Nutritional factors influencing tissue omega-3 metabolism and endocannabinoids levels in experimental models and humans
Abstract

The nutritional role of dietary fatty acids is quite controversial and often the object of mediatic campaigns either as panacea against all diseases or detrimental for human health. The most recent data are instead showing that it should be considered, in an optimal dietary regimen, the balance among all dietary fatty acids based on the current physiological needs, applying what it is currently known as personalised nutrition. It has been paid particular attention to the ratio between highly polyunsaturated fatty acids (HPUFAs) n-6, mainly arachidonic acid (ARA, 20:4n-6) and n-3 HPUFAs, mainly eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) in tissues, since an inverse correlation between circulating n-3 HPUFAs and several chronic pathological states has been observed, possibly mediated by modulation of the endocannabinoid system (ECS). However, to assess this balance and controlling it by dietary means remains a great challenge for nutritionists. In addition, other nutritional factors may influence their tissue concentration. It is therefore compelling to individuate the mechanism(s) of action and what are the most efficient nutritional conditions to maximize n-3 HPUFAs health-related beneficial activities.

The aim of this thesis was to evaluate the effects of some nutritional factors, such as conjugated linoleic acid (CLA), naturally occurring in the diet or produced by the probiotic Bifidobacterium breve (B. breve), in influencing n-3 PUFA metabolism to optimize the n-3 HUFA score (a biomarker of n-3 status in tissues) and possible interactions with the ECS in rodents and humans.

We evaluated whether CLA, from different dietary sources, was able to enhance DHA biosynthesis, from its precursor alpha linolenic (ALA), in experimental models and humans. We found that different dietary ALA/CLA ratios affected n-3 PUFA and EC profile with a graded decrease of ALA and EPA and corresponding increase of DHA, while the EC arachidonoylethanolamine (AEA) decreased parallel to ratio ALA/CLA, whereas dietary ALA, in absence of CLA, was not able to increase significantly circulating DHA levels. We also evaluated whether supplementation with B. Breve as probiotic, along with ALA, in mice, as a nutritional factor that may increase dietary CLA, modulated n-3 fatty acid metabolism. We found that dietary probiotics increased DHA biosynthesis in liver and epididymal adipose tissue. Actually, we showed an increased of DHA biosynthesis in liver, but not an increase of CLA, while the ratio CD 16:2/CLA, a biomarker of peroxisomal beta oxidation, increased significantly but not associated to a higher PPAR α gene expression. The observed parameters suggested that these effects may be related to a pro-inflammatory event possibly triggered by the activation of toll like receptors by B. breve. In fact, in an ancillary experiment on rats challenged with LPS, we obtained similar results on fatty acid metabolism. In addition, dietary probiotics decreased AEA and its congener palmitoylethanolamine (PEA) levels in liver, while in adipose tissue we found a significant increased levels of AEA, PEA and the other AEA congener oleylethanolamide (OEA).

Our studies strongly suggest that background diet may play an important role in modulating fatty acid metabolism and in particular in modulating n-3/n-6 fatty acid balance, thereby differently affecting the ECS. Therefore, the synergistic effects of different nutritional factors, as it occurs in daily life in humans are somewhat difficult to predict and may explain the contradictory results in the literature on the effects of dietary fatty acids, in particular when they are singularly considered without taking into account the whole dietary regimen. The discover that gut microbiota may directly interfere and modify fatty acid metabolism opens to novel nutritional strategies in shaping the optimal milieu for contrasting several metabolic disorders by modulating the ECS.

Future studies in humans will be focus on evaluating possible synergistic effects of CLA-enriched products with B. breve in maintaining and/or re-equilibrating energy and lipid metabolism, by modulating EC and congeners tissue profile, acting on energy metabolism and EPA and DHA related molecules, and also to assess if there is an optimal level of dietary EPA and DHA to allow a synergistic activity in different physio-pathological conditions.
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<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
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<tr>
<td>ACC1</td>
<td>acetyl coenzyme-A carboxylase-1</td>
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<tr>
<td>AEA</td>
<td>anandamide or arachidonylethanolamide</td>
</tr>
<tr>
<td>ALA</td>
<td>alpha-linolenic acid</td>
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<td>ARA</td>
<td>arachidonic acid</td>
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<td><em>B. breve</em> NCIMB 702258</td>
<td><em>Bifidobacterium breve</em> NCIMB 702258</td>
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<td><em>Bifidobacterium breve</em> DPC 6330</td>
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<td><em>B. breve</em> C50</td>
<td><em>Bifidobacterium breve</em> C50</td>
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<td>CB</td>
<td>cannabinoid receptor</td>
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<tr>
<td>CD</td>
<td>conjugated dienes</td>
</tr>
<tr>
<td>CD14</td>
<td>protein co-receptor (cluster of differentiation 14)</td>
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<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
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<td>coronary heart disease</td>
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<td>CLA</td>
<td>conjugated linoleic acid</td>
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<td>eicosapentaenoylethanolamine</td>
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<td>ER</td>
<td>endoplasmatic reticulum</td>
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<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FA or FAs</td>
<td>fatty acid or fatty acids</td>
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<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
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<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FIAF</td>
<td>fasting-induced adipose factor</td>
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<td>FO</td>
<td>fish oil</td>
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<td>GLA</td>
<td>gamma linolenic acid</td>
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<td>HDL</td>
<td>high density lipoprotein</td>
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<tr>
<td>HPUFA</td>
<td>highly polyunsaturated fatty acids</td>
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<tr>
<td>hs-CRP</td>
<td>high-sensitivity c-reactive protein</td>
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<td>IL-1β</td>
<td>interleukin-1beta</td>
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<td>KO</td>
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<td>LA</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>LNA</td>
<td>linolenic acid</td>
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<td>LOX</td>
<td>lipoxygenases</td>
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<td>LPL</td>
<td>lipoprotein lipase</td>
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<td>LPS</td>
<td>lipopolysaccharides</td>
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<td>LTB</td>
<td>leukotrienes</td>
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<tr>
<td>MAGL</td>
<td>monoacylglycerol lipase</td>
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<tr>
<td>MAMPs</td>
<td>microbes associated molecular patterns</td>
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<tr>
<td>MCFA</td>
<td>medium-chain fatty acids</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MUFA</td>
<td>monounsaturated fatty acids</td>
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<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
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<tr>
<td>NAPEPLD</td>
<td>acylphosphatidylethanolamine phospholipase-D</td>
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<tr>
<td>NASH</td>
<td>Nonalcoholic steato-hepatitis</td>
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<tr>
<td>OA</td>
<td>oleic acid</td>
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<tr>
<td>OEA</td>
<td>oleoylethanolamide</td>
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<tr>
<td>PA</td>
<td>palmitic acid</td>
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<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
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<td>PC</td>
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<td>phosphatidylethanolamine</td>
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<td>PEA</td>
<td>palmitoylethanolamide</td>
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<td>PGE</td>
<td>prostaglandins e</td>
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<td>PI</td>
<td>phosphatidylinositol</td>
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<tr>
<td>PI 3-kinase</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase b</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipid</td>
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<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptors</td>
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<tr>
<td>PS</td>
<td>phosphatidylserine</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
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<tr>
<td>Raf</td>
<td>rapidly accelerated fibrosarcoma (kinase)</td>
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<td>RBC</td>
<td>red blood cells</td>
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<tr>
<td>rTAG</td>
<td>re-esterified triacylglycerols</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid x receptor</td>
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<tr>
<td>SA</td>
<td>stearic acid</td>
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<tr>
<td>SFA</td>
<td>saturated fatty acids</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>sterol regulatory element-binding protein 1</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerols</td>
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<tr>
<td>TBX</td>
<td>thromboxane</td>
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<td>TH</td>
<td>t helper cells</td>
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<tr>
<td>TLRs</td>
<td>toll-like receptors</td>
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<tr>
<td>TNF α</td>
<td>tumour necrosis factor alpha</td>
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<tr>
<td>TRPV1</td>
<td>transient receptor potential of vanilloid type-1</td>
</tr>
<tr>
<td>VA</td>
<td>vaccenic acid</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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1. Introduction

Dietary fatty acids, present in the diet in a certain balance, have several physiological functions. For this reason, every dietary recommendation has to suggest the assumption of a proper amount and balance among all families of fatty acids (FAs). It has been paid particular attention to the ratio between highly polyunsaturated fatty acids (HPUFAs) n-6, mainly arachidonic acid (ARA, 20:4n-6) and n-3 HPUFAs, mainly eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), in tissues in relation to their dietary intake, since an inverse correlation between circulating n-3 HPUFAs and the occurrence of coronary heart disease has been observed (Harris, 2008). Furthermore, it has been shown that dietary n-3 HPUFAs contribute to retinal and brain development, prevent and modulate autoimmune disorders and cancers at different sites (Riediger, 2009), they have also been shown to be beneficial in a wide range of psychiatric disorders (Richardson, 2004). Some of these properties seem directly linked to the ability to reduce the biosynthesis of a range of molecules derived from ARA such as endocannabinoids (EC) and oxygenated eicosanoids, which also explains why it is crucial the balance between the n-6 and n-3 PUFAs.

However, to assess this balance and controlling it by dietary means remains a great challenge for nutritionists. In fact, some doubts have been casted by epidemiological studies on some of these beneficial effects (Kwak et al., 2012), particularly when the putative intake of n-3 FAs has not been evaluated or has been assessed by methodologies like food questionnaire or 24h dietary recall, which have been questioned because unreliable (Prentice, 2010). In addition, other nutritional factors may influence their tissue concentration irrespective of their dietary intake. It is therefore compelling to individuate the mechanism(s) of action and what are the most efficient source of n-3 PUFAs in the diet and nutritional factors able to maximize their nutritional activities.
1.1 n-3 PUFAs: ALA, EPA and DHA

Alpha-linolenic acid (ALA, 18:3 n-3) is a n-3 PUFA abundant in some vegetable oils, it represents the putative essential precursor of the longer chain n-3 HPUFAs (or omega-3 HPUFAs). The n-3 PUFA family is characterized by a double bond placed at the third carbon from the methyl end. Since mammals cannot insert double bonds more proximal to the methyl end, up to the ninth carbon atom (Δ9 desaturase), ALA should be present in the diet to produce EPA and DHA.

The n-6 PUFAs are characterised by a double bound placed at the sixth carbon from the methyl end and its precursor is linoleic acid (LA, 18:2n-6). Neither ALA or LA can be synthesized de novo, thus they are identified as essential fatty acids (EFAs) for mammals and they serve as precursor molecules for the rest of FAs belonging to the n-3 or n-6 FAs family, synthesized through a series of elongation and desaturation reactions.

The enzymatic system consists in fatty acyl-CoA synthetases, Δ6 and Δ5 desaturases and respective elongases (Barceló-Coblijn et al., 2009). Several studies have demonstrated that these two FA families share the same metabolic-pathway, thus they compete for the same enzymes (Brenner et al., 1966; Holman, 1968; Mohrhauer et al., 1967) (Fig.1). ALA, besides its conversion into longer FAs, is subjected to β-oxidation and extensive carbon recycling (Burdge 2006).

It has been demonstrated how EPA and DHA, and to a lesser extent docosapentaenoic acid (DPA n-3), are positively associated with health benefits, e.g. improvements in cardiovascular diseases (CVD) (Sun et al., 2008) and impact cellular function via oxidation to the 3-series of prostaglandins (Smith, 2005). Several scientific evidences in different models have confirmed that ALA is significantly accumulated and converted to n-3 HPUFAs (Barceló-Coblijn et al., 2009), even though not very efficiently (Burdge 2006), in a tissue-dependent manner (Barceló-Coblijn et al., 2009), suggesting that the metabolism is based upon a tissue-selective need for n-3 HPUFAs.

ALA, as a precursor, is considered as not adequate dietary source to provide and maintain the required levels of EPA and DHA in humans (Burdge 2006). It has been shown that the liver is the major site of conversion (Barceló-Coblijn et al., 2009), and, unlike rat brain, rat liver is able to increase the conversion of ALA to DHA during n-3 deficiency (Igarashi et al., 2007), whereas the heart is not capable of this conversion (Igarashi et al., 2008).

Conversion of ALA to DHA is quite critical, is desaturated to 18:4 n-3 by D-6 desaturase, chain-elongated to 20:4n-3, and then converted to EPA by D-5-desaturase. Thus, EPA can be elongated by elongase-2, forming 22:5 n-3, and then to 24:5 n-3 followed by a D-6 desaturation, forming 24:6 n-3.
(Fig.1). Then, 24:6 n-3 is transferred to the peroxisomes where it undergoes one round of b-oxidation forming DHA (Moore et al., 1995, Ferdinandusse et al. 1995).

N-3 FAs synthesized in the ER are incorporated into phospholipids (PLs) or further chain-elongated/desaturated to form DHA. It has been suggested that expression of elongase-2 and elongase-5 are critical to this process. While elongase-5 is regulated by peroxisome proliferator-activated receptors alpha (PPAR α), elongase-2 is regulated by sterol regulatory element-binding protein 1 (SREBP-1), suggesting different genetic control mechanisms for the tissue-selective accumulation of DHA in ALA-fed rats (Barceló-Coblijn et al., 2009). Moreover, it has been demonstrated the lack of influence of dietary n-3 status on the conversion of ALA to DHA in brain (Demar et al., 2005, Igarashi et al., 2007) and the plasticity of liver to dietary modulation of this process (Wang et al., 2005, Igarashi et al., 2008). It has been indicated in vitro and in vivo, as said before, that n-3 and n-6 PUFAs utilize the same enzymes for their elongation and desaturation, thus LA and ALA levels can influence the metabolic outcome of each other. Competition between these two FAs for enzymes involved in elongation and desaturation in liver microsomes was demonstrated at several steps of the metabolic pathway (Brenner et al., 1966; Mohrhauer et al., 1967). In addition, elongation and desaturation of FAs is subjected to feedback regulation because both ARA and DHA suppress endogenous conversion of LA and ALA into longer chain FAs, respectively (Emken et al., 1999, Enser et al., 1998). However, these reactions appears to be tissue-selective modulating FAs (Barceló-Coblijn et al., 2009).
It is controversial whether the health benefits of ALA depend on the fact that it acts as the precursor of EPA and DHA. In fact, in some experimental conditions it has been shown that an increase of ALA consumption elevates tissue ALA, EPA and DPA content and, in some cases, DHA content. Health benefits may also be linked to competition for the same metabolic enzymes with LA. Then, ALA consumption may be a good strategy to decrease elongation of n-6 FAs leading to reduced ARA content. n-6 FAs family components are present at very high levels in the Western diet, which is thought to promote an unhealthy balance between the n-3 and n-6 FAs families. Finally, ALA could directly interact with ion channels or nuclear receptors such as PPAR or retinoid X receptor (RXR) (Barceló-Coblijn et al., 2009).

However, it has been put in evidence that ALA may act differently than EPA and DHA (Mantzioris et al., 1995). All together these data suggest that the anti-inflammatory effect of ALA-enriched diets may provide a possible additional mechanism for its beneficial effect in primary and secondary prevention of coronary artery disease (Rallidis et al., 2003). In fact, it has been demonstrated that subjects who consumed flaxseed oil-based diet for 4 weeks produced 30% less TNF-α and interleukin-1 beta (IL-1β) and, 20% less TBX2 and PGE2 in mononuclear cells, when they were stimulated with lipopolysaccharide (LPS) (Caughey et al., 1996).
1.1.1 n-3 PUFA natural sources

- ALA-enriched sources

Commercial sources that have significant amounts of ALA are:

- *Linum usitatissimum*, linseed or flaxseed oil (53% ALA)
- *Brassica spp.* Canola or rapeseed oil (9% ALA)
- *Glycine max*, soybean oil (7% ALA)
- *Perilla frutescens*, Perilla oil (approx. 60% ALA) although its consumption is restricted to Asia
- *Camelina sativa*, camelina oil (38% ALA) consumed in Nordic countries

However, variable amounts of ALA are found in animal zooplankton, phytoplankton, marine species and in plant, where ALA is found in leaves, mainly in glycolipids, and as TAG in certain seed oils (rapeseed, flaxseed, perilla seed, chia seed), beans (soybeans, navy beans) and nuts (walnuts) (Lands, 2005).

It is not easy to increase n-3 PUFA intake because of the scarcity of n-3 natural sources and their limited availability. It has been shown that it is possible to increase n-3 PUFA levels by selecting n-3 natural or enriched sources and substituting in the diet for common food items (Mantzioris et al., 1994, Mantzioris et al., 2000). Some studies demonstrated that the incorporation of enriched nutrients meant a daily intake of approximately 1.8 g/d of EPA plus DHA and 9.0 g/d of ALA, leading to an average 3-fold increase in EPA in plasma, platelet, and mononuclear cell PLs and a decrease in TBX2 (36%), PGE (26%), and IL-1b (20%) at the end the treatment (Mantzioris et al., 2000). However, the consumption in this study is not sustainable because it is above the current recommendations. In fact, inclusion of ALA-containing sources such as flaxseed, walnuts and other ALA sources in baked products and cereals, flaxseed oil in salad dressings, as well as n-3-enhanced dairy products (Franklin et al., 1999, Petit et al., 2002), is a more functional and sustainable approach of introducing ALA into the diet.

Hence, strategically or naturally enriched in n-3 PUFA foods can be useful to achieve beneficial biochemical effects without the ingestion of supplements or dramatic changes in dietary habits and this kind of foods can be developed to support therapeutic and disease-preventive effects of n-3 PUFAs (Mantzioris et al., 2000, Metcalf et al., 2003).
- **Vegetable sources of n-3 PUFAs**

Algae are the main source of n-3 PUFAs because they are able to synthesize them de novo and they are also the basis for the enrichment of n-3 PUFAs in the lipids of other marine organisms over the aquatic food chain.

- **Animal sources of n-3 PUFAs**

The main dietary source of EPA and DHA is cold-water oily fish like salmon, sardine, anchovy, herring, or mackerel. Fish contains between 1%–1.5% PLs and 10%–15% TAG (Hjaltsason et al., 2006) and, depending on the kind of fish, up to one third of the EPA and DHA content might exist in the form of PLs (Xu et al., 1996). For example, it has been shown that in Atlantic salmon, EPA and DHA are bound to PLs and TAG in a 40:60 ratio (Polvi et al., 1992). Recently, a dietary form of EPA and DHA similar to that present in fish and marketed as supplement is krill oil (KO) (Winther et al. 2011). KO has been shown to have effects on several pathological conditions in obese rats, mice and humans and apparently more efficiently than fish oil (Burri et al. 2012, Murru et al. 2013).

Several studies on the comparison between n-3 TAG and n-3 PLs revealed that the PL form is better in improving the metabolic profile in obese mice than the TAG form (Rossmeisl et al., 2012). It has been shown that EPA and/or DHA taken up in tissues like liver, white adipose tissue and heart were more efficient when given in the PL form (Batetta et al. 2009). In mice, the intake of extracted herring roe lipids led to an increase in plasma adiponectin levels and a decrease in plasma TAG, PL, total cholesterol, and glucose amounts (Higuchi et al. 2006) and it has also been shown that, in a maze-behaviour experiment, extracted lipids improve learning capacity in mice (Shirai et al. 2006). Some effects were attributed to the suppression of FA synthesis and increase of FA β-oxidation (Shirouchi et al. 2007). In some human studies, phosphatidylycholine (PC) obtained from salmon roe were tested for their potential to alleviate chronic liver disease showing a significantly decreased serum globulin and increased HDL and apolipoprotein A–I and E levels (Hayashi et al. 1999). It has also been shown that marine PLs from salmon roe might help in the prevention of tumour-associated weight loss with a daily 1.5 g marine PLs dose for six weeks (Taylor et al., 2010).

Hence, fish represents a potential source of marine PLs and the amount of by-products from fish represents a valuable source of marine PLs. However, the FA content and also the overall amount of fat in farmed fish can be quite different in comparison to wild fish. Since vegetable oils and oilseeds are used in aquaculture feed, the n-3 / n-6 ratio can be as low as 2.9 for farmed versus 12.4 for wild salmon (Strobel et al., 2012), and the corresponding ratio in KO is 12.0 (Ulven et al., 2011).
It has been suggested that the food matrices may affect the bioavailability. For example, it has been observed that the administration of as low as 300mg/day of EPA+DHA to healthy subjects, incorporated into milk, subdivided into smaller doses throughout the day, for 3–6 weeks, determined a significant increase in EPA and DHA levels in plasma lipids as well as in HDL concentrations, while it determined a decrease of TAG concentrations without affecting total cholesterol concentration (Visioli et al., 2000). Interestingly, the same effects on TAG and HDL concentrations have been obtained with 1 to 7 g/day of EPA and DHA (Horrocks and Yeo, 1999). It has been suggested that variable blood levels of n-3 HPUFAs are due to their presence as common dietary components and, possibly, are responsible for the variability of the results observed; hence, it is recommended to apply a selection of subjects with uniform n-3 background levels before a trial begins (Galli et al., 2012). For this reason, it is difficult to recommend a precise n-3 HPUFA dose since the matrices, the form, and the quality may greatly influence tissue bioavailability. However, American Heart Association gave some dietary guidelines for n-3 HPUFAs and fish for primary prevention of coronary diseases, on a food-based approach (Kris-Etherton et al., 2007), which are two servings of fatty fish per week (Lichtenstein et al., 2006), 250–500 mg EPA+DHA per day (Mozaffarian and Rimm, 2006), while it is suggested a larger daily intake, 1 g or more, for a TAG-lowering effect (Murru et al., 2013).

1.1.2 Digestion and Absorption of n-3 PUFAs

Dietary fat is mainly composed of TAG, however a small portion of PLs (3–6%) is present (Thomson et al., 1989), which consist of a daily intake of about 2–8 grams/day. The most common PL in the intestinal lumen is phosphatidylcholine (PC) which is derived mostly from bile (10–20 g/day in humans) and also from the diet, while other PLs, such as phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), are present in much smaller amounts. Fat digestion, absorption, transport in the blood, and metabolism at cellular level are influenced by FA chain length and unsaturation number. Medium-chain fatty acids (MCFAs) are better absorbed than longer FAs because they can be dissolved in the aqueous phase and then they are able to be absorbed bound to albumin and transported to the liver directly by the portal vein (Ramirez et al., 2001). Bioavailability of FAs is influenced by dietary TAG structure. In fact, PA from human milk fat is well absorbed because of its incorporation in the sn-2 position of glycerol backbone (Tomarelli et al.,1968). TAG and PLs are digested and absorbed in different ways in the small intestine: TAG require emulsification by bile salts, while PLs can spontaneously form micelles and be transported in an aqueous environment. Some studies in humans and animals showed that oral administration of labelled PC leads to the rapid appearance of labelled PC in plasma, while a weak formation of labelled TAG (Galli et al., 1992).
It has been shown that dietary PLs may affect the lipoprotein composition and metabolism, especially the distribution of lipoproteins in the body and FA tissue incorporation. Some studies on infants have indicated that dietary PLs may be better absorbed than TAG. In particular, it has been shown that, in a group of preterm infants fed with different formulae, the absorption of DHA was higher in those infants receiving the HPUFA-PL formula than in infants receiving breast milk or the HPUFA-TAG formula (Carnielli et al., 1998). It has also been shown that EPA and DHA from KO, mainly bound to PLs, were absorbed at least as efficiently as EPA and DHA from FO, which are in the TAG form (Maki et al., 2009).

In this context, the uptake of three EPA + DHA formulations derived from FO (re-esterified (rTAG), ethyl esters (EE), and KO, mainly PL) has been compared and it showed that the bioavailability of n-3 HPUFAs may vary according to their esterified form. In fact, the highest incorporation of EPA + DHA into plasma PLs was obtained by KO, followed by FO rTAG and then by EE (Schuchardt et al., 2011).

The form of PUFAs may affect not only their lipid incorporation but also their nutritional activities. It has been shown that PL-bound EPA and DHA have distinct effects with respect to TAG-bound EPA and DHA. FO supplementation reduces plasma LDL, cholesterol and TAG levels, while increases HDL, improving lipoprotein profiles, through inhibition of TAG and VLDL synthesis in the liver (Phillipson et al., 1985, W.S. Harris et al., 1990). These effects are obtained by prolonged supplementation of 1 or more g/day of EPA and/or DHA, marketed either as TAG or EE (5-week supplementation of 4 g EPA or DHA, as EE, added to the ordinary diet) (Hansen et al., 1998). On the other hand, it has been demonstrated that EPA and DHA in krill powder form are able to significantly decrease plasma triglycerides only at about 400 mg per day (Berge et al., 2013). However, it is very difficult to establish if some of the effects may be related to different actions of EPA and/or DHA, since some authors reported several different result about EPA and DHA effects with different doses (Murru et al., 2013). For this reason, it is important to use a similar EPA/DHA ratio comparing the effects of the form (Batetta et al., 2009).

1.1.3 PUFAs Tissue Distribution

Intestinal absorption efficiency of lipids is affected by FA chain length and unsaturation, whereas their digestion products before absorption are determined by their chemical structure (TAG versus PLs) (Burri et al., 2012). It has been suggested that digestion of TAG start in the stomach, where they are hydrolyzed by lingual and gastric lipases, while the major portion of PLs is hydrolyzed in the small intestine by pancreatic phospholipase A2 with the help of other lipases. In contrast to the hydrolysis products of TAG, the corresponding products from PLs are not dependent on bile salts to form micelles. In fact, after uptake into the enterocytes, the PLs are mainly incorporated into the
chylomicron surface layer, while the TAG are solubilized in the interior of chylomicrons (Iqbal et al., 2009). Several studies with human intestinal CaCo-2 cells (Krimbou et al., 2005), rat (Magun et al., 1988) and swine (Wang et al., 2001) have shown that an important part of the dietary PL fraction is integrated into HDL particles already in the intestine that later join the plasma HDL pool. In addition, others have suggested an important role for the intestine in the synthesis of HDL (Brunham et al., 2006, Forester et al., 1983, Green et al., 1978). Chylomicrons are catabolised by the endothelial-bound enzyme lipoprotein lipase (LPL) in the bloodstream. This enzyme hydrolyzes TAG, releasing FAs, which can later be captured by tissue (Preiss-Landl et al., 2002). During delipidation of chylomicrons, chylomicron remnant particles are formed and they are finally taken up and metabolized by the liver. During circulation in the blood stream, lipid components of chylomicrons can also be transferred to HDL, during a process catalyzed by cholesteryl ester transfer protein (CETP) that exchanges cholesteryl esters from HDL to the chylomicron remnant particles and TAG to HDL (Yamashita et al., 2000).

It has been shown that FA absorption in the small intestine is equivalent for both the PL- and TAG-derived compounds (Burri et al., 2012). It has, however, been proposed that the different PL and TAG ester forms could direct the FAs to a particular blood pool (free FAs, outer versus inner layer of chylomicrons, different lipoprotein particles) (Amate et al., 2001), influencing the distribution of FAs into different tissues. It has also been evidenced that PLs might be a more efficient delivery form of PUFAs to the brain than TAG (Lagarde et al., 2001, Picq et al., 2010). For example, it has been found by radioactive labelling of DHA in PC and TAG esters that DHA from PC is more efficiently taken up into different tissues than DHA from TAG (Graf et al., 2010). The incorporation of DHA was more than twice as high from PLs as from TAG in brain, liver, and kidney, and higher DHA incorporation was also seen in adrenal gland, brown fat, thymus and uveal tract/retina. Specifically, in the brain the DHA uptake was significantly increased in 11 out of 14 brain regions after PL administration, compared to supplementation in TAG form. The increased tissue accumulation of radioactivity in the PC form appeared only in ten weeks old animals and was absent in two week old animals. Another observation of the study was a strong decrease of 14C-DHA coming from either ester form in the brain in older animals compared to younger animals. It has also been shown that radiolabeled ARA esterified in PLs accumulates more efficiently in baboon neonate tissues (brain, liver and lung) than do the TAG structures (Wijendran et al., 2002). The incorporation of ARA into the brain tissue was 2.1 fold higher when baboons were supplemented with PLs, as compared to TAG administration. Moreover, a study in obese Zucker rats compared n-3 PUFAs either given in the form of FO (n-3 PUFAs in TAG) or KO (majority of n-3 FAs in PLs) (Batetta et al., 2009). In this study, it has been demonstrated that KO led to a significantly higher incorporation of EPA and DHA into tissue PLs. In heart, there was a 96% and 42% higher incorporation of EPA and DHA, respectively, into the PLs after
KO compared to FO supplementation. Similar observations were identified in the liver, where EPA and DHA levels were 47% and 13% higher after KO administration, which is in agreement with the studies in rats and baboons (Graf et al., 2010, Wijendran et al., 2002). There was also a significantly higher presence of DHA in the brain after KO supplementation to the diet as compared to FO supplementation (Di Marzo et al., 2010). This finding was supported by another study with KO, demonstrating increased DHA amount in the brain and beneficial effect on memory function in rats (Gamoh, 2011).

n-3 HPUFAs are determinants in metabolism and also in diet of human and animals, especially in the PL form. Commercial PL products from vegetable sources are usually processed from soybean oil (also called soy lecithin), and are largely used as functional ingredients in pharmaceutical industries, cosmetics, and food. Moreover, PLs are extracted from non-marine animal sources like egg, milk and brain, or marine sources like krill, fish by-products, and roe. Pharmaceutical main applications of marine PLs are for nutritional use and for drug delivery. In drug delivery contest, PLs are interesting for their ability to form liposomes after mixture into an aqueous media. Liposomes have been investigated as drug carriers for decades and several different types have been developed.

The main application of PLs in foods is as an emulsifier but modern diets are poor in PLs due to increased use of refined oils and purified raw materials, which had led to a lower intake of PLs. Thus, the supplementation of marine PLs appears important for emulsifying properties, supplementation of n-3 PUFAs and beneficial nutritional effects of the PLs themselves. The increased accessibility of marine PLs during recent years opens up new possibilities for the use of PLs not only as a superior nutritional source of n-3 PUFAs, but also for use in the pharmaceutical, cosmetic and functional food industries (Burri et al., 2012).

1.1.4 Biological Activities of n-3 HPUFAs (EPA and DHA)

As described above, n-3 HPUFAs in fish products are mainly bound to PLs, while in fatty fish, such as salmon, n-3 HPUFAs are bound to PLs and TAG in a 40 : 60 ratio (Polvi and Ackman, 1992). Conversely, most of n-3 HPUFAs available as supplement, for example FO, is represented by almost exclusively TAG-bound n-3 HPUFAs, overlooking that dietary PLs have consistently shown to affect plasma and liver lipid levels in experimental animals (Cohn et al., 2008). Instead KO is rich in n-3 HPUFAs in the form of PLs, mainly phosphatidylcholine (PC), rather than TAG; the proportion of PLs in the total lipids of krill has been reported to vary between 30 and 60%, depending on krill species, age, season, and harvest time (Tou et al., 2007). Moreover, KO contains a lipid-soluble antioxidant, astaxanthin, that may preserve KO from oxidation. The peculiar composition may be responsible for the tissue bioavailability of n-3 HPUFAs and for the major part of health-promoting effects of KO,
such as its anti-inflammatory and hypo-lipidemic properties in humans (Bunea et al., 2004, Deutsch, 2007). It has been demonstrated that a daily intake of 3 g KO (543 mg EPA + DHA) increased the plasma level of EPA and DHA to the same extent as dietary FO (864 mg EPA + DHA) (S.M. Ulven et al., 2011), suggesting a comparable absorption for EPA and DHA after KO treatment, if not better than after FO treatment. However, it has been shown any significant difference between KO, FO and control treatments for TAG and lipoprotein response, confirming a previous study that also failed to detect any change in plasma lipids (Maki et al., 2009). It was probably due to the short period of treatment, the normo-lipidemic status of the subjects, or the low amount of EPA and DHA. On the other hand, other studies demonstrated a small increase in LDL and no effect on HDL found after FO treatment (Harris, 1997). Another controversial aspect is the high-sensitivity C-reactive protein (hs- CRP), a marker of systemic inflammation, which showed no change among KO, FO, or placebo treatments (Murru et al., 2013), while in individuals with a pro-inflammatory status, it has been reported an association among DHA/EPA and hs-CRP reduction (De Luis et al., 2009). In mice fed with high-fat diet, it has been demonstrated that n-3 HPUFA in PL is more efficacious than TAG in reducing hepatic steatosis, low-grade inflammation in white adipose tissue (Rossmeisl et al., 2012), blood lipid levels, and glycaemia (Tandy et al., 2009; Piscitelli et al., 2011). It has also been observed an increased hepatic expression of FA oxidation genes and down-regulation of lipogenic genes. This last effect was stronger in the PL-treated mice as a reduced plasma insulin and adipocyte hypertrophy was shown only with the PL form (Rossmeisl et al., 2012). This suggests that supplementation of n-3 HPUFA as PLs exerts stronger biological effects compared with the TAG form. It is due to the fact that various PL species can also act as ligands for nuclear receptors involved in the transcriptional regulation of steroidogenesis and cholesterol metabolism and the PL form has been shown to augment the bioavailability of DHA and EPA in both rodents and humans (Murru et al., 2013).

It has also been demonstrated that EPA and DHA have an influence in neuropsychiatric disorders, as several epidemiologic studies suggested that dietary consumption of n-3 HPUFAs affects neuropsychiatric disorders, presumably because of their structural and neurochemical involvement in pathophysiological processes (Murru et al., 2013).

Several studies demonstrated that EPA and DHA possess antithrombotic properties, they are able to decrease both the formation and tissue incorporation of ARA and they are also able to reduce the release of inflammatory acute-phase proteins. PGE-2 and LT-B4 are pro-inflammatory eicosanoids synthesized from ARA on phospholipase-mediated release from cellular PLs. n-3 HPUFAs are prostaglandin and leukotriene precursors (PGE-3 and LT-B5), characterized by a quite low conversion coefficient and anti-inflammatory properties (Fig.2). It has been shown that EPA and DHA inhibit production of ARA-derived eicosanoids in vitro, while in vivo eicosanoids formation seems to be less
affected by EPA and DHA dietary intake. Some studies suggest that eicosanoids formation is not promptly influenced by ARA dietary intake, at least within the range of traditional human diets, but it has been shown that very high amounts of FO may decrease PG formation improving weak anti-inflammatory and analgesic effects (Murru et al., 2013).

Fig. 2 Synthesis of pro and anti-inflammatory factors by Omega-3 and Omega-6 FAs.
1.2 n-3 PUFAs and Endocannabinoid Biosynthesis

Several studies have demonstrated that the composition of dietary FAs can affect energy homeostasis through changes of endocannabinoid system (ECS) (Banni and Di Marzo, 2010).

1.2.1 Focus on Endocannabinoids

The endogenous cannabinoid system (ECS) is an ubiquitous lipid signalling system that appeared early in evolution and exerts important regulatory functions throughout the body in all vertebrates. EC are molecules derived from ARA hydrolyzed from membrane PLs. The main EC are anandamide (arachidonoylethanolamide or AEA) derives from hydrolysis of membrane PLs at the sn-1 position, and 2-arachidonoylglycerol (2-AG) derives from hydrolysis at the sn-2 position (Di Marzo et al., 1994). They are ligands of G-protein-coupled receptors, called cannabinoid receptors (CB1 and CB2). CB1 is largely distributed in different brain areas and peripheral tissues. However, AEA shares same biosynthetic pathway with other structure related lipid messengers, palmitoylethanolamide (PEA) or oleoylethanolamide (OEA), that are formed by palmitic acid (PA, c16:0) or oleic acid (OA, C18:1 c9), respectively, esterified in sn-1. Being the latter FAs preferentially incorporated in sn-1, PEA, and OEA are more abundant than AEA. Their biological activity results by interacting with different receptors: OEA, an anorectic mediator that affects lipid and glucose metabolism, activates PPAR α (Thabuis et al., 2008), while PEA exerts anti-inflammatory actions by activating PPAR α (LoVerme et al., 2005), transient receptor potential of vanilloid type-1 (TRPV1), or peroxisome proliferator-activated receptors gamma (PPAR γ) (Costa et al., 2008).

![Fig. 3 Endocannabinoids and congeners.](image-url)
1.2.2 Diet influences ECS

Different studies have been shown that tissue levels of ECs are in part regulated by the activity of the corresponding biosynthetic precursors and in part by catabolic enzymes, fatty acid amide hydrolase (FAAH) (Bracey et al., 2002; McKinney and Cravatt, 2005), or monoacylglycerol lipase (MAGL) (Karlsson et al., 1997; Dinh et al., 2002).

Product of hydrolysis of 2-AG and AEA, ARA, is rapidly incorporated into membrane PLs (Di Marzo et al., 1994; Di Marzo et al., 1998). EC levels in tissues depend also on the availability of their biosynthetic precursors ARA in PLs (Berger and Roberts, 2005; Berger et al., 2002; Berger et al., 2006). For this reason, diet is able to change corresponding FAs esterified to individual PLs. It has been shown that mice fed with an n-3-PUFA-deficient diet exhibited higher brain 2-AG levels and a short-term supplementation of DHA rich FO reduced brain 2-AG levels as compared with the diet supplemented with low n-3 PUFAs. It has also been observed that ARA levels decreased concomitantly to an increase in DHA levels in the major brain PL species of mice fed with the FO diet as compared with those fed with the low n-3 PUFA diet (Watanabe et al., 2003). Hence, the dietary content of HPUFAs and their essential biosynthetic precursors could modify concentrations of EC and their congeners, mainly in peripheral tissues. It has been suggested that dietary HPUFAs might modulate FA composition of adipocyte PLs, and then it can be assumed that n-3 HPUFAs could improve effects in abdominal obesity, dyslipidaemia, and insulin resistance by CB1-mediated lipogenic actions of EC in adipocytes (Matias et al., 2007, Matias et al., 2008, Matias, Vergoni et al., 2008).

1.2.3 ECS and energy metabolism

Some studies demonstrated that EC can affect energy metabolism and exerts a regulatory control on every aspect related to the search, the intake, the metabolism and the storage of calories (Gatta-Cherifi and Cota 2015). It has been shown that increase of peripheral EC levels in both fasted and postprandial obese and overweight individuals correlates with intra-abdominal obesity, glucose intolerance, dyslipidaemia, and dyslipoproteinaemia (Silvestri and Di Marzo, 2013). Dietary FAs could influence endocannabinoid biosynthesis by modulating ARA levels in tissue PLs and thereby down-regulate an overactive endocannabinoid system (Banni and Di Marzo, 2010).

It has been shown that dietary DHA and EPA in the form of PLs are superior to TAG with respect to the preservation of glucose homeostasis and the reversal of hepatic steatosis, adipocyte hypertrophy, and low-grade inflammation (Rossmeisl et al., 2012). It was associated with a better DHA and EPA bioavailability in PL and with a suppression of the major EC levels in white adipose
tissue and plasma, suggesting that modulation of the endocannabinoid system activity contributed to their greater efficacy when compared to TAG form of n-3 HPUFAs. It has been described the role of the endocannabinoid system in the development of adipose tissue inflammation (Matias et al., 2008) or hepatic steatosis and it has also been suggested that adipocyte-derived N-acyl ethanol amines eicosapentaenoyl ethanolamine (EPEA) or docosahexaenoyl ethanolamine (DHEA), that is, the amides of EPA and DHA, play a role in some anti-inflammatory effects (Balvers et al., 2010, Meijerink et al., 2011).

It has been found through dietary experiments in mice fed with various diets differing in the LA content a positive correlation between the levels of ARA in liver and erythrocytes PLs and tissue 2-AG and AEA, which promoted accumulation of body fat (Alvheim et al., 2012), supporting the increase in obesity prevalence in the United States associated with the increased consumption of LA, the precursor of ARA, and hence the precursor of endocannabinoids (Blasbalg et al., 2011, Alvheim et al., 2012). On the contrary, EPA and DHA supplementation in diet resulted in a decrease of endocannabinoid levels in the liver and also in hypothalamus of mice fed with experimental diets with a high LA content (Alvheim et al., 2012). In addition, it has been shown that dietary KO increases DHA and reduces 2-AG, but not NAEs levels, in the brain of obese Zucker rats (Di Marzo et al., 2010).

The Influence of dietary FAs on brain endocannabinoid concentrations may have important implications by possible modulation of CB1, which is involved in the regulation of synaptic plasticity, control of movement, sensory perceptions to mood and neurogenesis (Lutz, 2009). AEA seems to be the most involved in adaptive mechanisms against stress and its consequences, while 2-AG is the one clearly involved in hyperphagia in animal models of obesity. In mice brain, decreased levels of 2-AG, but not AEA, were induced by high dose of dietary n-3 HPUFA as FO, whereas the opposite effect was obtained with an n-3-HPUFA-deficient diet (Watanabe et al., 2003).

Thus, the amount of dietary n-3 HPUFAs and the dietary form, as FO or KO, are able to influence EPA and DHA incorporation into brain lipids and consequently either the biosynthesis and/or incorporation into PLs of ARA through its partial replacement with EPA and DHA.

### 1.2.4 ECS in peripheral organs

Activation of CB1 receptors on white adipocytes stimulates fat deposition by facilitating adipocyte differentiation and increasing expression of adipogenic enzymes and activity of the lipoprotein lipase (Bensaid et al., 2003; Cota et al., 2003; Matias et al., 2006; Muccioli et al., 2010), confirming the role of the ECS in the regulation of lipid metabolism and use of substrates by peripheral organs. Some studies suggest that the ECS may control the trans-differentiation of the adipose tissue, since in vitro
blockade of CB1 directly promotes trans-differentiation of white adipocytes into a brown fat phenotype, rich in mitochondria and thermogenic (Perwitz et al., 2010). It has also been reported that block of CB1 receptors increases mitochondrial biogenesis in white adipocytes via an endothelial nitric oxide synthase mechanism (Tedesco et al., 2008). These aspects might determine an increase in energy expenditure, FA oxidation and thermogenesis observed after administration of CB1 antagonists in vivo (Cota et al., 2009; Herling et al., 2008; Nogueiras et al., 2008; Verty et al., 2009/A). It has also been shown that an increase in lipid oxidation and thermogenesis caused by an enhanced sympathetic tone determines a lean phenotype and are resistant to diet-induced obesity in mice selectively lacking CB1 expression in forebrain neurons and with a 60% reduction of the expression of the receptor in the cervical sympathetic ganglia (Quarta et al., 2010).

In hepatocytes, activation of CB1 receptors induces the expression of lipogenic enzymes, such as acetyl coenzyme-A carboxylase-1 (ACC1) and fatty acid synthase (FAS), which increase de novo FA synthesis and determine the development of liver steatosis, particularly during exposure to high-fat diets (Osei-Hyiaman et al., 2005; Osei-Hyiaman et al., 2008). In fact, CB1 receptors in liver might be involved in regulation of lipid metabolism, since mice lacking the receptor in hepatocytes, although still susceptible to diet-induced obesity, are protected against liver steatosis, hyperglycaemia, dyslipidaemia and insulin resistance (Osei-Hyiaman et al., 2008).

Some studies on isolated soleus muscle have shown that pharmacological blockade of CB1 improves both basal and insulin-stimulated glucose transport activity, while CB1 activation has the opposite effect (Lindborg et al., 2010). It has also been demonstrated that CB1 receptor can affect the responsiveness of skeletal muscle toward insulin through the modulation of the PI 3-kinase (phosphatidylinositol-4,5-bisphosphate 3-kinase)/PKB (protein kinase B) and the Raf (rapidly accelerated fibrosarcoma kinase)-MEK1/2 (mitogen-activated protein kinase) - ERK1/2 (extracellular-signal-regulated kinase) signalling pathways in muscle cell cultures (Lipina et al., 2010).

Several studies have demonstrated the presence of CB1 and CB2 receptors in rodent and human islets, with a species-dependent degree of expression (Li et al., 2011) and some reports indicate that CB receptors modulate insulin secretion by regulating glucose-induced calcium transients (Bermudez-Silva et al., 2009; Li et al., 2011; Nogueiras et al., 2009).

Several studies have focused on endocannabinoid signalling, which might link gut microbiota to the regulation of adipogenesis (Muccioli et al., 2010). It has been shown that gut microbiota influences whole body metabolism and energy balance (Turnbaugh et al., 2006) and controls the intestinal ECS tone, which in turn modulates gut permeability (Muccioli et al., 2010). In fact, CB1 receptors stimulation in a colonic epithelial monolayer cell model determines an increase of permeability,
caused by higher lipopolysaccharide levels, which worsen gut permeability and ECS tone in both the gastrointestinal tract and the white adipose tissue (Muccioli et al., 2010).
1.3 Conjugated Linoleic acid (CLA)

Another nutritional factor is represented by conjugated linoleic acid (CLA), a FA with a unusual structure of conjugated dienes (CD), with several positive activities in different experimental models (Koba and Yanagita, 2014). CLA is abundant in dairy products and it has been demonstrated improving health by metabolic regulation and ECS regulation (Pintus et al., 2013). The predominant isomer found in food is c9,t11 CLA, specially in the meat and dairy products derived from ruminant since it is formed in the rumen from LA by the biohydrogenation process (Fig.4), thanks to fermentative activity of the anaerobic bacterium, Butyrivibrio fibrisolvens (Kepler et al., 1966). Biohydrogenation consists in several enzymatic reactions, in particular an isomerisation reaction by the enzyme linoleic isomerase, that convert LA directly into CLA isomers. ALA and γ Linolenic acid (GLA, 18:3 c6,c9,c12) can be also converted into CLA by different reactions. Vaccenic acid (VA, 18:1 t11) is an intermediate of all these reactions, which could be saturated, forming Stearic acid (SA, 18:0), or absorbed in the gut and transported in tissues and desaturated to c9,t11 CLA isomer. This is the major responsible pathway for the presence of c9,t11 CLA in cow’s milk (Griinari et al., 1999) (Fig. 4).

![Biohydrogenation Diagram](image)

Fig. 4 Synthesis of CLA and VA by LA
1.3.1 Dietary sources of CLA

The main sources of CLA in the human diet are represented by dairy products like milk and cheese, and meat of ruminants (Chin et al., 1992), which provide about 0.4 g/day (Fritsche et al., 1998). The main isomer found in these foods is the c9,t11 CLA (Grinari et al., 1999). Concentration of CLA in dairy products typically ranges from 2.9 to 8.9 mg/g fat, with c9,t11 isomer representing from 73% to 93% of total CLA. Homogenized cow’s milk typically contains 5.5 mg/g fat. Low-fat yogurt has 4.8 mg/g fat, butter 4.7 mg/g fat and cottage cheese 4.5 mg/g fat.

Grass-fed cows produce milk with the highest CLA content (Kelly et al., 1998), but a grain diet supplemented with full-fat rapeseed or soybean concentrate increases CLA (MacDonald et al., 2000). Beef also has CLA in a similar range as dairy products, with the c9,t11 isomer contributing from 57% to 85% of total CLA (Chin et al., 1992, Shantha et al., 1993, Shantha et al., 1994) and, interestingly, CLA in meat is not destroyed by cooking (MacDonald et al., 2000). Vegetable oils and margarine have small amounts of CLA, about the same as the meat of non-ruminant animals and birds. It has been shown that CLA causes weight loss and reduce fat mass (Shen et al., 2013, Smedman et al., 2001, Bhattacharya et al., 2006, Park et al., 2007). The recommended daily amount of total CLA in human diet is about 0.15 g for women and 0.2 g for men (Ritzenthaler et al., 2001, Jiang et al., 1999). About 60% of the CLA intake derives from dairy products, and 37% from meat products. C9,t11 CLA represents about 90% of total CLA intake (about 190mg/d for men and 140 mg/d for women (Ritzenthaler et al., 2001). It has not been demonstrated any relationship between body composition and total CLA or c9,t11 isomer intake, suggesting that this isomer has little effect on body composition in humans (Ritzenthaler et al., 2001). In fact, CLA effects on body weight and body fat in humans were considerably less than those seen in mice, although the doses of CLA used in mouse and human were comparable (Terpstra et al., 2004). It has been shown that diets rich in high-fat dairy foods (containing c9,t11 CLA) significantly influence lipid and c9,t11 CLA concentrations in human milk (Park et al., 1999). Moreover, c9,t11 CLA intake in men is correlated with TAG c9,t11 CLA content and, for this reason, TAG c9,t11 CLA may serve as a biomarker for c9,t11 CLA intake. On the contrary, there are no correlations between c9,t11 CLA intake and the c9,t11 CLA content of any esterified plasma lipid fraction in females. Moreover, it has been shown that there is not a relation between dietary c9,t11 CLA intake or plasma c9,t11 CLA concentration and circulating lipoprotein cholesterol level in both sex (Ritzenthaler et al., 2012). Although some adverse effects of CLA intake on liver function have been reported in multiple studies, including different animal models (De Deckere et al., 1999, Belury et al., 1997, West et al., 1998), a very high daily intake of CLA did not produce clinically relevant effects on markers of liver and kidney function in healthy volunteers.
High intake of a mixture of c9,t11 CLA and t10,c12 CLA (80:20 ratio) raised the total to HDL ratio in healthy subjects (Wanders et al., 2010/B).

### 1.3.2 CLA metabolism

In PL of lamb liver naturally exposed to a diet rich in CLA, were found different FAs with a structure of conjugated dienes (CD) such as conjugated linolenic acid (CALA, CD 18:3), conjugated eicosatrienoic acid (CD 20:3), and conjugated arachidonic acid (CD 20:4) (Banni et al., 1996). Animal experiments demonstrated that CLA is metabolized similarly to LA, and competes for the same enzyme system (Banni, 2002) (Figure 5). The CLA can undergo desaturation reaction by the Δ6-desaturase enzyme, the subsequent elongation of the carbon chain by the elongase, and finally another desaturation by the Δ5-desaturase, while preserving the structure of conjugated double bonds (Banni et al., 1996, Belury et al., 1997; Sébédio et al., 1997). These findings were also confirmed in human studies (Lucchi et al., 2000).

Enzymatic competition between CLA and LA occurs at liver level, while incorporation competition takes place in extra hepatic tissues (Fig. 5). It has been shown that in those tissues rich in neutral lipids, CLA is able to decrease the concentration of the LA metabolites, but not in liver, where CD 20:4 has not been found (Banni et al., 1999). Moreover, it has been shown that CLA is able to modulate the formation and incorporation of the LA metabolites, such as GLA, eicosatrienoic acid (20:3), and ARA (all substrates for the biosynthesis of eicosanoids), replacing them with its metabolites (CD 18:3 and CD 20:3) (Banni et al., 1999), which are able to inhibit the metabolic pathways of COX and lipoxygenase (LOX), resulting in a reduction of the eicosanoids biosynthesis (Nugteren et al., 1987).
Also, there are several evidences that CLA and its metabolites accumulate in rat tissues in a dose dependent manner, and AA significantly decreased, suggesting that the effects explicated by the CLA metabolism may interfere with the metabolism of eicosanoids (Banni et al., 1999).

FA oxidation also occurs in peroxisomes, in FAs too long to be handled by the mitochondrial enzymes, eicosanoids (Reddy et al., 2001), isoprostanes (Chiabrando et al., 1999), and ARA (Gordon et al., 1994). Even for the biosynthesis of DHA the peroxisomal β-oxidation is required (Moore et al., 1995). CLA and CD 20:4 are converted to CD 16:2 and CD 16:3 through β-oxidation in peroxisomes (Banni et al., 2004).

CLA is able to influence lipid metabolism by altering the FA composition of the cell membrane through the reduction of monounsaturated fatty acids (MUFAs). In fact, it has been demonstrated that CLA directly reduces the activity of Δ9-desaturase enzyme rather than influencing its protein or mRNA synthesis (Park et al., 2000). It is possible to estimate this process by the index of desaturation that is calculated by the palmitoleic acid (16:1 c9)/ PA and the OA/SA ratios. A balanced ratio between MUFAs and saturated fatty acids (SFAs) is important to preserve the fluidity of the cell membrane. Unbalanced MUFA/SFA ratio can lead to significant changes of insulin sensitivity (Storlien et al., 1991), metabolic rate (Field et al., 1990), and determine conditions such as obesity (Jones et al., 1996). It has been shown that a mixture of equal amounts of c9,t11 and t10,c12 CLA isomers added to a diet based on vegetal fat (palm oil) compared to a diet with animal fat (ovine fat) and enriched with the same CLA mixture, improved serum profile of adipokines and inflammatory markers in obese Zucker rats (Martins et al., 2010). Particularly, rats fed with diets enriched in CLA exhibited lower daily feed intake, final body and liver weights, and hepatic lipid content. Total and LDL levels were increased in CLA supplemented groups. CLA also promoted higher adiponectin and lower plasminogen activator inhibitor-1 (PAI-1) serum concentrations. In contrast to palm oil diets, ovine fat increased insulin resistance and serum levels of leptin, TNF-α and interleukin-1beta (IL-1β). Adipose tissue from epididymis and retroperitoneum showed similar FA profile and t10,c12 CLA isomer was highly associated with adiponectin and PAI-1 levels. (Martins et al., 2010).

CLA, VA and ALA have been shown to possess peculiar nutritional properties as modifiers of cardiovascular risk. Pasture and lipid supplementation of the ruminant’s diet represent reliable dietary strategies in ruminant nutrition, increasing to 4-fold the content in the milk fat of n-3 PUFA, CLA and VA, and decreasing up to 23% that of SFA (Shen et al., 2013; Ritzenthaler et al., 2001). Several studies demonstrated that CLA enriched dairy products, on healthy subjects, did not increase CVD risk (Wanders et al., 2010/A; Wanders et al., 2010/B), and they determined significant reductions of inflammatory markers, such as IL-6, IL-8 and TNF-α (Sofi et al., 2010).
In human studies, it has been shown that an intake of 90 g/d of an enriched cheese resulted in significant increases in the plasma concentrations of CLA, VA and ALA, increases reflecting their high content in the cheese (Pintus et al., 2013) and an increase in the n-3 HPUFA score along with a decrease in the n-6:n-3 HPUFA ratio.
1.4 Gut Microbiota

Human gut microbiota consists of 10 to 100 trillion microorganisms (Savage et al., 1977) which it has been shown to interfere with energy homeostasis (Cani et al., 2008; Vijay-Kumar et al., 2010). It has also been demonstrated that microbiota provides important metabolic and biological functions that cannot be carried out by human metabolism (Jia et al., 2008). Gut microbiota participates in whole-body metabolism by affecting energy balance (Bäckhed et al., 2004; Turnbaugh et al., 2006; Cani et al., 2007/A) glucose metabolism (Cani et al., 2008; Cani et al., 2007/A; Cani et al., 2005/A; Cani et al., 2005/B; Cani et al., 2004) and low-grade inflammation (Cani et al., 2008; Cani et al., 2009; Cani et al., 2007/A; Rabot et al., 2010; Everard et al., 2011) associated with obesity and related metabolic disorders. It has been shown that gut microbiota-derived LPS may represent a cause for the onset and progression of inflammation and metabolic diseases (Cani et al., 2007/A) and, in pathological situations as obesity and type 2 diabetes, specific microbes associated molecular patterns (MAMPs) such as LPS play a major role in the onset the diseases associated with obesity (Cani et al., 2008; Cani et al., 2009; Cani et al., 2007/A; Cani et al., 2004; Everard et al., 2011; Cani et al., 2006).

LPS is regarded as a component of Gram-negative bacteria cell walls and as a potent inducer of inflammation. It has been demonstrated that dietary fats facilitate the development of metabolic endotoxemia (e.g., increased plasma LPS levels) (Cani et al., 2007/A; Cani et al., 2007/B) and it could occur through physiological mechanisms, such as the transport of LPS from the gut lumen to target tissues by newly synthesized chylomicrons from epithelial intestinal cells in response to fat feeding (Vreugdenhil et al., 2003; Ghoshal et al., 2009). Therefore, these mechanisms could contribute to higher plasma LPS levels and low-grade inflammation, as observed with a high-fat diet (Cani et al., 2007/A; Amar et al., 2008; Erridge et al., 2007; Laugerette et al., 2009; Al-Attas et al., 2009; Pussinen et al., 2011), suggesting that the hypothesis of metabolic endotoxemia could be linked to gut microbiota (Cani et al., 2012).

1.4.4 Gut Microbiota and the Innate Immune System

Toll-like receptors (TLRs) participate in the process of the innate immunity by integrating signals from microbiota-host interactions (e.g., pro-inflammatory signals), detecting LPS via its interaction with specific proteins that complex with TLR4 (CD14/TLR4 complex) (Bäckhed et al., 2003). Different studies have shown that metabolic endotoxemia represents a factor involved in the development of low-grade inflammation associated with insulin resistance and type 2 diabetes and high-fat diet increased fat mass, body weight and low-grade inflammatory state (in the liver, adipose tissues and muscle) through a LPS-dependent mechanism. It has also been demonstrated that mice that lack
functional LPS receptors (CD14 knockout mice) are resistant to diet-induced obesity and related disorders, including hepatic insulin resistance (Cani et al., 2007/A). It has been demonstrated a significant inflammation and insulin resistance after chronic subcutaneous infusion of LPS (mimicking metabolic endotoxemia), similarly to data observed following high-fat diet feeding (Cani et al., 2012).

Moreover, mice fed a high-fat diet that received broad-spectrum antibiotics showed a reduced metabolic endotoxemia, inflammation, insulin resistance and fat mass development (Cani et al., 2008) and same effects were confirmed in genetically obese (ob/ob) mice (Cani et al., 2008; Membrez et al., 2008). It was also observed that that germ-free mice did not develop inflammation following a high-fat diet, supporting the role of gut microbiota in the development of low-grade inflammation (Rabot et al., 2010). Different studies proposed that FAs stimulate the innate immune system, but probably in conjunction with initial stimulation by LPS of the TLR-4/CD14 complex and subsequent TLR-2 stimulation. For instance, antibiotic treated mice didn't show the development of high-fat diet-induced inflammation and insulin resistance. These mice fully digest and absorb the ingested fat and showed functional TLR-4/2 receptors (Cani et al., 2008; Rabot et al., 2010; Bäckhed et al., 2007), suggesting an initiation of signalling cascade by an LPS/TLR-4/CD14-dependent mechanism, which in turn activates TLR-2 expression to support innate immune system inflammatory responses. These studies suggest that the innate immune system and metabolic pathways are functionally intertwined (Olefsky et al., 2010) and involved in obesity and diabetes. It has been indicated that TLR 2 controls low-grade inflammation and insulin resistance (Ehses et al., 2010; Himes et al., 2010; Davis et al., 2011) and that TLR5 (Vijay-Kumar et al., 2010) and TLR2 (Caricilli et al., 2011) knockout mice exhibited altered gut microbiota composition, playing a central role in the development of obesity and associated disorders.

### 1.4.2 Gut Microbiota and Non-Alcoholic Fatty Liver Disease

It has been suggested that endotoxemia and gut-derived toxins may be the cause of the onset and progression of liver inflammation and damage in chronic liver diseases (Nolan et al., 1978; Nolan et al., 1975), as well non-alcoholic fatty liver disease (NAFLD) (Le Roy et al., 2013). This is a typical chronic liver complication observed in obesity and metabolic syndrome that involves a complex spectrum of pathological changes, including steatosis, nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis (Bedogni et al., 2005). It has been shown that diet-induced intestinal bacterial overgrowth, gut leakiness and increased endotoxin absorption have all been associated with NAFLD/NASH in rodents and human patients (Cani et al., 2009; Brun et al., 2007; Farhadi et al., 2008; Wigg et al., 2001). It has also been demonstrated that the impairment of gut barrier function and
subsequent alterations in gut permeability observed in NAFLD patients are determined by changes in tight junction protein expression and distribution. Therefore, it is supposed that hepatic exposure to gut-derived endotoxins may increase TLR activation, especially on Kupffer cell membranes, may activate nuclear transcription factors resulting in the release of numerous pro-inflammatory cytokines that ultimately lead to hepatic injury and fibrosis (Seki et al., 2007; Seki et al. 2008; Miura et al., 2010).

It has been demonstrated that probiotic treatment improves insulin resistance, hepatic histology and total FA content in mice with NASH, improving NAFLD/NASH and metabolic syndrome, confirming the close relationship between gut and liver (Li et al., 2003). Furthermore, it has been shown a decrease of TNF-α levels and hepatic inflammatory signalling in liver steatosis after supplementation with a mix of probiotic strains (VSL#3) (Loguercio et al., 2005; Velayudham et al., 2009). Similarly, it has been shown that prebiotic administration could ameliorate oxidative and inflammatory liver damage associated with NAFLD in humans (Loguercio et al., 2005).

1.4.3 Gut Microbiota and Endocannabinoid System

Given that obesity, inflammation and the ECS are interconnected, it has been also investigated how the gut microbiota and the gut barrier function might be related through modulation of the ECS (Cani et al., 2015). It has been shown that the gut microbiota controls the ECS activity in the colon and in adipose tissue in obesity conditions (Muccioli et al., 2010, Geurts et al., 2011). It has also been discovered, in vivo and in vitro, that the ECS controls gut barrier function via a CB1 dependent mechanism, given that the pharmacological blockade of the CB1 receptor in obese mice improves the gut barrier and reduces metabolic endotoxemia, independently of food intake behaviour (Muccioli et al., 2010).

It has also been found that the gut microbiota ‘interacts’ with adipose tissue development and metabolism through EC–gut microbiota crosstalk, determining, for example, the normalization of ECS tone in both the gut and adipose tissue by prebiotic treatment (Muccioli et al., 2010). In fact, an increase in endogenous production and content of N-arachidonoylethanolamine in obese animals (colon and both visceral and subcutaneous adipose tissue) (Muccioli et al., 2010, Geurts et al., 2011), was blunted by prebiotic treatment (Muccioli et al., 2010).

It is directly controlled via the balance between synthesis and degradation through N-acylphosphatidylethanolamine phospholipase-D (NAPE-PLD) and the FAAH controls N-arachidonoylethanolamine content. It has been found that modulation of the gut microbiota (high-fat diet or prebiotics) affects this pathway in both the intestine and in adipose tissue; in particular,
high-fat diet feeding increased NAPE-PLD and decreased FAAH mRNA expression, whereas prebiotics blunted these effects (Cani, 2012).

**1.4.4 Microbiota, metabolic endotoxemia, gut barrier function and ECS**

Gut permeability is linked to low-grade inflammation and insulin resistance by gut microbiota, explaining the development of obesity-related metabolic endotoxemia (Cani et al., 2008, Cani et al., 2007, Everard et al., 2011, Muccioli et al., 2010). Specifically, it has been shown that the tight-junction proteins (Zonula Occludens-1 and Occludin) involved in the gut barrier function may be altered by a high-fat diet, dependently on the gut microbiota because antibiotic treatment abolished diet-induced gut permeability (Cani et al., 2008; Cani et al., 2009). It has been also demonstrated that the specific modulation of gut microbiota composition with non-digestible carbohydrates (e.g., prebiotics) improves gut barrier integrity, reduces metabolic endotoxemia, and lowers inflammation and glucose intolerance (Cani et al., 2009; Everard et al., 2011; Cani et al., 2007; Muccioli et al., 2010). It has been proposed a ECS role as a link between the gut microbiota and the development of obesity and related disorders (Geurts et al., 2011; Muccioli et al., 2010). For instance, it has been demonstrated that the gut microbiota controls the EC tone in intestine and adipose tissue (Geurts et al., 2011; Muccioli et al., 2010) and that the gut microbiota regulates the CB1 expression, AEA content and its degrading enzyme FAAH, not only in the intestine but also in mouse adipose tissue (Muccioli et al., 2010). Several studies suggested a relationship between LPS and the ECS as the LPS control on EC synthesis in macrophages, the macrophage infiltration of adipose tissue and the liver during obesity, a key factor in metabolic disorder development (Weisberg et al., 2003; Lanthier et al., 2009).

It has been demonstrated that the ECS contributes to gut barrier function (*in vivo* and *in vitro*) and metabolic endotoxemia via CB1 receptor-dependent mechanisms and, precisely, the ECS controls gut barrier function through the distribution and localization of tight junction proteins (ZO-1 and occludin), independently of food intake behaviour. The ECS has also been identified in the gut and adipose tissue as a prominent pathway for adipogenesis regulation (Muccioli et al., 2010). Then, gut microbiota, the innate immune system and the endocannabinoid system are involved all together in the development of obesity and related disorders.

**1.4.5 Gut microbiota, adipose tissue metabolism and ECS**

Adipose tissue is considered as a organ with a central role in metabolism regulation (Gallic et al., 2009). In fact, it has been shown its role in energy storage and its function as an endocrine organ affecting energy homeostasis, innate immunity and inflammation (Gallic et al., 2009). The gut...
microbiota can also be considered as an “exteriorized organ” that contributes to host metabolism and homeostasis via different functions and mechanisms (Cani et al., 2011).

Several studies support a communication axis between the gut microbiota and adipose tissue, which influences the development of metabolic alterations associated with obesity. In fact, mice with gut microbiota (conventionally raised) have over 40% more fat mass than germ-free mice (without gut microbiota) (Bäckhed et al., 2004) and the transplantation of cecal content (gut microbiota) isolated from obese mice to germ-free mice resulted in a greater increase in total body fat mass compared with colonization with gut microbiota isolated from lean donors (Turnbaugh et al., 2006).

An increase of lipolysis and a decrease lipogenesis has been shown in the brown adipose tissue of germ-free compared with conventionally raised mice, suggesting that gut microbiota stimulates brown adipose tissue lipid metabolism (Mestdagh et al., 2012). It has been demonstrated that ob/ob mice fed with prebiotics have a different gut microbiota composition and a decreased adiposity index compared with obese mice fed with a normal chow diet (Everard et al., 2011). It has also been highlighted an increase of lipoprotein lipase enzyme (LPL) activity in germ-free mice. This enzyme catalyzes the release of FAs from circulating triglycerides and lipoproteins in muscle and adipose tissue, suggesting a relation with suppression of the fasting-induced adipose factor in the gut, an inhibitor of LPL activity that determines the accumulation of FAs in adipose tissue (Bäckhed et al., 2004). Finally, it has been demonstrated that germ-free mice are resistant to high-fat diet-induced body weight gain and fat mass accumulation, suggesting that gut microbiota promotes fat storage (Bäckhed et al., 2007) and determines development of non-alcoholic fatty liver disease in mice (Le Roy et al., 2013).

EC activation stimulates adipogenesis, while LPS stimulates ECS tone (Muccioli et al., 2010, Liu et al., 2003). Therefore, EC, LPS and inflammation regulating adipose tissue metabolism in physiopathological situations (e.g., obesity with endotoxemia, inflammation and EC activation), set a relationship between gut microbiota and adipose tissue mediated by the ECS (Cani et al., 2015).
1.5 Utilization of *Bifidobacterium breve* as probiotic

It has been shown that probiotics modulate the intestinal symbiotic bacteria, determining the maintenance of intestinal homeostasis (Martin et al., 2008; Sonnenburg et al., 2006). It has also been demonstrated that modulation of microbiota by probiotics can antagonize pathogenic bacteria through the reduction of luminal pH, inhibition of bacterial adherence, or production of antimicrobial molecules (Ng et al., 2009). Moreover, probiotics can enhance barrier functions of intestinal epithelial cells (Mennigen et al., 2009). Some studies have also shown that intestinal commensal microbiota has a great influence on the host intestinal immune system (Round et al., 2009; Hooper et al., 2010).

Commensal micro flora is very important for the development of the gut’s immune system (Macpherson et al., 2004) and, in this contest, some bacteria (*Lactobacillus* and *Bifidobacterium* species) are describe as “living microorganisms exerting health benefit” (Guarner et al., 1998; Shornikova et al., 1997), defining the concept of probiotics. It has been reported that these bacteria could prevent and treat rotavirus infections and post-antibiotic diarrhea (Shornikova et al., 1997; Cruchet et al., 2003), allergic diseases (Kalliomaki et al., 2001; Viljanen et al., 2005), and recurrence of inflammatory bowel disease (Rembacken et al., 1999; Guslandi et al., 2000), suggesting that the intestinal immune system could be a privileged target of probiotics. Despite several studies reported that probiotic bacteria induced modifications of immunologic parameters, the interactions between the cells of the intestinal immune system and bacteria remain largely unknown (Isolauri et al., 2001).

However, it has been observed that killed probiotic bacteria can affect the maturation and cytokine secretion profile of dendritic cells (DCs) (Christensen et al., 2002; Hart et al., 2004), which represent potent antigen-presenting cells, and DCs also have properties to induce negative regulation, with generation of regulatory T cells (Smits et al., 2005). Bacterial components, through the interaction between pathogen-derived immune-stimulatory molecules from bacteria and membrane receptors called pattern-recognition receptors, including the Toll-like receptor (TLR) family, are able to activate DCs (Takeda et al., 2005). Several microbial structures, such as proteins, lipids, glycoproteins, and nucleic acid motifs, contained in the wall, cytoplasm, and nucleus of bacteria, are recognized by different TLRs, suggesting that TLR engagement could have different types of effects on DC activation according to the bacteria strain (Qi et al., 2003).

It has been shown that active bacterial products from *Bifidobacterium breve* C50 (*B. breve* C50) could cross an intestinal monolayer of epithelial cells (Cruchet et al., 2003) and it was able to mature and fully activate DCs in vitro, inducing a particular cytokine synthesis profile and prolonged DC survival through a TLR2 pathway (Hoaurau et al., 2006).
Thus, it has been shown that DCs can represent a sensor of infection, generating specific immune responses with activation of effectors T cells (Banchereau et al., 1998) and that maturation, activation, and survival of DCs can be differentially affected according to the bacteria strain, the cytokine environment, and the presence or absence of inflammation. It has been observed that soluble bacteria products presented in the intestinal lumen are able to interact with mucosal DC and it has also demonstrated that *B. breve* C50SN-DCs produced high IL-10 and low IL-12 levels in contrast to LPS-DCs (Hoaurau et al 2006). This study was performed using supernatant of a viable *B. breve* culture and LPS contamination of *B. breve* C50SN was excluded because polymyxin B had no effect on *B. breve* C50SN-DCs and because the TLR4- transfected cell line was not activated by the supernatant (Hoaurau et al 2006).

IL-12 has been described as a pro-inflammatory cytokine that can induce a TH1 response, while IL-10 acts as a general inhibitor of proliferative and cytokine responses of both TH1 and TH2 cells and its production by DCs can induce regulatory T cells (O’Garra et al., 2003). It has been reported that some strains of probiotic bacteria enhance IL-10 synthesis from PBMCs, splenocytes, Peyer’s cells, and DCs in rat, murine, and human models (Roller et al., 2004, Lammers et al., 2003, Schultz et al., 2003, Colino et al., 2003), explaining the results of probiotic bacteria on allergic and inflammatory bowel diseases observed in clinical studies (Rosenfeldt et al., 2003; Viljanen et al., 2005; Guslandi et al., 2000).

It has also been demonstrated that some products of *B. breve* C50 determine different effects through TLR2 action (Hoaurau et al 2006). *B. breve* is a Gram-positive bacillus and, for this reason, it has been describe as ligand of TLR2 (Takeda et al., 2004). In fact, cell wall of Gram-positive bacilli comprises a thick peptidoglycan layer and a bilayer PL membrane with lipoproteins, which constitute the mean ligands of TLR2. Moreover, it has been reported a DC IL-10 secretion after TLR2 engagement (Agrawal et al., 2003; Agrawal et al., 2004). It has also been confirmed by human studies that IL-10 production induced by concomitant TLR2 engagement can block IL-12 production induced by TLR4 in human DCs (Re et al., 2004).

In conclusion, all these results suggest that the micro flora (including *B. breve*) is present not only to initiate and develop the gut’s immune system, but also to regulate the immune responses for some specific strains (Hoaurau et al 2006).
2. **Aim of the study**

With the studies described in this thesis we aimed at evaluating the effects of some nutritional factors, such as CLA naturally occurring in cheese, or produced by *B. breve*, in influencing n-3 PUFA metabolism to optimize the n-3 HUFA score, a biomarker of omega-3 status in tissues, and possible interactions with the ECS, in experimental models and humans.
3. **Materials and Methods.**

3.1 **Clinical studies.**

All protocols involving human subjects were performed according to the Good Clinical Practice rule, and each study was submitted to the appropriate Ethical Committee.

3.1.1 **Study on cheese enriched in CLA in healthy human volunteers (BIOCLA)**

The study was carried out at The Catholic University Sacred Heart in Rome, Italy, under the medical supervision of Dr. Maria Cristina Mele and Giorgio Cannelli. Thirty six volunteers were recruited, within the personnel of the Policlinico Universitario “A. Gemelli” of the Catholic University Sacred Heart, Rome, with the following inclusion criteria: age between 30 and 40 years; BMI (body mass index)=20-26 kg/m²; systolic blood pressure (SBP)=<150mmHg and diastolic blood pressure (DBP)<95mmHg; total cholesterol concentration in serum of ≤6mM; glucose level in serum of ≤4.2-6mM.

Exclusion criteria included subjects with BMI out of the normal range, with systemic diseases such as diabetes mellitus, liver disorders, neoplasms, collagen-vascular diseases, smokers, plasma total cholesterol or triglycerides higher than 250 mg/dl, blood transfusions within six months prior of the study and history of recent acute illness. Furthermore, the use of alcohol, tobacco and medicines that can influence the absorption or the metabolism of lipids were not permitted. The participants gave their informed consent after carefully reading the protocol. It was made clear that the participants could withdraw at any stage of the study. The participants were apparently healthy volunteers, male and female, free of prescribed drugs and without dietary restrictions. Prior to enrolment in the trial, the health status of each subject was checked with a questionnaire and screening tests (blood pressure, urinary protein and glucose, serum cholesterol, triglycerides and gamma-glutamyltransferase). Throughout the study, subjects registered side effects, intercurrent illnesses and use of medicines. Except for diet, they were advised to keep their customary living habits unchanged. Blood samples were taken from the antecubital veins after an overnight fast.

Subjects were randomly assigned to one of the following four groups of eight persons per group and the double blind procedure will be applied. Each member of the four groups will receive a given quantity of control (non-CLA enriched) or a CLA-enriched dairy product, 50g/d for two months. After 2 months of wash out, for a further two months, the dairy product will be switched, such that the control group will ingest the CLA-enriched product and vice versa, in order to verify the wash-out period, following ingestion of CLA-enriched dairy product. After the wash out, the study will be repeated, using the same subjects, with one different CLA-enriched dairy products and controls,
using the same experimental design as described above. Each subject ingested each control and each enriched cheese. Blood samples was drawn after each treatment and after wash out. The 3 control and enriched cheeses were from cows, sheep and goats. Table 1 describes the cheese FA composition.

<table>
<thead>
<tr>
<th>% in cheese fat</th>
<th>mean of control cheeses</th>
<th>Cow en</th>
<th>Sheep en</th>
<th>Goat en</th>
</tr>
</thead>
<tbody>
<tr>
<td>short chain (c4-c10)</td>
<td>18.4</td>
<td>12.4</td>
<td>16.5</td>
<td>17.2</td>
</tr>
<tr>
<td>12:0</td>
<td>5.0</td>
<td>3.0</td>
<td>4.0</td>
<td>3.4</td>
</tr>
<tr>
<td>14:0</td>
<td>10.7</td>
<td>6.3</td>
<td>8.5</td>
<td>8.9</td>
</tr>
<tr>
<td>15:0</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>16:0</td>
<td>23.8</td>
<td>18.4</td>
<td>17.3</td>
<td>16.2</td>
</tr>
<tr>
<td>17:0</td>
<td>0.7</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>18:0</td>
<td>8.2</td>
<td>10.8</td>
<td>9.6</td>
<td>8.7</td>
</tr>
<tr>
<td>14:1</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>18:1</td>
<td>0.8</td>
<td>0.8</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>14.8</td>
<td>20.7</td>
<td>15.9</td>
<td>15.9</td>
</tr>
<tr>
<td>VA</td>
<td>1.5</td>
<td>6.9</td>
<td>4.9</td>
<td>8.5</td>
</tr>
<tr>
<td>18:2n-6</td>
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<td>1.8</td>
<td>1.6</td>
<td>3.3</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.7</td>
<td>1.2</td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>CLA 9 cis, 11 trans</td>
<td>0.6</td>
<td>2.4</td>
<td>2.5</td>
<td>3.9</td>
</tr>
<tr>
<td>CLA+VA conversion</td>
<td>0.9</td>
<td>3.8</td>
<td>3.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Table 1 grams of FAs in 100 grams of fat

This study was part of the European Project “BIOCLA” EU Project QLKI-2002-02362, coordinated by Dr. Catherine Stanton. Enriched cheeses with CLA used in this study were provided by:

- Cow cheese: Dr. Catherine. Stanton, Food Research centre, Food Biosciences Department, Moorepark, Fermoy, co.Cork, Ireland
- Goat Cheese: Dr. Yves Chillard, INRA, Clermont-Ferrand-Theix, France.
- Sheep cheese: Dr. Giovanni Piredda, AGRIS, Bonassai, Italy.

In an ancillary study 24 healthy subjects of the above trial were randomly divided into two groups, one group was treated for 2 months with 4 grams of linseed oil (with an intake of 2g of ALA) and the other group with 4 grams of olive oil.
3.1.2 Study on fish oil and sheep enriched cheese CLA and ALA (fish and cheese)

15 healthy subjects were treated with increasing stepwise doses of FO. They started with 1g/d for 2 weeks increasing with 2g/d for 2 more weeks and 3g/d for other 2 weeks. After a 3 week wash out period, they were treated with 90g/d of commercially available ALA and CLA enriched sheep cheese (see Table 2 for FA composition). FO capsules were kindly provided by Aker Biomarine, Oslo, Norway; while ALA and CLA enriched sheep cheese was kindly provided by Argiolas Formaggi s.r.l., Dolianova, Italy.

<table>
<thead>
<tr>
<th>FAs (mg)</th>
<th>1 pill FO</th>
<th>2 pills FO</th>
<th>3 pills FO</th>
<th>90g CLA ENRH Sheep Cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2n6</td>
<td>79,30</td>
<td>158,60</td>
<td>237,90</td>
<td>537,89</td>
</tr>
<tr>
<td>20:4n6</td>
<td>6,20</td>
<td>12,39</td>
<td>18,59</td>
<td>43,84</td>
</tr>
<tr>
<td>18:3n3</td>
<td>7,27</td>
<td>14,54</td>
<td>21,81</td>
<td>484,06</td>
</tr>
<tr>
<td>20:5n3</td>
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<td>-</td>
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<td>-</td>
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<td>566,54</td>
<td>528,88</td>
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<td>259,04</td>
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<td>0,46</td>
<td>0,46</td>
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<tr>
<td>TOT SFA</td>
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<td>0,57</td>
<td>0,86</td>
<td>5800,00</td>
</tr>
<tr>
<td>tot Lipids</td>
<td>477,15</td>
<td>954,31</td>
<td>1431,46</td>
<td>12498,91</td>
</tr>
</tbody>
</table>

Table 2 mg of FAs in 1,2, 3 pills and in CLA- enriched sheep cheese. These amounts correspond to volunteers intake.
3.2 Preclinical studies

Animals were housed in climate- and light- (12h light/12 h dark) controlled quarters and allowed unlimited access to food and water.

All experiments were performed in strict accordance with the guidelines and protocols approved by the European Union (EU Council 86/609; D.L. 27.01.1992, No. 116) and by the Animal Research Ethics Committee of the University of Cagliari, Italy.

3.2.1 Study on rats fed different ratios of ALA/CLA

36 male Wistar rats (Harlan, Milan, Italy), with an average initial weight of 150-170g, were fed a chow diet for seven days, divided into 6 groups, and fed for 3 weeks 6 different diets. The diets were based on the AIN-93G formulation, with substitution of soybean oil with a blend of oils, olive oil, linseed oil and CLA (see Table 3 for ratios) which allowed the 6 diets to be equal for total FAs. Rats were fasted for 12 h prior to sacrifice. Upon necropsy, blood was immediately collected from the aorta for isolation of plasma, liver and perirenal adipose tissue were removed and rinsed in phosphate buffered saline, blotted and weighed, then immersed in liquid nitrogen for storage at -80°C until analysis.

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>OA</th>
<th>CLA</th>
<th>ALA</th>
<th>ALA/CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0,5</td>
<td>1,8</td>
<td>3,5</td>
</tr>
<tr>
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<td>2</td>
<td>1,0</td>
<td>1,5</td>
<td>1,5</td>
</tr>
<tr>
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<td>2</td>
<td>1,5</td>
<td>1,5</td>
<td>0,8</td>
</tr>
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<td>4</td>
<td>2</td>
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<td>1,0</td>
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</tr>
<tr>
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<td>2</td>
<td>2,5</td>
<td>0,8</td>
<td>0,3</td>
</tr>
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<td>6</td>
<td>2</td>
<td>3,0</td>
<td>0,5</td>
<td>0,2</td>
</tr>
</tbody>
</table>

Table 3 Diets composition (% of weight of the diet)

3.2.2 Study on mice treated with PPAR α agonist WY-14,643 or CLA

Female SV/129 mice (21-25 days old) (Harlan, Milan, Italy) were fed a chow diet for seven days then assigned to diet groups (n=12 mice/group). Mice were fasted for 12 h prior to sacrifice. Upon necropsy, livers were removed, rinsed in phosphate buffered saline, blotted and weighed, then immersed in liquid nitrogen for storage at -80°C until analysis.
3.2.3 Study on rats treated with LPS

Adult male Wistar rats (Harlan, Milan, Italy), weighing 150-200 g, were 24 h fasted at the time of the experimental procedure. LPS from Escherichia coli (serotype 055:B5; Sigma-Aldrich s.r.l., Milan, Italy) was freshly prepared in 0.9% NaCl. LPS was injected i.p (2 mg/100 g b.w) 6 hour before sacrifice. Control groups received 0.9% NaCl. Six hours after LPS injection rats were sacrificed by decapitation. Blood was collected from the inferior cava vein in EDTA rinsed syringes, centrifuged at +4°C to separate plasma for total lipids and n-3 PUFAs measurements. Livers were perfused in situ with 0.9% NaCl, removed from the abdominal cavity, frozen in liquid nitrogen, and stored at –80°C.

For TNF-α assay, EDTA-treated plasma was added with aprotinin to inhibit proteases inactivation of the cytokine. 0.02 Trypsin inhibitory units (TIU) of aprotinin/ml plasma were necessary. A commercially available enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Inc., Woburn, MA) was used for TNF-α detection, according to the manufacturer’s directions. A standard curve was performed by using rat TNF-α.

3.2.4 Probiotic Study in mice

Probiotic preparation and the feeding study on mice was carried out at Teagasc Laboratories (Teagasc Food Research centre, Food Biosciences Department, Moorepark, Fermoy, co.Cork, Ireland).

Preparation and administration of B. breve NCIMB 702258 and B. breve DPC 6330

It was previously shown that B. breve NCIMB 702258 and B. breve DPC 6330 are efficient CLA producers, converting up to 65% and 76%, respectively, of linoleic acid to cis-9, trans-11 CLA when grown in 0.5 mg/mL linoleic acid in vitro (Barrett et al., 2007, Coakley et al., 2003). Rifampicin resistant variants of B. breve NCIMB 702258 and B. breve DPC 6330 were isolated by spread-plating ~10^9 colony forming units (CFU) from an overnight culture onto MRS agar (de Man, Rogosa & Sharpe; Difco Laboratories, Detroit, MI, USA), supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma, Wicklow, Ireland) (mMRS), containing 500 µg/mL rifampicin (Sigma). Following anaerobic incubation (anaerobic jars with Anaerocult A gas packs; Merck, Darmstadt, Germany) at 37°C for 3 days, colonies were stocked in mMRS broth containing 40% (v/v) glycerol and stored at -80°C. To confirm that the rifampicin resistant variants were identical to the parent strain, molecular fingerprinting using pulse-field gel electrophoresis was employed.

Prior to freeze drying, B. breve NCIMB 702258 and B. breve DPC 6330 were grown in mMRS by incubating for 48 hr at 37°C under anaerobic conditions. The culture was washed twice in phosphate
buffered saline (PBS) and re-suspended at a concentration of \( \sim 2 \times 10^{10} \) cells/mL in 15% (w/v) trehalose (Sigma) in dH\(_2\)O. One millilitre aliquots were freeze-dried using a 24 hr programme (freeze temp. -40°C, condenser set point -60, vacuum set point 600 m Torr). Each mouse that received the bacterial strains consumed approximately \( 1 \times 10^9 \) live microorganisms per day. This was achieved by re-suspending appropriate quantities of freeze-dried powder in water which mice consumed ad libitum. Mice that did not receive the bacterial strains received placebo freeze-dried powder (15% (w/v) trehalose in dH\(_2\)O). Water containing either the bacterial strains or placebo freeze-dried powder was the only water supplied to the mice throughout the trial. Freeze dried powders with the bacterial strains underwent continuous quality control checks of cell counts for the duration of the trial, by plating serial dilutions on mMRS agar supplemented with 100 µg/mL of mupirocin (Oxoid) and 100 µg/mL rifampicin (Sigma) and incubating plates anaerobically for 72 hr at 37°C.

**Animals and treatment**

All animal experiments were approved by the University College Cork (UCC) Animal Ethics Committee and experimental procedures were conducted under the appropriate license from the Irish Government. Female BALB/c mice were purchased from Harlan Limited (Briester, Oxon, UK) at 8 weeks of age and housed under barrier-maintained conditions within the Biological Services Unit, UCC. Mice were allowed to acclimatise for 1 week prior to commencement of the study and were fed ad libitum with Teklad Global Rodent Standard Diet (Harlan Laboratories, Madison, WI, USA, #2018S), with free access to water at all times. Mice were housed in groups of five per cage and kept in a controlled environment at 25°C under a 12-hr-light/12-hr-dark cycle. After 1 week of acclimatisation, the mice were randomly divided into four groups (n 10 mice per group): (1) a flaxseed oil (FXO) supplemented dietary group (enriched in ALA) with *B. breve* NCIMB 702258 (approximate daily dose of \( 10^9 \) microorganisms); (2) a FXO supplemented dietary group (enriched in ALA) with *B. breve* DPC 6330 (approximate daily dose of \( 10^9 \) microorganisms); (3) FXO supplemented dietary group (enriched in ALA) with placebo freeze-dried powder (15% w/v trehalose in drinking water); (4) a control- unsupplemented group fed a standard rodent diet with placebo freeze-dried powder (15% w/v trehalose in drinking water).

The unsupplemented control diet contained the following nutrient composition: corn starch (32.45%), casein (20.0%), sucrose (15.0%), maltodextrin (12.0%), cellulose (5.0%), mineral mix (3.5%), vitamin mix (1.5%), L-cysteine (0.3%), choline bitartrate (0.25%), TBHQ antioxidant (0.002%) and the following composition of fat: palm oil (3.0%), safflower oil (3.0%), olive oil (3.0%), FXO (1.0%). The n-6 : n-3 ratio of this diet was \( \sim 5.3 \) and the diet contained \( \sim 0.5\% \) ALA. The FXO supplemented diet contained the following nutrient composition: corn starch (32.45%), casein (20.0%), sucrose (15.0%),
maltodextrin (12.0%), cellulose (5.0%), mineral mix (3.5%), vitamin mix (1.5%), L-cysteine (0.3%), choline bitartrate (0.25%), TBHQ antioxidant (0.002%) and the following composition of fat: FXO (5.5%), palm oil (1.5%), safflower oil (1.5%), olive oil (1.5%). The n-6 : n-3 ratio of this diet was ~0.75 and the diet contained ~3% ALA.

Body weight and food intake were assessed weekly. Following 6 weeks on experimental diets, the animals were killed by cervical dislocation. Liver and epididymal adipose tissue were blotted dry on filter paper, weighed and flash-frozen immediately in liquid nitrogen. All samples were stored at -80°C until processed.

**RNA extractions and complementary DNA synthesis**

Total RNA was isolated from the liver and adipose tissue using the commercial RNeasy Mini-Kit (Qiagen, West Sussex, UK), according to the manufacturer’s instructions. Total isolated RNA was quantified using the Nanodrop (Thermo Scientific). Single stranded complementary DNA (cDNA) was synthesised from 1μg of total RNA using 2.5ng/μL random primers (Promega, WI, USA), 10mM PCR nucleotide mix (Promega), 40units/μL RNasin Plus RNase inhibitor (Promega) and the Im-Prom II reverse transcriptase (Promega).

**Real-time PCR analysis**

Amplification of generated cDNA was performed in the Lightcycler 480 system (Roche Diagnostics Ltd., West Sussex, UK) using 0.25μM primers (MWG Eurofins, Ebersberg, Germany), 1μL cDNA and the Lightcycler 480 SYBR Green I Master kit (Roche Diagnostics Ltd). Real-time PCR conditions were set as previously described for delta 6 desaturase (Devlin et al., 2007), PPAR α and PPAR γ (Yatsuga and Suomalainen, 2012). All samples were analysed in duplicate and normalised to β-actin, as a constitutively expressed control gene. Melting curve analysis allowed the validation of the authenticity of the real-time PCR products. Basic relative quantification of expression was determined using the comparative $2^{-\Delta\Delta C_t}$ method.

3.3 **Lipid analysis**

Acetonitrile (CH$_3$CN), methanol (CH$_3$OH), chloroform (CHCl$_3$), n-hexane (C$_6$H$_{14}$), ethanol (C$_2$H$_5$OH), acetic acid (CH$_3$COOH) were HPLC grade and purchased from Sigma Chemicals Co., St. Louis, MO, USA. All standards of SFAs, UFAs, and c9,t11 CLA and t10,c12 CLA isomers, were purchased from the same company.
Ascorbic acid, potassium hydroxide (KOH), hydrochloric acid (HCl) were purchased from Carlo Erba, Milano, Italy. Deferoxamine mesylate (desferal) was purchased from CIBA-Geigy, Basel, Switzerland. Internal deuterated standards for AEA, 2-AG, PEA and OEA quantification by isotope dilution ([\(^2\text{H}\)_8AEA, \([\(^2\text{H}\)_52AG, \([\(^2\text{H}\)_4PEA, \([\(^2\text{H}\)_4OEA) were purchased from Cayman Chemicals, MI, USA.

**Extraction of total lipids**

Total lipids were extracted by the method of Folch (Folch et al., 1957). Briefly, samples of human plasma (1 ml) or rat liver (about 0.3 g), were homogenized each into a 2:1 chloroform-methanol solution containing 2 μg of vitamin E and deuterated AEA (200 ng), 2-AG (300 ng), OEA (200 ng), and PEA (100 ng).

Tubes containing lipids under extraction were kept one hour in the dark, added an equal volume of double-distilled water (ddH\(_2\)O) to that of methanol present, then left another hour in the dark. Samples were centrifuged for one hour at 900 x g to facilitate the separation of the chloroform phase from the aqueous-methanol.

The lower chloroform phase containing lipids was collected, divided in different aliquots for subsequent analyses and evaporated under vacuum by a rotator evaporator at room temperature.

**Quantitative determination of total lipids**

Total lipid quantification was performed by the method of Chiang (Chiang et al., 1955) on evaporated aliquots of chloroform phase containing lipids, collected after initial lipid extraction.

1.5 ml of the Chiang reagent (2 g of K\(_2\)Cr\(_2\)O\(_7\), into 4 ml ddH\(_2\)O, make up to a final volume of 100 ml adding H\(_2\)SO\(_4\)) was added, and the samples incubated for 30 min. at 100 °C. Finally, 1.5 ml of ddH\(_2\)O was added, and the absorbance measured at the wavelength of 600 nm by a colorimeter. To determine the concentration of total lipids a standard curve of corn oil was used, and the range of reliability of the method was between 100 and 800 mg of lipids.

**Fatty acid analysis of tissue lipid fraction**

An aliquot of the lipid fraction for each sample was mildly saponified using a procedure in order to obtain FFAs for HPLC analysis. Lipid extracts were dissolved in 5 ml of ethanol, 100 μl of desferal (25 mg/ml ddH\(_2\)O), 1 ml of a 25% solution of ascorbic acid in water, 0.5 ml of 10N KOH, and left 14 hours in the dark at room temperature. Later, 10 ml of n-hexane and 7 ml of ddH\(_2\)O were added, then the samples were acidified with 0.35 ml of 37% HCl, to a pH 3-4. Samples were centrifuged for 1h at 900
x g. The hexane phase containing FFAs was collected, the solvent evaporated, and the residue was dissolved in 0.5 ml of CH$_3$CN/0.14% of CH$_3$COOH (v/v).

Separation of FAs, including CLA and its metabolites, was carried out with an Agilent 1100 HPLC system (Agilent, Palo Alto, Calif., USA) equipped with a diode array detector. A C-18 Inertsil S ODS-2 Chrompack column (Chrompack International BV, Middleburg, The Netherlands), 5 μm particle size, 150 x 4.6 mm, was used with a mobile phase of CH$_3$CN/H$_2$O/CH$_3$COOH (70/30/0.12, v/v/v) at a flow rate of 1.5 ml/min (Melis et al., 2001). Conjugated diene unsaturated FAs were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were electronically stored. Second-derivate UV spectra of the conjugated diene FAs were generated using Phoenix 3D HP Chemstation software (Agilent, Palo Alto, CA). These spectra were acquired to confirm identification of the HPLC peaks (Angioni et al., 2002).

Since SFAs are transparent to UV, after derivatization, they were measured as fatty acid methyl esters (FAMEs), by a gas chromatograph (Agilent, Model 6890, Palo Alto, CA) equipped with split ratio of 20:1 injection port, a flame ionization detector (FID), an autosampler (Agilent, Model 7673), a 100 m HP-88 fused capillary column (Agilent). Finally, data were analyzed by the Agilent ChemStation software system. The injector and detector temperatures were set up at 250°C and 280°C, respectively. H$_2$ served as carrier gas (1 ml/min), and the FID gases were H$_2$ (30 ml/min), N$_2$ (30 ml/min), and purified air (300 ml/min). The temperature program was as follows: initial temperature was 120°C, programmed at 10°C/min to 210°C and 5°C/min to 230°C, then programmed at 25°C/min to 250°C and held for 2 min (Batetta et al. 2009).

**Analysis of the EC and their congeners**

Aliquots of organic phase (chloroform) containing extracted lipids were evaporated to dryness under vacuum, and reconstituted with 0.3 ml and 0.4 ml of 100% methanol for human plasma and rat tissue samples, respectively.

Quantification of AEA, 2-AG, PEA and OEA, was carried out by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), and using selected ion monitoring (SIM) at M+1 values for the four compounds and their deuterated homologues (Di Marzo et al., 2001). A C-18 Zorbax Eclipse Plus column (Agilent, Palo Alto, CA) 5 μm particle size, 50 x 4.6 mm, was used with a mobile phase of CH$_3$OH/H$_2$O/CH$_3$COOH (80/20/0.3, v/v/v) at a flow rate of 0.5 ml/min.
3.4 **Statistical analysis**

The data are expressed as the mean±SD or s.e.m as specified in the legends. Differences between two groups were assessed using an unpaired, two-tailed Student’s t-test. Data sets involving more than two groups were assessed by ANOVA followed by a Tuckey’s post hoc test. Data with different superscript letters were significantly different (P<0.05) according to the post hoc ANOVA statistical analysis. Data were analysed using statistiXL (Broadway – Nedlands, Western Australia). The results were considered statistically significant for P<0.05.
### 4. Results

We aimed first at evaluating whether CLA was able to enhance DHA biosynthesis from its precursor ALA. Previous results failed to demonstrate such effect (Attar-Bashi et al., 2007), however in that paper they use only one concentration of ALA and CLA. We therefore carry out a preclinical study using different ratio ALA/CLA (Table 3). We found that the highest yield in plasma DHA was obtained with a ratio ALA/CLA between 0.2-0.3 (Fig. 6) and increased linearly in relation to ALA/CLA ratio (Fig. 7). Plasma levels of ALA and CLA were proportional, even though not linearly, to the amount present in the diet (Fig. 6).

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>DHA mean</th>
<th>DHA SD</th>
<th>ALA mean</th>
<th>ALA SD</th>
<th>CLA mean</th>
<th>CLA SD</th>
</tr>
</thead>
<tbody>
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<td>315.64</td>
<td>69.43</td>
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</tr>
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<td>34.34</td>
<td>280.26</td>
<td>28.38</td>
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</table>

Table 4 Plasma levels (nmoles/ml of plasma) of DHA, ALA and CLA.

**Fig. 6** Logarithmic regression of ALA and CLA plasma concentrations in function to ALA/CLA ratio in the diet. Data represent means ±SEM.

**Fig. 7** Linear regression of DHA plasma concentration in function to ALA/CLA ratio in the diet. Data represent means ±SEM.
In the experimental groups 2, 4 and 6 we also analysed liver and visceral adipose tissue FAs, EC, PEA and OEA. Figure 8 shows that different ALA/CLA ratios affect n-3 PUFA profile with a graded decrease of ALA and EPA and corresponding increase of DHA. CLA increases in a dose dependent manner (Fig. 9). Figure 10 shows a decrease of AEA paralleled to ratio ALA/CLA, while PEA, OEA and 2-AG did not change significantly.

**n-3 PUFA profile in Liver**

![Graph showing changes of n-3 PUFA profile in liver. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).]

Fig. 6 Changes of n-3 PUFA profile in liver. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

![Graph showing changes of CLA levels in liver. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).]

Fig. 9 Changes of CLA levels in liver. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
In adipose tissue, ALA and CLA levels (Fig. 11 and 12) are modified according to their dietary concentration as shown in the liver, however, EPA and DHA (Fig. 10) and EC, PEA and OEA did not change significantly (Fig. 13).

Fig. 10 Changes of EC levels in liver. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

In adipose tissue, ALA and CLA levels (Fig. 11 and 12) are modified according to their dietary concentration as shown in the liver, however, EPA and DHA (Fig. 10) and EC, PEA and OEA did not change significantly (Fig. 13).

Fig. 11 Changes of n-3 PUFA profile in visceral adipose tissue. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
Fig. 12 Changes of CLA levels in visceral adipose tissue. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

Endocannabinoids levels in Visceral Adipose Tissue

Fig. 13 Changes of EC levels in visceral adipose tissue. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
We therefore evaluate whether similar results were reproducible in humans. We analyzed plasma FAs from a clinical study where 36 volunteers were treated with 50 grams of 3 control or CLA-enriched cheeses made from milk of cows, sheep and goats respectively. Since the milk was enriched in CLA by dietary means, by enhancing the rumen biohydrogenation, they were also enriched in VA, which may be converted in humans to CLA by SCD with a yield of about 20% (Turpeinen et al., 2002). The three enriched cheeses had different concentrations of CLA, particularly goat cheese was particularly rich in CLA and VA (Table 1).

As shown in Table 5 the daily 50g of cheese, taking into account the contribution of VA conversion, will supply CLA in the range of 0.4-0.7g a day.

<table>
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<tr>
<th>g/50g of cheese</th>
<th>mean of control cheeses</th>
<th>Cow en</th>
<th>Sheep en</th>
<th>Goat en</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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</tr>
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<tr>
<td>CLA+VA conversion</td>
<td>0.1</td>
<td>0.5</td>
<td>0.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 5 Grams of FAs in daily dose of 50 grams of cheese
We also analyze plasma FAs from a study where 12 volunteers were treated with 2g/d of ALA for 2 months, compared with 12 volunteers treated with olive oil. As shown in Table 6, treatment with ALA only, failed to increase DHA plasma concentration, while ALA and EPA increased significantly. On the other hand, feeding CLA enriched goat and cow cheese increased significantly DHA, while feeding sheep cheese increased but not significantly.

Table 6 Plasma levels of FAs in experimental groups after different cheeses intake and after 2g/d of ALA intake. In red significant differences (p<0.05)
We also measured the n-3 HUFA score, calculated by the ratio \((20:5n3+22:6n3)/(20:5n3+22:6n3+20:4n6+20:3n6+20:3n9+22:4n6)\)*100, which essentially evaluates the percentage of the HUFAs n3 with respect to all the HUFAs that can potentially act as eicosanoids precursors and compete for incorporation into tissue PLs. Therefore, it can better evaluate the impact of a nutritional treatment on the balance of n-3/n-6 HUFAs (Stark, 2008). We found that 2g/d of ALA fails to increase this parameter while all three CLA enriched cheeses n-3 HUFA score increased significantly (Fig. 16 and 17).

![Fig. 14 Changes of n-3 PUFA profile in plasma. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).](image1)

![Fig. 15 Changes of n-6 PUFA profile in plasma. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).](image2)
Fig. 16 Changes of CALA and CLA levels in plasma. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

Fig. 17 Changes of n-3 score in plasma. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
Since we recently found that sheep cheese enriched in CLA VA but also ALA, not only increased n-3 HUFA score but also decrease AEA in hypercholesterolemic patients (Pintus et al., 2013), we evaluated whether supplementation with a similar sheep cheese was comparable, and to what extend, to supplementation with FO, in terms of n-3 HUFA score. In figure 18 it is shown that 90g/d of the enriched sheep cheese corresponded to 1 pill and a half of FO or intake of about 170mg/d of a mixture of EPA+DHA.

![Graph showing dose response curve of n-3 HUFA score levels in plasma after fish oil intake. The green dot represents the mean of the n-3 HUFA score level after intake of 90g of CLA and ALA enriched sheep cheese.](image)

In these studies on humans, EC and congeners did not change significantly with any of the treatments (data not shown).
Another nutritional factor that may increase dietary CLA, is supplementation with probiotics and in particular *Bifidobacteria*. In fact, it has been shown that *B. breve* NCIMB 702258 and *B. breve* DPC 6330 are efficient CLA producers, converting up to 65% and 76%, respectively, of linoleic acid to cis-9, trans-11 CLA when grown in 0.5 mg/mL linoleic acid *in vitro* (Barrett et al., 2007, Coakley et al., 2003).

We therefore treated mice with different diets and strains:

1) a control-unsupplemented group fed a standard rodent diet with placebo freeze-dried powder (15% w/v trehalose in drinking water).
2) a flaxseed-oil (FXO) supplemented dietary (containing about 50% of ALA) group with placebo freeze-dried powder (15% w/v trehalose in drinking water).
3) a FXO supplemented dietary group (enriched in ALA) with *B. breve* NCIMB 702258 (approximate daily dose of $10^9$ microorganisms)
4) a FXO supplemented dietary group (enriched in ALA) with *B. breve* DPC 6330 (approximate daily dose of $10^9$ microorganisms)

We used these treatments in order to evaluate whether *B. breve* were able, by producing CLA and CALA, to increase DHA biosynthesis in Liver and Epididymal adipose tissue.

Data on liver lipids, actually showed an increased of DHA biosynthesis, but surprisingly, we found a significantly decrease of CLA (Fig 19 and 20).

![Fig. 19 Changes of n-3 PUFA profile in liver of female mice fed an ALA-enriched diet either alone in combination with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).](image-url)
Fig. 20 Changes of ALA and CALA levels in liver of female mice fed an ALA-enriched diet either alone in combination with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

Fig. 21 n-3 PUFA score in liver of female mice fed an ALA-enriched diet either alone in combination with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
However we found an increase, but not significantly, of CD 16:2 (Fig. 22), a product of CLA peroxisomal beta oxidation (Banni et al., 2004), while the ratio CD 16:2/CLA, biomarker of peroxisomal beta oxidation, increased significantly (Fig. 23).

**Fig. 22** Changes of C16:2 levels in liver of female mice fed an ALA-enriched diet either alone in combination with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

**Fig. 23** Peroxisomal beta oxidation in liver of female mice fed an ALA-enriched diet either alone in combination with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
We also measured 15:0, as a possible elongation product of propionic acid produced by *B. breve*. In the liver we found increased levels in the groups of mice treated with *B. Breve* strains. However, due to the high variability was not statistically significant (Fig. 24).

Fig. 24 Changes of C15:0 levels in liver of female mice fed an ALA-enriched diet either alone in combination with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
On the other hand, in adipose tissue we did find an increase of CLA, CALA and 15:0 (Fig. 25 and 26).

Fig. 25 Changes of CLA and CALA levels in epididymal adipose tissue of female mice fed an ALA-enriched diet either alone or in combination with B. breve NCIMB 702258 or B. breve DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

Fig. 26 Changes of C15:0 levels in epididymal adipose tissue of female mice fed an ALA-enriched diet either alone in combination with B. breve NCIMB 702258 or B. breve DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
The increase in peroxisomal beta oxidation in the liver, however, was not associated to a higher PPAR α gene expression, which actually increase after ALA enriched diet administration, but it decreases when mice were fed with the probiotics. In adipose tissue treatments with *B. breve* strains PPAR α and γ gene expression did not change significantly (data not shown).

![Graph](image)

**Fig. 27** Relative mRNA expression of PPAR α. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

In addition, it was found a decrease of liver lipids (Fig. 28) confirming a previous study (submitted for publication), where it was also found an increase of plasma lipids.

![Graph](image)

**Fig. 28** Changes of total lipids in liver (mg/g tissue) of female mice fed an ALA-enriched diet either alone in combination with B.Breve NCIMB 702258 or B.Breve DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
All these parameters rather than an effect of CLA and relative PPAR α activation may be related to an effect induced by pro-inflammatory cytokines through activation of TLRs (Plantinga et al., 2011).

We therefore analyzed tissue lipids of rats treated with LPS, and we actually found a similar pattern with increased DHA biosynthesis in the liver (Fig. 31) and plasma (Fig. 32), but not in adipose tissue (Fig. 33), while ARA was found increased only in the liver (Fig. 31) Interestingly, to the increase of DHA corresponded a decrease of ALA and EPA (the latter only in the liver) (Fig. 31), and the increase of ARA and c20:3 was accompanied by a decrease of LA and GLA. Altogether these data suggest an induction of desaturation and elongation of n-3 and n-6 PUFAs. We found a decrease of liver and increase of plasma lipids (Fig. 29). TNF-α plasma levels confirmed the induction of inflammation by LPS treatment (Fig. 30).

Fig. 29 Concentration of total lipids in liver and plasma during LPS intoxication. Data represent mean and SD of 6 rats per experimental group. (*) denotes statistically significant differences (p<0.05)

Fig. 30 Plasma concentration of TNF-α during LPS treatment, confirming intoxication. Data represent mean and SD of 6 rats per experimental group. (*) denotes statistically significant differences (p<0.05)
Fig. 31 PUFA profile in liver during LPS intoxication. Data represent mean and SD of 6 rats per experimental group. (*) denotes statistically significant differences (p<0.05)

Fig. 32 PUFA profile in plasma during LPS intoxication. Data represent mean and SD of 6 rats per experimental group. (*) denotes statistically significant differences (p<0.05)
Fig. 33 PUFA profile in visceral adipose tissue during LPS intoxication. Data represent mean and SD of 6 rats per experimental group. (*) denotes statistically significant differences (p<0.05)
In addition, we evaluated whether similar effects on DHA biosynthesis take place in mice after inducing PPAR α, by treating mice with a PPAR α agonist, WY – 14,643, or with CLA. The results clearly show that PPAR α activation or CLA feeding were able to induce DHA biosynthesis, which corresponded also an increase of EPA and decrease of ALA (Fig. 34), and an increase of ARA (Fig. 35), suggesting an increase of delta 6 desaturase, further confirmed by the increase of its mRNA (Fig. 36). As expected, CLA levels increased proportionally to its levels in the diet (Fig.37). Interestingly, we also found a decrease of liver lipids with both treatments (Fig. 38). We did not find any change in plasma (data not shown).

**n-3 PUFAs in Liver**

![Graph showing n-3 fatty acids in liver](image)

Fig. 34 Changes of n-3 PUFA profile in liver of female mice fed 1.5 % CLA or 0.01 % Wy-14,643. Data represent mean and SD of experimental group. Different letters denote significant statistic differences (p<0.05).

![Graph showing ARA levels in liver](image)

Fig. 35 Changes of ARA levels in liver of female mice fed 1.5 % CLA or 0.01 % Wy-14,643. Data represent mean and SD of experimental group. Different letters denote significant statistic differences (p<0.05).
Fig. 36 Induction of \(\Delta 6\) desaturase mRNA by dietary CLA or Wy-14,643 in mouse liver. Different letters denote significant statistic differences (\(p<0.05\)).

Fig. 37 Changes of CLA levels in liver of female mice fed 1.5 % CLA or 0.01 % Wy-14,643. Data represent mean and SD of experimental group. Different letters denote significant statistic differences (\(p<0.05\)).

Fig. 38 Changes of total lipid concentration in liver of female mice fed 1.5 % CLA or 0.01 % Wy-14,643. Data represent mean and SD of experimental group. Different letters denote significant differences (\(p<0.05\)).
As it regards, the EC and congeners, we also evaluated whether supplementation with *B. breve* was able to modify their concentration. We found a significant decrease of AEA and PEA levels after both probiotics supplementation in liver (Fig. 39), while in adipose tissue we found a significant increase of AEA, PEA and OEA levels (Fig. 40).

**Endocannabinoids levels in Liver**

![Graph showing EC levels in liver](image)

**Fig. 39** EC levels in liver of female mice fed an ALA-enriched diet either alone in combination with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).

**Endocannabinoids levels in Epididymal Adipose Tissue**

![Graph showing EC levels in adipose tissue](image)

**Fig. 40** EC levels in epididymal adipose tissue of female mice fed an ALA-enriched diet either alone in combination with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. Data represent mean and SD of experimental group. Different letters denote significant differences (p<0.05).
5. Discussion

Dietary lipids have often been in the centre of debates or as detrimental or beneficial for human health. Usually the “bad guys” are claimed to be the SFAs and the “good ones” the PUFAs, particularly the n-3 family. Rather than attach moral terms to nutritional factors, it should be considered, in an optimal dietary regimen, the balance among all dietary FAs based on the current physiological needs. In addition, most of the studies, in particular the epidemiological ones, rely on the dietary intake assessed by food questionnaires which, has been demonstrated, are poorly reliable (Prentice, 2010). Also in intervention studies, often are not evaluated the changes in plasma and/or RBC FA profile, the larger are the studies the higher are the chances that FA analyses are not carried out due to the relative high costs, hampering the possibility to unravel the controversial data on the nutritional effects of dietary FAs. One of the parameters of n-3 PUFAs status in humans, believed more reliable that have been proposed, is the blood or RBC omega-3 index (Harris, 2008) and/or the plasma n-3 score (Stark, 2008) that have been widely used to evaluate the effect of dietary highly n-3 PUFAs (Harris and Von Schacky, 2004). In fact, plasma and RBC EPA and DHA concentration are generally directly correlated to dietary EPA and DHA (see fig. 18).

Most of the effects attributed to n-3 PUFA family are mainly related to dietary EPA and DHA, while ALA seems to have other beneficial effects disjointed from its putative property as precursor of EPA and DHA (Murru et al., 2013). In fact, ALA has been shown generally to be a poor precursor particularly for DHA, first because is the major beta-oxidised FA in mitochondria (Burdge, 2006), rendering it less available for desaturation and elongation, and second, because biosynthesis of DHA required a crucial step in peroxisome for a partial beta-oxidation (Burdge, 2006). Therefore, any other event that increase desaturases and peroxisomal beta oxidation may also favour DHA biosynthesis. In our studies we found that dietary CLA, in a specific range of ratio with ALA, is able to significantly increase DHA biosynthesis, rendering some food, as for example cheese, particularly if enriched in CLA, an unexpected source of DHA, even though not comparable to the direct intake of EPA and DHA through fish products or supplementation with fish or KOs. In Countries like Italy where the intake of fish products is quite low (Leclercq et al., 2009), far below the recommended daily dose, other nutritional factors may exert a significant impact in increasing the n-3 HUFA score. The effects of dietary EPA and DHA have been mainly associated to beneficial outcome in cardiometabolic and neurological diseases (Murru et al., 2013). The mechanism(s) of action is mainly related to the decrease of ARA in tissue membranes, decreasing the substrate for eicosanoids and EC biosynthesis and forming related n-3 compounds such as resolvins and neuroprotectins (Serhan et al., 2015). Recently the activity of EPA and DHA on the EC biosynthesis has been proposed mainly in alleviating the metabolic syndrome in experimental animals and humans (Banni and Di Marzo, 2010). Recently
we have also shown that the decrease of n-3 HUFA score associated to intake of CLA enriched cheese was able to decrease AEA and improves blood lipid profile (Pintus et al., 2013). Interestingly, we have also recently shown that CLA, in a model of fatty liver, is able to increase liver levels of PEA and OEA (Piras et al., 2015). In our study we found that at the ratio of ALA/CLA that maximize DHA formation, there was also a decrease of AEA and an increase, even though not significant, of PEA and OEA in the liver. However, in adipose tissue we did not find neither an increase of DHA nor a decrease of AEA. Again, also in the human studies we did not detect any change in EC and congeners levels. Our data clearly indicate that metabolic changes by dietary FAs seems to be tissue specific and affected by other factors such as background diet, energy and lipid metabolism and genetic factors. As a matter of fact, we did find changes in AEA by dietary ALA and CLA enriched cheese in hypercholesterolemic overweight subjects (Pintus et al. 2013), or by dietary krill powder in hypertrigliceridemic and obese (Berge et al., 2013) and dietary KO in obese subjects (Banni et al., 2011). Furthermore, we found changes in plasma EC profile in healthy normo-weight subjects in relation to taste sensitivity to 6-n-propylthiouracil (Barbarossa et al., 2013), a phenotype genetically determined. Therefore, relationship between dietary FAs and EC tissue levels may be determined by several nutritional, metabolic and genetic factors.

Based on these data we were prompted to evaluate whether treatment with *B. breve*, possibly by increasing CLA formation, was able to modify tissue FA profile and thereby EC and congeners biosynthesis. We found CLA increased significantly in adipose tissue, despite no change in CLA concentration in the liver probably because it was beta-oxidised in peroxisomes to CD 16:2 as demonstrated by the significant increase of the ratio CD 16:2/CLA, however, we did find an increased formation of DHA. In addition, there was a decrease of liver lipids, suggesting an activation of PPAR α, even though the increase of plasma lipids did not. As a matter of fact, gene expression of PPAR α resulted decrease with *B. breve* intake, and also analysis of the EC revealed a decrease of all the amides, while AEA increased in adipose tissue. These effects suggested to us that treatment with *B. breve* strains may be similar to those triggered by inflammation. In fact, rats challenged with LPS showed similar changes in terms of increased DHA, while total lipids decreased in the liver and increased in plasma. It seems therefore that in our experimental conditions intake of *B. breve* triggers a series of changes probably linked to activation of TLRs (Plantiga et al., 2011), but we cannot rule out that some of the effects might be mediated by CLA, such as increase in adipose tissue of PEA and OEA as shown previously in liver of rats fed CLA (Piras et al., 2015). Interestingly, increase of CLA was evident only in adipose tissue. However, only one strain, *B. breve* NCIMB 702258, seems to be able to enhance PEA and OEA adipose tissue levels, which may suggest that also among strains there might be different metabolic responses. On the other hand, the increase of AEA in adipose tissue might be related to TLRs activation as shown in lymphocytes (Maccarrone et al., 2001), where it has
been shown to down-regulate FAAH. Therefore, these data suggest that concurrent effects of these nutritional factors may result in changes of EC and congeners profile. This may well represent what it occurs in humans with multiple challenges that may contribute to the changes in FA metabolism and thereby in EC biosynthesis. Interestingly, we also found an increase of c15:0 in adipose tissue of B. breve treated mice, which may derive from elongation of propionic acids produced by B. breve (Floch, 2010). In our experimental conditions it appears that part of propionic acid is elongated up to 15:0. This may be used as a marker of B. breve viability after ingestion. Whether 15:0 possesses specific nutritional activity is not known. It has been used as marker of intake of dairy products since it is as well produced in the rumen (Smedman et al., 1999). It has been claimed that plasma 15:0 and 17:0 are negatively correlated to cardiovascular diseases (Chowdhury et al., 2014). However, it is not possible to determine whether it is related to changes in the microbiota and/or to intake of dairy products. Targeted experiments should be carried out to evaluate the specificity of these FAs as markers of specific nutritional factors.

In the last 50 years the focus on dietary lipids has been mainly devoted to the detrimental effect of dietary SFA, because of its ability to increase cholesterolemia, inflammation, insulin resistance and it has also been claimed to predispose for obesity and related metabolic disorders (Kennedy et al., 2009). Our data, on the other hand, suggest that balance between HUFAs of the n-3 and n-6 families may play a crucial role, and this balance may be modified not only by controlling their dietary intake, but also other factors may well influence this balance such as CLA, gut microbiota and inflammatory status. One can therefore speculate that excessive dietary intake of SFA may be detrimental because of not sufficient intake of the other dietary FA families or in determining a wrong balance among the different type of dietary FAs. Interestingly, it has been shown that dietary FAs may modify gut microbiota in mice (Caesar et al., 2015), with dietary SFA aggravating adipose tissue inflammation through TLR signalling mediated by changes in gut microbiota, while transferring microbiota from fish oil-fed mice dampens dietary SFA-induced inflammation (Caesar et al. 2015). Therefore, the detrimental effects of dietary SFA may not be due to a direct effect of tissue SFA on TLR pathway, as previously suggested in coculture of adipocytes and macrophages (Suganami et al., 2007), also because not dietary SFA, but rather dietary carbohydrates, has been shown to increase significantly SFA levels in human plasma via de novo lipogenesis (Volk et al., 2014). Therefore, several variables influence SFA nutritional activity, rendering difficult to establish their optimal dietary intake (Chowdhury et al. 2014), without taking into account the other dietary components.

Furthermore, our data suggest that the major 3 n-3 PUFAs, ALA, EPA and DHA are crucial nutrients to create the optimal environment to respond adequately to metabolic or inflammatory challenges, and can influence, under a metabolic point of view, the beneficial effects of probiotics.
From our studies emerge that synergistic effects of different nutritional factors, as it occurs in daily life in humans are somewhat difficult to predict and may explain the contradictory results in the literature on the effects of dietary FAs (Chowdhury et al. 2014). The discover that gut microbiota may directly interfere and modify FA metabolism opens to new nutritional strategies in shaping the optimal milieu in several metabolic disorders, acting on the EC system.

There is a wide consensus on the beneficial properties on human health by probiotics. Our studies however indicate that background diet, along with other nutritional metabolic and genetic factors, may play an important role in modulating FA metabolism and thereby EC biosynthesis. In addition, as recently reviewed (Cani et al., 2015), other factors such as gut-barrier function and the possibility to modulate intestinal integrity by manipulating the ECS may open to novel mechanisms by which gut microbiota and ECS interact.

Future studies will be devoted to evaluate in humans the possible synergistic effects of CLA-enriched products with *B. breve*: i) in maintaining and/or re-equilibrating energy and lipid metabolism, by modulating EC and congeners tissue profile, ii) acting on energy metabolism and EPA and DHA related molecules, and iii) to assess if there is an optimal level of dietary EPA and DHA to allow a synergistic activity.

This nutritional approach may help in particular metabolic disorders as those causing fatty liver, where CLA (Piras et al.,2015), n-3 PUFAs (Batetta et al., 2009, Berge et al., 2013) and manipulation of the gut microbiota (Le Roy et al., 2013) have been shown to decrease liver fat accumulation.

We may conclude that it is not scientifically correct to focus on a single class of dietary FAs, but several factors may influence their absorption deposition and fate as precursors of bioactive molecules that can in turn affect lipid and energy metabolism. It is also emerging a crucial role of the gut microbiota and the possibility to modify it by using pre and probiotics, opening a novel and extensive area of research in nutrition.
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