Alvarez, & Fernández-López, 2018). Different digestion models have been developed by the scientific community that try to mimic the complex physicochemical and physiological conditions of the human gastrointestinal tract, along with *in vivo* models in living organisms (Hur, Lim, Decker, & McClements, 2011).

3. Digestion models

The fate of food in the gastrointestinal tract can be studied using several methods or models including static and dynamic *in vitro* models, various cell and ex vivo cultures, animals and humans (Bohn et al., 2017).

3.1. *In vitro* digestion: static and dynamic models

In vitro digestion models are widely used in food and nutritional sciences for predicting compound bioaccessibility due to several advantages as compared to the *in vivo* models, since they are relatively inexpensive and simple, more rapid, do not present ethical restrictions, conditions can be controlled, sampling is easy and results are reproducible (Minekus et al., 2014).

Static or dynamic *in vitro* models can be used to simulate different phases of digestion. Most of the numerous protocols described in the literature are static ones, where the physicochemical and enzymatic environment of each digestive step is recreated (Dupont et al., 2018). These methods include two or three digestion steps (oral, gastric, and intestinal). In each phase, the food product is incubated for a specific time and at a specific temperature with simulated salivary, gastric and intestinal fluids, respectively, while the pH is generally maintained at a fixed value by using a buffer (Lucas-González et al., 2018). While this may seem a simple method, the lack of consensus concerning the physiological conditions applied has led to different models and hence impeding the possibility to compare results across research groups. To harmonize the conditions for simulated digestion of foods, an international standardised protocol for static *in vitro* digestion has been published (Minekus et al., 2014). After intestinal phase, different adaptations, such as centrifugation, filtration or dialysis of the digestate have been carried out to analyse the bioaccessible fraction (Rodrigues, Mariutti, & Mercadante, 2016). Even though *in vitro* static models are oversimplistic and do not reproduce all the dynamic aspects of the gastrointestinal tract, they are increasingly useful in predicting *in vivo* digestion (Bohn et al., 2017).

Compared to static models, dynamic models have the advantage that they can simulate the continuous changes of the physicochemical conditions including

variation of pH from the mouth to the stomach and the intestine, altering enzyme secretion concentrations, and peristaltic forces in the gastrointestinal tract (Alminger et al., 2014). Different dynamic gastric models have been developed and designed to mimic the dynamics related to human gut physiology. The Dynamic Gastric Model was developed at the Institute of Food Research (Norwich, UK) (Vardakou et al., 2011). It is composed of two successive compartments, that mimic the fundus and the antrum of the stomach, and simulates the gastric mixing, transit, breakdown forces (including flow, shear, and hydration), pH gradients, and gastric secretions found in the human stomach (Alminger et al., 2014). The Human Gastric Simulator (HGS), a model developed at the University of California-Davis was designed to mimic the gastric shear forces and stomach grinding (Kong & Singh, 2010). It is composed of a mono-compartmental system where the gastric secretion rate, pH, and gastric emptying can be controlled and varied as needed to represent different physiological conditions (Dupont et al., 2018). The TNO Gastro-Intestinal Model (TIM-1 and TIM-2), developed by The Netherland Organization (TNO), combines all the stages of the gastrointestinal tract, from the stomach to the colon (Minekus et al., 1999; Minekus, Marteau, Havenaar, & Huis in 't Veld, 1995). The TIM-1 system consists of four successive compartments that represent the stomach, duodenum, jejunum and ileum. A computerized control modulates intraluminal pH, enzymatic activity, bile salt concentrations, peristaltic movements, and gastrointestinal transit of the food (Arranz, Corredig, & Guri, 2016). On the other hand, in TIM-2, there is a simulator of the colon (upper, transverse and descending) regions, where the presence of microbial culture allows further assessment of the digestate disruption and fermentation, and the effects of components on the microbial community can be observed (Arranz et al., 2016). In addition to TIM-2, a higher level of complexity can be found in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model, starting with the stomach and ending with the colon (Molly, Vande Woestyne, & Verstraete, 1993). The SHIME model consists of a succession of five reactors simulating the different parts of the gastrointestinal tract. The system has been improved and nowadays, it is a computer-controlled device that can be used to simulate the gastrointestinal microbial ecology and physiology of healthy

humans, babies, elderlies, and some specific disease conditions (Dupont et al., 2018).

3.2. Cell and *ex vivo* cultures

The bioaccessible fraction obtained after *in vitro* digestion simulation, can be used to address further mechanistic questions, such as estimation of absorption or transport through the intestinal epithelium by employing absorptive cell culture systems or *ex vivo* intestinal tissue (Cardoso, Afonso, Lourenço, Costa, & Nunes, 2015).

The human epithelial cell line Caco-2 has been widely used as a model of the intestinal epithelial barrier and have been applied to uptake and transport studies for both hydrophilic and lipophilic bioactive compounds (Alminger et al., 2014).The Caco-2 cell line, which is originally derived from a colon carcinoma, exhibits some morphological and functional characteristics similar to those of differentiated epithelial cells of the intestinal mucosa (Sambruy, Ferruzza, Ranaldi, & De Angelis, 2001).

Ex vivo methodologies, include procedures with viable functional tissues or organs isolated from an organism and incubated outside the organism in an artificial environment under highly controlled conditions (Verhoeckx et al., 2015). The most common intestinal tissue models are intestinal rings, intestinal segments, everted gut sac system (the intestinal section is everted and both ends are tied after filling the sac with buffer), and gut organoids (isolated crypt cells and differentiated to multiple organoids) (Verhoeckx et al., 2015).

3.3. *In vivo* digestion: animals and humans

Although a great development has been achieved on the *in vitro* approaches, these are especially intended for initial screening and should be complemented with *in vivo* studies (Fernández-García et al., 2009). *In vivo* feeding methods, using animals or humans models, generally offer the most precise results and are still considered the “gold standard” for bioavailability of nutrients and bioactive compounds at specific target populations (Lucas-González et al., 2018).

Within *in vivo* studies, balance studies and tissue concentration are two approaches that allow determination of the absorbed amount of nutrients, bioactive compounds, or their metabolites. Balance studies determine the absorbed amount by measuring the difference between the fed and excreted amounts of the nutrient or bioactive compound (Fernández-García et al., 2009). Tissue concentration consists of monitoring the increase in plasma/serum concentration of the nutrient or bioactive compound (Fernández-García et al., 2009). Alternatively, imaging methods, such as magnetic resonance, ultrasound or X-rays, can be used to visualize the processes occurring within the gastrointestinal tract as food component passes through (McClements & Li, 2010). These strategies have been applied in experimental animals or humans to determine absorption of different bioactive compounds (Carbonell-Capella et al., 2014; Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). In spite of all the beneficial contributions that *in vivo* studies can have in nutrition and health related issues, their application has been limited mainly due to ethical issues, but also because they are time consuming and present inter-subject variability (Norton, Gonzalez Espinosa, Watson, Spyropoulos, & Norton, 2015).

Regarding animal models, the nematode *Caenorhabditis elegans* has been developed into an important model for biomedical research, filling the gap between the *in vitro* cell culture and the mammalian models because of its physiological relevance (Arya, Das, & Subramaniam, 2010). Some of the advantages of this animal model as compared to mouse models are its short generation time and lifespan, morphological simplicity, and ease of maintenance and of genetic manipulation (Gruber, Ng, Poovathingal, & Halliwell, 2009). Indeed, in contrast to cellular models, it allows studying a whole organism, with many different organs and tissues and increases the chance of identifying synergistic or off-target effects (Kaletta & Hengartner, 2006). Furthermore, *C. elegans* shares a substantial number of genes and pathways with humans, making it a suitable model for human health conditions and diseases (Ma et al., 2018). In particular, in recent years *C. elegans* has been used as an *in vivo* model to study the biological effects of different bioactive compounds, especially

aspects linked to oxidative stress (Peixoto et al., 2017; Sobeh et al., 2016; Su & Wink, 2015).

Using studies merely *in vitro* or *in vivo* models will not elucidate all the details, and a combination of methods is needed to fully understand the complexity of the reactions occurring during bioactive compounds digestion (Arranz et al., 2016).

4. Delivery systems

Different approaches have been evaluated for the improvement of bioaccessibility/bioavailability of bioactive ingredients. The potential health benefits of many bioactive compounds are not fully reached because they can be chemically degraded during processing, storage or digestion. Consequently, there is a need to develop food-grade delivery systems to encapsulate and protect them until they reach an appropriate location within the human body (mouth, stomach, small intestine, or colon) (McClements, 2017). Emulsion technology is particularly suited for the design and fabrication of these vehicles for use in food applications (Cofrades et al., 2017). Many types of emulsion-based delivery can be used as edible delivery systems, including conventional emulsion, nanoemulsions, multiple emulsions, multilayer emulsions, solid lipid particles, and filled hydrogel particles, each with its own advantages and disadvantages (McClements, 2010). Among all of them, conventional oil-in-water emulsions (O/W), oil droplets dispersed in an aqueous medium, are currently the most widely used method because of their relative ease of preparation, compared to more sophisticated systems (Cofrades et al., 2017). In fact, our research group have been extensively used different O/W emulsions, including gelled emulsions (oil droplets trapped within gel particles), as delivery systems for n-3 fatty acids and natural antioxidants in the formulation of functional foods (Berasategi et al., 2014; Poyato, Ansorena, Berasategi, Navarro-Blasco, & Astiasarán, 2014).

On the other hand, the dispersion of lipids in the form of droplets, the size of the lipid droplets and the composition of their interface may affect the kinetics of lipid digestion and consequently, their absorption (Michalski et al., 2013). In this

sense, emulsification has been proposed as a strategy that could improve the bioavailability of lipidic bioactive compounds, such as n-3 PUFAs (Lin, Wang, Li, & Wright, 2014). Studies comparing the bioavailability of n-3 PUFAs from emulsion-based formulas and from non-emulsified oil have reported higher plasma n-3 PUFAs following consumption of emulsified oil (Haug et al., 2011; Raatz, Redmon, Wimmergren, Donadio, & Bibus, 2009). In an emulsified form, n-3 PUFAs are more easily exposed to pancreatic lipase, resulting in better digestion and absorption (Schuchardt & Hahn, 2013).

A better understanding of the physicochemical properties and stability during storage of the emulsion-based delivery system, as well as the changes that occur during the gastrointestinal conditions, would be of interest to design and optimize new vehicles for a successful delivering of bioactive compounds.

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OBJECTIVES/OBJETIVOS

The general aim of this thesis was to design and to evaluate novel functional ingredients able to protect and deliver bioaccessible bioactive compounds.

In particular, this work was focused on the following objectives:

1. To evaluate the influence of *in vitro* gastrointestinal digestion models on the bioaccessibility of three phenolic compounds (rutin, caffeic acid and rosmarinic acid).
2. To explore plant extracts as potential sources of antioxidant compounds, to characterize these compounds from the chemical standpoint and to evaluate their bioaccessibility.
3. To study the behavior of different delivery systems (O/W emulsions and gelled emulsions) during gastrointestinal digestion and their influence on bioaccessibility of bioactive compounds (plant extracts and long-chain omega-3 fatty acids).
4. To study the *in vivo* activity of bioactive compounds using different approaches:
    4.1. Animal model (*Caenorhabditis elegans*) to evaluate antioxidant activity of plant extracts.
    4.2. Human nutritional intervention to evaluate metabolic implications of eicosapentaenoic acid and α-lipoic acid.

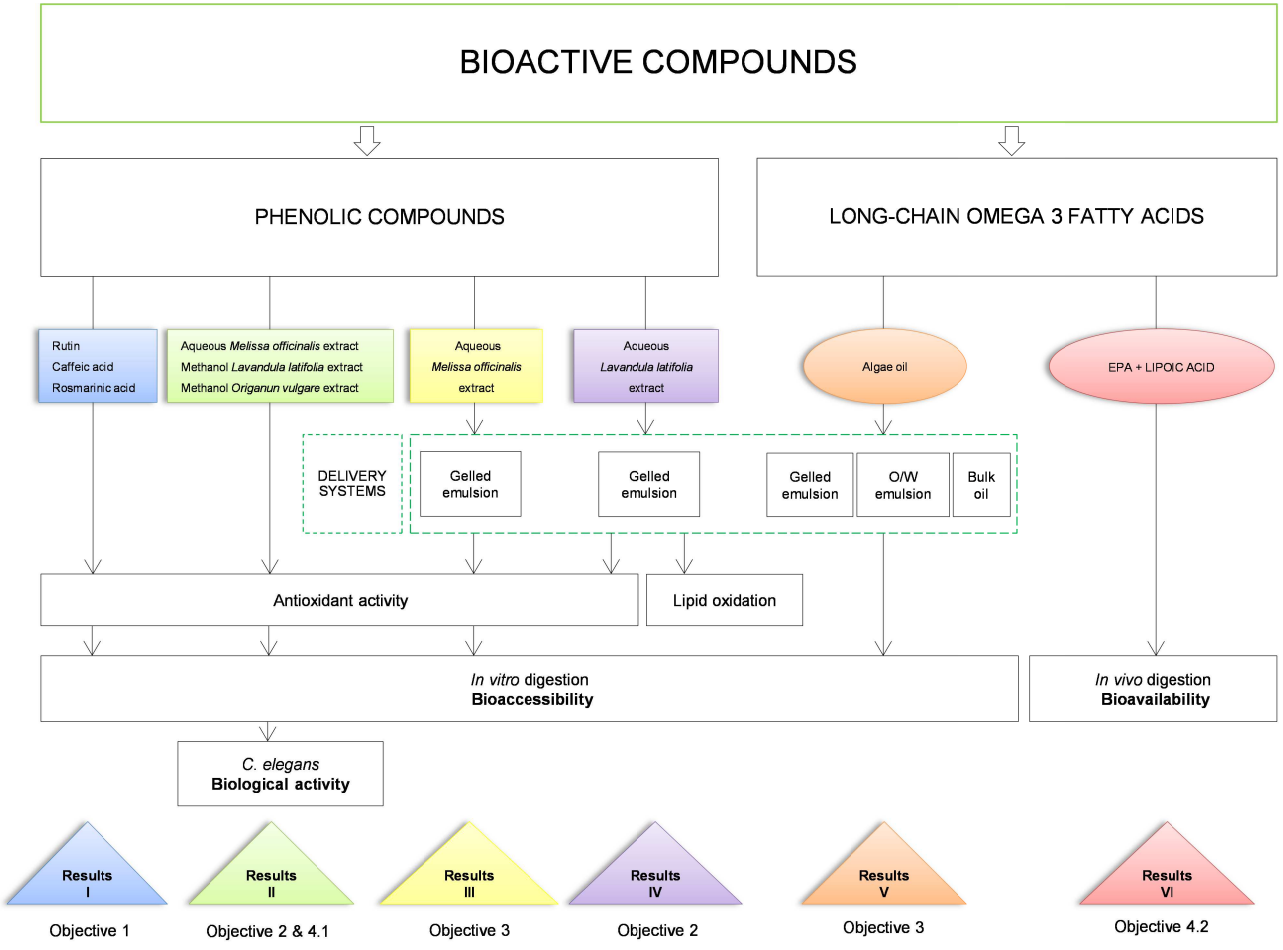
El objetivo general de la presente tesis fue diseñar y evaluar nuevos ingredientes funcionales capaces de proteger y vehiculizar compuestos bioactivos bioaccesibles.

En particular, este trabajo se centró en los siguientes objetivos:

1. Evaluar la influencia de modelos de digestión *in vitro* en la bioaccesibilidad de tres compuestos fenólicos (rutina, ácido cafeico y ácido rosmarínico).
2. Explorar extractos de plantas como fuentes potenciales de compuestos antioxidantes, caracterizar estos compuestos desde un punto de vista químico y evaluar su bioaccesibilidad.
3. Estudiar el comportamiento de diferentes sistemas de vehiculización (emulsiones O/W y emulsiones gelificadas) durante la digestión gastrointestinal y su influencia en la bioaccesibilidad de distintos compuestos bioactivos (extractos de plantas y ácidos grasos omega 3 de cadena larga).
4. Estudiar la actividad *in vivo* de compuestos bioactivos desde diferentes enfoques:
    4.1. *Caenorhabditis elegans* como modelo animal para evaluar la actividad antioxidante de los extractos de plantas.
    4.2. Intervención nutricional en humanos para evaluar las implicaciones metabólicas del ácido eicosapentaenoico y ácido α-lipoico.

EXPERIMENTAL DESIGN

MATERIAL AND METHODS


BIOACTIVE COMPOUNDS
PHENOLIC COMPOUNDS
LONG-CHAIN OMEGA 3 FATTY ACIDS
Rutin
Caffeic acid
Rosmarinic acid
Aqueous Melissa officinalis extract
Methanol Lavandula latifolia extract
Methanol Origanun vulgare extract
Aqueous Melissa officinalis extract
Acueous Lavandula latifolia extract
Algae oil
EPA + LIPOIC ACID
DELIVERY SYSTEMS
Gelled emulsion
Gelled emulsion
Gelled emulsion
O/W emulsion
Bulk oil
Antioxidant activity
Lipid oxidation
In vitro digestion
Bioaccessibility
In vivo digestion
Bioavailability
C. elegans
Biological activity
Results I
Results II
Results III
Results IV
Results V
Results VI
Objective 1
Objective 2 & 4.1
Objective 3
Objective 2
Objective 3
Objective 4.2

PLANT EXTRACTS

The plant extracts analysed in this thesis were:

- Aqueous *Melissa officinalis* extract (Results II, III).
- Methanol *Lavandula latifolia* extract (Results II).
- Methanol *Origanum vulgare* extract (Results II).
- Aqueous *Lavandula latifolia* extract (Results IV).

These plant extracts were selected based on their antioxidant activity evaluated in preliminary studies in collaboration with the members of our research group from Laboratory of Pharmacognosy and Laboratory of Botanic (Universidad de Navarra).

METHODS

In vitro gastrointestinal digestion

The *in vitro* gastrointestinal digestion model included oral, gastric and intestinal phases, according to the procedure described in Results I. To digest fat containing samples, an adaptation of the method in the composition of the duodenal juice was performed (Results III, V).

In vitro antioxidant activity

Total phenolic compounds (TPC), DPPH and ABTS were determined following the procedures described by García-Herreros, García-Iñiguez, Astiasarán, and Ansorena (2010) (Results I, II, III, IV). FRAP was determined by the method proposed by Benzie and Strain (1996), adapted to a 96 well plate (Results II).

In vivo antioxidant activity

The nematode *C. elegans* was used to evaluate the *in vivo* antioxidant activity of the plant extracts (Results II). The following assays were performed:

- Intracellular reactive oxygen species (ROS) accumulation was measured in wild type worms using H_2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) (Peixoto et al., 2016).

- Survival assay: wild type worms were exposed to a lethal dose of juglone for 24 h (Peixoto et al., 2016).
- Glutathione-S-transferase (GST-4) under juglone-induced oxidative stress: CL2166, a transgenic *C. elegans* strain expressing green fluorescent protein (GFP), was used to quantify the expression of GST-4 (Results II).

HPLC and UPLC analysis

High performance liquid chromatography with diode array (HPLC-DAD) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) have been used to identify the main phenolic compounds in the plant extracts (Results II).

Volatile compounds

In gelled emulsions, the volatile compounds were analysed by headspace solid phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS) (Results IV).

Lipid profile

Emulsions and oils: lipid extraction was performed with chloroform: methanol, followed by methylation, and identification and quantification of fatty acids using a gas chromatography flame ionization detector (GC-FID) (Results IV, V).

Human plasma: plasma fatty acids were determined according to the procedure described by Ostermann, Müller, Willenberg, and Schebb (2014) (Results VI).

Free fatty acids (FFAs)

The FFAs released during the *in vitro* intestinal digestion in oily samples was determined with a commercial kit (NEFA-HR2, Wako Diagnostics, VA, USA) (Results V).

MDA (malondialdehyde)

Oxidative stability of the micellar fraction in digested oily samples was determined by measuring the end product of lipid peroxidation chain, MDA, with a commercial kit (MAK085, Sigma-Aldrich, Steinheim, Germany) (Results V).

In Table 1, it is summarized the samples and methods applied in this work.

Table 1. Samples and methods used in the different papers of this work.

Samples	Methods	Results
Standard compounds: • Rutin • Caffeic acid • Rosmarinic acid	o *In vitro* gastrointestinal digestion o *In vitro* antioxidant activity: ABTS, DPPH o HPLC-DAD	**Results I** **Paper:** Bioaccessibility of rutin, caffeic acid and rosmarinic acid: Influence of the *in vitro* gastrointestinal digestion models.
Plant extracts: • Aqueous extract of *M. officinalis* • Methanol extract of *O. vulgare* • Methanol extract of *L. latifolia*	o *In vitro* gastrointestinal digestion o *In vitro* antioxidant activity: ABTS, DPPH, FRAP, TPC o *In vivo* antioxidant activity: ROS intracellular, survival assay, GST-4 o HPLC-DAD, UPLC-MS	**Results II** **Paper:** Bioaccessibility and biological activity of *Melissa officinalis*, *Lavandula latifolia* and *Origanum vulgare* extracts: Influence of an *in vitro* gastrointestinal digestion.
Plant extract + gelled emulsion: • Aqueous extract of *M. officinalis* • Linseed oil gelled emulsion	o *In vitro* gastrointestinal digestion o *In vitro* antioxidant activity: ABTS, DPPH, TPC	**Results III** **Poster:** Bioaccessibility of an aqueous extract of *Melissa officinalis* delivered by a carrageenan omega-3 fatty acids gelled emulsion.
Plant extract + gelled emulsion: • Aqueous extract of *L. latifolia* • Sunflower oil gelled emulsion • Algae oil gelled emulsion	o *In vitro* antioxidant activity: ABTS. DPPH, TPC o Lipid profile (GC-FID) o Volatile compounds	**Results IV** **Paper:** Volatiles formation in gelled emulsions enriched in polyunsaturated fatty acids during storage: type of oil and antioxidant.
Algae oil: • Bulk algae oil • Algae oil O/W emulsion • Algae oil gelled emulsion	o *In vitro* gastrointestinal digestion o Lipid profile (GC-FID) o Free fatty acids o MDA	**Results V** **Paper:** DHA rich algae oil delivered by O/W or gelled emulsions: strategies to increase its bioaccessibility.
Human plasma samples	o Lipid profile (GC-FID)	**Results VI** **Paper:** Effects of EPA and lipoic acid supplementation on circulating FGF21 and the fatty acid profile in overweight/obese women following a hypocaloric diet.

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RESULTS

Results I

Journal of Functional Foods

Bioaccessibility of rutin, caffeic acid and rosmarinic acid: Influence of the *in vitro* gastrointestinal digestion models

Lucía Gayoso, An-Sophie Claerbout, María Isabel Calvo, Rita Yolanda Cavero, Iciar Astiasarán, Diana Ansorena

Gayoso L, Claerbout AS, Calvo MA, Cavero RY, Astiasarán I, Ansorena D. Bioaccessibility of rutin, caffeic acid and rosmarinic acid: Influence of the in vitro gastrointestinal digestion models. Journal of Functional Foods, 2016, 26: 428-438. https://doi.org/10.1016/j.jff.2016.08.003

Results II

Journal of Functional Foods

Bioaccessibility and biological activity of *Melissa officinalis*, *Lavandula latifolia* and *Origanum vulgare* extracts: Influence of an *in vitro* gastrointestinal digestion

Lucía Gayoso, Mariana Roxo, Rita Yolanda Cavero, María Isabel Calvo, Diana Ansorena, Iciar Astiasarán, Michael Wink

Gayoso L, Roxo M, Cavero RY, Calvo MA, Ansorena D, Astiasarán I, Wink M. Bioaccessibility and biological activity of Melissa officinalis, Lavandula latifolia and Origanum vulgare extracts: Influence of an in vitro gastrointestinal digestion. Journal of Functional Foods, 2018, 44: 146-154. https://doi.org/10.1016/j.jff.2018.03.003

Results III

Poster: XI Jornada de Investigación en Ciencias Experimentales y de la Salud

Bioaccessibility of an aqueous extract of *Melissa officinalis* delivered by a carrageenan omega-3 fatty acids gelled emulsion

Lucía Gayoso, Iciar Astiasarán, Diana Ansorena

XI JORNADA DE INVESTIGACIÓN EN CIENCIAS EXPERIMENTALES Y DE LA SALUD

BIOACCESSIBILITY OF AN AQUEOUS EXTRACT *OF Melissa officinalis* L. DELIVERED BY A CARRAGEENAN OMEGA-3 FATTY ACIDS GELLED EMULSION

Lucía Gayoso,Iciar Astiasarán,Diana Ansorena

Departamento de Ciencias de la Alimentación y Fisiología
Facultad de Farmacia y Nutrición, Universidad de Navarra

INTRODUCTION

Melissa officinalis has been long used for its therapeutic properties, many of them associated to bioactive antioxidant compounds. Emulsions have been proposed to be particularly effective delivery systems for functional ingredients, such as plant extracts or lipidic compounds. However, the antioxidant properties of these bioactive ingredients may be modified by the gastrointestinal tract during the digestion process. In this sense, *in vitro* digestion models are being increasingly used to evaluate the gastro-intestinal behaviour of functional ingredients.

OBJECTIVES

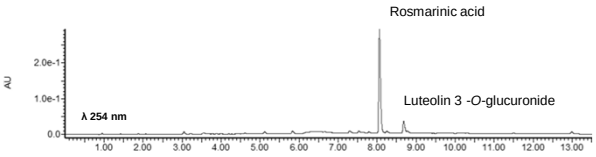
- Characterize the aqueous extract of *M. officinalis* by UPLC-DAD.
- Evaluate the influence of the incorporation of an aqueous extract of *M. officinalis* into a carrageenan linseed oil gelled emulsion on its antioxidant properties (ABTS, DPPH and TPC (total phenolic compounds)) after an *in vitro* digestion process.

EXPERIMENTAL DESIGN & RESULTS

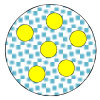
Melissa officinalis | ***Melissa officinalis* + delivery system** | **Delivery system**

Aqueous extract of *M. officinalis* was prepared as described by Berasategi et al. (2014).

UPLC-DAD

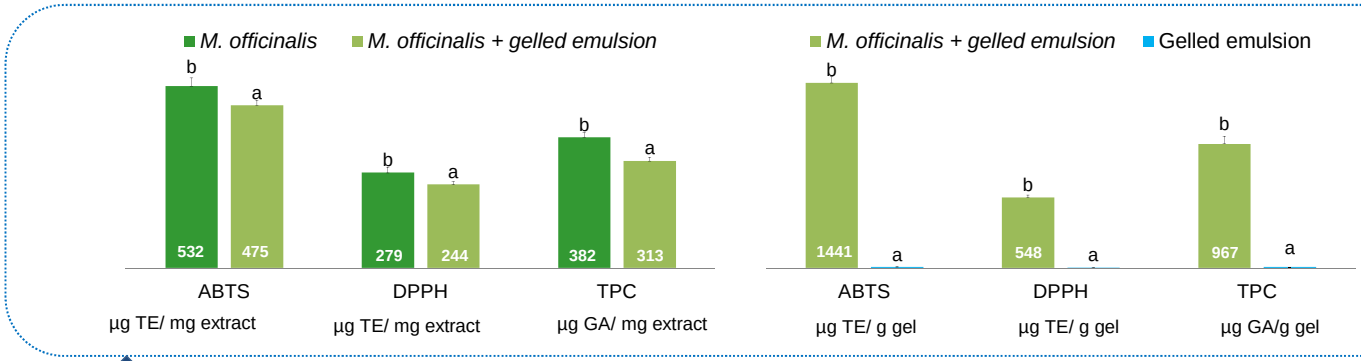


M. officinalis GELLED EMULSION
40% linseed oil + 1.5% carrageenan + 0.3% plant extract



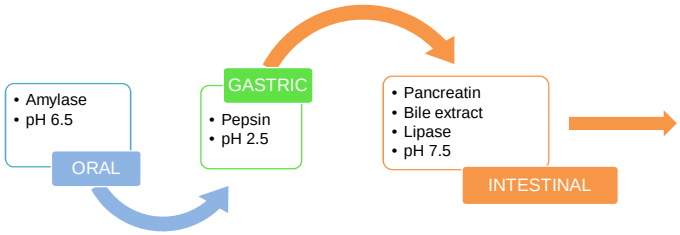
GELLED EMULSION
40% linseed oil + 1.5 % carrageenan

The preparation of the gelled emulsion was made following the methodology described by Poyato et al. (2014).



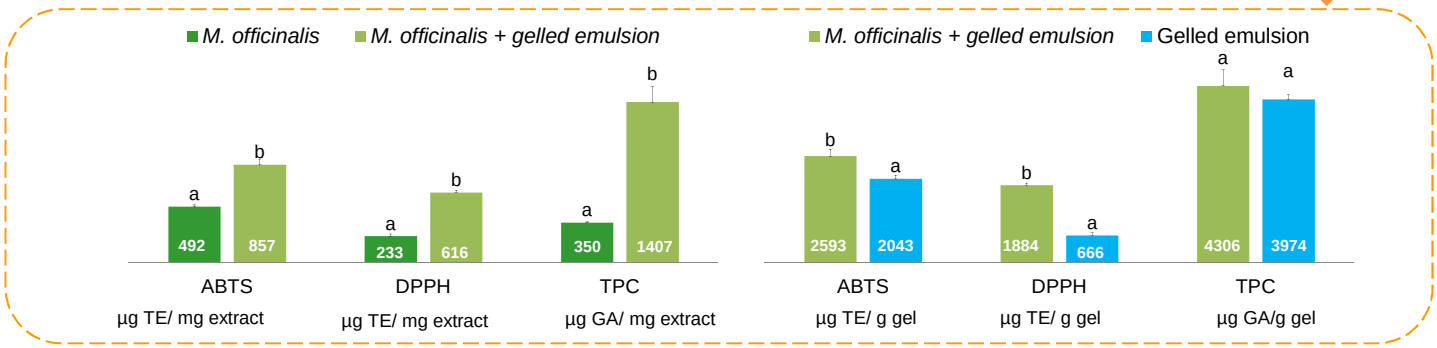
Different letters indicate significant differences ($p \leq 0.05$)

BEFORE DIGESTION
Antioxidant activity:
ABTS, DPPH, TPC



AFTER DIGESTION
Antioxidant activity:
ABTS, DPPH, TPC

The *in vitro* digestion process was performed following the procedure described by Gayoso et al. (2016), with modifications.



Different letters indicate significant differences ($p \leq 0.05$)

CONCLUSION

Rosmarinic acid and luteolin 3 -*O*-glucuronide were the main compounds identified in the aqueous extract of *M. officinalis*. The antioxidant activity of the plant extract after the digestion process was up to 93% of the activity found before digestion. Moreover, when the plant extract was delivered into the gel, this value increased up to 4.5 fold the activity found before digestion. Therefore, the carrageenan gelled emulsion is a potential ingredient to develop bioaccessible functional foods.

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Gayoso, L., Claerbourt, A.S., Calvo, M.I., Cavero, R.Y., Ansorena, D., Astiasarán, I. (2016). Journal of Functional Foods 26, 428-438.
Poyato, C., Ansorena, D., Berasategi, I., Navarro-Blasco, Í., Astiasarán, I. (2014). Meat Science, 98(4), 615-621.

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Gobierno de Navarra (Departamento de Educación)

Results IV

Journal of Food Science and Technology

Volatile formation in gelled emulsions enriched in polyunsaturated fatty acids during storage: Type of oil and antioxidant

Lucía Gayoso, Candelaria Poyato, María Isabel Calvo, Rita Yolanda Cavero, Diana Ansorena, Iciar Astiasarán

Gayoso L, Poyato C, Calvo MA, Cavero RY, Ansorena D, Astiasarán I. Volatiles formation in gelled emulsions enriched in polyunsaturated fatty acids during storage: type of oil and antioxidant. Journal of Food Science and Technology, 2017, 54(9):2842–2851. https://doi.org/10.1007/s13197-017-2722-5

Results V

Journal of the Science of Food and Agriculture (under review)

DHA rich algae oil delivered by O/W or gelled emulsions: strategies to increase its bioaccessibility

Lucía Gayoso, Diana Ansorena, Iciar Astiasarán

ABSTRACT

Background: Bioaccessibility of bioactive compounds for functional food deserves to be evaluated. An *in vitro* gastrointestinal digestion model was applied to provide information about the extent of lipid hydrolysis, oxidative stability and bioaccessibility of algae oil (42% of docosahexaenoic acid - DHA), comparing three lipid delivery systems: bulk oil, soy protein stabilized O/W emulsion and carrageenan gelled emulsion.

Results: Lipid digestion kinetics was slightly influenced by the delivery systems. Nevertheless, at the end of intestinal digestion, lipolysis in the three samples ranged between 49-52%, showing a partial oil digestion. Lipid oxidation, measured by malondialdehyde (MDA), was significantly lower ($p < 0.01$) in both emulsified oils after intestinal digestion, as compared to the bulk oil. Bioaccessibility of DHA was 58%, 71% and 84% for bulk oil, O/W emulsion and gelled emulsion, respectively.

Conclusion: This suggests that both emulsified delivery systems used in this work enhanced the solubilization of free fatty acids, in particular omega 3 fatty acids, and therefore their potential intestinal absorption.

Keywords: DHA; algae oil; bioaccessibility; emulsion; *in vitro* digestion.

INTRODUCTION

The enrichment of foods with bioactive lipids, such as omega 3 fatty acids, is an increasingly important area in the food industry [1]. Omega 3 fatty acids are widely used compounds due to their beneficial health effects. It has been described their role in the improvement of the cognitive function, their influence on the reduction of the risk of cardiovascular and inflammatory diseases, and their relationship with the reduction of certain types of cancer [2–4]. Although marine oils are the main source of long chain omega 3 fatty acids, algae oil has emerged as an environmentally-friendly and vegan source of these fatty acids, with DHA being the most abundant one. DHA is a key omega 3 fatty acid with an important role in the development of the neurological system [5], and algae oil is a main dietary source of this fatty acid [6].

In the human body, lipid digestion involves lipolysis of the dietary triglycerides by gastric and pancreatic lipase and their solubilisation in mixed bile salt micelles, so that they can be available for their absorption by the intestinal cells [7]. *In vitro* digestion models mimic the conditions of fluids during the gastrointestinal tract, including digestive enzymes, salt concentrations, pH and digestion time, among other factors [8]. Our group has previously applied an *in vitro* model system for determining the bioaccessibility of antioxidant compounds [9]. Moreover, these models are being increasingly used to evaluate lipid-based delivery systems and can be used as a screening tool for designing lipid-based formulations [10].

The digestion of dietary lipids in the gastrointestinal tract is a critical process determining their bioaccessibility, which is a critical precursor to bioavailability [11]. Strategies to improve omega 3 bioaccessibility have been explored, including those based on emulsification [12]. In terms of supramolecular structure, emulsification can favour the action of digestive lipases by simplifying the emulsification that occurs within the gastrointestinal tract and increasing the oil-water interfacial area for lipase adsorption [11,13]. Indeed, in contrast to encapsulation technologies based on polymeric particles, emulsion technology is versatile, non-proprietary and cheap [14].

Food emulsions may be stabilized by a great variety of emulsifiers, such as proteins, surfactants, phospholipids and polysaccharides, and it has been reported that the nature of the emulsification may influence the gastrointestinal fate of emulsified fish oils [15]. The technological development and application of soy protein stabilized emulsion and Tween 80 stabilized carrageenan gelled emulsion were previously studied by our research group as delivery systems for omega 3 fatty acids in functional foods [16,17]. In the present study, these two delivery systems were selected to emulsify algae oil. Unlike fish oil [7,15,18–20], emulsification of algae oil has been less studied and few studies have addressed their impact on bioaccessibility [11,12]. The aim of this work was to evaluate the extent of lipid digestion, oxidative stability and bioaccessibility of algae oil depending on the lipid delivery system applied (bulk oil, soy protein stabilized O/W emulsion and a carrageenan gelled emulsion). An *in vitro* model system was applied for that purpose.

MATERIAL AND METHODS

Material

Algae oil derived from the marine alga *Schizochytrium* sp. (O55-O100 life's OMEGA 60) was kindly donated by DSM Nutritional Products (Kaiseraugst, Switzerland). *K*-carrageenan (Satiagel™ RPM 87R1) was provided by Cargill (Saint-Germain-en-Laye, France). Soy protein was obtained by Apasa (Guipozkoa, Spain). Alpha-amylase from human saliva (A1031, 852 U/mg protein), pepsin from porcine gastric mucosa (P7000, 674 U/mg protein), pancreatin from porcine pancreas (P1750, 4× United States Pharmacopeia specifications), lipase from porcine pancreas (L3226, 419 U/mg protein, activity using olive oil substrate), bile extract (B8631) and Tween 80 were purchased from Sigma-Aldrich (Steinheim, Germany).

Emulsion preparation

The O/W emulsion was prepared as described by García-Íñiguez de Ciriano *et al*. [21]. Eight parts of hot water (50 °C) were mixed with one part of isolated soy protein for 2 min and then with 10 parts of algae oil for other 3 min.

Homogenization was made using a homogenizer (16.000 rpm, Ultra-Turrax® T25 basic).

Gelled emulsion preparation

The formulation of the gelled emulsion included algae oil (40%), *k*-carrageenan (1.5%) and Tween 80 (0.12%). The preparation was made following the methodology described by Poyato *et al*. [16]. The carrageenan was dissolved in water and the surfactant was added to the oil. Both phases were heated separately up to 70 °C and mixed using a homogenizer (16.000 rpm, Ultra-Turrax® T25 basic). The emulsion was cooled to room temperature in a sealed flask, allowing the carrageenan to polymerize. The gel was kept overnight under refrigeration (4 °C) until being used or analyzed.

***In vitro* digestion**

The amount of sample subjected to digestion was calculated so there was 1 g of oil in every system: 1 g of bulk oil, 1.9 g of O/W emulsion and 2.5 g of gelled emulsion. Samples were placed in a Falcon tube, mixed with 20 mL of water and homogenized for 5 seconds with an Ultra-Turrax® (T25 basic). *In vitro* digestion, including oral, gastric and intestinal phases, was performed following the procedure described by Gayoso *et al.* [22] with slight modifications in the composition of the duodenal juice to adapt the protocol to fat containing samples. In oral step, 250 µL of alpha-amylase/g of sample (stock solution: 1.3 mg/mL in 1 mM $CaCl_2$) was added, pH was adjusted to 6.5 with 1 M $NaHCO_3$ and samples were incubated in a water bath at 37 °C for 2 min with magnetic stirring. In the gastric digestion, on the same tube, 330 µL of pepsin/g sample (stock solution: 160 mg/mL in 0.1 M HCl) was added, pH was adjusted to 2.5 with 3 M HCl and the incubation time was 120 min at 37 °C. After gastric digestion, 5 mL of pancreatin-bile extract- lipase (25 mg/mL of pancreatin + 160 mg/mL of bile extract + 33 mg/mL of lipase in 0.1 M $NaHCO_3$) were added to the gastric mixture, pH was adjusted to 7.5 with $NaHCO_3$ and the incubation time was the same as that of gastric digestion (120 min at 37 °C). Lipase concentration was calculated to achieve 2000 U/mL in the intestinal mixture as stated in the international consensus protocol described by Minekus *et al.* [8].

Bile extract concentration was selected based on the dose used by Ortega *et al.* [24] to digest samples with 15% of fat content.

Micellar fraction

After 120 min of intestinal digestion, samples were centrifuged at 4000 rpm for 40 min at room temperature [18]. Three phases were formed after centrifugation: oily phase containing undigested lipids (upper), aqueous phase (micellar phase), and a precipitated pellet phase. The oily phase was carefully collected with a Pasteur pipette and weighed. The intermediary phase corresponding to the micellar fraction of the digesta represents the bioaccessible fraction, and it was collected in order to determine the fatty acid composition. The difference between the initial amount of oil and the oil in the oily phase was assumed to be the amount of oil in the micellar fraction.

Lipid bioaccessibility was defined as the weight percentage of oil that, from the initial amount of oil in the sample before digestion, was incorporated into the micellar fraction after centrifugation [11]. It was calculated by the following formulae:

Fat bioaccessibility (%) = (amount of oil (g) in the micellar fraction/initial amount of oil (g) before digestion) x100

DHA bioaccessibility (%) = (DHA (g) in the micellar fraction/initial amount of DHA (g) before digestion) x100

Fat extraction and fatty acid composition

Extraction of lipids in samples (bulk oil, O/W emulsion, gelled emulsion) before digestion and in the micellar fraction was based on the Folch method. Briefly, samples were mixed with chloroform/methanol (2:1, v/v) and then homogenized with an Ultra-Turrax® (samples before digestion) or by shaking vigorously in a separation funnel (micellar fraction samples), allowing then to separate into two phases. Organic phase was collected and a second extraction with chloroform was performed. Finally, organic phases were combined and purified using a 0.88% KCl solution. The solvent was evaporated using a rotary evaporator.

The fatty acids were determined in the oil and in the lipid extracts by gas chromatography FID detection according to the procedure described by Poyato *et al.*[25]. Results were expressed as g fatty acids/100 g oil from three independent replicates. After the quantification of the individual fatty acids, the sums of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and omega 3 fatty acids were calculated.

Determination of free fatty acids (FFAs)

The non-esterified fatty acid (NEFA) kit (NEFA-HR2, Wako Diagnostics, VA, USA) was used to determine FFAs released during the *in vitro* intestinal digestion, as previously described by Malaki *et al.* [26]. Aliquots of the intestinal digestate were taken at 0, 2, 5, 15, 30, 60 and 120 min of intestinal digestion process. 100 µL of intestinal sample was added to 100 µL of 0.1 M HCL to stop lipolysis. The FFAs were extracted by adding 900 µL of hexane. The mixture was then vortex for 10 s and centrifuged at 12000 rpm for 30 min. After centrifugation, as kit manufacture recommendations indicate, 5 µL of sample was mixed with 200 µL of reagent A into a 96-well plate, then incubated at 37 °C for 5 min. Afterwards, 100 µL of reagent B was added to each well, followed by a second incubation at 37 °C for 5 min. A standard curve (oleic acid from 0.05 to 1 mM) was also performed. FFAs were determined measuring absorbance at 550 nm, using a UV-Vis spectrophotometer (FLUOStar Omega spectrofluorometric analyser, BMG Labtechnologies, Offenburg, Germany). The average molecular weight of the algae triglycerides was calculated as 908.4 g/mol, based on their fatty acid profile. The percentage of lipid hydrolysis was calculated based on the moles of FFAs present at each time point with respect to the total moles of fatty acids initially present. Results shown are the mean of three independent replicates.

MDA

Oxidative stability of the micellar fraction was determined by measuring the end product of lipid peroxidation chain, malondialdehyde (MDA), with a commercial kit (MAK085, Sigma-Aldrich, Steinheim, Germany). The manufacturer´s instructions were followed. MDA forms with thiobarbituric acid a colorimetric

product, proportional to the MDA concentration. Samples of intestinal digestate (10µL) were taken at 0, 2, 5, 15, 30, 60 and 120 min of intestinal digestion process and mixed with 500 µL of 42 mM H_2SO_4 and 125 µL of phosphotungstic acid solution. Then, samples were incubated at room temperature for 5 min and centrifuged at 11000 rpm for 3 min. The supernatant was discarded and the pellet was resuspended on ice with water/BHT solution. After the addition of 600 µL of TBA solution, samples were incubated at 95 ºC for 60 min and subsequently cooled on ice. Finally, the absorbance was measured at 532 nm using a UV-Vis spectrophotometer (FLUOStar Omega spectrofluorometric analyser, BMG Labtechnologies, Offenburg, Germany). Results were expressed in µmol MDA/g oil and are the mean of three independent replicates.

Microscopic image analysis

The microstructure of the samples after gastric and intestinal digestion was checked by a Nikon E-800 (Kawasaki, Japan) brightfield light microscopy with 10× magnification. After the gastric and intestinal steps, a drop of sample was placed on microscope slide and covered with a cover slip just before observation. The images were monitored and captured by digital Nikon DXM-1200.

Statistical analysis

The statistical analysis of data was done using Stata v.12.0 software (StataCorp LP, Texas, USA). Each parameter was measured in three independent replicates. For the evaluation of the significant differences in lipolysis and MDA at different times and among samples, one-way analysis of variance (ANOVA) with Bonferroni *post hoc* test (p <0.05) was applied. In the analysis of the lipid profile of samples, the effect of the delivery system and digestion, and their interaction was studied by a 2x2 factorial ANOVA. T-student test was applied in case of significant interaction between the variables. A principal component analysis (PCA) was performed on the lipid profile, lipolysis and oxidation data applying orthogonal rotation.

RESULTS AND DISCUSSION

Lipid hydrolysis

The lipolysis of bulk oil was compared with that observed when the oil was incorporated into an O/W emulsion and a gelled emulsion. The extent and rate of lipolysis was determined by measuring the FFAs released during the *in vitro* intestinal digestion process (Figure 1).The initial amount of FFAs at time 0 (initial point of intestinal digestion) was negligible in all cases. From the initial point, during the first 15 min a rapid release of FFAs from the lipid droplets was observed in all samples. This release was modelled and the resulting curves fit a first order kinetic model in the three cases (R^2 =0.87 for bulk oil; R^2 =0.97 for O/W emulsion, R^2 =0.99 for gelled emulsion). Modelling also revealed that lipid kinetics was influenced by the delivery system. The fastest release of FFAs was observed in the gelled emulsion and the O/W emulsion in comparison with the bulk oil. This was confirmed by the k values of the kinetic model of the lipolysis that were 4.4851, 4.423 and 4.3788 for the bulk oil, O/W emulsion and gelled emulsion, respectively. The different behaviour on the rate of hydrolysis between the bulk algae oil and the two delivery systems might be related to the smaller droplet size and a larger surface area of lipid droplet exposed to digestive enzymes. To confirm this hypothesis, microscopic evaluation of the samples after the gastric digestion, just before starting the intestinal step, was performed. The images confirmed the reduction of the drop size with the emulsification process. Effectively, figure 2 shows how the drop size in bulk oil after gastric digestion (a) was bigger than those of O/W emulsion (c) and gelled emulsion (e). The smaller droplets have a larger surface area and therefore, a greater number of sites for lipase binding contribute to facilitate lipolysis. This results are in agreement with those that reported that there is a reasonable correlation between the droplet size and the rate of lipolysis [14,27].

In all samples, the velocity of lipolysis reaction markedly decreased after the first 30 min and reached a plateau approximately after 60 min of intestinal digestion. This behaviour was also observed by Malaki *et al*. [28], where the saturation of the oil-water interface with bile salts, along with the accumulation

of lipolitic products, as FFAs and monoglycerides, inhibited further access of pancreatin to its substrate.

Despite the initial differences in droplet size and lipolysis rate among samples at the beginning of the intestinal digestion, a similar reduction in droplet size was observed at the end of intestinal digestion in the three samples (Figure 2b, 2d, 2f). Moreover, there were no significant differences in the % of lipolysis among samples, reaching values around 49-52% indicating a partial oil digestion. In fact, it has been described a high resistance of long chain polyunsaturated fatty acids to *in vitro* lipolysis [12,19]. This effect has been related to the resistance of EPA and DHA to pancreatic lipase, at least partly due to the presence of a double bond close to the carboxyl end [29].

Lipid oxidation

Algae oil is highly susceptible to oxidation due to the presence of highly unsaturated long-chain fatty acids and some emulsion-based delivery systems have been shown to be suitable to protect polyunsaturated fat from lipid oxidation [30]. Indeed, the gastrointestinal tract appears to be a pro-oxidative environment [31], so the evaluation of the algae oil oxidation delivered by the conventional O/W emulsion or the gelled emulsion and as a bulk oil was studied under intestinal digestive conditions by measuring the lipid oxidation product MDA (Figure 3). MDA can be derived from several precursor molecules, but the main origin is PUFA [32].

In our study, MDA values were low in all samples during the first min of intestinal digestion, which indicated minimum gastric oxidation (Figure 3). Although gastric environment has been described as a pro-oxidant because of low pH [33], other authors [32] also described no oxidation at gastric step during *in vitro* digestion of cold liver oil. The hypothesis proposed by these authors was the lack of gastric lipolysis because no gastric lipase was added, as it was also confirmed in our study where no FFAs were detected just after gastric digestion.

The maximum value of MDA was observed after 15 min of intestinal digestion, without noticing significant differences among the three samples (23.88 ± 3.32, 21.60 ± 3.46, 16.48 ± 1.20 µmol/g oil in bulk oil, gelled emulsion and O/W

emulsion, respectively, $p > 0.05$). This fact could be explained by the fast liberation of FFAs during the first min of intestinal digestion, as it has been previously pointed out. Larsson *et al.* [31] described that lipolysis process play a role on gastrointestinal oxidation of cod liver oil, because of FFAs are more susceptible to oxidation than the intact triglycerides and could act as pro-oxidative.

At the end of the intestinal digestion, MDA levels in the O/W emulsion (8.94 ± 1.22 µmol/g oil) and gelled emulsion (8.28 ± 0.6 µmol/g oil) were in line with levels found in marine oil emulsions reported by Kenmogne-Domguia *et al*. [34]: 7.8 µmol/g oil and 7.2 µmol/g oil during *in vitro* digestion of bovine serum albumin-stabilised emulsion and phospholipid-stabilised emulsion, respectively. In the bulk oil, a significantly ($p < 0.05$) higher lipid oxidation was observed (22.94 µmol/g oil), which could have contributed to explain the increase in SFAs in the micellar fraction of bulk oil, as it will be further discussed (Table 1). In addition, soy protein and Tween 80 are emulsifiers that could have protective effects against lipid oxidation at the end of the digestion process. In fact, soy protein has shown antioxidant properties during *in vitro* digestion, affecting not only the extent of lipid oxidation, but also reaction pathways [35]. Apart from soy protein, other protein isolates have also shown antioxidant activity in emulsions [20,36]. Regarding surfactants such as Tweens, it has been reported their role protecting emulsified lipids against oxidation [37]. In the particular case of the gelled emulsion, in addition to Tween 80, an additional antioxidant effect could be attributed to carrageenan. In fact, the potential lipid antioxidant effect of carrageenan by means of hydroxyl radical formation, such as other sulfated polysaccharides has been reported [38]. Moreover, the decrease of MDA observed at the end of intestinal digestion in the W/O emulsion and gelled emulsion could be due to its degradation or metabolism into other compounds, as suggested by other authors [39].

Lipid profile and bioaccessibility

As there is a relationship between lipid composition and oxidative stability, the different behaviour of the bulk oil as compared to the delivery systems with regard to lipid oxidation susceptibility observed at the end of intestinal digestion

may have an impact on the lipid composition of the bioaccessible fraction. The lipid profile of the bulk oil, O/W emulsion and gelled emulsion before the gastrointestinal digestion process and in the micellar fraction obtained after the digestion process was compared (Table 1). Results show for the first time complete lipid profile of the micellar fraction of algae oil, not only DHA data.

Algae oil contains large proportion of EPA and DHA that accounted approximately for 18% and 42% of the total fatty acids, respectively. The PUFA fraction represents 68%, followed by SFA (24%) and MUFA (7%). In general, although some statistical differences were found among the three samples before digestion, these were not quantitatively relevant. On the other hand, in order to determine the influence of the delivery system and digestion process on the lipid profile a 2x2 factorial ANOVA was carried out, comparing the samples before and after digestion. This test showed that there was a significant interaction between delivery system and digestion for the most abundant fatty acids found in the algae oil (DHA, palmitic acid and EPA), affecting their bioaccessibility.

When the bioaccessibility of algae oil was calculated, it was observed that the gelled emulsion showed the highest value (84%), followed by the O/W emulsion (73%) and the bulk oil (71%). Namely, the micellar fraction contained higher amount of fat when it was supplied with than without a delivery system. Even though both emulsions and bulk oil presented the same rate of lipolysis at the end of intestinal digestion (Figure 1), it seems that emulsification played a role in enhancing the transfer of fatty acids from oil to the aqueous phase, as suggest by Lin *et al*. [12]. Differences between bulk oil and emulsified oils (gelled emulsion and O/W emulsion) in the micellar fraction were reflected in the lipid profile. SFAs were around 31% in the case of bulk oil and 21% for emulsified oils, which may be explained by the higher level of MDA in bulk oil than in emulsified samples at the end of intestinal digestion (Figure 3). PUFAs were around 59% for bulk oil and 70% for emulsified oils. In the case of omega 3 fatty acids, bulk oil only showed a 53%, whereas emulsified oils showed higher values (63-64%). Regarding DHA, it was totally transferred to the micellar fraction in emulsified oils, whereas there was a significant loss in the

digestion of bulk oil, with approximately 18% of loss. In terms of bioaccessibility, DHA showed the following values: 58%, 71% and 84% for bulk oil, O/W emulsion and gelled emulsion, respectively. A slight loss of EPA (approximately 7%) was found in emulsified samples, whereas in the bulk oil it was 23%. Thus, the amount of long-chain unsaturated fatty acids transferred to the micellar fraction was higher in the emulsified samples, which agrees with the higher bioaccessibility of omega 3 from emulsified algae oil *vs*. bulk oil described *in vivo* [40] and *in vitro* [12]. These differences are highly interesting from the nutritional perspective, taking into account that the micellar fraction is considered the bioaccessible fraction.

PCA

A multivariate analysis taking into account the measured parameters (fatty acid profile, lipolysis and oxidation) was carried out to summarize the variability of data. Figure 4 shows the bidimensional representation of these variables by the loading plot and score plot, according to two principal components. Despite all the variables had similar loadings, altogether defined two components adequately. Principal component 1 (PC1) and principal component 2 (PC2) explained up to 85% of the total variance (57% and 27%, respectively). According to these two components, a clear difference can be observed between samples before and after digestion, mainly due to PC1, which was mainly defined by FFAs, linoleic acid and palmitoleic acid. Moreover, bulk oil can be clearly distinguished from emulsified oils just only after digestion. Differences found in oxidation intensity and lipid profile after digestion justifies the observed distribution of samples.

CONCLUSIONS

Algae oil delivered by the O/W emulsion and the gelled emulsion showed some advantages, in terms of bioaccessibility and lipid oxidation in comparison with the bulk oil, whereas no differences were observed in the extent of lipolysis at the end of intestinal stage. Emulsification increased solubilization of digestion products in mixed micelles, especially of DHA, which could be an effective strategy to increase omega 3 fatty acids bioaccessibility. Therefore, both the

emulsion and the gel used in this work, in comparison with the bulk oil, represent an interesting advantage to take into account to design functional foods.

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Figure 1. Release of FFAs (% Lipolysis) from bulk oil, O/W emulsion and gelled emulsion during *in vitro* intestinal digestion.

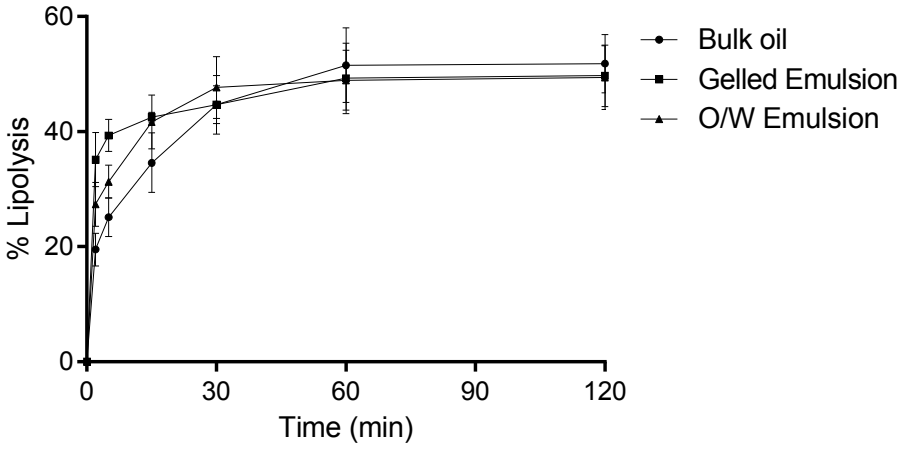
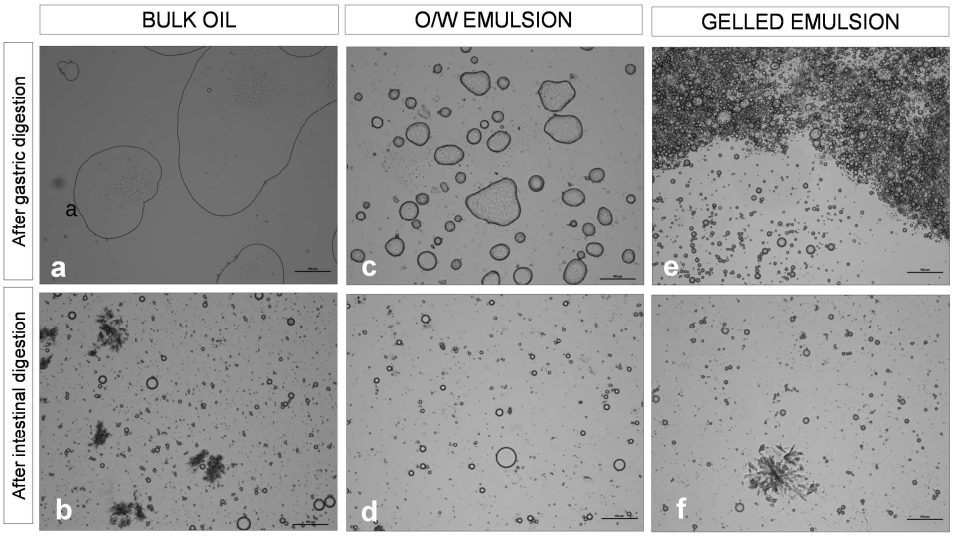


Figure 2. Microstructure of bulk oil, O/W emulsion and gelled emulsion after *in vitro* gastric digestion and intestinal digestion. Scale bar represents 100 μm.



a-Bulk oil after gastric digestion, b-Bulk oil after intestinal digestion, c-O/W emulsion after gastric digestion, d-O/W emulsion after intestinal digestion, e-Gelled emulsion after gastric digestion, f-Gelled emulsion after intestinal digestion.

Figure 3. Formation of MDA in bulk oil, O/W emulsion and gelled emulsion during in vitro intestinal digestion.

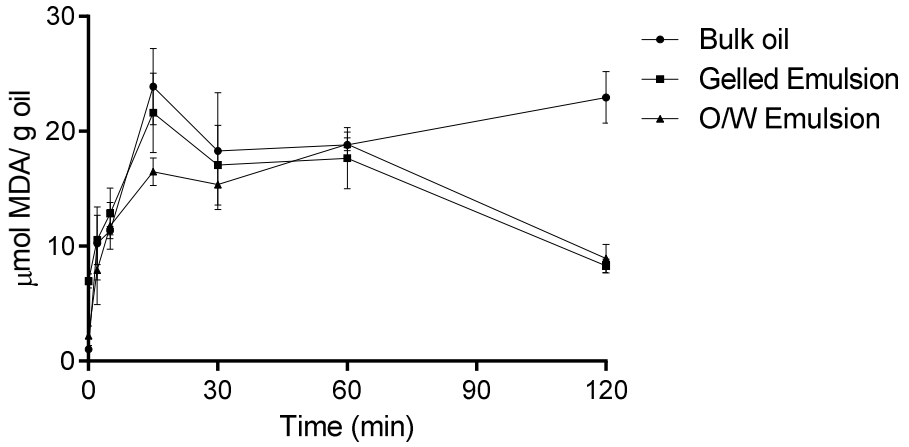
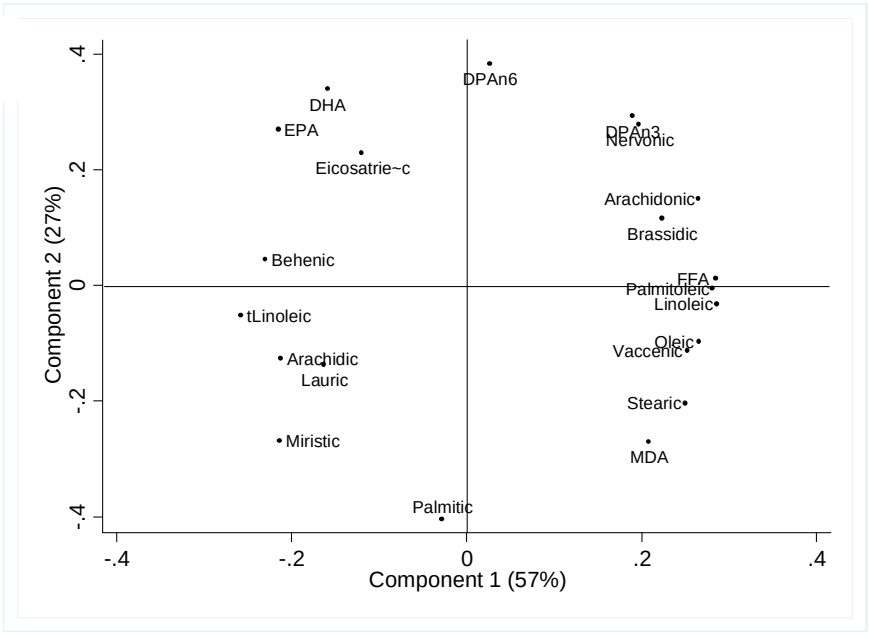
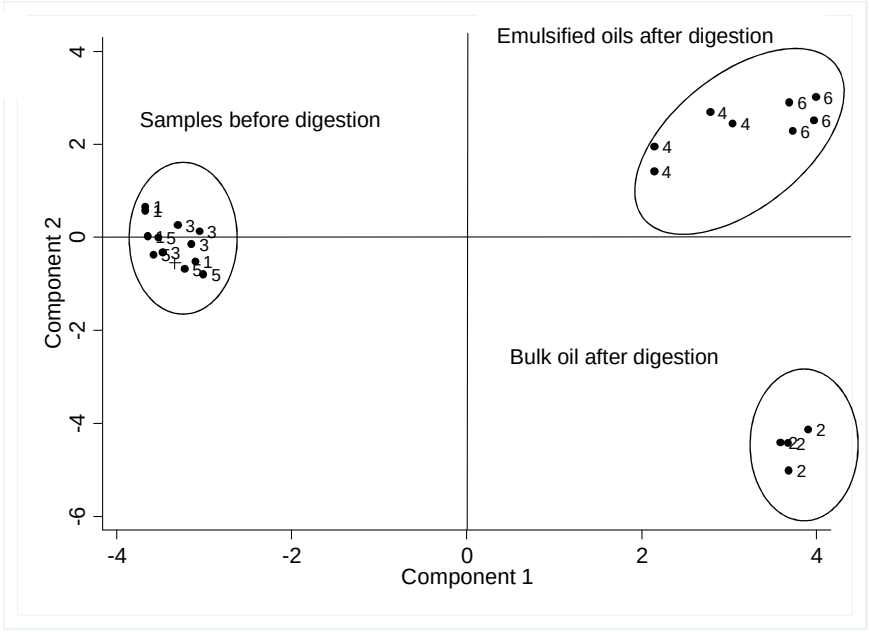


Figure 4. Loading plot (a) and score plot (b) of PC1 and PC2 carried out on fatty acid profile in bulk oil, O/W emulsion and gelled emulsion before and after in vitro digestion.

a)



b)



1: bulk oil before digestion; 2: bulk oil after digestion; 3: O/W emulsion before digestion; 4: O/W emulsion after digestion; 5: gelled emulsion before digestion; 6: gelled emulsion after digestion.

Table 1. Lipid profile of bulk oil, O/W emulsion and gelled emulsion before and after *in vitro* digestion expressed in g fatty acids/100 g fatty acids (mean ± sd).

	Bulk oil			O/W Emulsion			Gelled Emulsion			*P* value†		
	Before digestion	Micellar Fraction	T-test (*p*)	Before digestion	Micellar Fraction	T-test (*p*)	Before digestion	Micellar Fraction	T-test (*p*)	Delivery system	Digestion	Ds x D
Lauric C12:0	0.09±0.00 a	0.10±0.01 A	0.1296	0.12±0.00 c	0.09±0.00 AB	0.0000	0.10±0.00 b	0.08±0.01 B	0.000	0.0000	0.0000	0.0000
Myristic C14:0	1.48±0.04 a	1.47±0.05 A	0.6773	1.55±0.02 b	1.15±0.00 C	0.000	1.57±0.01 b	0.99±0.11 B	0.000	0.0000	0.0000	0.0000
Palmitic C16:0	20.46±0.43 a	25.92±0.61 A	0.000	20.91±0.17 ab	16.87±1.79 B	0.0041	21.44±0.34 b	16.72±1.08 B	0.000	0.0000	0.0149	0.0000
Palmitoleic C16:1	0.07±0.00 a	0.17±0.01 A	0.000	0.09±0.00 b	0.16±0.02 A	0.0003	0.09±0.01 b	0.16±0.02 A	0.000	0.3786	0.0000	0.0223
Stearic C18:0	1.67±0.03 a	3.22±0.08 A	0.000	1.68±0.01 a	2.30±0.03 B	0.000	1.80±0.05 b	2.38±0.13 B	0.000	0.0000	0.0000	0.0000
Oleic C18:1 (ω-9)	6.57±1.01 a	9.25±0.10 A		6.04±0.04 a	8.18±0.41 B		6.40±0.09 a	8.15±0.39 B		0.0080	0.0000	0.2551
***c*-Vaccenic C18:1 (ω-7)**	0.17±0.01 a	0.46±0.03 A	0.000	0.29±0.01 c	0.35±0.01 B	0.0001	0.18±0.00 a	0.38±0.02 B	0.000	0.0000	0.0000	0.0000
***t*-linoleic C18:2Δ9t.12t**	0.11±0.01 a	0.08±0.01 A		0.12±0.00 a	0.09±0.00 A		0.11±0.02 a	0.06±0.00 B		0.0099	0.0000	0.0568
Linoleic C18:2Δ9c.12c	0.51±0.05 a	2.22±0.04 A	0.000	0.61±0.01 a	1.93±0.13 B	0.000	0.58±0.01 a	1.88±0.15 B	0.000	0.0006	0.0000	0.0001
Arachidic C20:0	0.40±0.02 a	0.29±0.02 A	0.0002	0.29±0.00 b	0.29±0.01 A	0.9548	0.39±0.00 a	0.21±0.03 B	0.0062	0.0068	0.0000	0.0007
Behenic C22:0	0.15±0.02 a	0.06±0.01 A	0.0002	0.09±0.01 b	0.10±0.01 B	0.3495	0.15±0.00 a	0.06±0.01 A	0.0000	0.2376	0.0000	0.0000
Brassidic C22:1t Δ13t	0.07±0.00 a	0.08±0.00 A	0.0289	0.06±0.01 a	0.09±0.01 A	0.0007	0.07±0.01 a	0.08±0.00 A	0.0121	0.8924	0.0000	0.0074
Eicosatrienoic C20:3 (ω-3)	0.13±0.01 a	0.12±0.01 A		0.14±0.01 a	0.14±0.01 B		0.13±0.01 a	0.13±0.00 AB		0.0659	0.0508	0.1605
Arachidonic C20:4 (ω-6)	1.50±0.03 a	1.76±0.05 A	0.0001	1.50±0.01 a	1.93±0.08 B	0.0000	1.51±0.01 a	1.96±0.01 B	0.0000	0.0001	0.0000	0.0005
Eicosapentaenoic C20:5 (ω-3)	18.01±0.40 a	13.83±0.14 A	0.0000	18.07±0.06 a	16.88±0.33 B	0.0004	18.14±0.20 a	16.95±0.24 B	0.0003	0.0000	0.0000	0.0000
Nervonic C24:1 (ω-9)	0.28±0.01 a	0.28±0.01 A	0.7057	0.27±0.01 a	0.34±0.00 B	0.0000	0.28±0.01 a	0.35±0.01 B	0.0002	0.0000	0.0000	0.0000
Docosapentaenoic C22:5 (ω -6)	2.05±0.04 a	1.87±0.04 A	0.0008	2.16±0.02 b	2.18±0.04 C	0.5259	2.08±0.01 a	2.32±0.02 B	0.0000	0.0000	0.0584	0.0000
Docosapentaenoic C22:5 (ω -3)	3.94±0.12 a	4.04±0.13 A	0.3035	4.09±0.10 a	4.71±0.12 B	0.0002	4.05±0.01 a	4.82±0.08 B	0.0000	0.0000	0.0000	0.0000
Docosahexaenoic C22:6 (ω-3)	42.30±0.46 a	34.89±0.34 A	0.0000	41.96±0.32 b	41.71±0.07 B	0.3894	41.11±0.05 b	41.03±0.01B	0.1302	0.0000	0.0000	0.0000
Σ SFA	24.28±0.52 a	31.06±0.57 A	0.000	24.63±0.18 a	20.77±1.70 B	0.0040	25.49±0.43 b	21.10±0.53 B	0.0000	0.0000	0.1503	0.0000
Σ MFA	7.08± 1.00 a	10.16±0.12 A		6.69±0.04 a	9.03±0.42 B		6.94±0.09 a	9.19±0.19 B		0.0107	0.0000	0.1715
Σ PUFA	68.49± 0.68 a	58.67±0.48 A	0.0000	68.50±0.22 a	70.03±1.27 B	0.0568	67.81±0.04 b	69.56±0.72 B	0.0024	0.0000	0.0000	0.0000
Σ Omega 3	64.37± 0.67 a	52.83±0.44 A	0.0000	64.24±0.26 a	64.00±1.03 B	0.6685	63.60±0.04 b	63.29±0.73 B	0.8561	0.0000	0.0000	0.0000

†Results from 2x2 factorial ANOVA. When interaction was significant, t-student test was applied, showing the *p* value in the column T-test. Different small letters (a,b,c) indicate significant differences ($p < 0.05$) among samples before digestion by *post hoc* Bonferroni test. Different capital letters (A, B, C) indicate significant differences ($p < 0.05$) among samples in the micellar fraction by *post hoc* Bonferroni test. Ds: delivery system; D: digestion.

Results VI

Effects of EPA and lipoic acid supplementation on fatty acids profile in overweight/obese women following a hypocaloric diet

The results of this chapter are part of a study published in Food &Function in collaboration with a research group from the Department of Nutrition, Food Science and Physiology (led by Dr. Moreno-Aliaga). The entire paper is included in **Annex I**: Escoté X, Félix-Soriano E, Gayoso L, Huerta AE, Alvarado MA, Ansorena D, Astiasarán I, Martínez JA, Moreno-Aliaga MJ. **Effects of EPA and lipoic acid supplementation on circulating FGF21 and fatty acids profile in overweight/obese women following a hypocaloric diet.** Food Funct., 2018, 9, 3028.

ABSTRACT

Dietary supplementation with eicosapentaenoic acid (EPA) and/or α-lipoic acid (LIP) has shown beneficial effects on obesity. In this study, we evaluated EPA and/or LIP effects on fatty acids (FA) profile in overweight/obese women following hypocaloric diets. EPA supplementation decreased plasma n-6-PUFAs content and increased n-3-PUFAs, mainly EPA and DPA, but not DHA. In the LIP-alone supplemented group a decrease in the total SFA and n-6-PUFAs content was observed after the supplementation. Furthermore, EPA affected desaturase activity, lowering Δ4D and rising Δ5/6D. These effects were not observed in the LIP-supplemented groups.

INTRODUCTION

Obesity is a complex disease characterized by an enlargement of adipose tissue mass and caused by an interaction of several factors, including genetics and epigenetics, but also dietary environmental, age and physical activity (WHO, 2017). In the last decades, obesity has become a worldwide epidemic with million deaths each year, and it is predicted that 1.12 billion adults will be obese by 2030 (WHO, 2017). Moreover, obesity is strongly associated with several comorbidities such as type 2 diabetes, insulin resistance, hypertriglyceridemia, nonalcoholic fatty liver disease, cardiovascular diseases and certain types of cancer, which reduce life expectancy and life quality (extensively reviewed in Prieto-Hontoria et al. (2011).

Therefore, understanding the molecular basis of obesity is necessary to halt the worldwide obesity increase. The classical strategy to fight against obesity combines low-calorie diets and physical activity (Huerta, Navas-Carretero, Prieto-Hontoria, Martínez, & Moreno-Aliaga, 2015). However, new food recommendations and diets are constantly analysed as promising therapies to achieve weight loss (Calder, 2012). Some trials in overweight and obese subjects have also suggested that supplementation with omega-3 polyunsaturated fatty acids (n-3 PUFAs) and antioxidants, such as α-lipoic acid (LIP), might help in promoting weight loss, reducing fat mass and increasing satiety (Ana E. Huerta et al., 2015).

Furthermore, n-3 PUFAs are essential nutrients with relevant benefits for health, which include the ability to decrease hypertriglyceridemia, reducing the risk of cardiovascular diseases and exerting anti-inflammatory effects (Lorente-Cebrián et al., 2013). It has been suggested that metabolic actions of n-3 PUFAs partially occur due to a modulation of adipose tissue metabolism and its secretory functions (Martínez-Fernández, Laiglesia, Huerta, Martínez, & Moreno-Aliaga, 2015; Moreno-Aliaga, Lorente-Cebrián, & Martínez, 2010). Although eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be produced endogenously from other fatty acids, these processes have a low efficiency (Calder, 2012), making necessary a dietary consumption of these marine n-3 PUFAs to achieve optimal levels.

On the other hand, LIP is a short-chain fatty acid, and an essential co-factor for mitochondrial function. LIP can be synthesized in the body through the lipoic acid synthase (Padmalayam, Hasham, Saxena, & Pillarisetti, 2009). LIP can be also found in small amounts in some vegetables such as broccoli, tomatoes, Brussels sprouts, potatoes, garden peas and rice bran as well as in animal-derived foods (Huerta et al., 2015). Some studies have suggested that LIP supplementation is able to promote body weight loss and improve glucose homeostasis (Huerta et al., 2015), partially by the modulation of adipose tissue metabolism (Fernández-Galilea et al., 2014) and associated secretory functions (Huerta et al., 2015b).

Obesity is characterized by a chronic low-grade inflammation and a high n-6/n-3 PUFAs ratio, which is associated with an inflammation profile in obesity (Yang et al., 2016). Moreover, fatty acid desaturases (FAD) are critical regulators of the fatty acid metabolism (Dasilva et al., 2016). In this context, production of oleic, palmitoleic and arachidonic acids by high FAD activities have been associated with obesity and insulin resistance. In contrast, high FAD activity to produce EPA and DHA was associated with an increase in insulin sensitivity (Vessby, Gustafsson, Tengblad, & Berglund, 2013).

Therefore, we hypothesize that supplementation with LIP and/or EPA can help to reduce the negative effects of obesity and associated comorbidities by their influence on the fatty acids profile in overweight and obese women following a hypocaloric diet. For this purpose, the fatty acids profile and the n-6/n-3 PUFAs ratio were characterized in overweight/obese women at baseline and after the intervention.

MATERIALS AND METHODS

Subjects

This study is an ancillary analysis of the OBEPALIP trial (Huerta et al., 2015), which principal outcome was to study the effects of LIP and EPA on the amount of weight loss in humans. Briefly, from this ancillary analysis 57 women were included as volunteers. Medical history and physical examination were conducted by the physician of the Metabolic Unit of the University of Navarra. The inclusion and exclusion criteria were as previously described (Huerta et al., 2015). This study was performed in compliance with the Helsinki Declaration, approved by the Research Ethics Committee of the University of Navarra (007/2009) and registered at clinicaltrials.gov as NCT01138774. After their written consent, they were allocated to one of the four experimental groups by simple randomization.

Study design and intervention

The OBEPALIP study was a short-term randomized double blind placebo-controlled weight loss trial with 4 parallel nutritional intervention groups: Control group: 3 placebo–I capsules (containing sunflower oil) and 3 placebo-II capsules (containing same excipients as the LIP capsules); EPA group: 1300 mg/day of EPA distributed in 3 capsules of EPA 80 (provided by Solutex, Madrid, Spain) containing 433.3 mg of EPA and 13.8 mg of DHA as ethyl-ester and 3 placebo-II capsules; LIP group: 300 mg/day of LIP from 3 capsules containing 100 mg of LIP (Nature's Bounty®, NY, USA) and 3 placebo-I capsules; and finally EPA + LIP group: 1300 mg/day of EPA (distributed in 3 capsules of EPA 80) and 300 mg/day of LIP (from 3 capsules containing 100 mg of LIP) (Huerta et al., 2015).

All intervention groups followed a moderate caloric restricted diet (55% carbohydrates, 30% lipids and 15% proteins) in accordance with the American Heart Association Guidelines and prescribed individually by a dietitian. During the first visit (baseline), each participant was instructed to follow an energy-restricted diet accounting for 30% less than her total energy expenditure, and to not change the physical activity pattern during the 10-week intervention period.

Volunteers were advised to avoid marketed n-3 PUFAs-enriched products. At baseline and at the end point, the volunteers underwent 10-12 h of fasting and met with the physician, the dietitian and the nurse at the Metabolic Unit. A catheter was inserted into the antecubital vein for a fasting blood sample extraction. All data and measurements were taken by the same person using standard protocols to attenuate bias. Follow-up visits were scheduled with each volunteer every two weeks to monitor weight, assess the adherence to the diet and ensure that the capsules were consumed adequately.

Blood samples

Fasting blood samples were drawn on weeks 0 and 10 into Serum Clot Activator tubes (4 mL Vacuette®) and into tubes containing tripotassium EDTA (4 mL Vacuette®). Plasma was extracted from EDTA tubes after centrifugation at 1500 g during 15 min at 4 °C. All samples were separated appropriately and stored at -80 °C for analysis.

Plasma fatty acids determination

Plasma fatty acids were determined according the procedure described by Ostermann, Müller, Willenberg, and Schebb (2014), with slight modifications. 50 µL of internal standard (C23:0, 180 µg/mL in heptane) was added to 150 µL of plasma and lipid extraction was performed with 2250 µL of a mixture of chloroform:methanol (1:2). Afterwards, 750 µL of chloroform and 750 µL of water were added; the sample was mixed for 1.5 min and centrifuged for 10 min at 6,000 rpm at room temperature. The lower organic phase was collected and the upper phase was re-extracted with 750 µL of chloroform. The organic phases were combined and evaporated under nitrogen, in a water bath at 40 °C. The derivatization for the preparation of fatty acid methyl esters (FAMEs) was carried out following NaOH+boron trifluoride protocol. The dried lipid extract was dissolved in 300 µL of methanolic NaOH (0.5 N) and heated for 15 min at 90-95 °C in a tightly closed vial. Then, 1,500 µl of boron trifluoride solution (20% in methanol) was added and the sample was heated for another 30 min at 90-95 °C. After cooling the sample, 2,250 µL of saturated NaCl solution and 1,500 µL of heptane were added. Then, the sample was vortex-

mixed for 4 min and centrifuged at 4,600 rpm for 5 min. The upper organic phase was collected, evaporated under nitrogen in a water bath at 40 °C and reconstituted in 75 µL of heptane. The FAMEs were analysed by gas chromatography- flame ionization detector (GC-FID) using a Perkin-Elmer Clarus 500 gas chromatograph (Madrid, Spain), equipped with a split–splitless injector, automatic autosampler, and coupled to a computerized system for data acquisition (TotalChrom, version 6.3.2). A capillary column SP™-2560 (100 m × 0.25 mm × 0.2 µm; Sigma-Aldrich) was used. The temperature of the injection port and detector were 250 °C and 260 °C, respectively. The oven temperature was: 175 °C for 10 min, heated to 200 °C at a rate of 7 °C/min, raised to 220 °C at a rate of 3 °C/min, and finally 220 °C for 20 min. The carrier gas was hydrogen, 30 psi. The volume injection was 1 µL and the split ratio was 25:1. The identification of the FAMEs was done by comparison of their retention times with those of pure FAMEs and previous works (Ansorena, Echarte, Ollé, & Astiasarán, 2013). The quantification of individual fatty acids was based on the internal standard method using calibration curves for every compound and results were expressed as µg/mL.

Fatty acid desaturase indexes measurement

Desaturase activities indexes in dietary supplemented groups were measured using a validated methodology from the fatty acids (FA) data (Warensjö et al., 2009). FAD indexes were calculated as product/ precursor ratio for: Stearoyl-CoA desaturase (SCD16 or SCD18) = [palmitoleic (16:1 n7)/palmitic (16:0)] or [oleic (18:1 n-9)/stearic (18:0)]; ∆4D = [DHA (22:6 n-3)/ DPA (22:5 n-3)]; and ∆5/6D = [EPA (20:5 n-3)/ALA (18:3 n-3)]. Figure 1 shows biosynthesis routes of long-chain fatty acids. The long chain saturated fatty acids and unsaturated fatty acids of the n-7 and n-9 series can be synthesized from palmitic acid (16:0) produced by the fatty acid synthase. Long-chain fatty acids of the n-6 and n-3 series can only be synthesized from precursors obtained from dietary precursors (Naudí et al., 2013).

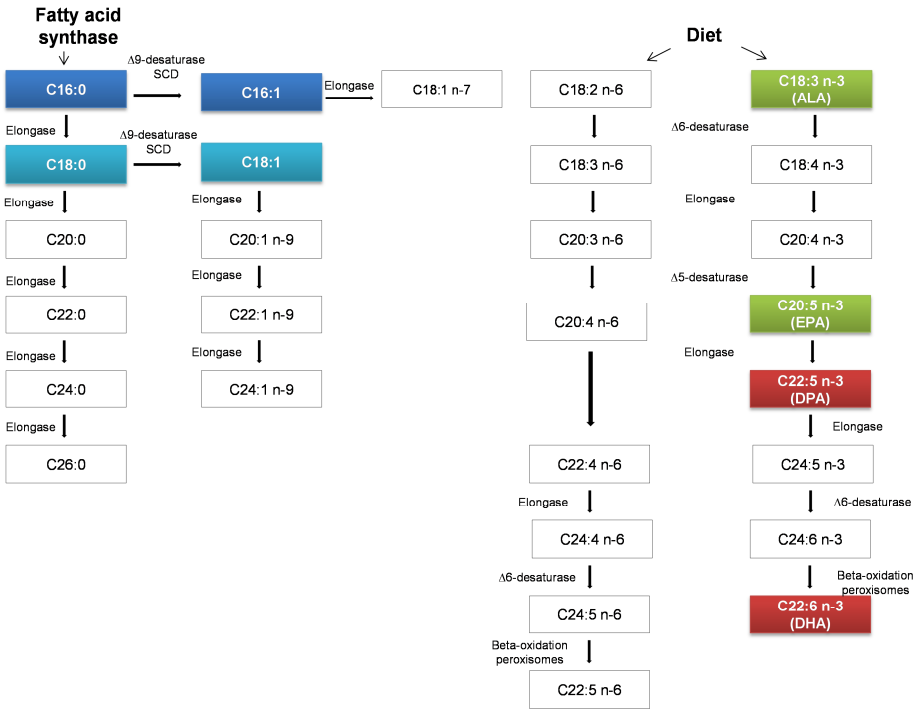


Figure 1. Biosynthesis of long-chain fatty acids. Adaptation from Naudí et al., (2013).

Statistical analysis

Statistical analyses were performed using Stata Statistical Software (College Station, TX: StataCorp LP) version 12.1 for Windows. Graphics were represented using GraphPad Prism Software v. 6.0 for Windows (GraphPad Software, San Diego, CA). Data are expressed as mean ± standard error of the mean (SEM). Normality was calculated by Shapiro-Wilk test and the differences after the nutritional intervention trial within each group were assessed by paired Student's *t* test when variables were normally distributed and Wilcoxon's sign-test when not. The differential effects between the intervention groups were estimated by two-way ANOVA. Differences were considered significant if $P < 0.05$.

RESULTS

Effect on plasma FA profile and n-6/n-3 ratio

Noticeable differences were observed in plasma FA profile between the different interventional groups (Table 1). After the hypocaloric diet, it was observed a significant decrease in the levels of stearic acid, α-linoleic acid and eicosadienoic acid in the control group. Interestingly, LIP supplementation promoted a significant decrease in circulating levels of saturated FA (SFA): myristic, pentadecanoic, palmitic, heptadecanoic, stearic and lignoceric acids. Therefore, the levels (Σ) of SFA as well as of n-6 PUFAs, and total PUFAs were also decreased in the LIP group. In contrast, nervonic acid levels were increased after the intervention in the LIP group. The EPA supplemented groups (EPA and EPA + LIP) showed lower levels of α- linoleic, γ-linolenic, behenic and arachidonic acids after the intervention. In contrast, EPA and DPA levels, as well as the total n-3-PUFAs content (Σ), were strongly increased in these supplemented groups. In parallel, total n-6 PUFAs content and the n-6/n-3 ratio were significantly decreased in both EPA-supplemented groups.

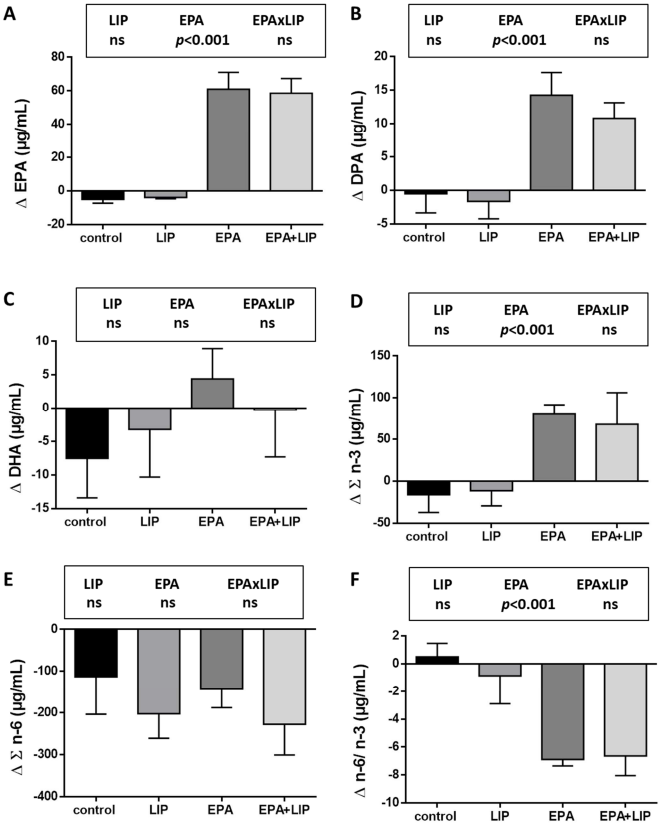
Furthermore, the two-way ANOVA analysis of changes (endpoint-baseline) between groups confirmed that EPA, DPA and n-3 PUFAs content increased, whereas the n-6/n-3 PUFAs ratio decreased in the groups supplemented with EPA (EPA and EPA + LIP) *vs*. those without EPA supplementation (Fig. 2A, 2B, 2D and 2F). No significant changes between groups were observed for DHA and n-6 PUFAs (Fig. 1C and 1E).

Table 1. Plasma fatty acid profile at the baseline and at the endpoint in the four intervention groups.

	control		LIP		EPA		EPA+LIP	
	baseline	**endpoint**	**baseline**	**endpoint**	**baseline**	**endpoint**	**baseline**	**endpoint**
14:0 (myristic)[b]	23.2 ± 10.4	13.0 ± 4.7	**26.4 ± 6.9**	**14.1 ± 4.6***	14.6 ± 3.0	11.2 ± 2.2	18.1 ± 4.8	10.7 ± 1.8
15:0 (pentadecanoic)[b]	4.6 ± 1.4	3.6 ± 0.6	**6.2 ± 1.5**	**4.9 ± 1.6***	3.3 ± 0.3	3.3 ± 0.4	3.7 ± 0.7	3.1 ± 0.4
16:0 (palmitic)[b]	682.1 ± 111.0	602.0 ± 49.4	**780.7 ± 171.0**	**693.8 ± 170.0***	522.8 ± 41.5	508.9 ± 32.8	612.4 ± 88.7	535.7 ± 51.5
16:1 n-7 (palmitoleic)[b]	46.9 ± 12.6	36.6 ± 7.6	**75.8 ± 31.9**	**57.5 ± 24.3***	32.5 ± 2.9	32.1 ± 5.7	47.3 ± 10.8	34.2 ± 4.0
17:0 (heptadecanoic)[b]	8.2 ± 1.0	7.4 ± 0.8	**9.2 ± 1.7**	**7.3 ± 1.4***	6.8 ± 0.7	6.4 ± 0.3	6.6 ± 0.7	7.1 ± 0.9
18:0 (stearic)[b]	**240.8 ± 23.9**	**201.0 ± 14.3***	**242.4 ± 29.1**	**188.6 ± 31.8***	212.1 ± 14.2	179.6 ± 14.1	198.6 ± 11.8	167.0 ± 6.7
18:1 n-9 (oleic)[b]	717.6 ± 122.6	614.6 ± 45.3	749.1 ± 162.7	718.6 ± 194.1	521.8 ± 61.7	490.5 ± 28.8	549.1 ± 67.7	492.2 ± 53.5
18:1 n-7 (cis-vaccenic)[b]	61.9 ± 10.4	60.3 ± 7.4	65.5 ± 14.4	68.8 ± 17.9	46.0 ± 6.8	51.4 ± 8.9	57.3 ± 7.5	49.9 ± 3.3
18:2 n-6 (linoleic)[a]	1017.1 ± 109.5	906.8 ± 68.7	**1025.3 ± 99.5**	**844.7 ± 131.6***	**884.6 ± 40.3**	**763.8 ± 63.6***	**811.9 ± 73.4**	**624.4 ± 59.9***
18:3 n-3 (α-linolenic)[b]	**11.4 ± 2.6**	**8.2 ± 1.0***	**10.7 ± 1.9**	**8.1 ± 1.9***	8.02 ± 1.2	8.3 ± 0.6	7.7 ± 0.9	6.4 ± 0.6
18:3 n-6 (γ-linolenic)[a]	11.9 ± 2.6	9.0 ± 2.4	**17.4 ± 4.4**	**9.5 ± 2.3***	**11.5 ± 0.7**	**6.2 ± 1.4***	**13.4 ± 3.1**	**6.1 ± 1.3***
20:0 (arachidic)[a]	3.6 ± 0.3	3.5 ± 0.3	3.9 ± 0.5	3.8 ± 0.5	3.6 ± 0.2	3.4 ± 0.2	2.9 ± 0.3	3.1 ± 0.3
22:0 (behenic)[a]	5.9 ± 0.9	5.7 ± 0.7	6.8 ± 1.3	5.2 ± 0.7	**6.0 ± 0.8**	**5.1 ± 0.7****	5.1 ± 0.6	4.1 ± 0.4
20:1 n-9 (eicosenoic)[b]	4.0 ± 0.9	3.1 ± 0.5	3.7 ± 1.0	4.4 ± 2.1	2.3 ± 0.3	2.2 ± 0.3	2.4 ± 0.4	2.5 ± 0.6
20:2 n-6 (eicosadienoic)[b]	**7.2 ± 1.4**	**6.3 ± 1.0***	8.0 ± 2.1	7.0 ± 2.6	5.8 ± 0.3	4.9 ± 0.5	5.9 ± 0.9	4.6 ± 0.6
20:4 n-6 (arachidonic)[a]	227.3 ± 20.4	227.6 ± 23.1	310.9 ± 45.4	296.9 ± 52.3	**218.3 ± 25.4**	**200.8 ± 26.1***	282.9 ± 38.5	250.1 ± 33.9
20:5 n-3 (EPA)[b]	23.5 ± 5.0	18.5 ± 4.7	**12.4 ± 1.8**	**8.5 ± 1.5***	**10.6 ± 0.7**	**71.7 ± 10.0***	**12.6 ± 1.7**	**71.3 ± 7.4***
22:5 n-3 (DPA)[b]	10.3 ± 1.6	9.8 ± 0.9	10.4 ± 1.3	8.7 ± 1.9	**9.0 ± 1.2**	**23.3 ± 4.3***	**8.6 ± 0.4**	**19.4 ± 2.0***
22:6 n-3 (DHA)[a]	80.6 ± 9.8	73.1 ± 6.8	72.5 ± 11.0	69.3 ± 13.0	60.0 ± 3.4	64.4 ± 3.9	66.5 ± 7.5	66.2 ± 11.1
24:0 (lignoceric)[b]	6.0 ± 0.7	5.8 ± 0.5	**7.0 ± 1.1**	**5.0 ± 0.8***	**7.5 ± 0.6**	**5.5 ± 0.4***	6.1 ± 0.7	4.8 ± 0.7
24:1 n-9 (nervonic)[b]	12.2 ± 0.9	16.2 ± 2.3	**12.8 ± 2.5**	**17.4 ± 3.4***	11.9 ± 1.2	12.9 ± 0.8	13.0 ± 1.4	13.4 ± 0.7
Σ n-3[b]	125.8 ± 16.3	109.6 ± 12.3	105.9 ± 14.8	94.6 ± 15.8	**87.6 ± 5.0**	**167.6 ± 15.5 ****	**95.4 ± 8.0**	**163.3 ± 17.8***
Σ n-6[b]	1256.3 ± 122.0	1143.4 ± 84.7	**1353.6 ± 139.9**	**1151.0 ± 179.5***	**1114.3 ± 51.6**	**970.8 ± 86.4***	**1108.1 ± 88.9**	**880.6 ± 81.7***
n-6/n-3[a]	10.0 ± 1.3	10.9 ± 1.2	13.3 ± 1.4	12.5 ± 1.1	**12.8 ± 0.7**	**5.9 ± 0.7*****	**12.1 ± 1.8**	**5.5 ± 0.5****
Σ n-9[b]	733.8 ± 123.0	633.9 ± 44.2	765.6 ± 165.9	740.4 ± 198.9	535.9 ± 61.1	505.6 ± 29.3	564.5 ± 67.3	508.1 ± 54.4
Σ SFA[b]	974.5 ± 147.1	841.9 ± 67.3	**1082.6 ± 211.9**	**922.7 ± 209.7***	775.8 ± 58.4	723.3 ± 48.8	853.5 ± 105.5	735.6 ± 60.1
Σ MUFA[b]	842.5 ± 139.7	730.8 ± 50.7	906.8 ± 211.0	866.7 ± 240.2	614.4 ± 65.9	589.1 ± 41.7	669.2 ± 83.8	592.2 ± 59.0
Σ PUFA[a]	1389.3 ± 127.6	1259.3 ± 87.3	**1467.5 ± 155.2**	**1252.6 ± 196.5***	1188.9 ± 66.0	1084.3 ± 94.4	1209.4 ± 86.3	1078.6 ± 94.1

Data are represented as mean ± SEM (μg/mL). Analysis was performed with paired *t* test[a] (for variables with normal distribution) and Wilcoxon's test[b] (for variables with non-normal distribution). LIP, lipoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. (n= 5 per group). *P<0.05, **P<0.01, ***P<0.001 *vs*. baseline levels.

Figure 2. Changes at the indicated plasma fatty acids after 10 weeks of nutritional intervention. (A) EPA, (B) DPA, (C) DHA, (D) Σ n-3, (E) Σ n-6 and (F) ratio n-3/ n-6. Data (mean ± SEM) were analyzed by two-way ANOVA. Significant differences were observed in groups supplemented with EPA ($p<0.001$). LIP, lipoic acid; EPA, eicosapentaenoic acid, DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; ns, non-significant.



Effects of LIP and/or EPA supplementation on FAD activity indexes

FAD indexes from FA data of plasma were calculated as product/precursor ratio (Dasilva et al., 2016). LIP and EPA supplementation did not influence SCD16 (palmitoleic/palmitic) (Table 2). SCD18 (oleic/stearic) was slightly increased in the LIP group, but the two-way ANOVA analysis revealed no significant differences between groups. In contrast, EPA supplementation (EPA and EPA + LIP) clearly influenced the n-3 PUFAs-related desaturases by significantly decreasing the Δ4D (DHA/DPA) and increasing the Δ5/6D (EPA/ALA) (Table 2).These results were corroborated by the two-way ANOVA analysis observing significant differences in the Δ4D and Δ5/6D activities in EPA-supplemented groups (Table 2).

Table 2. Fatty acid desaturases activity indexes at baseline and at endpoint in the four intervention groups.

						Anova 2x2		
		control	**LIP**	**EPA**	**EPA+LIP**	**LIP**	**EPA**	**LIPxEPA**
Δ4D[a] (DHA/DPA)	**baseline**	8.48 ± 1.36	6.95 ± 0.32	6.94 ± 0.57	7.81 ± 0.99			
	endpoint	7.60 ± 0.67	7.88 ± 1.24	**3.21 ± 0.69*** **	**3.47 ± 0.54** **			
	change	-0.88 ± 0.91	0.93 ± 1.01	-3.73 ± 0.57	-4.34 ± 0.66	0.465	**<0.001**	0.154
Δ5/6D[b] (EPA/ALA)	**baseline**	2.30 ± 0.44	1.29 ± 0.27	1.44 ± 0.20	1.80 ± 0.35			
	endpoint	2.31 ± 0.61	1.22 ± 0.29	**8.56 ± 0.97** **	**11.31 ± 1.12***			
	change	0.01 ± 0.34	-0.08 ± 0.19	7.13 ± 1.01	9.51 ± 1.28	0.210	**<0.001**	0.181
SCD16[b] (palmitoleic /palmitic)	**baseline**	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01			
	endpoint	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.00			
	change	-0.01± 0.01	-0.01± 0.01	0.00 ± 0.01	-0.01 ± 0.01	0.135	0.442	0.626
SCD18[b] (oleic/stearic)	**baseline**	2.93 ± 0.32	2.98 ± 0.25	2.45 ± 0.13	2.74 ± 0.22			
	endpoint	3.08 ± 0.19	**3.65 ± 0.32***	2.76 ± 0.11	2.92 ± 0.23			
	change	0.14 ± 0.16	0.66 ± 0.18	0.27 ± 0.19	0.18 ± 0.11	0.219	0.315	0.050

Fatty acid desaturases indexes were calculated as product/precursor ratio from the total fatty acid data. Data are represented as mean ± SEM (µg/mL). Differences between baseline and endpoint were analyzed with *t*-test[a] (for variables with normal distribution) and Wilcoxon test[b] (for variables with non-normal distribution). Data changes (endpoint-baseline) were analyzed by two-way ANOVA (right panel). LIP, lipoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; ALA, α-linolenic acid. (n=5 per group). *P<0.05, **P<0.01, ***P<0.001 *vs*. basal levels.

DISCUSSION

As expected, lipidomic studies demonstrated that n-3 PUFAs levels (EPA and DPA) were higher in the EPA supplemented groups, whereas this effect was not observed at the DHA level.

The n-6/n-3 PUFAs ratio has been associated with an increasing prevalence of chronic inflammatory diseases (Simopoulos & P., 2016). Indeed, an increase in the n-6/n-3 PUFAs ratio increases the risk for obesity (Simopoulos & P., 2016). Our data indicate that after the intervention, EPA supplemented groups presented lower n-6/n-3 ratio, which may be associated with a lower inflammation and a healthier metabolic profile (Liu et al., 2013). In fact, a previous study in this cohort revealed that EPA supplementation decreases markers of macrophage infiltration and attenuates inflammation in human adipose tissue (Huerta et al., 2016).

Furthermore, fatty acid desaturase levels are limiting steps in the synthesis of different fatty acids, and thus promising targets for the treatment of metabolic diseases (Dasilva et al., 2016). In animals models fed with diets rich in EPA and DHA, desaturases involved in the transformation of α-linolenic acid (ALA) into EPA and DHA are up-regulated, meanwhile the activity of these enzymes to produce ARA from LA are reduced (Dasilva et al., 2016). These are in accordance with the results observed in the present study, and it was related with healthier insulin sensitivity (Das, 2005).

Regarding LIP supplementation, the present study showed a clear reduction in the systemic SFAs levels as well in the n-6 PUFAs content. Saturated fatty acids (SFAs), especially saturated long chain fatty acids such as myristic acid, palmitic acid and stearic acid, common in Western diets, have been related to a higher risk of developing cardiovascular diseases. Palmitic acid, as well as n-6 PUFAs, has also been related with the promotion of inflammatory responses (Ghosh, Gao, Thakur, Siu, & Lai, 2017). Consequently, the reduction in the levels of these types of FAs observed after LIP supplementation can promote a healthier metabolic profile, which may be in relation with a recent study showing that LIP not only induces weight loss, but also reduces systemic inflammatory

and cardiovascular disease–related risk biomarkers (Huerta, Prieto-Hontoria, Sainz, Martinez, & Moreno-Aliaga, 2016).

Nevertheless, it is important to mention some limitations of this study, including the sample size, gender (only women), and length of the nutritional intervention. Moreover, our trial was performed in healthy overweight/obese women. Therefore it would be of interest to validate the results of our study in larger cohorts and to assess the role of EPA and/or LIP in the regulation of FA and fatty acid desaturases.

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GENERAL DISCUSSION

The search and assesment of natural bioactive compounds is currently a main topic in functional foods development. Among other sources of bioactive ingredients, we have focused on plant extracts, rich in phenolic compounds, and on bioactive lipid components, such as omega 3 fatty acids and α-lipoic acid. The evidence of the association of biocompounds with health benefits is growing, and for that purpose, accurate identification of chemical structures and evaluation of their funcionality under different circunstances is required. In the present work, the bioactive compounds were studied in different contexts: technological (antioxidant protection against lipid oxidation in lipid delivery systems) and physiological (bioaccessibility and biological activity).

Chemical characterization of plant extracts

Medicinal and aromatic herbs have long been consumed for their therapeutic effect, and nowadays continue being one of the best sources of potential bioactive compounds. Plants of *Lamiaceae* family, such as *Melissa officinalis* L., *Origanun vulgare* L. and *Lavandula latifolia* Medicus are traditionally used in Spain and in other European countries for their medical, culinary and aromatic properties. In this work, different extracts obtained from these plants were evaluated as potential sources of natural antioxidants: aqueous *L. latifolia* extract, aqueous *M. officinalis* extract, methanol *L. latifolia* extract and methanol *O. vulgare* extract. The selection of these plant extracts was made considering their antioxidant activity, which was tested in preliminary tests in our research group. All the samples showed *in vitro* antioxidant effect measured by ABTS and DPPH assays, being the two aqueous extracts those with the highest antioxidant values (Results II, III and IV). In addition, it has been pointed out that aqueous extracts present advantages in terms of environment and safety with respect to other extracts obtained with organic solvents (Chemat, Vian, & Cravotto, 2012).

A significant positive correlation was observed between total phenolic compounds (TPC) and DPPH and ABTS in the aqueous *M. officinalis* extract and the methanol *L. latifolia* and *O. vulgare* extracts, indicating that polyphenols significantly contribute to the antioxidant activity of these extracts. In order to

identify the main phenolic compounds that contribute to this antioxidant activity, LC-MS analysis was performed. Two compounds were identified in the aqueous extract of *M. officinalis*: rosmarinic acid and luteolin-3´-*O*-glucuronide; 5 compounds in the methanol extract of *L. latifolia:* two glycoside derivatives of coumaric acid, a glycoside of ferulic acid, luteolin-7-*O*-glucoside and rosmarinic acid; and 3 compounds in the methanol extract of *O. vulgare*: a glycoside of luteolin, 4-(3,4-dihydroxybenzoyloxymethyl)-phenyl-β-D-glycopyranoside and rosmarinic acid (Results II).

After the chemical determination of the antioxidant activity of plant extracts, understanding gastrointestinal digestion process is a relevant factor in the design of strategies to incorporate antioxidant compounds to functional foods in an efficient way. The aqueous *M. officinalis* extract and the methanol *L. latifolia* and *O. vulgare* extracts were submitted to an *in vitro* gastrointestinal digestion in order to evaluate their bioaccessibility. Before proceeding with the extracts, the first step of this evaluation was to select the digestion model conditions needed for that purpose.

Methodological aspects related to *in vitro* digestion models

In vitro digestion models have been extensively used to study the bioaccessibility of different food matrices and plant extracts. However, a major obstacle for the interpretation of phytochemical bioaccessibility based on *in vitro* studies is the large number of published models since the description of the first model developed for studying iron bioaccessibility (Alminger et al., 2014; Miller, Schricker, Rasmussen, & Van Campen, 1981). In this context of lack of uniformity in the methodology, the COST action INFOGEST, an international network joined by more than 200 scientists from 32 countries working in the field of digestion, proposed a standardised static *in vitro* digestion method suitable for food (Minekus et al., 2014). However, at intestinal level, there is no specific recommendation in that consensus for the conditions to be used and, in fact, in recent literature, multiple protocols have been applied to isolate the portion that can be considered equivalent to the bioaccessible fraction: filtration (Chen et al., 2016; Pinacho, Cavero, Astiasarán, Ansorena, & Calvo, 2015), dialysis

membrane (Carbonell-Capella, Buniowska, Esteve, & Frígola, 2015; Mosele, Macia, Romero, & Motilva, 2016) and centrifugation (He et al., 2016; Pineda-Vadillo et al., 2016). The bioaccesible fraction is considered the entire intestinal fraction in the filtration method, the supernatant after centrifugation of the intestinal fraction in the centrifugation method and the sample that goes through the semi-permeable membrane during the dialysis method.

Therefore, one of the objectives of this work was to study the influence of the digestion model applied on the bioaccessibility of different pure standard phenolic compounds, rutin, caffeic acid and rosmarinic acid, which are widely present in plant extracts. The three common digestion models were tested to compare their influence on the characteristics and amount of the bioaccesible fraction in each case: filtration, centrifugation and dialysis membrane (Results I).

If it is assumed that bioaccessibility is defined as the amount of compound available for absorption, some methodological considerations should be made in order to calculate this value when applying digestion models. For instance, the addition of salts ($NaHCO_3$) during the digestion process to reach the physiological intestinal pH, involves what it could be called “dilution effect”, which means a decrease in the concentration of the compound (lower proportion of the analysed compound per gram of digested lyophilised sample). This effect was clearly noticeable when bioaccessibility was calculated with data expressed as concentration (mg of pure standard/mg lyophilised digested sample) because a significant loss of all compounds was observed. However, when bioaccessibility was calculated with data expressed as absolute amount (total mg of standard in the digested sample) this degradation was much lower, or even absent in the case of rutin. Hence, the expression of results as concentration of a compound in the digested fraction could be compromised by a dilution effect.

In addition to the units used to express the results, bioaccessibility was also influenced by the digestion model applied. Bioaccessibility was higher using filtration and centrifugation methods than when using dialysis membrane. These results are in agreement with previous studies that reported lower recovery of

phenolic compounds by dialysis (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007; Bouayed, Deusser, Hoffmann, & Bohn, 2012).

Therefore, studying different *in vitro* digestion models allowed establishing the optimal conditions to further evaluate the bioaccessibility of the plant extracts (*M. officinalis*, *L. latifolia* and *O. vulgare*) and bioactive lipidic compounds. The model chosen in the following studies was centrifugation.

Bioaccessibility and biological activity of plant extracts

The bioaccessibility of a compound can be influenced by many factors, such as the chemical state of the compound, the food matrix, the cooking processes, interactions with other components and the presence of suppressors or cofactors that may increase or decrease, respectively, its intestinal absoption (De Santiago, Pereira-Caro, Moreno-Rojas, Cid, & De Peña, 2018; Juániz et al., 2017; Parada & Aguilera, 2007). The bioaccessibility of the individual phenolic compounds in the plant extracts studied showed that some compounds were more stable than others during the digestion process, and the bioaccessibility (% of compound that remains in the bioaccessible fraction after the intestinal digestion) ranged between 41-107%. Among the compounds characterized in the three plant extracts, the most bioaccessible ones were: rosmarinic acid in the aqueous extract of *M. officinalis* (98%); glycoside derivatives of coumaric acid and glycoside of ferulic acid (approximately 100%) in the methanol extract of *L. latifolia*; 4-(3,4-dihydroxybenzoyloxymethyl)-phenyl-β-D-glycopyranoside (93%) and rosmarinic acid (83%) in the methanol extract of *O. vulgare* (Results II).

Regarding antioxidant activity, there was no decrease (in *O. vulgare* samples) or a slight loss (up to 18% in *M. officinalis* and up to 19% in *L. latifolia* samples) after gastrointestinal digestion, indicating that the bioaccessible fraction remained potentially bioactive. However, after the intestinal digestion of standard phenolic compounds (rutin, caffeic acid and rosmarinic acid), the decrease in antioxidant activity was significant in the three compounds, especially in the case of caffeic acid (up to 86%). These results point out to a certain protective effect of the food matrix on polyphenols during digestion, as

reported by other authors (Ortega, Reguant, Romero, Macià, & Motilva, 2009; Siracusa et al., 2011).

As previously mentioned, *in vitro* digestion models are excellent tools to predict bioaccessibility of phytochemicals. However, the final uptake of bioactive compounds in the gut (bioavailability) is a prerequisite for further physiological functions. In this work, the nematode *C. elegans* model was used to assess the biological effect of these plant extracts before and after *in vitro* digestion. The purpose was to evaluate the biological effect of the crude extracts and also to check if the digested extracts were still bioactive after the *in vitro* digestion process (Results II).

The assays carried out were mainly focused on the evaluation of the antioxidant activity: survival assay under a lethal dose of juglone (pro-oxidant naphthoquinone), intracellular reactive oxygen species (ROS) and expression of the antioxidant enzyme glutathione-S-transferase (GST-4) under juglone-induced oxidative stress. Results showed that *L. latifolia* and *O. vulgare* at the tested doses were not able to attenuate significantly the deleterious effects of juglone in the survival assay, while *M. officinalis* showed a hormetic effect (positive outcomes at low doses and toxic effects at high doses). All the extracts had a significant effect decreasing endogenous ROS levels and GST-4 expression, both when testing in crude and digested extracts. The observed effects were similar to those published by other authors for antioxidants such as guarana extract (Peixoto et al., 2017), lignans from *Arctium lappa* (Su & Wink, 2015), grape pomace (Jara-Palacios et al., 2013), quercetin (Büchter et al., 2015; Kampkötter, Nkwonkam, et al., 2007) or *Ginkgo biloba* extract (Kampkötter, Pielarski, et al., 2007). These findings suggest that the antioxidant activity is not only achieved by scavenging radicals but also by modulation of the expression of the GST-4 antioxidant enzyme.

This part of the work regarding the use of *C. elegans* as model to assess *in vivo* antioxidant effects was carried out at the Heildelberg University (Institute of Pharmacy and Molecular Biotechnology).

Lipid delivery systems

Emulsion technology is particularly suited for the design and fabrication of different types of delivery systems for lipidic and non-lipidic bioactive compounds (Leal-Calderon & Cansell, 2012). In this work, two lipid delivery systems were studied: soy protein stabilized O/W emulsion and carrageenan gelled emulsion (Results III, IV, V). Both types of emulsions were previously proposed as carriers of unsaturated fatty acids in functional food formulations (Berasategi et al., 2014; Poyato, Ansorena, Berasategi, Navarro-Blasco, & Astiasarán, 2014).

It is well known that one of the most important causes of deterioration of unsaturated fatty acids is lipid oxidation. The aqueous *L. latifolia* extract was tested as a potential natural antioxidant in gelled emulsions formulated with 40% of sunflower or algae oil (Results IV). The identification of volatile compounds related to lipid oxidation was performed during their storage (1 and 49 days, at 25 ºC). A protective effect of *L. latifolia* extract was observed in both gelled emulsions, with a significant decrease in the aldehydes fraction during this period. In terms of total volatiles, the plant extract had a significant lowering effect only in sunflower oil gels. Therefore, further studies are needed to verify higher doses of *L. latifolia* extract.

On the other hand, emulsification has been explored as a strategy to improve bioaccessibility of bioactive compounds (Lin, Wang, Li, & Wright, 2014; Mao & Miao, 2015). In this context, O/W and gelled emulsions were used to study the delivery of food bioactives during the *in vitro* gastrointestinal digestion.

Digestion of a food matrix with lipidic fraction, such as an emulsion, presents some particularities regarding *in vitro* digestion conditions. Fat-soluble compounds differ in their digestion behaviour from water-soluble compounds as they require incorporation into mixed micelles, consisting of bile acids, partially digested lipids (monodiglycerides, diglycerides), phospholipids, and other lipophilic/amphipathic constituents (Bohn et al., 2017). Pancreatic lipase and bile salts play a predominant role for micellarization, so supplying sufficiently lipase and bile salt concentration is an important consideration in the *in vitro*

digestion of samples with lipophilic compounds (Biehler & Bohn, 2010). Consequently, in our work, in order to digest emulsions with ≥40% of oil, it was necessary to adapt the protocol of the *in vitro* gastrointestinal procedure, with the incorporation of lipase (2000 U/mL) and increase the bile concentration up to 800 mg/g of fat (Minekus et al., 2014; Ortega et al., 2009). After the intestinal digestion step, the methodology applied to obtain the bioaccessible fraction containing the mixed micelles was centrifugation, as previously mentioned.

Aqueous *M. officinalis* extract was incorporated into a carrageenan linseed oil gelled emulsion (Results III). It was noticeable that when the plant extract was delivered into the gel, the antioxidant activity after the digestion process increased 1.8-fold ABTS, 3.5-fold DPPH and 4.5-fold TPC values. Carrageenan is a complex polymer that loses stability with pH values below 4.3. At low pH values, the polymer can breakdown at 3,6-anhydrogalactose linkage leading to the formation of new products or to the liberation of certain sulphated functional groups with antioxidant activity (Imeson, 2009), which could also contribute to explain these results. Nevertheless, further studies would be necessary for a better understanding of the physical and chemical changes occurring in the digestion of the gelled emulsion to confirm this hypothesis.

Algae oil, a source of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), was delivered by soy protein stabilized O/W emulsion, carrageenan gelled emulsion or as a bulk oil (Results V). The delivery of the algae oil by O/W emulsion or gelled emulsion increased the bioaccessibility of n-3 fatty acids with respect to the bulk oil. Namely, the bioaccessibility of DHA in O/W emulsion and gelled emulsion was significantly higher than bulk oil (1.2- and 1.4-fold, respectively), which agrees with the higher bioaccessibility of n-3 from emulsified algae oil *vs*. bulk oil described *in vivo* (Lane, Li, Smith, & Derbyshire, 2014) and *in vitro* (Lin et al., 2014). In addition, lipid oxidation, measured by malondialdehyde (MDA), was significantly lower in both emulsified oils at the end of the intestinal digestion, as compared to the bulk oil. Previous studies pointed out the protective effect against lipid oxidation of emulsifiers, such as soy protein (used in O/W emulsion) (Nieva-Echevarría, Goicoechea, & Guillén, 2017) and Tween 80 (used in gelled emulsion) (Berton, Ropers, Viau, & Genot,

2011). In the gelled emulsion, in addition to Tween 80, an additional antioxidant effect could be attributed to carrageenan (Rocha De Souza et al., 2007).

Nutritional intervention

Bioavailability studies are a key step regarding health claims related to food components in the formulation of functional foods (Rein et al., 2013). In this sense, in the evaluation of health claims by the European Food Safety Authority (EFSA), data from human studies addressing the relationship between the consumption of the food component and the claimed effect are required for substantiation (EFSA, 2016). Thus, *in vivo* studies are required to complement *in vitro* ones, that should be regarded as a screening, ranking, or categorizing tool (Etcheverry, Grusak, & Fleige, 2012).

After having evaluated bioactive compounds by *in vitro* digestion models and animal models (*C. elegans*), this part of the work was focused on the "gold standard" technique to study the metabolism of food compounds: nutritional intervention studies (Results VI).

The tested compounds in this part of the work included: EPA and α-lipoic acid (LIP). Among other properties, these lipidic bioactive components have shown a positive role managing weight loss and fat mass reduction in overweight and obese subjects (Huerta, Navas-Carretero, Prieto-Hontoria, Martínez, & Moreno-Aliaga, 2015).

In this study, the lipid profile of plasma samples of overweight/obese women following supplementation with EPA and/or LIP during 10-week hypocaloric diet was evaluated. Samples were taken at baseline and endpoint. The nutritional intervention was designed with 4 groups: EPA group (1300 mg/day of EPA), LIP group (300 mg/day of LIP), EPA+LIP group (1300 mg/day of EPA and 300 mg/day of LIP) and control group (placebo capsules).

Short-term bioavailability of long-chain fatty acids is commonly determined by analysing the lipid profile in plasma samples (Schuchardt & Hahn, 2013). The composition and incorporation of fatty acids in plasma is the result of distinct processes: intake, metabolism and peripheral utilization, reflecting the fat

intakes with the diet (Risé, Eligini, Ghezzi, Colli, & Galli, 2007). As expected, EPA supplementation (EPA and EPA+LIP groups) increased significantly EPA and docosapentaenoic acid (DPA) in plasma, as well as the total n-3-PUFAs content, but DHA was unaffected. Conversion rate of DPA or EPA to DHA has been reported to be extremely low, so DHA levels appear largely determined by direct dietary consumption (Mozaffarian & Wu, 2012). Similar to other nutrients, the bioavailability of n-3 fatty acids is highly variable and determined by numerous factors (Schuchardt & Hahn, 2013). Higher bioavailability may translate into greater biological effects (Schuchardt & Hahn, 2013) and these results showed that consuming 1300 mg/day of EPA from supplementation resulted in significant appearance of EPA and DPA in human plasma.

Lipid profile, n-6/n-3 ratio and fatty acid desaturases (FAD) activity are considered biomarkers of oxidative stress and inflammation (Dasilva et al., 2016). EPA and EPA+LIP groups decreased significantly the n-6/n-3 ratio and affected desaturase activity, lowering ∆4D(DHA/DPA) and rising ∆5/6D(EPA/ALA). In the lipoic-alone supplemented group, a decrease in the total saturated fatty acids and n-6 PUFAs content was observed. The reduction in the levels of these types of FAs can promote a healthier metabolic profile, as suggested by other studies (Huerta, Prieto-Hontoria, Sainz, Martínez, & Moreno-Aliaga, 2016).

Concluding remarks

The field of study of bioactive compounds has been a major area of research in these recent years. In this work, different scenarios have been addressed in the study of these compounds. Chemical characterization, *in vitro* and *in vivo* digestion models and the use of delivery systems have offered an overall picture of the potential effects of some plant extracts and omega 3 fatty acids. However, new insights dealing with the multiple factors that affect the absorption and metabolism of these molecules, such as food matrices effect and colonic microbiota action, as well as toxicological studies, would contribute with meaningful information about the physiological behaviour and safety of bioactive compounds.

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CONCLUSIONS/CONCLUSIONES

1. Bioaccessibility studies are difficult to be compared due to the many variables that may influence the gastrointestinal digestion. The methodology applied at intestinal level and the units used to express results are two critical variables to be taken into account to interpret bioaccessibility results.

2. Bioaccessibility of rutin, caffeic acid and rosmarinic acid was significantly higher in filtration and centrifugation than in dialysis method. In the filtration and centrifugation conditions, the bioaccessibility of rutin was 100%, followed by rosmarinic acid (76-69%) and caffeic acid (60-46%).

3. Aqueous *M. officinalis* extract, methanol *L. latifolia* extract and methanol *O. vulgare* extract were rich sources of phenolic compounds, including phenolic acids and flavonoids as the most abundant ones. All of them exhibited antioxidant activity in the *in vitro* assays and also biological activity in the *C. elegans* model, before and after the *in vitro* gastrointestinal digestion. Thus, these plant extracts are sources of bioaccessible and bioactive natural antioxidants.

4. The delivery of the aqueous *M. officinalis* extract by a carrageenan omega-3 fatty acids gelled emulsion, increased up to 4.5-fold the antioxidant activity after its *in vitro* gastrointestinal digestion.

5. Aqueous *L. latifolia* extract showed a protective effect against lipid oxidation (significant decrease in the volatile aldehydes fraction) in gelled emulsions enriched in polyunsaturated fatty acids.

6. Emulsification of algae oil using O/W emulsions or gelled emulsions was an effective strategy to increase DHA bioaccessibility compared to bulk oil (1.2-fold and 1.4-fold, respectively).

7. EPA supplementation (1300 mg/day for 10 weeks) in overweigth/obese women following a hypocaloric diet increased plasma omega-3 PUFAs, mainly EPA and DPA, and decreased omega-6 PUFAs. In the lipoic-alone supplemented group (300 mg/day), a decrease in the total saturated fatty acids and omega-6 PUFAs content was observed after the supplementation.

1. Los estudios de bioaccesibilidad son difíciles de comparar debido a las múltiples variables que pueden influir en la digestión gastrointestinal. Dos variables críticas a tener en cuenta cuando se interpretan los resultados de bioaccesibilidad son la metodología aplicada a nivel gastrointestinal y las unidades de expresión de resultados.

2. La bioaccessibilidad de la rutina, del ácido cafeico y del ácido rosmarínico fue significativamente mayor en los métodos de filtración y centrifugación que en el método de diálisis. En las condiciones de filtración y centrifugación, la bioaccesibilidad de la rutina fue 100%, del ácido rosmarínico 76-69% y del ácido cafeico 60-46%.

3. Los extractos de *M. officinalis* (acuoso), *L. latifolia* (metanólico) y *O. vulgare* (metanólico) fueron una fuente rica en compuestos fenólicos, siendo los ácidos fenólicos y los flavonoides los más abundantes. Todos los extractos mostraron actividad antioxidante en los ensayos *in vitro* y actividad biológica en el modelo *C. elegans*, antes y después de la digestión gastrointestinal *in vitro*. Por ello, estos extractos de plantas son una fuente de antioxidantes naturales bioaccesibles y bioactivos.

4. La vehiculización del extracto acuoso de *M. officinalis* mediante una emulsión gelificada de carragenato y ácidos grasos omega 3, incrementó hasta 4,5 veces la actividad antioxidante después de su digestión gastrointestinal *in vitro*.

5. El extracto acuoso de *L. latifolia* mostró un efecto protector frente a la oxidación lipídica (descenso significativo de la fracción de aldehídos) en emulsiones gelificadas enriquecidas en ácidos grasos poliinsaturados.

6. La emulsificación del aceite de alga usando emulsiones O/W o emulsiones gelificadas fue una estrategia efectiva para incrementar la bioaccesibilidad del DHA en comparación con el aceite sin emulsionar (1,2 y 1,4 veces, respectivamente).

7. La suplementación de EPA (1300 mg/día durante 10 semanas) en mujeres con sobrepeso/obesidad siguiendo una dieta hipocalórica incrementó los niveles plasmáticos de los ácidos grasos poliinsaturados omega-3, principalmente EPA y DPA, y disminuyó los ácidos grasos poliinsaturados omega-6. En el grupo suplementado solo con ácido α-lipoico (300 mg/día), se observó un descenso en el total de ácidos grasos saturados y ácidos grasos poliinsaturados omega-6 después de la suplementación.

LIST OF ABBREVIATIONS

ABTS	2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ALA	α-linolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EFSA	European Food Safety Authority
EGCG	epigallocatechin gallate
EPA	eicosapentaenoic acid
FA	fatty acids
FFA	free fatty acids
GA	gallic acid
GC	gas chromatography
GST-4	glutathione-S-transferase
HPLC	high-performance liquid chromatography
HS-SPME	headspace-solid phase microextraction
IF	intestinal filtration
LC-MS	liquid chromatography-mass spectrometry
LIP	α-lipoic acid
MDA	malondialdehyde
MUFA	monounsaturated fatty acids
O/W	oil-in-water emulsion
PCA	principal component analysis

PUFA	polyunsaturated fatty acids
RA	rosmarinic acid
RF	residual fraction
ROS	reactive oxygen species
SF	soluble fraction
SFA	saturated fatty acids
TE	trolox equivalent
TPC	total phenolic compounds
UPLC	ultra-performance liquid chromatography
WHO	World Health Organization

ANNEX I

Food & Function

PAPER

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Effects of EPA and lipoic acid supplementation on circulating FGF21 and the fatty acid profile in overweight/obese women following a hypocaloric diet†

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FGF21 has emerged as a key metabolism and energy homeostasis regulator. Dietary supplementation with eicosapentaenoic acid (EPA) and/or α-lipoic acid (LIP) has shown beneficial effects on obesity. In this study, we evaluated EPA and/or LIP effects on plasma FGF21 and the fatty acid (FA) profile in overweight/obese women following hypocaloric diets. At the baseline, FGF21 levels were negatively related to the AST/ALT ratio and HMW adiponectin. The weight loss did not cause any significant changes in FGF21 levels, but after the intervention FGF21 increased in EPA-supplemented groups compared to non-EPA-supplemented groups. EPA supplementation decreased the plasma n-6-PUFA content and increased n-3-PUFAs, mainly EPA and DPA, but not DHA. In the LIP-alone supplemented group a decrease in the total SFA and n-6-PUFA content was observed after the supplementation. Furthermore, EPA affected the desaturase activity, lowering Δ4D and raising Δ5/6D. These effects were not observed in the LIP-supplemented groups. Besides, the changes in FGF21 levels were associated with the changes in EPA, n-3-PUFAs, Δ5/6D, and n-6/n-3 PUFA ratio. Altogether, our study suggests that n-3-PUFAs influence FGF21 levels in obesity, although the specific mechanisms implicated remain to be elucidated.

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Introduction

Obesity is a complex disease characterized by an enlargement of adipose tissue mass[1] and caused by an interaction of several factors, including genetics and epigenetics, and also dietary, environmental and physical activity.[1] In the last few decades, obesity has become a worldwide epidemic with millions of deaths each year with a prediction that 1.12 billion adults will be obese by 2030.[1] Moreover, obesity is strongly associated with several comorbidities such as type 2 diabetes, insulin resistance, hypertriglyceridemia, nonalcoholic fatty liver disease, cardiovascular disease and certain types of cancer, which reduce the life expectancy and life quality (extensively reviewed in the work by Prieto-Hontoria *et al.*[2]).

Therefore, understanding the molecular basis of obesity is necessary to halt the worldwide obesity increase. The classical strategy to fight against obesity combines low-calorie diets and physical activity.[3] However, new food recommendations and diets are constantly analyzed as promising therapies to achieve weight loss.[4] Some trials on overweight and obese subjects have also suggested that supplementation with omega-3 polyunsaturated fatty acids (n-3-PUFAs) and antioxidants, such as α-lipoic acid (LIP), might help in promoting weight loss, reducing fat mass and increasing satiety.[3]

Furthermore, n-3-PUFAs are essential nutrients with relevant benefits for health, which include the ability to decrease hypertriglyceridemia, reducing the risk of cardiovascular disease and exerting anti-inflammatory effects.[5] It has been suggested that metabolic actions of n-3-PUFAs partially occur due to a modulation of adipose tissue metabolism and its secretory functions.[6,7] Although eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be produced endogenously from other fatty acids, these processes have a low efficiency,[4] making necessary a dietary consumption of these marine n-3-PUFAs to achieve optimal levels.

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On the other hand, LIP is a short-chain fatty acid and an essential co-factor for mitochondrial function. LIP can be synthesized in the body through the lipoic acid synthase.[8] LIP can also be found in small amounts in some vegetables such as broccoli, tomatoes, Brussels sprouts, potatoes, garden peas and rice bran as well as in animal-derived foods.[3] Some studies have suggested that LIP supplementation is able to promote body weight loss and improve glucose homeostasis,[3] partially by the modulation of adipose tissue metabolism[9] and associated secretory functions.[10]

In recent years, much attention has been given to Fibroblast growth factor 21 (FGF21) and its role as a metabolic regulator of glucose, lipids and energy balance.[11] FGF21 is mainly produced in the liver and facilitates glucose uptake and lipolysis in white adipose tissue.[12] However, it was described that FGF21 reduces the release of FFAs to the circulation.[12] On the other hand, FGF21 has been shown to be regulated by a mixture of EPA and DHA[13] and by LIP[14] in rodents and in culture cells, respectively. Nevertheless, there are few intervention studies analyzing the regulation of the plasma levels of FGF21 by diet and the metabolic status in humans and the pathophysiological role.[15]

Obesity is characterized by a chronic low-grade inflammation and a high n-6/n-3 PUFA ratio, which is associated with an inflammation profile in obesity.[16] Moreover, fatty acid desaturases (FAD) are critical regulators of the fatty acid metabolism.[17] In this context, production of oleic, palmitoleic and arachidonic acids by high FAD activities has been associated with obesity and insulin resistance.[18] In contrast, high FAD activity to produce EPA and DHA was associated with an increase in insulin sensitivity.[17]

Therefore, we hypothesize that supplementation with LIP and/or EPA can help in reducing the negative effects of obesity and associated comorbidities by their influence on the regulation of FGF21 and the fatty acid profile in overweight and obese women following a hypocaloric diet. For this purpose, the potential relationship between the circulating levels of FGF21, the fatty acid profile and the n-6/n-3 PUFA ratio was characterized in overweight/obese women at the baseline and after the intervention.

Materials and methods

Subjects

This study is an ancillary analysis of the OBEPALIP trial,[3] the principal outcome of which was to study the effects of LIP and EPA on the amount of weight loss in humans. Briefly, from this ancillary analysis 57 women were included as volunteers. Medical history was determined and physical examination was conducted by the physician of the Metabolic Unit of the University of Navarra. The inclusion and exclusion criteria were as previously described.[3] This study was performed in compliance with the Helsinki Declaration, approved by the Research Ethics Committee of the University of Navarra (007/2009) and registered at clinicaltrials.gov as NCT01138774. After their written consent, they were allocated to one of the four experimental groups by simple randomization. The baseline and endpoint anthropometric and biochemical features of the 57 overweight/obese women analyzed, as well as their hormonal characteristics, are detailed in ESI Table 1.†

Study design and intervention

The OBEPALIP study was a short-term randomized double blind placebo-controlled weight loss trial with 4 parallel nutritional intervention groups: Control group: 3 placebo–I capsules (containing sunflower oil) and 3 placebo-II capsules (containing the same excipients as the LIP capsules); EPA group: 1300 mg day^{-1} of EPA distributed in 3 capsules of EPA 80 (provided by Solutex, Madrid, Spain) containing 433.3 mg of EPA and 13.8 mg of DHA as ethyl-ester and 3 placebo-II capsules; LIP group: 300 mg day^{-1} of LIP from 3 capsules containing 100 mg of LIP (Nature's Bounty®, NY, USA) and 3 placebo-I capsules; and finally EPA + LIP group: 1300 mg day^{-1} of EPA (distributed in 3 capsules of EPA 80) and 300 mg day^{-1} of LIP (from 3 capsules containing 100 mg of LIP).[3]

All intervention groups followed a moderate caloric restricted diet (55% carbohydrates, 30% lipids and 15% proteins) in accordance with the American Heart Association Guidelines and prescribed individually by a dietitian. During the first visit (baseline), each participant was instructed to follow an energy-restricted diet accounting for 30% less than her total energy expenditure, and to not change the physical activity pattern during the 10-week intervention period. Volunteers were advised to avoid marketed n-3 PUFA-enriched products. At the baseline and at the end point, the volunteers underwent 10–12 h of fasting and met with the physician, the dietitian and the nurse at the Metabolic Unit. Anthropometric measurements and body composition analysis were performed. A catheter was inserted into the antecubital vein for a fasting blood sample extraction. All data and measurements were taken by the same person using standard protocols to attenuate bias. Follow-up visits were scheduled with each volunteer every two weeks to monitor weight, assess the adherence to the diet and ensure that the capsules were consumed adequately.

Anthropometric measurements and body composition

Anthropometric measurements of weight, height, waist and hip circumference were performed in concordance with the established protocols. Before starting the measurements, the material was calibrated and standardized; height was evaluated using a stadiometer (Seca 220, Vogel & Halke, Germany) and the waist and hip circumference were assessed with a flexible tape with a precision of 1 mm and were used to calculate the waist to hip ratio. The body composition was estimated at the baseline and endpoint by Dual X-ray Absorptiometry (DXA), using the Lunar, Prodigy, software version 6.0 (Madison, WI, USA).

Blood samples and biochemical determination

Fasting blood samples were drawn on weeks 0 and 10 into Serum Clot Activator tubes (4 mL Vacuette®) and into tubes containing tripotassium EDTA (4 mL Vacuette®). Plasma was extracted from EDTA tubes after centrifugation at 1500*g* for 15 min at 4 °C. All samples were separated appropriately and stored at −80 °C for analysis. Serum levels of glucose, total-cholesterol, HDL-cholesterol, triglycerides (TG), β-hydroxybutyrate, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the auto analyzer PENTRA C200 (HORIBA medical, Madrid, Spain). The values of LDL-cholesterol were calculated using the Friedewald equation defined as LDL-cholesterol = Total cholesterol − HDL-cholesterol − TG/5. Plasma samples were used for assessing insulin, leptin, adiponectin and FGF21 concentration by enzyme-linked immunosorbent assay (ELISA) using appropriate kits and following the manufacturer's instructions. Insulin was determined using a Human Sensitive ELISA Kit from Mercodia (Uppsala, Sweden). HOMA-IR was defined as fasting serum insulin (mU L^{-1}) × fasting plasma glucose (mmol L^{-1})/22.5. Leptin and total adiponectin were determined using ELISA kits from BioVendor (Brno, Czech Republic). High molecular weight (HMW) adiponectin was determined by Elisa kits from ALPCO Diagnostics (Salem, NH, USA), as previously described.[3] FGF21 levels were determined in plasma samples obtained at the baseline and at the end of the trial, using a commercially available Human FGF21 DuoSet ELISA kit from R&D Systems (MN, USA) following the manufacturer's instructions.

Plasma fatty acid determination

Plasma fatty acids were determined according to the procedure described by Ostermann *et al.*,[19] with slight modifications. 50 μL of internal standard (C23:0, 180 μg mL^{-1} in heptane) was added to 150 μL of plasma and lipid extraction was performed with 2250 μL of a mixture of chloroform : methanol (1 : 2). Afterwards, 750 μL of chloroform and 750 μL of water were added; the sample was mixed for 1.5 min and centrifuged for 10 min at 6000 rpm at room temperature. The lower organic phase was collected and the upper phase was re-extracted with 750 μL of chloroform. The organic phases were combined and evaporated under nitrogen, in a water bath at 40 °C. The derivatization for the preparation of fatty acid methyl esters (FAMEs) was carried out following the NaOH + boron trifluoride protocol. The dried lipid extract was dissolved in 300 μL of methanolic NaOH (0.5 N) and heated for 15 min at 90–95 °C in a tightly closed vial. Then, 1500 μl of boron trifluoride solution (20% in methanol) was added and the sample was heated for another 30 min at 90–95 °C. After cooling the sample, 2250 μL of saturated NaCl solution and 1500 μL of heptane were added. Then, the sample was vortex-mixed for 4 min and centrifuged at 4600 rpm for 5 min. The upper organic phase was collected, evaporated under nitrogen in a water bath at 40 °C and reconstituted in 75 μL of heptane. The FAMEs were analysed by using a gas chromatography-flame ionization detector (GC-FID) using a PerkinElmer Clarus 500 gas chromatograph (Madrid, Spain), equipped with a split–splitless injector, automatic autosampler, and coupled to a computerized system for data acquisition (TotalChrom, version 6.3.2). A capillary column SP™-2560 (100 m × 0.25 mm × 0.2 μm; Sigma-Aldrich) was used. The temperature of the injection port and detector was 250 °C and 260 °C, respectively. The oven temperature was 175 °C for 10 min, heated to 200 °C at a rate of 7 °C min^{-1}, raised to 220 °C at a rate of 3 °C min^{-1}, and finally 220 °C for 20 min. The carrier gas was hydrogen, 30 psi. The volume injection was 1 μL and the split ratio was 25 : 1. The identification of the FAMEs was performed by comparison of their retention times with those of pure FAMEs and previous studies.[20] The quantification of individual fatty acids was based on the internal standard method using calibration curves for every compound and the results are expressed as μg mL^{-1}.

Fatty acid desaturase index measurement

Desaturase activity indexes in dietary supplemented groups were measured using a validated methodology from the fatty acid (FA) data.[21] FAD indexes were calculated as the product/precursor ratio for: Stearoyl-CoA (SCD16 or SCD18) = [palmitoleic (16:1 n7)/palmitic (16:0)] or [oleic (18:1 n-9)/stearic (18:0)]; Δ4D = [DHA (22:6 n-3)/DPA (22:5 n-3)]; and Δ5/6D = [EPA (20:5 n-3)/ALA (18:3 n-3)].

Statistical analysis

Statistical analyses were performed using Stata Statistical Software (College Station, TX: StataCorp LP) version 12.1 for Windows. Graphics were represented using GraphPad Prism Software v. 6.0 for Windows (GraphPad Software, San Diego, CA). Data are expressed as mean ± standard error of the mean (SEM). Normality was calculated by the Shapiro–Wilk test and the differences after the nutritional intervention trial within each group were assessed by paired Student's *t* test when variables were normally distributed and Wilcoxon's sign-test when not. The differential effects between the intervention groups were estimated by two-way ANOVA. Differences were considered significant if $P < 0.05$. The potential relationships between FGF21 and several anthropometric parameters and metabolic biomarkers were analyzed by the bivariate Pearson's and Spearman's correlation.

Results

FGF21 circulating levels and their relationship with anthropometric and biochemical parameters at the baseline

Plasma FGF21 levels and correlation analyses between plasma FGF21 levels and anthropometric and biochemical parameters at the beginning of the study are shown in Table 1. FGF21 was negatively correlated with HMW adiponectin, the ratio of HMW adiponectin/total adiponectin and the AST/ALT ratio. However, no other significant associations were identified at the baseline.

Table 1 FGF21 levels at the baseline and correlations with anthropometric and metabolic biomarkers of the volunteers

Variable	$n = 57$	r/rho	P
Age (years)[b]	38.49 ± 0.97	−0.027	0.845
Body weight (kg)[a]	84.59 ± 1.55	0.202	0.139
BMI (kg m^{-2})[b]	32.24 ± 0.50	0.219	0.102
Fat mass (kg)[b]	39.00 ± 1.04	0.241	0.076
Fat free mass (kg)[a]	45.61 ± 0.69	0.179	0.191
Total cholesterol (mg dL^{-1})[b]	193.26 ± 4.60	−0.159	0.238
Triglycerides (mg dL^{-1})[b]	88.91 ± 5.68	0.141	0.304
FFAs (mmol L^{-1})[b]	0.53 ± 0.03	−0.077	0.568
β-Hydroxybutyrate (mmol L^{-1})[b]	0.34 ± 0.03	0.210	0.118
Glucose (mg dL^{-1})[a]	91.29 ± 1.07	0.012	0.931
Insulin (mU L^{-1})[b]	8.46 ± 0.94	0.042	0.759
HOMA-IR[b]	1.85 ± 0.22	0.060	0.660
AST (IU L^{-1})[b]	16.84 ± 0.59	0.054	0.689
ALT (IU L^{-1})[b]	18.76 ± 1.20	0.258	0.053
AST/ALT ratio[a]	0.98 ± 0.03	−0.264	**0.047**
Leptin (ng mL^{-1})[a]	21.44 ± 1.02	0.232	0.083
Adiponectin (μg mL^{-1})[b]	13.08 ± 0.59	0.207	0.125
HMW adiponectin (μg mL^{-1})[b]	2.17 ± 0.17	−0.270	**0.046**
HMW adiponectin/total adiponectin[b]	0.17 ± 0.01	−0.382	**0.004**
FGF21 (pg mL^{-1})	287.8 ± 11.8	—	—

Values are means ± SEM. Spearman correlations. *$P < 0.05$.

Data are represented as mean ± SEM. Correlations were performed with Pearson[a] (for variables with normal distribution) and Spearman[b] (for variables with non-normal distribution). ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BMI, body mass index; FGF21, fibroblast growth factor 21; HDL, high density lipoprotein; HMW, high molecular weight; HOMA-IR, homeostasis model assessment for insulin resistance; LDL, low density lipoprotein.

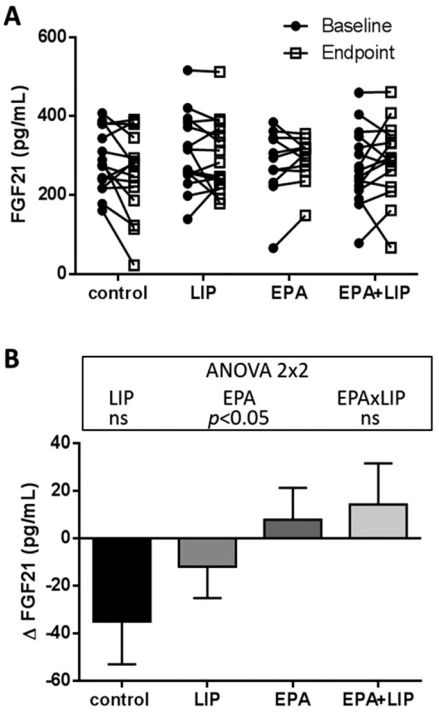

Fig. 1 Circulating FGF21 levels in plasma. (A) FGF21 plasma levels at the beginning and at the endpoint in the four intervention groups. Data were analyzed by paired t-test after testing for normality. No significant differences were observed. (B) Changes (endpoint-baseline) in FGF21 after 10 weeks of nutritional intervention. Data (mean ± SEM) were analyzed by two-way ANOVA. Significant differences were observed in groups supplemented with EPA ($P < 0.05$). LIP, lipoic acid; EPA, eicosapentaenoic acid; ns, non-significant.

Effects of weight loss, EPA and LIP supplementation on circulating plasma levels of FGF21

As previously reported by the research group,[3,10,22,23] data in this subpopulation of the OBEPALIP study also showed differences in the decrease of body weight, BMI, fat mass, free fat mass, waist circumference, hip circumference and waist to hip ratio after the intervention with a hypocaloric diet in all experimental groups (ESI Table 1†). In parallel, several systemic biomarkers of glucose and lipid metabolism, including fasting insulin, HOMA-IR and total cholesterol, showed a better profile in all experimental groups. Interestingly, plasma concentrations of FGF21 did not change before and after the 10 weeks on a hypocaloric diet in any group (Fig. 1A). However, it is important to note that the analysis of changes in FGF21 (endpoint-baseline), by a two-way ANOVA, revealed that FGF21 increased in both groups supplemented with EPA (EPA and EPA + LIP) compared to groups without EPA (control and LIP). These changes were not related to changes in BMI or total fat mass (data not shown), suggesting that EPA could be somehow involved in the induction of FGF21 in humans (Fig. 1B).

Effect on plasma FA profile and n-6/n-3 ratio

Noticeable differences were observed in the plasma FA profile between the different interventional groups (Table 2). After the hypocaloric diet, a significant decrease in the levels of stearic acid, α-linoleic acid and eicosadienoic acid was observed in the control group. Interestingly, LIP supplementation promoted a significant decrease in the circulating levels of saturated FA (SFA): myristic, pentadecanoic, palmitic, heptadecanoic, stearic and lignoceric acids. Therefore, the levels (Σ) of SFA as well as of n-6-PUFAs, and total PUFAs were also decreased in the LIP group. In contrast, nervonic acid levels were increased after the intervention in the LIP group. The EPA supplemented groups (EPA and EPA + LIP) showed lower levels of α-linoleic, γ-linolenic, behenic and arachidonic acids after the intervention. In contrast, EPA and DPA levels, as well the total n-3-PUFA content (Σ), were strongly increased in these supplemented groups. In parallel, the total n-6-PUFA content and the n-6/n-3 ratio were significantly decreased in both EPA-supplemented groups.

Furthermore, the two-way ANOVA analysis of changes (endpoint-baseline) between groups confirmed that EPA, DPA and n-3-PUFA contents increased, whereas the n-6/n-3 PUFA ratio decreased in the groups supplemented with EPA (EPA and EPA + LIP) *vs.* those without EPA supplementation (Fig. 2A, B, D and F). No significant changes between the groups were observed for DHA and n-6-PUFAs (Fig. 2C and E).

Effects of LIP and/or EPA supplementation on FAD activity indexes

FAD indexes from the FA data of plasma were calculated as the product/precursor ratio.[17] LIP and EPA supplemen-

Table 2 Plasma fatty acid profile at the baseline and at the endpoint in the four intervention groups

	Control		LIP		EPA		EPA + LIP	
	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint
14:0 (myristic)[b]	23.2 ± 10.4	13.0 ± 4.7	**26.4 ± 6.9**	**14.1 ± 4.6***	14.6 ± 3.0	11.2 ± 2.2	18.1 ± 4.8	10.7 ± 1.8
15:0 (pentadecanoic)[b]	4.6 ± 1.4	3.6 ± 0.6	**6.2 ± 1.5**	**4.9 ± 1.6***	3.3 ± 0.3	3.3 ± 0.4	3.7 ± 0.7	3.1 ± 0.4
16:0 (palmitic)[b]	682.1 ± 111.0	602.0 ± 49.4	**780.7 ± 171.0**	**693.8 ± 170.0***	522.8 ± 41.5	508.9 ± 32.8	612.4 ± 88.7	535.7 ± 51.5
16:1 n-7 (palmitoleic)[b]	46.9 ± 12.6	36.6 ± 7.6	**75.8 ± 31.9**	**57.5 ± 24.3***	32.5 ± 2.9	32.1 ± 5.7	47.3 ± 10.8	34.2 ± 4.0
17:0 (heptadecanoic)[b]	8.2 ± 1.0	7.4 ± 0.8	**9.2 ± 1.7**	**7.3 ± 1.4***	6.8 ± 0.7	6.4 ± 0.3	6.6 ± 0.7	7.1 ± 0.9
18:0 (stearic)[b]	**240.8 ± 23.9**	**201.0 ± 14.3***	**242.4 ± 29.1**	**188.6 ± 31.8***	212.1 ± 14.2	179.6 ± 14.1	198.6 ± 11.8	167.0 ± 6.7
18:1 n-9 (oleic)[b]	717.6 ± 122.6	614.6 ± 45.3	749.1 ± 162.7	718.6 ± 194.1	521.8 ± 61.7	490.5 ± 28.8	549.1 ± 67.7	492.2 ± 53.5
18:1 n-7 (*cis*-vaccenic)[b]	61.9 ± 10.4	60.3 ± 7.4	65.5 ± 14.4	68.8 ± 17.9	46.0 ± 6.8	51.4 ± 8.9	57.3 ± 7.5	49.9 ± 3.3
18:2 n-6 (linoleic)[a]	1017.1 ± 109.5	906.8 ± 68.7	**1025.3 ± 99.5**	**844.7 ± 131.6***	**884.6 ± 40.3**	**763.8 ± 63.6***	**811.9 ± 73.4**	**624.4 ± 59.9***
18:3 n-3 (α-linolenic)[b]	**11.4 ± 2.6**	**8.2 ± 1.0***	**10.7 ± 1.9**	**8.1 ± 1.9***	8.0 ± 1.2	8.3 ± 0.6	7.7 ± 0.9	6.4 ± 0.6
18:3 n-6 (γ-linolenic)[a]	11.9 ± 2.6	9.0 ± 2.4	**17.4 ± 4.4**	**9.5 ± 2.3***	**11.5 ± 0.7**	**6.2 ± 1.4***	**13.4 ± 3.1**	**6.1 ± 1.3***
20:0 (arachidic)[a]	3.6 ± 0.3	3.5 ± 0.3	3.9 ± 0.5	3.8 ± 0.5	3.6 ± 0.2	3.4 ± 0.2	2.9 ± 0.3	3.1 ± 0.3
22:0 (behenic)[a]	5.9 ± 0.9	5.7 ± 0.7	6.8 ± 1.3	5.2 ± 0.7	**6.0 ± 0.8**	**5.1 ± 0.7****	5.1 ± 0.6	4.1 ± 0.4
20:1 n-9 (eicosenoic)[b]	4.0 ± 0.9	3.1 ± 0.5	3.7 ± 1.0	4.4 ± 2.1	2.3 ± 0.3	2.2 ± 0.3	2.4 ± 0.4	2.5 ± 0.6
20:2 n-6 (eicosadienoic)[b]	**7.2 ± 1.4**	**6.3 ± 1.0***	8.0 ± 2.1	7.0 ± 2.6	5.8 ± 0.3	4.9 ± 0.5	5.9 ± 0.9	4.6 ± 0.6
20:4 n-6 (arachidonic)[a]	227.3 ± 20.4	227.6 ± 23.1	310.9 ± 45.4	296.9 ± 52.3	**218.3 ± 25.4**	**200.8 ± 26.1***	282.9 ± 38.5	250.1 ± 33.9
20:5 n-3 (EPA)[b]	23.5 ± 5.0	18.5 ± 4.7	**12.4 ± 1.8**	**8.5 ± 1.5***	**10.6 ± 0.7**	**71.7 ± 10.0***	**12.6 ± 1.7**	**71.3 ± 7.4***
22:5 n-3 (DPA)[b]	10.3 ± 1.6	9.8 ± 0.9	10.4 ± 1.3	8.7 ± 1.9	**9.0 ± 1.2**	**23.3 ± 4.3***	**8.6 ± 0.4**	**19.4 ± 2.0***
22:6 n-3 (DHA)[a]	80.6 ± 9.8	73.1 ± 6.8	72.5 ± 11.0	69.3 ± 13.0	60.0 ± 3.4	64.4 ± 3.9	66.5 ± 7.5	66.2 ± 11.1
24:0 (lignoceric)[b]	6.0 ± 0.7	5.8 ± 0.5	**7.0 ± 1.1**	**5.0 ± 0.8***	**7.5 ± 0.6**	**5.5 ± 0.4***	6.1 ± 0.7	4.8 ± 0.7
24:1 n-9 (nervonic)[b]	12.2 ± 0.9	16.2 ± 2.3	**12.8 ± 2.5**	**17.4 ± 3.4***	11.9 ± 1.2	12.9 ± 0.8	13.0 ± 1.4	13.4 ± 0.7
Σn-3[b]	125.8 ± 16.3	109.6 ± 12.3	105.9 ± 14.8	94.6 ± 15.8	**87.6 ± 5.0**	**167.6 ± 15.5****	**95.4 ± 8.0**	**163.3 ± 17.8***
Σn-6[b]	1256.3 ± 122.0	1143.4 ± 84.7	**1353.6 ± 139.9**	**1151.0 ± 179.5***	**1114.3 ± 51.6**	**970.8 ± 86.4***	**1108.1 ± 88.9**	**880.6 ± 81.7***
n-6/n-3[a]	10.0 ± 1.3	10.9 ± 1.2	13.3 ± 1.4	12.5 ± 1.1	**12.8 ± 0.7**	**5.9 ± 0.7*****	**12.1 ± 1.8**	**5.5 ± 0.5****
Σn-9[b]	733.8 ± 123.0	633.9 ± 44.2	765.6 ± 165.9	740.4 ± 198.9	535.9 ± 61.1	505.6 ± 29.3	564.5 ± 67.3	508.1 ± 54.4
ΣSFA[b]	974.5 ± 147.1	841.9 ± 67.3	**1082.6 ± 211.9**	**922.7 ± 209.7***	775.8 ± 58.4	723.3 ± 48.8	853.5 ± 105.5	735.6 ± 60.1
ΣMUFA[b]	842.5 ± 139.7	730.8 ± 50.7	906.8 ± 211.0	866.7 ± 240.2	614.4 ± 65.9	589.1 ± 41.7	669.2 ± 83.8	592.2 ± 59.0
ΣPUFA[a]	1389.3 ± 127.6	1259.3 ± 87.3	**1467.5 ± 155.2**	**1252.6 ± 196.5***	1188.9 ± 66.0	1084.3 ± 94.4	1209.4 ± 86.3	1078.6 ± 94.1

Data are represented as mean ± SEM (μg mL^{-1}). Analysis was performed with paired *t* test[a] (for variables with normal distribution) and Wilcoxon's test[b] (for variables with non-normal distribution). LIP, lipoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. (n = 5 per group). *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* baseline levels.

tation did not influence SCD16 (palmitoleic/palmitic) (Table 3). SCD18 (oleic/stearic) was slightly increased in the LIP group, but the two-way ANOVA analysis revealed no significant differences between the groups. In contrast, EPA supplementation (EPA and EPA + LIP) clearly influenced the n-3 PUFA-related desaturases by significantly decreasing the Δ4D (DHA/DPA) and increasing the Δ5/6D (EPA/ALA) (Table 3). These results were corroborated by the two-way ANOVA analysis observing significant differences in the Δ4D and Δ5/6D activities in EPA-supplemented groups (Table 3).

Relationship between changes in FGF21 levels and changes in FA levels

To better investigate the potential relationships between the plasma changes of FGF21 after the intervention with changes in the FA profile, a correlation analysis was performed. A positive association was observed between the changes in FGF21 levels with the changes in EPA levels, and with total n-3-PUFA (Σ) levels, while a negative association was found with the changes in the n-6/n-3 PUFA ratio (Table 4).

In addition, the changes in Δ5/6D activity were also positively associated with the changes in FGF21 levels (Table 4).

Discussion

Lately much attention has been given to FGF21 and its role as a metabolic regulator.[11] Nevertheless, the underlying mechanisms by which FGF21 exerts its effects on humans remain poorly understood, and only a few studies have evaluated the relationship between FGF21 and fatty acids.[24,25] FGF21 is considered a fasting hormone and, although seemingly paradoxical, some studies have reported that circulating FGF21 levels are elevated during obesity in rodents and humans.[26,27] In fact, some studies on humans support the idea that obesity represents a FGF21 resistant state.[28] Thus, FGF21 is considered a stress-response hormone, synthesized and released in obese patients as a compensatory mechanism to decrease cell damage,[15] which has been related to adiposity.[29]

The current study revealed that FGF21 levels were not apparently related to body weight, BMI or total fat mass in healthy overweight/obese women. It is important to mention that a recent study has reported that in insulin-sensitive obese individuals, serum FGF21 levels positively correlate with the subcutaneous adipose tissue area, but not with the visceral fat area or total fat.[30] Unfortunately, in our obese insulin-sensitive participants, the area of subcutaneous *vs.* visceral fat was not measured, and therefore we cannot rule out a potential relationship between the amount of subcutaneous fat and cir-

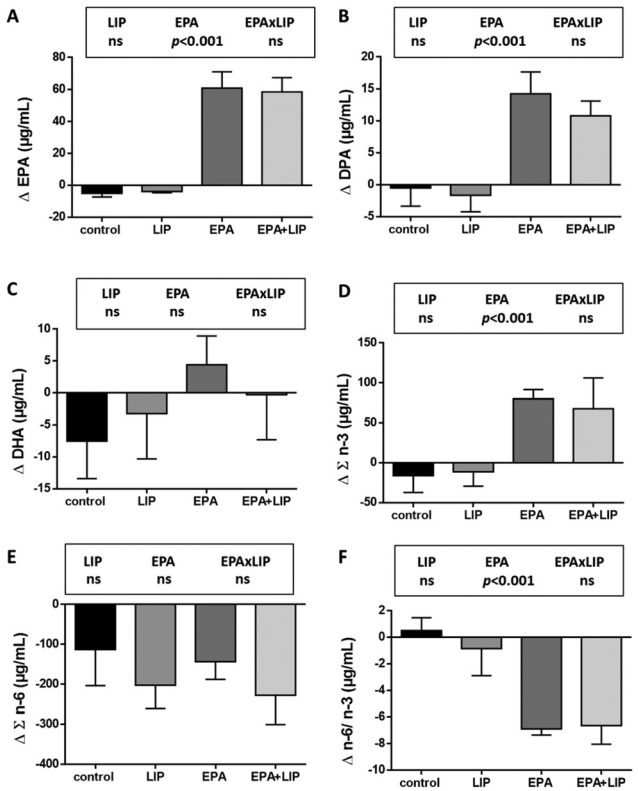

Fig. 2 Changes (endpoint-baseline) at the indicated plasma fatty acids after 10 weeks of nutritional intervention. (A) EPA, (B) DPA, (C) DHA, (D) Σn-3, (E) Σn-6 and (F) ratio n-6/n-3. Data (mean ± SEM) were analyzed by two-way ANOVA. Significant differences were observed in groups supplemented with EPA ($P < 0.001$). LIP, lipoic acid; EPA, eicosapentaenoic acid, DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; ns, non-significant.

Table 4 Correlations after the treatments in changes of plasma FGF21 levels and changes in the indicated plasma fatty acids and fatty acid desaturase indexes

		ΔFGF21	
	Change	*P*	*r*/rho
ΔEPA[b]	25.00 ± 7.95	**0.021**	0.526
ΔDPA[a]	4.90 ± 1.78	0.162	0.334
ΔDHA[a]	−1.81 ± 3.18	0.173	0.326
ΔΣn-3[a]	26.20 ± 11.43	**0.032**	0.494
ΔΣn-6[a]	−180.22 ± 33.82	0.467	−0.178
ΔΣn-9[a]	−63.18 ± 28.54	0.253	−0.276
Δ(n-6/n-3)[a]	−3.31 ± 1.02	**0.041**	−0.473
ΔΔ4D[a]	−1.85 ± 0.63	0.268	−0.268
ΔΔ5/6D[a]	3.97 ± 1.09	**0.021**	0.524
ΔSCD16[a]	−0.01 ± 0.00	0.459	0.181
ΔSCD18[a]	0.32 ± 0.09	0.193	−0.313

Correlations were performed with Pearson[a] (for variables with normal distribution) and Spearman[b] (for variables with non-normal distribution). Data changes (endpoint-baseline) were expressed as mean ± SEM; μg mL^{-1}. (n = 20) EPA, eicosapentaenoic acid, DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

culating FGF21 levels. However, circulating levels of FGF21 correlated negatively with HMW adiponectin. Adiponectin is an adipokine predominantly secreted by adipocytes, with a key role in the regulation of glucose and lipid metabolism.[25] Obese subjects show a decrease in adiponectin levels, which is related to insulin resistance and inflammation.[31] Therefore, adiponectin and FGF21 seem to behave differently in obesity, showing decreased and increased levels, respectively,[27,32] which is in agreement with the negative correlation found in our data. The relationships between FGF21 and adiponectin are complex and some mechanisms may explain the dissociation between the plasma levels of adiponectin and FGF21 in humans,[25] such as proinflammatory cytokines and oxidative stress, and may be further aggravated by the low-grade chronic inflammation associated with obesity. Therefore, it is likely that the ability of FGF21 to induce the production of adiponectin is diminished in obese subjects due to a FGF21 resistance.[25,27,28]

Table 3 Fatty acid desaturase activity indexes at the baseline and at the endpoint in the four intervention groups

						Anova 2 × 2		
		Control	LIP	EPA	EPA + LIP	LIP	EPA	LIP × EPA
Δ4D[a] (DHA/DPA)	Baseline	8.48 ± 1.36	6.95 ± 0.32	6.94 ± 0.57	7.81 ± 0.99			
	Endpoint	7.60 ± 0.67	7.88 ± 1.24	**3.21 ± 0.69*****	**3.47 ± 0.54****			
	Change	−0.88 ± 0.91	0.93 ± 1.01	−3.73 ± 0.57	−4.34 ± 0.66	0.465	**<0.001**	0.154
Δ5/6D[b] (EPA/ALA)	Baseline	2.30 ± 0.44	1.29 ± 0.27	1.44 ± 0.20	1.80 ± 0.35			
	Endpoint	2.31 ± 0.61	1.22 ± 0.29	**8.56 ± 0.97****	**11.31 ± 1.12***			
	Change	0.01 ± 0.34	−0.08 ± 0.19	7.13 ± 1.01	9.51 ± 1.28	0.210	**<0.001**	0.181
SCD16[b] (palmitoleic /palmitic)	Baseline	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01			
	Endpoint	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.00			
	Change	−0.01± 0.01	−0.01± 0.01	0.00 ± 0.01	−0.01 ± 0.01	0.135	0.442	0.626
SCD18[b] (oleic/stearic)	Baseline	2.93 ± 0.32	2.98 ± 0.25	2.45 ± 0.13	2.74 ± 0.22			
	Endpoint	3.08 ± 0.19	**3.65 ± 0.32***	2.76 ± 0.11	2.92 ± 0.23			
	Change	0.14 ± 0.16	0.66 ± 0.18	0.27 ± 0.19	0.18 ± 0.11	0.219	0.315	0.050

Fatty acid desaturase indexes were calculated as the product/precursor ratio from the total fatty acid data. Data are represented as mean ± SEM (μg mL^{-1}). Differences between the baseline and endpoint were analyzed with the *t*-test[a] (for variables with normal distribution) and Wilcoxon test[b] (for variables with non-normal distribution). Data changes (endpoint-baseline) were analyzed by two-way ANOVA (right panel). LIP, lipoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; ALA, α-linolenic acid. (n = 5 per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* basal levels.

Recent studies have also shown increased FGF21 levels in patients with non-alcoholic fatty liver disease (NAFLD), which represents a FGF21-resistant state.[11] This association indicates that an impaired FGF21 response occurs in diseases associated with chronic inflammation.[11] Unfortunately, hepatic fat amount was not measured in our participants. Instead, biochemical parameters related to liver injury were measured (AST, ALT and the AST/ALT ratio). The AST/ALT ratio is an indicator of liver injury especially relevant in NAFLD, and is also considered predictive of liver cirrhosis.[33] At the baseline, our participants did not report NAFLD or any other associated diseases, but a negative relationship between FGF21 and the AST/ALT ratio was observed, suggesting that FGF21 may be involved in protecting liver injury progression. In fact, FGF21 has preventive functions against lipotoxicity, oxidative stress and chronic inflammation.[11,15,34]

The energy-restricted diet alone or in combination with LIP, EPA or both promoted weight loss and fat mass reduction in healthy overweight/obese women, improving anthropometric and biochemical parameters associated with lipid and glucose metabolism.[3] In this sub-cohort of the OBEPALIP study, weight loss did not induce any significant change in circulating plasma of FGF21 levels after the intervention period. Currently, studies aiming to analyze the impact of weight loss on the circulating levels of FGF21 in humans are limited, even without agreement on the effects of different therapies for weight-loss on FGF21 levels.[26] Dietary treatments reduced FGF21,[26] whereas bariatric surgery induced an increase in FGF21 levels, suggesting that FGF21 concentrations are associated with other obesity-related factors rather than adiposity levels.[26] As mentioned above, FGF21 is considered a stress-response hormone, and some bariatric surgeries are accompanied by metabolic stress, which may induce FGF21 levels as a compensatory mechanism to decrease cell damage.[15] This study performed in healthy women, together with the inter-individual variability and the characteristics of the nutritional intervention, may explain the lack of significant effects of weight loss on FGF21.

As expected, lipidomic studies demonstrated that n-3-PUFA levels (EPA and DPA) were higher in the EPA supplemented groups, whereas this effect was not observed at the DHA level. On the other hand, *in vivo* n-3 PUFA supplementation ameliorates adipose tissue inflammation,[7] and it has been suggested that a mixture of EPA and DHA induces the expression and release of FGF21. However, some studies suggested that these increased FGF21 levels did not appear to be a major mechanism through which n-3-PUFAs ameliorate metabolic disorders.[13] Recently, it has been reported that n-3 PUFAs and EPA alone induce thermogenic activation[35] and other investigations described that thermogenic activation upregulates FGF21 levels.[36] Interestingly, our data suggested that EPA may prevent FGF21 tendency to decrease during weight loss. Besides, changes in EPA levels were related to changes in FGF21 levels. Contrarily, it was described that in patients with NAFLD, fish oil decreased the levels of FGF21, suggesting that fish oil may have an effect on the improvement of FGF21 resistance.[37] Thus, the effects of n-3-PUFAs on FGF21 remain still unclear and seem to be tissue specific or metabolic state dependent.

The n-6/n-3 PUFA ratio has been associated with an increasing prevalence of chronic inflammatory diseases.[38] Indeed, an increase in the n-6/n-3 PUFA ratio increases the risk for obesity.[38] Our data indicate that after the intervention, EPA supplemented groups presented a lower n-6/n-3 ratio, which may be associated with a lower inflammation and a healthier metabolic profile.[39] In fact, a previous study of our group in this cohort revealed that EPA supplementation decreases markers of macrophage infiltration and attenuates inflammation in human adipose tissue.[23] Interestingly, our current data unravel a negative relationship between the changes in FGF21 levels and the changes in the n-6/n-3 ratio.

Furthermore, fatty acid desaturase levels are the limiting steps in the synthesis of different fatty acids, and thus promising targets for the treatment of metabolic diseases.[17] In animals models fed with diets rich in EPA and DHA, desaturases involved in the transformation of α-linolenic acid (ALA) into EPA and DHA are up-regulated, meanwhile the activity of these enzymes to produce ARA from LA is reduced.[17] These are in accordance with the results observed in the present study, and it was related to healthier insulin sensitivity.[40]

Regarding LIP supplementation, the present study showed a clear reduction in the systemic SFA levels as well in the n-6 PUFA content. Saturated fatty acids (SFAs), especially saturated long chain fatty acids, such as myristic acid, palmitic acid and stearic acid, common in Western diets, have been related to a higher risk of developing cardiovascular disease. Palmitic acid, as well as n-6-PUFAs, has also been related to the promotion of inflammatory responses.[41] Consequently, the reduction in the levels of these types of FAs observed after LIP supplementation can promote a healthier metabolic profile, which may be in relation with a recent study of our group showing that LIP not only induces weight loss, but also reduces systemic inflammatory and cardiovascular disease-related risk biomarkers.[23]

Nevertheless, it is important to mention some limitations of this study, including the sample size, gender (only women), and length of the nutritional intervention. Moreover, our trial was performed in healthy overweight/obese women. Therefore it would be of interest to validate the results of our study in larger cohorts and to assess the role of EPA and/or LIP in the regulation of FGF21 as well as to better characterize the potential involvement of FGF21 in the metabolic actions of EPA and LIP in obese subjects with and without metabolic disturbances.

Conclusion

Altogether, our results show that circulating levels of FGF21 negatively correlate with the adiponectin system and the AST/ALT ratio in healthy overweight/obese women. Our study also suggests a role of n-3-PUFAs, especially EPA, in the regulation of FGF21 in humans. However, the mechanism underlying the

relationship between FGF21 and n-3-PUFAs in obesity needs to be further characterized.

Conflicts of interest

There are no conflicts to declare.

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DISSEMINATION OF RESULTS

Articles in scientific journals

Gayoso, L., Claerbout, A., Calvo, M. I., Cavero, R. Y., Astiasarán, I., Ansorena, D. (2016). Bioaccessibility of rutin, caffeic acid and rosmarinic acid: Influence of the *in vitro* gastrointestinal digestion model. *Journal of Functional Foods*, *26*, 428-438.
Q1- Food Science & Technology.

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Poster communication

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