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Structure and cardioprotective activities of polar lipids of olive pomace, olive pomace-enriched fish feed and olive pomace fed gilthead sea bream (*Sparus aurata*)

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ABSTRACT

Total lipids of olive pomace (OP), olive pomace diet (OP diet), fish oil diet (FO diet) and fish filets of farmed gilthead sea bream (fish fed with FO diet and OP diet respectively) were extracted and separated into polar (TPL) and neutral (TNL) lipids. All samples were assessed for their in vitro activity against washed rabbit platelets aggregation induced by platelet activating factor (PAF) and they were further analyzed by electrospray-mass spectrometry. The high levels of palmitic (16:0), oleic (18:1 cis ω−9), linoleic (18:2 ω−6) and docosapentaenoic acid (DPA 22:5 ω−3) contained in both OP and FO diets are reflected to the gilthead sea breams fed with the individual diet respectively, while the gilthead sea bream fed with FO diet displays a decrease in DPA. All samples contained various glycerophospholipids species. Two PE species were identified in OP, OP diet and fish fed with OP diet and not in FO diet, while that might be an indication that these substances are likely to be the key polar phospholipids that have the ability to be in vitro PAF inhibitors, i.e. inhibit the formation of atherosclerotic plaques in blood arteries.

1. Introduction

The requirement within the aqua feed industry to find and implement sustainable alternatives to fish oils (FO) is gradually increasing. Mostly due to the fact that the worldwide availability of fish, which is the main source of fishmeal and FO, is expected to remain stable or even to decrease in future decades. Meanwhile the consumption of fish and fish-derived products has considerably increased over recent decades partly due to their omega-3 (ω−3) fatty acids that confer benefits for the risk reduction of cardiovascular disease (CVD) (Hu et al., 2002; Levitan, Wolk, & Mittleman, 2009; Mozaffarian, 2008), making them very important components of adult nutrition, while there is an evidence that fish oil has the ability to decelerate the formation of plaque in the arteries (Wang et al., 2006). The use of FO in aquaculture is the key impediment on the future growth and sustainability of the industry. FO, being the most widely available dietary source of health-beneficial omega-3, long-chain polyunsaturated fatty acids (ω−3 LC-PUFA), ranges remarkably in supply and cost. The most severe product from a food security point of view, is that FO is extracted unsustainably from world oceans (Bimbo, 2015). As a result, in aquaculture today, we are facing this paradox: for the production of FO we need wild sardines whose overfishing is probably not sustainable. Current trends in aquaculture are now towards the lowering of our dependence on FO; research is now thus focusing towards alternative oil-sources in global level, in order to reduce the aquaculture industry's dependence on FO and the corresponding overfishing of sardines' stocks.

The state of the world fishery resources, which provides the raw material for fish meal and fish oil production, has been a bone of contention in many quarters (De Silva, Francis, & Tacon, 2011). Recent data show that the proportion of fisheries production used for direct human consumption increased from about 71% in the 1980s to more than 86% (136 million tons) in 2012, with the remainder 14% (21.7 million tons) destined to non-human food uses (FAO (Food and Agriculture Organization), 2014) (e.g. fishmeal and fish oil). FO from small pelagic fish represents a finite fishery resource and is not expected to be able to sustain the rapid expansion of the global aquaculture industry. The overwhelming evidence that ω−3 LC-PUFA have a significant impact on

Abbreviations: OP, olive pomace; FO, fish oil; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; TL, total lipids; TPL, total polar lipids; TNL, total neutral lipids; PL, phospholipid; TAG, triacylglycerol; DAG, diacylglycerol; FAME, fatty acid methyl ester; PAF, platelet activating factor; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; IC_{50}, inhibitory concentration at 50%; HPLC-UV, high performance liquid chromatography-ultra violet; GC-FID, gas chromatography-flame ionization detector; GC–MS, gas chromatography–mass spectrometry; ES-MS, electrospray-mass spectrometry; CVD, cardiovascular disease.

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many aspects of human health means that lipids/fatty acids derived from fish have an important role in consumer perception. This is the reason why managing the lipid resources that we use in fish aquaculture more sustainably is vital for the future of aquaculture production, as well as consumer perception that it is a product with health benefits.

Therefore, over the past few years there is a currently great urgency within the aqua feed industry for identifying and using alternative plant-derived oils in formulated fish feeds in order to reduce dependence upon marine FO as well as to reduce costs (Alexis, 1997; Izquierdo et al., 2005; Nasopoulou & Zabetakis, 2012; Wassef, Saleh, & El-Abd El-Hady, 2008). The most common vegetable oils used previously for fish feed production have been soybean, linseed, rapeseed, sunflower, palm oil and olive oil. Partial replacement of FO by these vegetable oils is only possible when the fatty acids present in the diets in sufficient quantities to meet the essential fatty requirements of the fish and ultimately the human.

A promising alternative plant lipid source is olive pomace (OP), which is a natural agricultural by-product of olive oil production, that contains constituents with atheroprotective activity such as PAF (platelet activating factor) inhibitors (Demopoulos, Pinckard, & Hanahan, 1979; Karantonis et al., 2008; Nasopoulou & Zabetakis, 2013) according to "The PAF implicated atherosclerosis theory"(Demopoulos, Karantonis, & Antonopoulou, 2003). Extensive research has been carried out in our laboratory on olive oil by-products and fish feeds supplemented with these products, with respect to their capacity to prevent or delay the process of atherogenesis and thus prevent the subsequent development of cardiovascular diseases (CVDs). Therefore, the presence of PAF-inhibitors or PAF-antagonists in OP highlights the importance of this resource in terms of cardio-protection. In addition, olive oil (Karantonis et al., 2006) and olive pomace polar lipids (Tsantila et al., 2007) possess in vivo antiatherogenic properties, while a separate in vivo study (Nasopoulou et al., 2010) confirmed that phospholipids from gilthead sea breams (*Sparus aurata*) can reduce the thickness of atherosclerotic lesions in hypercholesterolemic rabbits. In addition, it has been demonstrated that, the feeding of OP supplemented fish feeds to fish results in an improvement in its ability to prevent atherogenesis and therefore heart diseases (Nasopoulou, Stamatakis, Demopoulos, & Zabetakis, 2011).

Taking into consideration the benefits of fish oil replacement by alternative plant sources in fish feeds as reviewed recently by our team (Nasopoulou & Zabetakis, 2012) and the capacity of OP to prevent atherogenesis and therefore the onset of CVDs, our group has further focused to the use of OP in fish feeds. The effect of OP in fish feed (OP-diet) and in gilthead sea bream muscle (*S. aurata* fed with OP-diet) by HPLC-UV (Nasopoulou et al., 2013) has been studied. In the current paper, we have extended that work by carrying out ES-MS–MS lipidomic analysis of OP, OP-diet and gilthead sea bream fed with OP-diet with the scope to identify in fish feed and fish anti-atherogenic compounds of OP origin. Such data would suggest to upscale OP usage in the sustainable production of aquacultured gilthead sea bream (*S. aurata*); a fact that would lead to the improvement of the aquatic food security.

2. Materials and methods

2.1. Reagents and instrumentation

All reagents and solvents were of analytical grade and purchased by Merck (Darmstadt, Germany). Fatty acid methyl ester (FAME) standards were of gas chromatographic (GC) quality and supplied by Sigma-Aldrich (St. Louis, MO, USA), as well as bovine serum albumin (BSA) and PAF (1-O-alkyl-2-acetyl-sn-glycero-3- phosphocholine). Platelet aggregation was measured in a Chrono-Log (Havertown, PA, USA) aggregometer (model 400-VS) coupled to a Chrono-Log recorder (Havertown, PA, USA). The lipidomic analysis was conducted with an Absceix 4000 QTrap, a triple quadrupole mass spectrometer equipped with a nanoelectrospray source while the FAME products were analyzed by gas chromatography-flame ionization detector (GC-FID) on an Agilent Technologies J&W with a DB-23 fused silica capillary column (60 M × 0.251 mm i.d., 0.25 μm; Agilent, Santa Clara California, USA) and by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies (GC-6890 N, MS detector-5973) with a ZB-5 column (30 M × 0.25 mm i.d., ×0.25 μm film thickness, Phenomenex).

2.2. Fish diets analysis

Olive pomace (OP), the experimental fish feed enriched with OP (OP diet), compounded by adding 8% OP prior to the extrusion, following the principles of fish nutrition (Gatlin, 2010) and the reference fish feed – fish oil diet (FO diet) – containing 100% fish oil (anchovy oil), were analyzed for a number of nutritional parameters. Protein digestibility determination took place according to van Leeuwen, Kleef, Kempen, Huisman, and Verstegen (1991) (Leeuwen et al., 1991) and energy determination took place according to the following equation (Gatlin, 2010):

$$\text{Energy (MJ/kg)} = \{(\text{CPg} * 23.6\ \text{kJ}) + (\text{CFg} * 39.5\ \text{kJ}) + ([\text{CFig.} + \text{NFEg}] * 17.4\ \text{kJ})\}/1000$$

where CP: crude protein; CF: crude fat; CFi: crude fiber; NFE = 1000 − (CP + CF + Ash + Moist).

OP originated from a local olive oil production. Both diets were formulated at the facilities of the marine farm where the dietary experiment took place using a twin-screw extruder creating pellets, followed by the addition of oil mixture. The pellets were dried, sealed and kept in air-tight bags until use. The analysis of OP and both diets is given in Supplemental Table 1.

2.3. Fish sampling

Cultured gilthead sea bream (*S. aurata*), raised with different diets and of initial mean body weight 350–400 g were obtained from a commercial marine farm located in the suburbs of Athens. In total, two types of samples were used (gilthead sea breams fed with OP diet and FO diet). Approximately 100 fish specimens were collected per each dietary treatment and transported in ice to the laboratory. The mass of 30 fish samples was weighted and 5 of them were beheaded, chopped and filleted and only the filets were used in the subsequent experiments.

2.4. Isolation of lipids extracts

Total lipids (TL) of OP, OP diet, FO diet and fish filets of aquacultured gilthead sea bream fed with OP and FO diet, were extracted according to the Bligh–Dyer method (Bligh & Dyer, 1959). One tenth of the TL was weighed and stored at −20 °C while the rest of it was further separated into polar lipids (PL) and neutral lipids (NL) by counter-current distribution (Galanos & Kapoulas, 1962). The upper phase of petroleum ether contained the total neutral lipids while the lower phase of ethanol with total polar lipids were selected in a glass-stoppered flask, evaporated at 30 °C on the rotary evaporator, weighted, dissolved in chloroform/methanol (1:1), and stored at −20 °C until further analysis.

2.5. Biological assay

Purified polar lipid fractions of OP, OP diet, FO diet and fish filets of aquacultured gilthead sea bream fed with OP and FO diet respectively, were tested for their biological activity according to the washed platelet aggregation assay (Demopoulos et al., 1979). PAF as well as the examined samples were dissolved in 2.5 mg of bovine serum albumin (BSA) mL^{-1} of saline. The inhibitory activity of TPL was expressed as IC_{50} value (in μg of TPL) against PAF-induced aggregation (Nasopoulou, Nomikos, Demopoulos, & Zabetakis, 2007) (10^{-8} mol/L final concentration in the cuvette).