

Ana Sousa Ramos Ramalho Ribeiro de Magalhães Sant'Ana

# Farmed fish as a functional food: fortification strategies with health valuable compounds

Doutoramento em Ciências do Mar, da Terra e do Ambiente

Ramo de Aquacultura

Especialidade Nutrição



2019



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UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2019



## Farmed fish as a functional food: fortification strategies with health valuable compounds

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## Summary

The current trend in the aquaculture industry of replacing fish meal and fish oil by alternative ingredients is reducing the levels of n-3 LCPUFA and microelements such as iodine and vitamin D<sub>3</sub>, of which fish is the main natural food source. Exogenous feeding in aquaculture unlocks the possibility to tailor fish composition with healthy beneficial nutrients improving quality and nutritional value of edible fish. An on-line survey to inquire about the concept of fish fortification (**chapter 2**) showed that half of respondents agreed with it and were willing to consume fortified fish. Respondents less interested on fish origin (wild vs aquaculture) were more receptive to fortification. Subsequently, several experimental trials were undertaken targeting the muscle fortification of gilthead seabream and rainbow trout with health beneficial compounds. Seabream and trout were able to accumulate iodine and selenium in muscle as response to a dietary fortification of these trace minerals, supplied from distinct sources (**chapter 3**). At similar supplementation levels, the maximum fillet iodine content found in seabream was higher than that found in trout which is probably associated with the physiological needs for homeostasis of freshwater and marine fish. Efficacy of dietary fortifications aiming to raise iodine concentration in fish muscle may be more dependent on the targeted fish species than on the iodine source used. In trout, an effective muscle fortification with selenium was achieved with a selenised yeast supplemented diet. Fortification of fish muscle with vitamin D<sub>3</sub> via dietary supplementation is apparently a more complex process, showing variable results (**chapter 4**). Raising dietary vitamin D<sub>3</sub> from a basal level to the maximum permitted level did not significantly enhance fillet vitamin D<sub>3</sub> contents. In **chapter 5**, a study was undertaken to assess the potential of microalgae biomass, from the diatom *Phaeodactylum tricornutum*, as a functional ingredient in seabream feeds. Results showed that algae-fed seabream positively improved external appearance, originating a more vivid yellow pigmentation of the operculum and a lighter colouration of ventral skin. Moreover, an untrained consumer panel showed a clear preference for algae-fed fish when compared with those fed a commercial diet. Marine-derived raw materials traditionally used in aquafeeds are scarce resources, mainly fish oil rich in n-3 LCPUFA. To tackle the low retention of dietary EPA and DHA in fish, our work

focused on a nutritional strategy to foster the metabolic sparing of n-3 LCPUFA in gilthead seabream fillets (**Chapter 6**). The fatty acid composition of tissues is not solely dependent on the quantitative fatty composition of feeds but is also affected by their relative ratios. The initial fatty acid composition of fish seems to play a significant role on the EPA and DHA sparing efficiency. A higher MUFA intake led to a significant increase on EPA and DHA retention in fish with a vegetable nutritional background. Retention of combined EPA and DHA in seabream fillets was relatively low, with DHA retention generally more than double of EPA.

The use of natural/organic compound sources in fortification trials were equally or more efficacious than the synthetic ones and should be considered adequate alternatives. In the various fortification trials, the nutritional contribution of trout and seabream fillets (160 g serving) to the daily adequate intake of combined EPA and DHA was well above the recommended levels. The serving amount of trout fillets could contribute to 98% of selenium, over 100% of vitamin D<sub>3</sub> and 13% of iodine Daily Recommended Intakes, respectively. In turn, the consumption of 160g of seabream fillets would cover 60% of vitamin D<sub>3</sub> and 84% of iodine Daily Recommended Intake levels. The fortification strategies enhanced the nutritional contribution of seabream and trout fillets in health beneficial compounds resulting in novel products, and thus resulting in potential candidates to the functional foods market. Throughout our work we contributed towards the generation of new data on the efficacy of feed fortification strategies as tools to modulate several traits of farmed fish.

Keywords: Fish nutrition; Feed fortification; Fish quality; Nutritional contribution; Consumers' perception; Functional foods

## Resumo

A actual tendência na indústria de aquacultura para substituir a farinha de peixe e o óleo de peixe por fontes alternativas tem reduzido os níveis de n-3 LCPUFA e microelementos, como iodo e vitamina D<sub>3</sub>, dos quais o pescado é a principal fonte alimentar natural. A utilização de dietas compostas permite modular a alimentação dos peixes com nutrientes benéficos para a saúde de forma a melhorar a qualidade e o valor nutricional do pescado. A aplicação de um questionário on-line sobre o conceito de fortificação (**capítulo 2**) revelou que metade dos entrevistados concorda com a fortificação de peixes e estaria disposta a consumir peixe fortificado. Os entrevistados que mostraram um menor interesse em conhecer a origem do pescado (i.e. selvagem vs aquacultura) foram os mais receptivos ao conceito de fortificação. Seguidamente, foram realizados vários ensaios experimentais tendo como principal objectivo a fortificação dos músculos de dourada e de truta arco-íris com compostos benéficos para a saúde humana. Verificou-se um aumento da concentração de iodo e selénio no músculo de dourada e truta como resposta a uma fortificação desses minerais, provenientes de fontes distintas (**Capítulo 3**). Utilizando níveis semelhantes de suplementação, verificou-se que o teor máximo de iodo no filete de dourada foi maior do que o de truta, o que provavelmente estará associado às diferentes necessidades fisiológicas de homeostasia entre peixes de água doce e marinhos. A eficácia da suplementação alimentar para aumentar a concentração de iodo no músculo dos peixes poderá estar mais dependente da espécie do que da fonte de iodo utilizada. Na truta, obteve-se uma fortificação eficaz de selénio no músculo, após a utilização de uma dieta suplementada com levedura selenizada. A fortificação do músculo de peixes com suplementação alimentar de vitamina D<sub>3</sub> é, aparentemente, um processo mais complexo apresentando resultados variáveis (**capítulo 4**). O aumento de vitamina D<sub>3</sub> na dieta, de um nível basal para o nível máximo permitido, não resultou num aumento significativo da concentração de vitamina D<sub>3</sub> no músculo. No **capítulo 5**, discutem-se resultados de um estudo para avaliar o potencial da biomassa de microalgas, recorrendo à diatomácea *Phaeodactylum tricornutum*, como ingrediente funcional em alimentos para dourada. Observou-se que a utilização de microalgas na dieta permitiu tornar mais apelativa a aparência externa dos peixes,

originando uma pigmentação amarela mais viva no opérculo e uma coloração mais clara na região ventral. Além disso, uma avaliação visual realizada por um painel não treinado, mostrou uma clara preferência pelos peixes alimentados com microalgas comparativamente aos alimentados com uma dieta comercial. As matérias-primas provenientes de espécies marinhas, tradicionalmente usadas em alimentos para aquacultura, são recursos escassos, em particular o óleo de peixe rico em n-3 LCPUFA. De forma a estudar a baixa retenção de EPA e DHA alimentar pelos peixes, desenvolveu-se uma estratégia nutricional para promover a retenção metabólica de n-3 LCPUFA no músculo de dourada (**Capítulo 6**). A composição em ácidos gordos nos tecidos não depende apenas da concentração em gordura dos alimentos, mas é influenciada pelas suas proporções relativas. A composição inicial em ácidos gordos no peixe parece desempenhar um papel importante na eficiência de retenção de EPA e DHA. Observou-se um aumento significativo da retenção de EPA e DHA com a ingestão de MUFA em peixes inicialmente alimentados com uma dieta à base de ingredientes vegetais. A retenção de EPA e DHA no músculo de dourada foi relativamente baixa, sendo a retenção de DHA, geralmente, mais do dobro de EPA.

Em conclusão, relativamente a ensaios de fortificação, a utilização de compostos a partir de fontes naturais e/ou orgânicas foi considerada igualmente ou mais eficaz do que a partir de fontes sintéticas. Nos diversos ensaios de fortificação realizados verificou-se que o contributo nutricional dos filetes de truta e de dourada (dose de 160 g) para uma Ingestão Diária Adequada de EPA e DHA foi acima dos níveis recomendados. A mesma dose de filetes de truta tem um contributo de 98% em selénio, mais de 100% em vitamina D<sub>3</sub> e 13% em iodo, para a Dose Diária Recomendada. O consumo de 160g de filetes de dourada cobre 60% em vitamina D<sub>3</sub> e 84% em iodo dos níveis da Dose Diária Recomendada. As estratégias de fortificação promoveram o aumento do contributo nutricional dos filetes de truta e de dourada, em compostos benéficos para a saúde, resultando em novos produtos, potencialmente candidatos ao mercado de alimentos funcionais. Ao longo do nosso trabalho, foi possível gerar novos conhecimentos sobre a eficácia de fortificação de rações como ferramentas para modular características nutricionais e de qualidade do pescado de aquacultura.

Palavras-chave: Nutrição de peixes; Suplementação de alimentos; Qualidade do pescado;  
Contributo nutricional; Percepção do consumidor; Alimentos funcionais



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# GENERAL INTRODUCTION

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## Chapter 1



## 1.1. Introduction

The world is constantly evolving, new technologies are designed, and industrial processes are becoming more efficient. In food sector, new ingredients are produced, and novel methods are developed every day, responding to growing needs of world population. The fisheries sector presents no exception to this development; however, it is restricted by the limited availability of natural marine and freshwater resources. As world population grows, aquaculture production is expected to respond to the increasing demand towards fish and seafood products and therefore needs to tackle the issues adjacent to this rapid increase. The aquaculture industry, and in particular the fish farming of carnivorous fish, is dependent on marine derived ingredients extracted from wild fish stocks. The efficient use of these limited marine ingredients, mainly fish meal (FM) and fish oil (FO), is a huge and urgent challenge. The partial substitution of FM and FO in the manufacture feed sector by vegetable ingredients is already acceptable as a current practice without major impacts on animal growth performance. However, concerns arise with the effects of FM and FO substitutions on the nutritional value of fish muscle and concomitant beneficial nutrients for human diets. Food and nutrition science have moved from identifying and correcting nutritional deficiencies to designing foods that promote optimal health and reduce the risk of disease. Food has a direct implication on the human health and well-being with scientific evidences on the relation between food and health emerging at a rapid pace. Lately, an important research focus applied on personalized nutrition has been revealing a better understanding of the relation between food and gene expression, showing the magnitude of the nutritional effects on the functioning of human organisms.

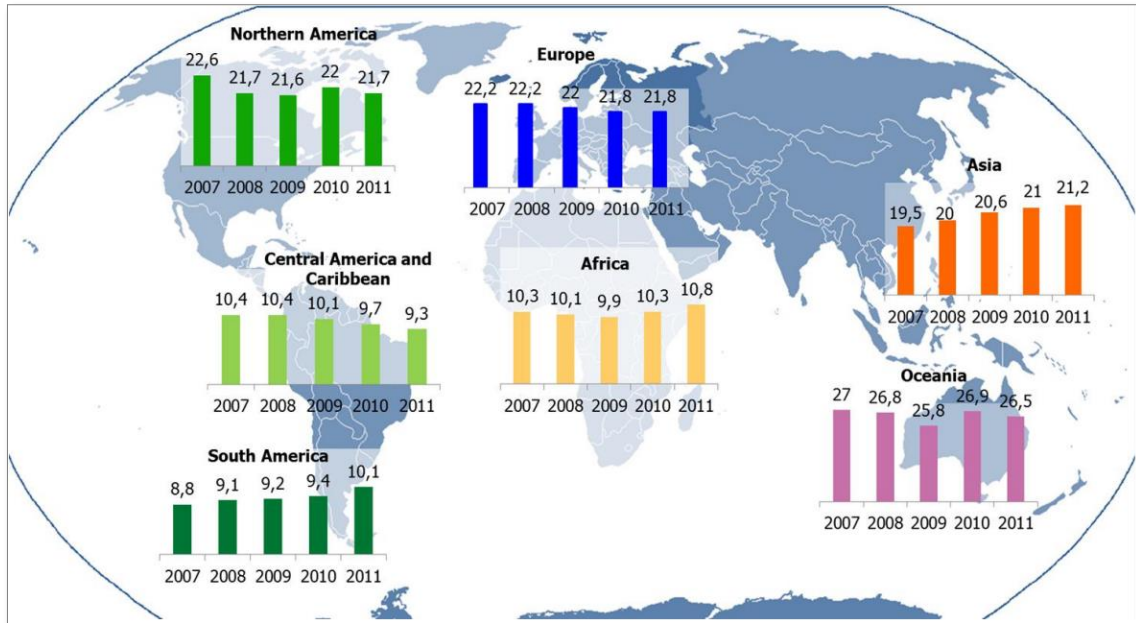
Eating is frequently a hedonistic and social act. However, consumers are becoming more aware of food beneficial effects and hazards and care for a balanced and nutritious diet. The impressive availability of different foodstuff and the increasing knowledge of food traits are leading consumers to have a more active role regarding their diets. Fish and seafood are generally considered nutritious products and contributing to a healthy eating behaviour. As stated by FAO (2014): “All foods have benefits and risks associated with their consumption, but very few foods provide the benefits to the same levels as do fish products”. Within this

context, aquaculture has a prominent role contributing for a better nutrition by improving fish as food for human consumption.

## 1.2. Seafood consumption trends and contribution to human dietary patterns worldwide: differences by regions

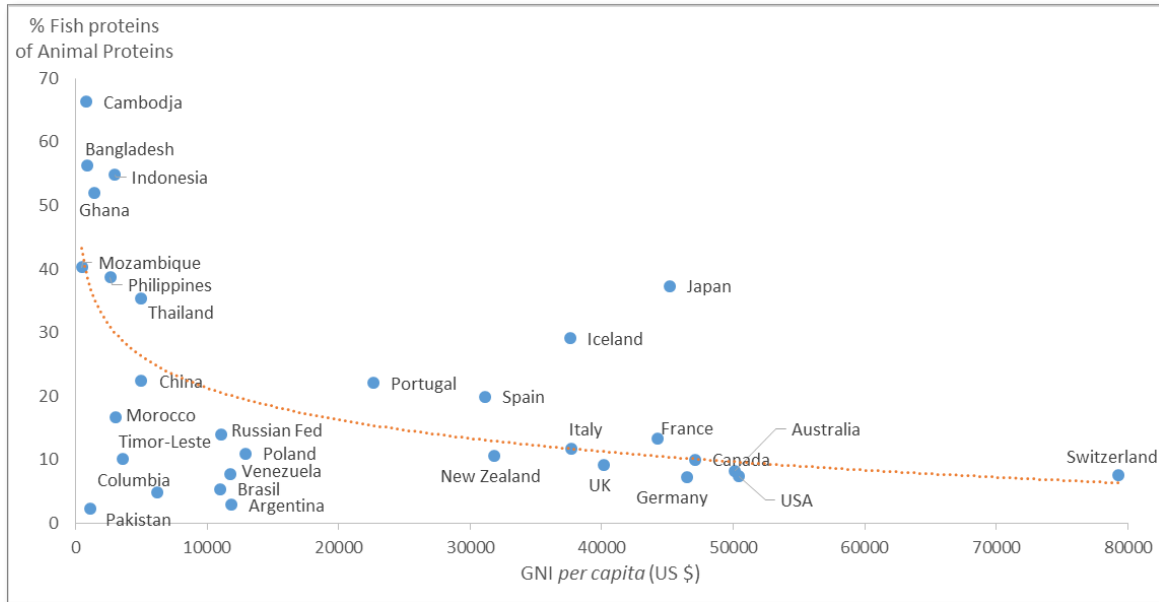
Fish is consumed worldwide. According to FAO's most recent data, in 2016, 170.9 million tonnes of fish as food were produced in the world and an apparent *per capita* consumption is estimated to have reached 20.3 kg in 2016 (FAO, 2018a). Twenty-five years earlier, in 1990, world production was 97.8 million tonnes, and *per capita* consumption nearly 13,3 kg/year (FAO, 2018b; FAOSTATS, 1990).

Fish as food supply have been steadily increasing mainly owed to aquaculture expansion and consumers demand having, since the year 2000, outpaced the world population growth. Fish and seafood are valuable sources of animal protein and the contribution of fish for global animal protein intake was 16.9 percent in 2013 and 6.7 percent of all plant and animal protein consumed (FAO, 2018b). However, fish distribution and availability have been uneven, the lowest supply is observed in Africa and two-thirds of the total fish supply is accounted in Asia, where China is responsible for the highest *per capita* growth. In developed regions fish consumption values have been oscillating with a tendency to decrease (fig. 1.1). FAO prospects (2014) on fish as food consumption refer an increase in all Continents except in Africa since supply will not be able to cope with population growth. In many developed countries consumption will show only a minor growth and developing countries are expected to have 91 percent of total fish consumption increase. In fact, African and Asian countries, where total protein intake are respectively 67 and 78 g/per capita/day, are much more dependent on aquatic animal protein (18.4% and 23%) than industrialized countries (e. g. 7.5% in Northern America) where protein consumption is over 109 g/per capita/day (FAO, 2018b). It is generally observed that the percentage of animal protein, within total protein consumed, increases with countries income. However, apart from Japan, this increase is mostly owed to the intake of terrestrial animal proteins.



**Figure 1.1.** Fish consumption per capita in the world (kg, 2007 – 2011) (from EUMOFA, 2015)

As observed in figure 1.2, in 2011, fish represented a very important parcel, over 50 percent, of total animal protein intake in several Asian low-income countries like Indonesia or Bangladesh and over 40 percent in Philippines, whereas in Australia, USA and some European countries fish intake accounted less than 10 percent of the protein intake (fig. 1.2). Particularly in the EU, the 2011 scenario was similar, the consumption of proteins from fish and seafood was, on average, 7 percent of total protein intake (EUMOFA, 2015).



**Figure 1.2.** Percent of fish protein of animal protein by countries per capita Gross National Income (GNI). Source: FAO (2012) and World DataBank (data from 2011)

Adapted from Jensen, 2006.

Fish consumption patterns vary among populations and are largely related with resource endowment and culture more than with income levels (Jensen, 2006). In developing populations, where diets are highly dependent on staple foods, fish consumption is driven by supply and seasonally available products. Moreover, the regular intake of fish is crucial to correct imbalanced diets at caloric, protein and mineral levels (FAO and WHO, 2011). In developed countries, as Europeans, the consumption of fish products is driven by consumers demand and is influenced by several social factors like habits, children in the household, convenience, age, and education (Myrland et al., 2000; Olsen, 2003; Olsen et al., 2007).

### 1.3. Aquaculture role in food supply

Aquaculture has been the fastest growing animal production industry in the world. In the 1980s and 1990s showed high annual growth rates (10.8 and 9.5); between 2001 and 2016 the annual average rate declined to 5.8 percent, yet aquaculture continues to grow faster than other major food production sectors (FAO, 2018a). In 2015, aquaculture accounted 46 percent of total fisheries production, and since 2014, aquatic farmed products for human

consumption overtook fish food supply from wild fisheries, for the first time (FAO, 2015), as illustrated in figure 1.3. Prospects are for aquaculture production to surpass total capture fisheries in 2023 (OECD - FAO, 2015).

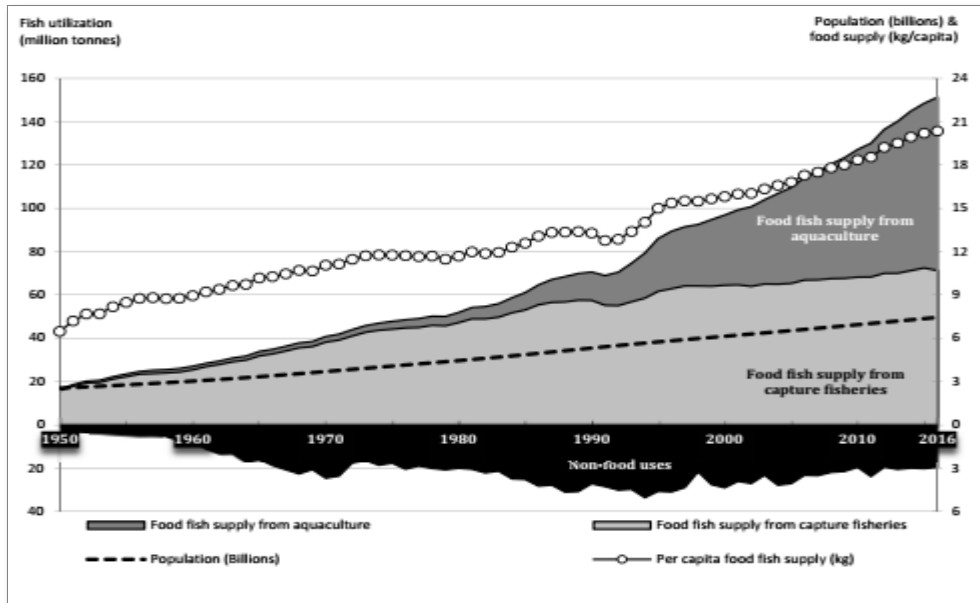


Figure 1.3. World fish utilization and supply (retrieved from FAO, 2018b)

Aquaculture expansion and the shift towards farmed products are also dictating a change in the species consumed. Aquaculture sector plays a prominent role on the future of fish as food consumption and dietary diversification. Increased production volumes usually decrease market prices and intensify commercialization, this was the case of salmon, shrimps, bivalves, tilapia, and catfish (*Pangasius spp.*) (FAO, 2014a). In 2016, excluding aquatic plants, 558 species were registered by FAO as cultured worldwide, reaching 80 million of produced tonnes and 231.6 billion dollars (FAO, 2018a). However, data from 2014, showed that only 10 species groups accounted for almost 90 percent of all production and 87 percent of trade value of farmed animal, being carps and ciprinids; shrimp and prawns; and salmon and trouts the most valuable species groups and carps and ciprinids the largely produced group (FAO, 2014b).

Population growth, urbanization, globalization, new lifestyles and markets have been leading to great changes in the food sector influencing consumption patterns. Convenience, variety, safety and health concerns are becoming increasingly important during consumers buying

decisions. Fish and seafood are no exception, however, factors such as the presence of bones and the time consumed in household procedures are considered undesirable fish characteristic on nowadays standards of life when fewer time is spend in food preparations (Brunso et al., 2009). Fishery products consumption will increase along with a thriving market of new ready-to-eat and convenience products, mainly supplied by the aquaculture industry, though the array of available fish species as exists nowadays may shrivel.

#### 1.4. Nutritional value of fish

Fish and seafood are generally considered nutritious food and associated to healthy dietary behaviours. Despite the large variations observed among species, aquatic animal food product is one of the most rich and nutritive food sources in terms of nutrient composition, as compiled by Tacon and Metian (2013). The main chemical components are protein and lipids whereas carbohydrates are present usually at low levels. Fish protein is highly digestible with high biological value, containing significant amounts of available essential amino acids in well balanced proportions. Usually, lysine and leucine in the essential, and aspartic and glutamic acids in the nonessential are the most abundant amino acids, being often the content of essential amino acids greater than in standard protein food sources (Nunes et al., 2010). Usually, fish are leaner than red meats, with lower saturated fat content and lower caloric density (Tacon and Metian, 2013).

**Table 1.1.** Protein and fat contents, fatty acids groups and energetic value in 100 g of selected food products

	Protein (g)	Fat (g)	SFA (mg)	MUFA (mg)	PUFA (mg)	PUFA $\omega$ 3 (mg)	PUFA $\omega$ 6 (mg)	Energetic value (kcal/kJ)
<b>Meat and egg</b>								
Chicken	20.3	2.7	656	816	627	70	557	110/462
Egg	12.0	9.4	2850	4000	1390	69	1320	136/571
Pork loin defatted	19.0	13.7	5050	5550	1390	115	1270	199/833
<b>Lean Fish</b>								
Desalted Atlantic cod	19.0	0.4	68	53	139	128	11	85/355
European hake	17.0	0.7	143	110	273	247	27	119/498
Meagre	20.4	1.4	342	465	234	386	10	100/417
<b>Moderately fatty fish</b>								
Farmed gilthead seabream	17.8	8.3	1674	2867	2763	1468	1295	202/844
Farmed seabass	19.8	7.9	1439	2467	2734	2016	718	116/486
Rainbow trout	18.3	7.3	1540	2740	2020	1750	267	139/581
<b>Fatty fish</b>								
Sardine	17.9	10.9	2746	2558	4071	3753	318	187/783
Atlantic salmon	16.2	21.9	4291	10037	5148	4326	766	267/1116
European eel	13.4	27.7	8604	2369	13296	12254	1042	307/1285

Sources: Nunes et al., 2006 and Danish Food Composition Databank (<http://frida.fooddata.dk/?lang=en>, accessed on August 9<sup>th</sup>, 2016). Note: Values are indicative for each species and may vary depending on the source.

**Table 1.2.** Fatty acids and cholesterol content in 100 g of selected food products (mg)

	Palmitic acid 16:0	Oleic acid 18:1	Linoleic acid 18:2 $\omega$ 6	Alpha- linoleic acid 18:3 $\omega$ 3	Arachidonic acid 20:4 $\omega$ 6	EPA 20:5 $\omega$ 3	DHA 22:6 $\omega$ 3	Cholesterol
<b>Meat and egg</b>								
Chicken	447	687	477	20	80	10	30	65
Egg	2060	3590	1170	68	152	1	0	359
Pork loin defatted	3050	4,62	1270	17	0	0	0	62
<b>Lean Fish</b>								
Desalted Atlantic cod	51	38	4	1	8	35	86	52
European hake	90	55	7	3	12	66	155	19
Meagre	160	257	5	3	0	157	153	50
<b>Moderately fatty fish</b>								
Farmed gilthead seabream	1114	1990	1143	117	46	236	790	51
Farmed seabass	970	1234	599	115	53	540	1104	52
Rainbow trout	1030	968	267	129	15	366	994	57
<b>Fatty fish</b>								
Sardine	1695	980	105	77	83	1672	1169	28
Atlantic salmon	2688	3810	691	182	56	1172	1773	40
European eel	5553	1155	121	15	785	4428	7270	26

Sources: Nunes et al., 2006 and Danish Food Composition Databank (<http://frida.fooddata.dk/?lang=en>, accessed on August 9<sup>th</sup>, 2016). Note: Values are indicative for each species and may vary depending on the source.

Fish with more than 10 percent of muscle fat, like salmon, sardine, mackerel and eel, are considered as fatty fish; with less than two percent muscle fat are lean fish, these are cod and hake; other species, such as seabass, seabream or trout, are considered as intermediate with muscle fat ranging between five and 10 percent (Nunes et al., 2010). Lipids, including fish lipids, comprise several lipid classes such as triacylglycerols, phospholipids, wax esters, and cholesterol. The fatty acids (FA), constituents of triacylglycerols are divided in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), depending on the number of double bonds present in each FA. PUFA consist of omega-3 and omega-6 fatty acids, being linoleic acid (LA, 18:2 n-6) the precursor of omega-6 series and alpha-linolenic (ALA, 18:3 n-3) the precursor of the omega-3 series, which includes eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Species and physiological status are main determinants of fat content in aquatic animal species, however it also varies with available feed, season and production method, with values ranging between 0.2 and almost 30 percent. Nevertheless, as a rule, higher amounts of long-chain omega-3 PUFA (n-3 LCPUFA) are found in the fattest species, whereas the percentage of SFA is nearly constant across species (Nunes et al., 2010). With some exceptions, as MUFA in meagre and some farmed fish, PUFA are commonly the predominating fatty acids group in fish species, which includes high levels of EPA and DHA. As described in table 1.1 average amounts of DHA and EPA levels are much higher in aquatic animals and almost inexistent in meat from terrestrial animals and eggs, whereas PUFA in meat and eggs consists almost in omega-6 linoleic acid.

Fish as food is an excellent and complete product, with numerous nutritional benefits further than the well-known omega-3 fatty acids. Fish is also a very rich source of taurine, several minerals and vitamins. Important minerals supplied are selenium, potassium, magnesium, zinc, calcium and iodine, the former usually absent in terrestrial meats (fig. 1.4 to 1.9).

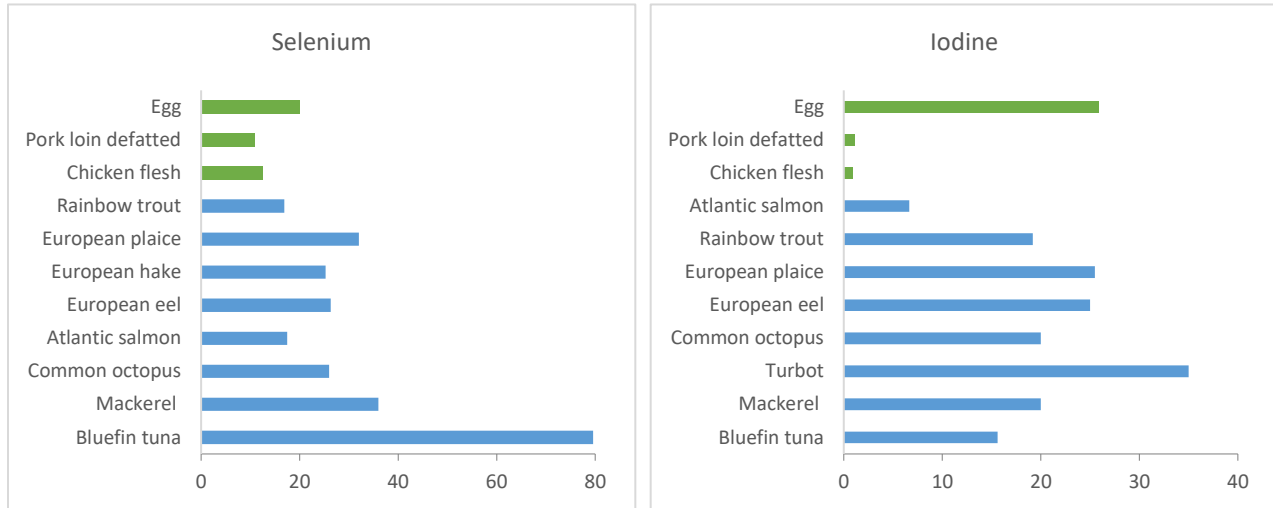


Figure 1.4. and Figure 1.5. Selenium and iodine content in selected food products (µg per 100g of edible part)

Source: Danish Food Composition Databank. Note: Values are indicative for each species and may vary depending on the source.

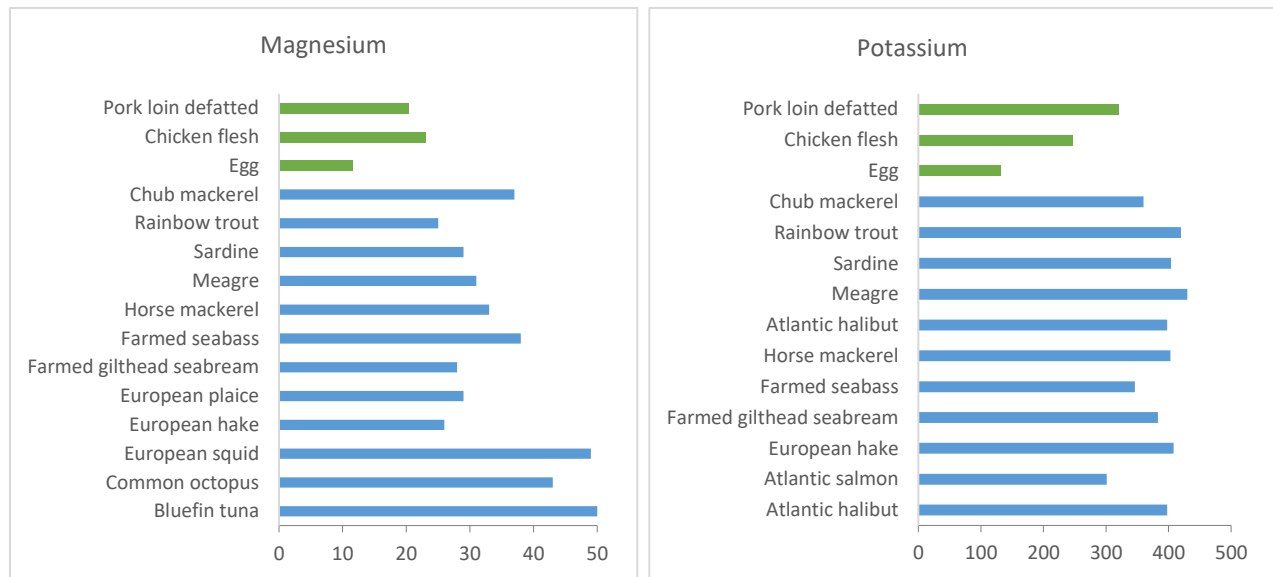
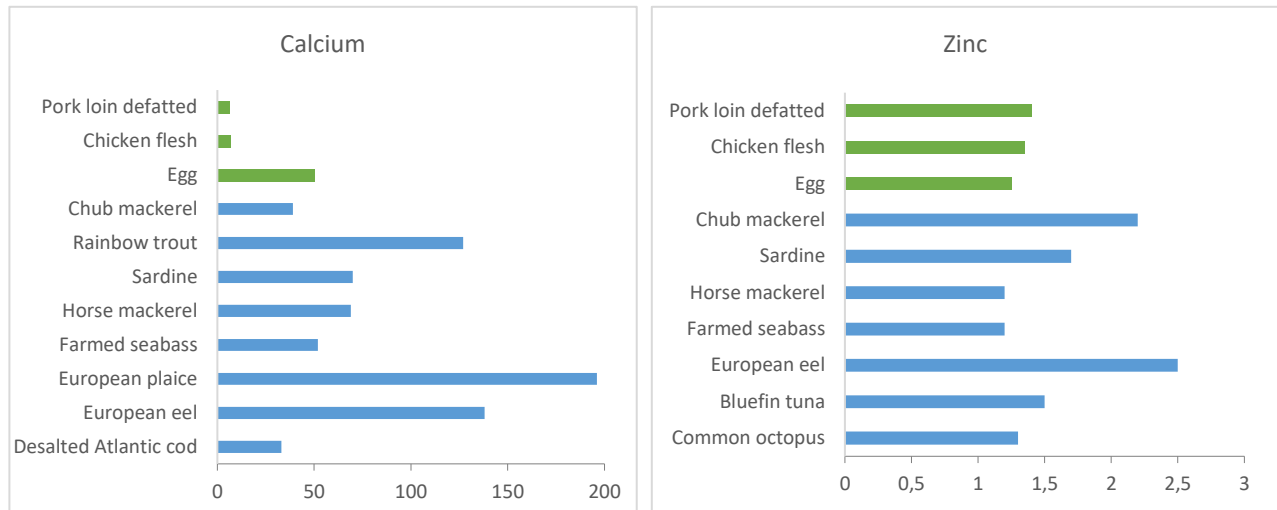


Figure 1.6. and Figure 1.7. Magnesium and potassium content in selected food products (mg per 100g of edible part)

Source: Nunes et al., 2006 and Danish Food Composition Databank. Note: Values are indicative for each species and may vary depending on the source.



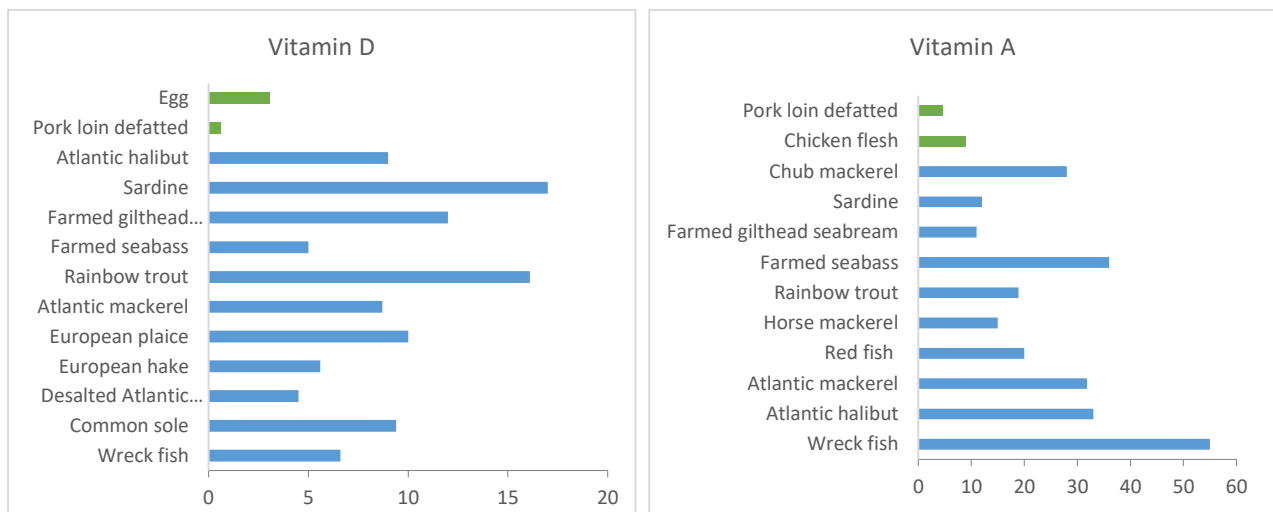
**Figure 1.8. and Figure 1.9.** Calcium and zinc content in selected food products (mg per 100g of edible part)

Source: Nunes et al., 2006 and Danish Food Composition Databank. Note: Values are indicative for each species and may vary depending on the source.

Iodine and selenium are usually present in lower amounts in terrestrial meats compared to fish and seafood. Iodine is an essential trace mineral prevailing in aquatic animals and seaweeds (Andersen et al., 2002; Teas et al., 2004). Though marine fish have much higher iodine content than freshwater fish (Lall, 1995), amounts may show a great variation among species as well as between individuals within species. For instance, iodine levels in wild cod from the Barents Sea, showed values ranging from 0.7 to 12.7 mg per kg fresh weight (Julshamn et al., 2001). Selenium is also an essential mineral present in high amounts in aquatic food products, which levels may range from 32-42 µg in seafood and may reach 80 µg in bluefin tuna (fig. 1.4), whereas in terrestrial meats mean values described are around 24 µg per 100 g of food (Tacon and Metian, 2013).

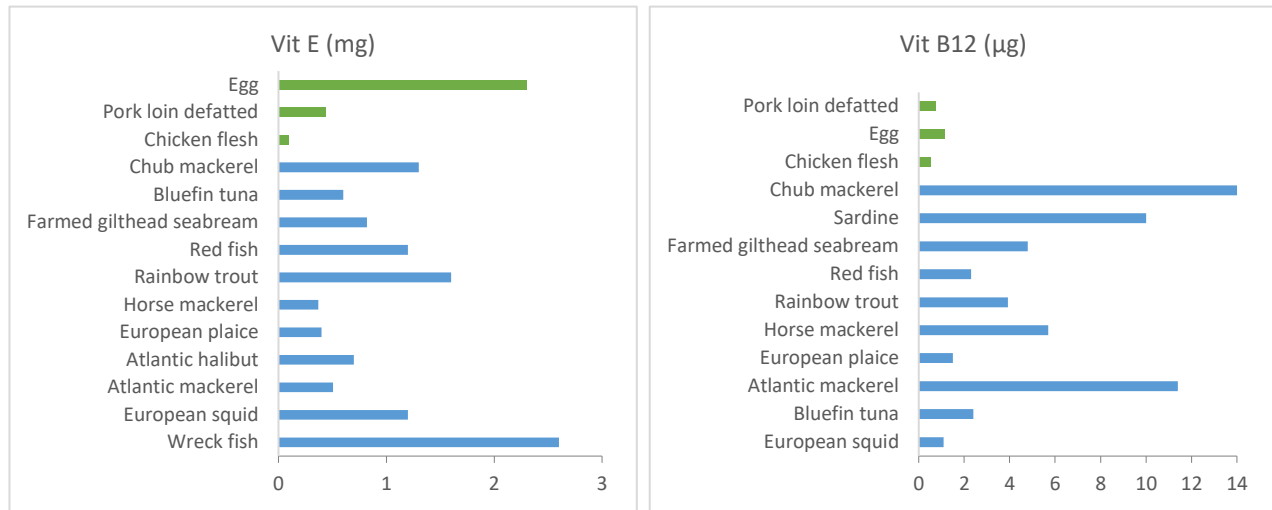
The most significant vitamins found in fish and seafood are the fat-soluble vitamins D and A, and water-soluble niacin, B12 and B6 (Nunes et al., 2010) (fig. 1.10 to 1.13). Seafood is the richest natural food source of vitamin D (Lamberg-Allardt, 2006). Vitamin D content in fish, as described in the literature, may range between 5.6 µg in European hake and 25.6 µg in eel, per 100 g fresh weight (Nunes et al., 2006; Lamberg-Allardt, 2006). It is also known that vitamin D in fish and aquatic animals is present only as cholecalciferol (vitamin D<sub>3</sub> form), in

terrestrial animals both forms are represented (vitamins D<sub>2</sub> and D<sub>3</sub> forms), whereas in plants only ergocalciferol (vitamin D<sub>2</sub> form) is present. Vitamins A, E and B12 can be found in terrestrial animals but are present in relevant amounts in aquatic animals (fig. 1.11 to 1.13). Data represented graphically shows average values of micronutrients. As previously mentioned, mineral and vitamin contents may show significant variations between species and among individuals of the same species. For example, data on raw rainbow trout showed large variations on vitamin D<sub>3</sub> (8.0-23.2 µg) or iodine (14-28 µg); also in wild salmon significant variations were found in vitamin D<sub>3</sub> (8-55 µg) and iodine (6-77 µg) contents; Atlantic halibut and bluefin tuna presented large variations on retinol values (3-63 µg and 123-834 µg, respectively); and similarly, selenium contents in bluefin tuna (48.8-232.7 µg) and wild salmon (2.5-52.3 µg) also varied considerably (Danish Food Composition Databank). As observed from the presented figures it is very important to diversify the types of fish, and food in general, to obtain the best available nutrients from each food item. As showed, fish has a high nutritional value and its consumption is recommended as part of a good dietary pattern to maintain good health through optimal nutrition.



**Figure 1.10. and Figure 1.11.** Vitamin D and Vitamin A content in selected food products (µg per 100g of edible part).

Source: Nunes et al., 2006 and Danish Food Composition Databank. Note: Values are indicative for each species and may vary depending on the source.



**Figure 1.12. and Figure 1.13.** Vitamin E and Vitamin B12 content in selected food products (mg and µg per 100g of edible part, respectively.)

Source: Nunes et al., 2006 and Danish Food Composition Databank. Note: Values are indicative for each species and may vary depending on the source.

### 1.5. Health benefits associated with fish consumption

Scientific evidence linking fish consumption and health beneficial effects have been growing enormously. Health authorities worldwide recommend the intake of at least two fish portions a week, and some suggest the consumption between one and five portions a week with cardio protective effects in adults; improvement of digestive health and beneficial functional outcomes of childrens' neurodevelopment during pregnancy; among other benefits (American Academy of Pediatrics, 2012; Department of Agriculture, Department of Health and Human Services, 2010; EFSA, 2014a; Kris-Etherton et al., 2002; USDA, 2015; World Gastroenterology Organisation, 2008). Recommendations are also applied to specific daily amounts of EPA and DHA, usually ranging from 250 mg to 500 mg per day for adults with an additional 200 mg of DHA per day for pregnant and lactating women (FAO, 2010; IOM, 2005a; ISSFAL, 2004). Fish and seafood consumption are usually associated with a higher intake of omega-3 fatty acids, in particular with EPA and DHA. However, fish have a very limited capacity to produce these fatty acids. EPA and DHA are produced by marine microalgae and accumulation in fish occurs via the food chain. Also, in humans the

conversion of alpha-linolenic acid (ALA, 18:3 n-3) into n-3 LCPUFA can occur but at a restricted rate of five percent for EPA and only 0.05 percent for DHA (Burdge and Calder, 2005; Wang et al., 2006). Both the intake of EPA and/or DHA supplements and the consumption of fish as food have been associated with numerous health benefits in several clinical fields (Shahidi and Ambigaipalan, 2018). The main physiological roles of n-3 LCPUFA are related to cell behaviour via surface or intracellular fatty acid “receptors” and optimal cell membrane structure via changes in the composition of membrane phospholipids (Calder, 2012). A correlation between n-3 LCPUFA and the prevention of cardiovascular diseases (CVD) have been suggested since the 1970s after the observation of lower cardiac events with high intake of fish, seal and whale meat by the Eskimos from Greenland. Some inconsistencies were found in results between scientific studies (Hooper et al., 2006), but these have been attributed to the variability of methods used and patients’ status. Many of these works conclude that fish intake is associated with the prevention of CVD, particularly with a reduced risk of death from cardiac events (Mozaffarian and Wu, 2012; Raatz et al., 2013; Ruxton et al., 2004; Saravan et al., 2010), which are the leading cause of death worldwide (WHO, 2016). Changes in risk indicators are generally evident after some weeks of fish consumption and may be consequence of higher incorporation of n-3 LCPUFA into phospholipids altering the membrane fluidity (Clandinin et al., 1991) and the fatty acid receptors responses. Another referred effect is the binding of n-3 LCPUFA to cytosolic receptors that regulate cell signalling and gene transcription affecting metabolic pathways associated with coronary heart disease (Vanden Heuvel, 2004). The association between maternal fish consumption and the physiological role of DHA in infant cognitive development (Hibbeln et al., 2007; Julvez et al., 2016; Sherry et al., 2015) and optimal visual acuity (Koletzko et al. 2008; Qawasmi et al., 2013) was also extensively described. Improved development scores were measured in 18 months children after mothers had a high fish intake during pregnancy compared with those who had no fish in their diets (Daniels et al., 2004). The role of EPA and DHA in inflammation include several actions leading to inflammation inhibition and rising anti-inflammatory resolvins and protectins, improving the body physiological response (Calder, 2012; 2013). There are also growing evidences of n-3

LCPUFA involvement in neurotransmission and neuroprotection, and thus preventing neurodegenerative disease of ageing and brain damage (Denis et al., 2015; Janssen and Kiliaan, 2014). Moreover, the intensification of produced research has been discriminating the specific effects of, for example, EPA in mood disorders and DHA on neurodegenerative conditions (Dyall, 2015).

As previously mentioned, fish is the main natural food source of iodine (Haldimann et al., 2005; Lee et al., 1994) and vitamin D (Lamberg-Allardt, 2006; Nakamura et al., 2002; Schmid and Walther, 2013), providing also important amounts of selenium (Rayman et al., 2008). These are essential nutrients with major roles on health physiological pathways. Selenium is a constituent of selenoproteins, has a structural and enzymatic role, being also a powerful anti-oxidant, acts as catalyst to produce active thyroid hormone, and it is important for the proper functioning of the immune system (reviewed by Rayman, 2000). Although most dietary selenium is absorbed efficiently, higher retention is observed from organic forms compared with inorganic ones (Finley, 2006). Iodine is an essential trace element used in the synthesis of thyroid hormones, which are important regulators of energy metabolism and crucial for the normal development of different tissues, as the brain (reviewed by Zimmermann, 2007). Iodine deficiency remains a major public health problem since it is the most common cause of preventable mental impairment worldwide (WHO et al., 2007). Universal iodisation of salt has been the main strategy for the control of iodine deficiencies disorders in most countries. However, the reduction of salt intake is also an important public health issue, and new complementary strategies are needing to be adopted. To promote the consumption of naturally iodine-rich food such as seafood and the fortification of alternative vehicles are considerable options (Land et al., 2013). Vitamin D has well established functions related to calcium absorption and homeostasis, bone mineralization and bone health, also particularly concerning osteomalacia and rickets (Bhan et al., 2010; Hess and Hunger, 1921). Increasing evidence correlates vitamin D status with a wide range of non-skeletal health outcomes, like CVD or cancer (Newberry et al., 2014). Vitamin D deficiency is a public health problem that affects individuals across ages, gender and status, in several countries. Optimal vitamin D status is dependent on factors like lifestyle, dietary patterns, sunlight exposure and

geographical region. Nevertheless, intake is critical to achieve the established dietary requirements for vitamin D (Cashman et al., 2011). Vitamin D<sub>3</sub> (cholecalciferol), the available form in fish products, was recently shown, to be more efficient at raising serum 25(OH)D concentrations than is vitamin D<sub>2</sub> (Tripkovic et al., 2012) suggesting that fish and other vitamin D<sub>3</sub> sources are preferential choices to improve vitamin D status.

The advantage of eating fish alternatively to the ingestion of pills and supplements was recently reinforced (Larsen et al., 2011; Lund, 2013; Stonehouse et al., 2011). An animal study showed that a large dose of n-3 LCPUFA once per week, like a meal intake, was more effective in increasing whole body n-3 LCPUFA content when compared with a smaller daily dose, such as pills (Ghasemifard et al., 2015). Also, EPA and DHA serum levels were higher after the intake of a salmon meal compared to cod liver oil supplements (Elvevoll et al., 2006). Moreover, in a human intervention trial, selenium from a fish source had a significantly higher apparent absorption than that from yeast, another organic source (Fox et al., 2004). Additionally, recent data recognized the beneficial effects of selenium (Berr et al., 2009) and iodine (Bath et al., 2013) present in fish, in studies where effects were previously solely attributed to LCPUFA.

A food-based approach seems to be the best dietary option and has been vastly claimed by nutritionists for achieving nutrient adequacy, preventing and treating diseases (Kieft-de Jong et al., 2012; American Dietetic Association, 2005).

### **1.6. Implications of the current substitution of fish meal and fish oil on the nutritional value of fish fillets**

The rapid growth observed in the aquaculture sector over the past decades was boosted by several technical, biological and scientific improvements. Of major importance was the supply of marine key ingredients like FM and FO which allowed the development of compound diets with balanced amino acids and fatty acids profiles. FM and FO were massively incorporated in compound feeds with high protein and lipid levels targeting the rapid growth of carnivorous fish. In 2008, almost 74 percent of the FO and 61 percent of FM available worldwide was used by the aquaculture industry (Tacon et al., 2011). The total

pelleted feed production increased at an annual average rate of 11.1 percent between 1995 and 2007 and is expected to grow from 7.6 million tonnes in 1995 to 70.9 million tonnes in 2020 (Tacon et al., 2011). Marine fish meal and oils are finite ingredients and over the years several approaches were tackled to reduce its use. But also, efforts have been made to improve its utilization efficiency and to find sustainable alternative protein and fat sources for inclusion in fish feeds. Significant improvements were achieved with alternative ingredients and the incorporation of FM have been substantially reduced in feeds of many farmed species (Olsen and Assan, 2012) without major negative impacts on growth performance and feed utilization. In 1990, Norwegian salmon feed contained 90 percent of marine origin ingredients, whereas in 2013 this was reduced to only 30 percent (Ytrestøyl et al., 2015). Projections are to reduce the inclusion of FM in several farmed species by half between 2010 and 2020 (Tacon et al., 2011). Plant protein and fats have been widely used as cost-effective alternative sources reducing the dependency of marine ingredients. Protein from plant sources when fed, to carnivorous fish, presented several problems like high fibre contents and anti-nutritional components that consequently reduced the absorption of nutrients and could interfere with the vitamins' functions. Technological advances allowed the removal or reduction of these components resulting in high protein ingredients. Salmon and gilthead seabream can now be produced with only 10 percent of FM (Bendiksen et al., 2011; Burr et al., 2012; Dias et al., 2009) regarding that essential amino acids are supplemented in a balanced profile. Plant oils have also been successfully used to replace FO in compound feeds of several fish species (Turchini et al., 2010). Up to 66 percent fish oil was efficiently replaced in seabream, that can now be produced using only 5 to 6 percent of FO (Dias et al., 2009; Benedito-Palos et al., 2008), and current practice (data referring to 2013) in salmon industry indicates the use of less than 54 percent fish oil when compared to what was used in 1990, corresponding to 10.9 percent of marine oil in salmon feeds (Ytrestøyl et al., 2015). However, plant oils are characterized by higher levels of n-6 fatty acids and are also poor sources of important long chain n-3 LCPUFA including EPA and DHA (reviewed by Turchini et al., 2009). It is generally observed in nutrition studies that the fatty acid profile of compound feeds is mimicked in fish proximate composition. The substitution of FO by plant

oils will reflect a higher content of n-6 fatty acids, lower levels of EPA and DHA, and increase n-6/n-3 ratio in fish muscle. Even if no negative impact is observed in fish performance, concerns are raised regarding the beneficial effects of farmed fish intake for consumers' health. Moreover, the changes in n-6/n-3 PUFA ratio exert physiological changes, since n-6 and n-3 PUFA are precursors of signalling molecules with opposing effects, that modulate membrane composition, receptor signalling and gene expression (Schmitz and Ecker, 2008). Excessive intake of n-6 PUFA, as found today, increases the risk of numerous chronic diseases including CVD, cancer, inflammatory and autoimmune diseases (Simopoulos, 2016). A balanced n-6/n-3 ratio is important to maintain body homeostasis and promote normal development. To date, there is an absence of a cost-effective alternative for FO replacement with the same high levels of omega-3 LCPUFA. Microalgae biomass could be a promising alternative source of omega-3 LCPUFA for the future. The incorporation of microalgae biomasses in feeds have been normally applied in fish larvae diets or when targeting a specific benefit. However, actual microalgae production volumes are still low with significantly high manufacture costs, preventing its use as a bulk ingredient in aquafeeds (Shields and Lupatsch, 2012; Chauton et al., 2015). Other alternative ingredients have been studied at experimental level with promising results. For instance, the transgenic oilseed of the crop *Camelina sativa* was successfully modified to produce EPA and DHA at similar levels found in fish oil (Ruiz-Lopez et al., 2014). Also, insect larvae biomass has been regarded for its high protein levels (Sánchez-Muros et al., 2014). Nevertheless, a significant scientific and legal efforts still must be tackled before these ingredients can be introduced in practical fish diets. As best practice, feed companies nowadays use a mixture of several protein and oil sources to minimize eventual negative impacts of a sole ingredient and maximize the contribution of each ingredient to the feed matrix.

### 1.7. Finishing strategies: approaches to recover LCPUFA levels

The substitution effects of FM and FO in aquafeeds have been studied for several years. Concerns like animal growth performance, feed utilization, immunity or welfare were addressed in many research works with outcomes depending on species, level of

replacement and feed composition. However, a common concern and a major impact of use of plant ingredients in fish feeds are the effects on the muscle nutritional value and its contribution to consumers' diet and wellbeing (Turchini et al., 2009). The restoration of high levels of n-3 LCPUFA, like EPA and DHA, in fish muscle has been attempted by using finishing diets during the last months of production (finishing period). The most common approach has been to supply a FO based diet aiming a wash-out effect of the plant oils used during the growth-out phase. Several studies showed that restoration of muscle lipid profile usually respond to a dilution model directly related to the fatty acids intake (Benedito-Palos et al., 2009). Despite these physiological processes being tissue and species specific, it has been verified that the fatty acids accumulation over time follows exponential functions (Glencross et al., 2003; Turchini et al., 2006), where the rate directly varies with the fatty acids concentration previously present in the tissue. Hence, by using a finishing FO-based diet, the n-3 LCPUFA, lacking in plant oils, will deposit at a faster rate when compared with a continuously fed FO treatment (Turchini et al., 2006). However, results of the utilization of finishing diets are far from consensual owed to differences on trials duration, species studied, and type of feed used during growth-out phase. Some studies described the full recovery of EPA but not DHA levels (Zhou et al., 2015), others were able to full restore DHA but only partial levels of EPA (Izquierdo et al., 2005; Trushenski and Boesenberg, 2009). In most trials, the recovery of both EPA and DHA, to levels found in a continuously fed FO group, was not fully achieved (Bell et al., 2003; Fountoulaki et al., 2009; Mourente and Bell, 2006; Regost et al., 2003; Torstensen et al., 2005). Benedito-Palos and co-workers (2009) performed a study comparing the FO utilization in different feeding strategies and observed that feeding seabream with 60 percent vegetable oil (VO) diet with a subsequent FO-based finishing diet had the same FO expenditure compared to a continuously fed seabream with 33 percent VO diet. From the present literature is perceived that more efficient finishing strategies are required than the simple transfer to a FO-based diet after the growth-out phase. Several authors described a sparing effect of n-3 LCPUFA and particularly EPA and DHA when fish are fed a mixture of FO with higher amounts of SFA and MUFA sources since the former are preferably catabolized for energy and maintenance (Codabaccus et al., 2012; Stubhaug et

al., 2007; Turchini et al., 2011). When supplied in excess, fish uses n-3 LCPUFA via  $\beta$ -oxidation for energy production. A reduction of EPA and DHA dietary levels, with the simultaneous higher intake of MUFA, and to a lesser extent SFA, have shown to promote the deposition of EPA and DHA. These concepts still need to be corroborated with new knowledge and validated in several species. New approaches are required to reduce and to efficiently improve the use of a valuable ingredient as FO, without disregarding the expected high levels of EPA and DHA in fish muscle.

### 1.8. Tailoring fish muscle with health beneficial compounds

The use of plant ingredients also affects the amounts of several minerals, as iodine and selenium, and vitamins such as A, D, E and B group, usually found in fish, since these compounds are scarce or not existing in many plant sources used in feeds. Aiming to mitigate this problem only few works were undertaken using dietary fortification as a tool to tailor muscle composition. In many trials with dietary supplementation, objectives were set with diverse purposes and often the final concentration of the supplemented compound was not measured in the muscle, missing relevant information for consumers. Works performed with selenium, showed that supplementation causes a concentration increase of the mineral in fish muscle tissues, positively related with dietary levels, as observed in channel catfish (Wang and Lovell, 1997), rainbow trout (Pacitti et al., 2015) or common carp (Elia et al., 2011). Some differences found on the muscle retention efficiency were associated with selenium chemical forms, being organic sources like selenomethionine more bioavailable than inorganic sources (Küçükbay et al., 2009; Lin, 2014; Rider et al. 2009; Saffari et al., 2016; Wang and Lovell, 1997). The few experimental trials performed with dietary iodine inclusion presented an increase in muscle contents on Atlantic salmon (Julshamn et al., 2006), chars (Schmid et al., 2003) and rainbow trout (Valente et al., 2015), though not linearly proportional to feed concentrations. In this case, the increased muscle levels were achieved both with inorganic iodine (KI) as with two iodine-rich macroalgae, *Laminaria digitata* and *Gracilaria vermiculophylla*. Supplementation with other micro-elements also induced modulation in muscle levels. For example, accumulation of copper in rainbow trout muscle

seem to be more dependent on dietary concentration rather than chemical form (Read et al., 2014). On the other hand, zinc concentration in muscle was raised by dietary inorganic zinc when compared to organically bound zinc in seabass (Fountoulaki et al., 2010) and in rainbow trout (Rider et al., 2009). Supplementation of aquafeeds with vitamins was also performed resulting in variable outcomes. In literature are reported trials with increasing dietary levels of vitamin D that present dissimilar results on muscle deposition levels. In rainbow trout muscle, vitamin D content varied from 5.7 to 15.6  $\mu\text{g}\cdot 100\text{ g}^{-1}$  not dependent on dietary doses (Mattila et al., 1999). On the contrary, Horvli et al. (1998) found muscle vitamin D concentrations reflecting feed increasing levels. Same findings were reported by Graff et al. (2002) on a study with Atlantic salmon fry. Studies with vitamin E supplementation presented more homogenous results with an observed gradation in muscle or whole-body content in response to dietary vitamin E, in seabass (Gatta et al., 2000), salmon (Menoyo et al., 2014), rainbow trout (Chaiyapechara et al., 2003), halibut and turbot (Stéphan et al., 1995; Tocher et al., 2002). Trials directed to observe the accumulation of specific FA in fish muscle were also undertaken. Kennedy et al. (2007 a, b) showed an increased deposition of conjugated linoleic acid (CLA) and tetradecylthioacetic acid (TTA) in commercial size rainbow trout and juvenile cod, aiming beneficial effects for consumers associated with the accumulation of the bioactive fatty acids.

Similarly, in terrestrial animals, dietary supplementation has been used as a tool to enhance meat products nutritional value and quality. The increased content of FA in diets resulted in higher muscle concentrations, in several terrestrial species. Feeds supplementation with CLA resulted in a higher deposition of these fatty acids in the muscle and adipose tissues of pork (Joo et al., 2002; Dugan et al., 2004) and chicken (Du and Ahn, 2002; Kawahara et al., 2009) with relatively high efficiency. Dietary supplementation of omega-3 fatty acids have been used to increase omega-3 PUFA in animal muscle tissues and eggs (reviewed by Fraeye et al., 2012). Broiler chickens fed fish-oil up to four percent showed an increased muscle content in n-3 LCPUFA, in particular EPA and DHA, and a concomitant decrease in SFA and MUFA concentrations (Lopez-Ferrer et al., 2001). The inclusion of only one percent fish oil in pig diets resulted in increased muscle levels of EPA, DHA and ARA with decreased levels of n-6

fatty acids and associated reduction of n6/n3 ratio (Leskanich et al., 1997). The dietary supplementation with selenium had largely increased muscle levels in pork (Mahan and Parret, 1996), beef (Juniper et al., 2008), lamb (Juniper et al., 2009) and chicken (Skřivan et al., 2008), resulting in higher contents when organic selenium was utilised in comparison with in inorganic sources, as observed in fish. However, it was observed a great variation on the accumulation levels of selenium between produced species after the similar dietary supplementation values. Increased levels of dietary iodine proportionally raised muscle iodine concentrations in pigs both with inorganic (Franke et al., 2008) and organic sources (He et al., 2002, Dierick et al., 2009). Vitamin E incorporation in animal feeds has been used to improve fresh meat quality by limiting protein and lipid oxidation during storage. Moreover, increased levels in muscle were observed with higher dietary amounts of  $\alpha$ -tocopherol in pork (Lanari et al., 1995), beef (Chan et al., 1996) and lamb (Guidera et al., 1997) animals. Regarding some essayed minerals, the muscle response for increased dietary levels of copper, iron and manganese was considered mostly absent. Also, for zinc, an effect between dose supplementation or chemical form and muscle concentration could not be verified.

Literature shows that the efficacy of dietary strategies is dependent on several factors like species, element source, chemical form or concentration levels, additionally, life stage, age, sex and environmental factors are also referred as important elements also influencing supplementation results. Minerals and vitamins are essential nutrients with key functions in several physiological pathways including animal health and disease resistance. Using high dietary levels of minerals and vitamins may be toxic and harmful for fish health, compromising its immunity and impairing growth. The supplements concentration in animal feeds must therefore be carefully selected, balancing between the animal needs and the consumers' additional benefits from eating fish and meat.

### **1.9. Fish as a functional food: carrier, benefits and consumers perception**

In developed countries, food items are regarded beyond the suppression of hunger and the supply of essential nutrients and are perceived as hedonistic products and sources of

improved wellbeing. Moreover, consumers' perception of food is evolving and awareness of the relation between food and health is emerging. These were both a challenge and an opportunity for the food industry to develop innovative products responding to consumers' expectations, and a novel field emerged, the functional foods. The Concerted Action on Functional Food Science in Europe (FuFoSE) defined as functional, a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of disease; also, it should be consumed as part of a normal food pattern (Diplock et al., 1999). The consumers' acceptance of a functional food is determined by a multitude of factors. Aspects such as familiarity with the functional ingredient; nature of the food carrier; the perceived health effect; interest in general health and price were highlighted as important factors conditioning the success of these products (Annunziata and Vecchio, 2011; Sirò et al., 2008; Verbeke, 2005). It is important for consumers to understand and trust the reward/benefit of consuming functional foods and to know that the health claims/efficacy are supported by scientific evidence (Teratanavat and Hooker 2006; Urala and Lähteenmäki, 2007; Verbeke, 2005). Moreover, the perceived healthiness of the food carrier together with the combination carrier-ingredient were also referred as determinant factors for consumers' acceptance (Annunziata and Vecchio, 2013; Krutulyte et al., 2011; van Kleef et al., 2005). Products that are perceived as intrinsically healthy and with an overall positive image, like yogurt or juice, were preferred as credible functional food carriers (Annunziata and Vecchio, 2011; Rozin et al., 2004; Siegrist et al., 2008). Functional foods associated to a more natural image with less manipulation also rises the receptivity and the purchase intention (Bearth et al., 2014; Teratanavat and Hooker, 2006). In a food choice questionnaire was observed that the enrichment with an already present functional ingredient was the most favourable option (Ares and Gámbaro, 2007). For example, consumers found more appropriate the enrichment with fish oil/omega-3 of fish balls or tuna salad comparatively with yogurt or a sports bar (Krutulyte et al., 2008). Main reasons suggested are taste modifications and an expected off-flavor from the fish oil inclusion in non-seafood products (Bech-Larsen and Grunert, 2003). Many studies, across countries, suggested that consumers are not willing to

compromise taste over health benefits (Bech-Larsen and Grunert, 2003; Tuorila and Cardello, 2002; Verbeke, 2006). Being a source of multiple beneficial compounds, previously described, fish is considered as an excellent carrier of healthy nutrients. Consumers already expect that fish intake will supply several bioactive ingredients, such as omega-3 fatty acids, and believe that these are effective at improving health (Patch et al., 2005; Vella et al., 2013). Studies performed among European consumers found that an interest in healthy eating was positively and directly related with fish consumption (Pieniak et al., 2008; Trondsen et al., 2004a; Verbeke et al., 2005). Combined with the growing development of the aquaculture industry and with the possibility to tailor muscle properties with health beneficial compounds, farmed fish could be considered as an excellent candidate for a functional food.

#### **1.10. Fish overall quality: influences of farming conditions and culinary procedures**

Food quality is a broad term encompassing the intrinsic traits of a food product, like nutritional value and safety, together with external cues, as appearance or colour, and organoleptic properties that are more dependent on individual perception such as texture, flavour and odour (Grunert, 1995; Oude-Ophuis and Van Trijp, 1995). The main characteristics focused when describing fish quality are: overall appearance; freshness; flavour; aroma; nutritional value and texture (Grigorakis, 2010). Fish quality may be influenced by several intrinsic and external factors namely size, sexual maturity, feed composition, farming conditions or rearing temperature (Børrensen, 1992). Freshness was referred as the single most important attribute when assessing fish quality and comprises several parameters previously mentioned as aroma, flavour, texture and sensory response (Alasalvar et al., 2001). Consumers considered freshness as a fundamental attribute, directly related with fish quality, and capable of influencing their decisions (Brunso et al., 2009). The visual appearance of fish is the first cue to draw consumers' attention and is determinant during purchasing (Vasconcellos et al., 2013). Skin colour and pigmentation are determinant factors for overall visual appearance and can be associated with feeding practices and the intake of carotenoid pigments. As reviewed by Shahidi et al. (1998) carotenoid requirements,

metabolism and deposition in fish are biological processes species specific. Salmonids skin and muscle colour are mainly dependent on astaxanthin dietary absorption and respond to astaxanthin concentrations in feed (Torrissen et al., 1989) whereas in seabream, effectiveness of pigmentation has been difficult to modulate by dietary means (Gomes et al., 2002). Adequate pigmentation during production is extremely important because will determine consumers acceptance of farmed products. Consumers' expectations about fish quality aspects, such as with pigmentation, flavour and texture, are usually linked to wild counterparts' characteristics. In countries where fish is commercialized as whole, visual criteria are more relevant than in regions or markets where fish is sold in portions. In portion size fish markets criteria like quality and safety are emphasised over appearance.

The edible part of fish is normally the fillet, which is primarily composed by water, protein and fat fractions. The main constituent is water, which usually accounts for about 70-80 per cent of the weight of a fresh fish fillet. It is tightly bound to the proteins and affects texture and flavour. The protein fraction is usually more constant and unchanged but the fat, which is a combination of intramuscular lipids and storage depots, can be modulated by dietary means. Moreover, the profile and amounts of fatty acids in the muscle are known to influence sensory traits as flavour, odour and texture (Grigorakis, et al., 2003). Farming practices, feeds' composition and husbandry conditions are some factors that can impact nutritional and quality criteria in fish, namely: odour, flavour and texture features. Flavour is a very important attribute for heavy fish consumers (Brunso et al., 2009), and a major concern of fish producers. Studies refer several sensory traits distinguishable between cultured and wild fish (Grigorakis, 2007). It was also described that fish from semi-intensive systems have traits closer to their wild counterparts compared to intensively raised fish (Valente et al., 2011) suggesting that farming conditions may modulate sensory characteristics of fish (Cardinal et al., 2011). However, in intensive reared fish modifications, feed composition has apparently minor impacts on flavour and odour traits (reviewed by Matos et al., 2017). Texture and muscle firmness can be affected by husbandry practices, slaughtering method, *rigor mortis* development and storage conditions but also by individual differences such as body size and sexual maturation (reviewed by Johnston, 2008). In

general, farmed fish has a softer texture compared to wild fish, partially owed to a more sedentary lifestyle that restrain muscle activity and development (Haard, 1992). Also, fat depots have a major influence in the muscle texture, decreasing when lipid storages increase (Grigorakis, 2007). Overall, valued fish quality attributes may be, within a limited range, modulate during production in aquaculture systems. Farming conditions, particularly feed composition, open the possibility to tailor fish quality in terms of its nutritional value, sensory properties and skin pigmentation.

Processing methods such as household culinary procedures also influence food quality by altering physical and chemical properties of fish (Alipour et al., 2010). The resulting changes affect the nutritional value and bioaccessibility of the present nutrients in fish muscle (Costa et al., 2015). Moisture content usually decreases with cooking processes with subsequent increase of solids and concentration of some nutrients (table 1.3). In general terms, boiling and grilling are satisfactory treatments regarding nutrients preservation. Frying methods increase oil absorption altering the fat content and profile of fish muscle. These changes are influenced by the vegetable oil used during frying (Nunes et al., 2010).

**Table 1.3.** Nutritional data of selected molluscs and fish products raw and subjected to culinary procedures (per 100 g of edible part)

	Farmed gilthead seabream			European squid		Horse mackerel			Sardine		
	Raw	Boiled	Grilled	Raw	Grilled	Raw	Fried	Grilled	Raw	Grilled	Canned
<b>Protein and lipids</b>											
Protein (g)	17.8	20.8	25.2	15.8	32.5	18.6	22.7	23.9	17.9	24.1	24
Total fat (g)	8.3	12.5	7.4	0.9	1.6	2.2	9.3	2.8	10.9	9.2	12.7
SFA (mg)	1674	2339	1466	242	497	560	1548	705	2746	2396	3001
MUFA (mg)	2867	3697	2484	58	121	626	2541	757	2558	2070	5582
PUFA (mg)	2763	4318	2561	369	672	610	4129	794	4071	3494	2806
20:05 ω3 (mg)	236	646	232	111	112	128	246	145	1672	1288	792
22:06 ω3 (mg)	790	1289	811	242	417	363	754	501	1169	1334	1256
<b>Minerals</b>											
Calcium (mg)	15	30	65	18	28	69	86	61	70	67	445
Magnesium (mg)	28	34	35	49	49	33	37	35	29	35	42
Potassium (mg)	383	367	494	225	73	403	465	468	404	496	369
Copper (mg)	<0.03	0.05	0.05	<0.03	0.7	0.08	0.09	0.11	<0.03	0.11	0.15
Zinc (mg)	0.8	0.7	1	1	0.7	1.2	1.1	0.9	1.7	1.2	2.5
<b>Vitamins</b>											
Vit A (μg)	11	12	8.8	9.6	n.a.	15	8.2	n.a.	12	9	9
Vit E (mg)	0.82	0.32	0.16	1.2	1.8	0.37	0.79	0.18	0.03	0.7	1.5
Vit D (μg)	12	8.4	7.9	3.5	<0.70	4.1	2.8	4	17	11	8.8
Vit B1 (mg)	0.2	0.23	0.23	0.07	0.04	0.15	0.13	0.14	0.02	0.05	<0.02
Vit B12 (μg)	4.8	n.a.	4.2	1.1	n.a.	5.7	6.6	6.4	10	9.3	n.a.
Vit A (μg)	11	12	8.8	9.6	n.a.	15	8.2	n.a.	12	9	9

Source: adapted from Nunes et al., 2006. Note: Values are indicative for each species and may vary depending on the source. n.a.: not analysed

### 1.11. Objectives

Fish produced from aquaculture systems will be the main source of dietary fish in a near future. Controlled farming conditions opens the possibility to tailor fish quality and nutritional value by the modulation of feeds composition, with clear benefits for consumers' health. However, little research has been done to enrich fish muscle with health promoting nutrients beyond guaranteeing the fish nutritional requirements. The general objective of this PhD is to develop aquaculture fish as a functional food. The concept relies on the use of health-beneficial nutrients, such as iodine, selenium, vitamin D, n-3 LCPUFA as natural feed supplements, and also using algae as a novel vehicle to attain the fillets enrichment of two major species produced in Europe: a saltwater species – gilthead seabream (*Sparus aurata*) and a freshwater species – rainbow trout (*Oncorhynchus mykiss*). For this purpose, the following goals were set:

- Assess, through a questionnaire approach, consumers' habits of eating fish and their perception of the theoretical concept "farmed fish as a functional food" (Chapter 2).
- Establish the efficacy of trace nutrients (e.g. iodine, selenium and vitamin D) and complex ingredients, such as microalgae, using dietary fortification strategies regarding supplemental dose, bioavailability of product forms, fish performance and muscle composition (Chapter 3 and 4).
- Evaluate the effects of enrichment strategies on overall (biochemical and sensory) fish quality criteria and skin pigmentation (chapter 5).
- Optimize the process for restoring the muscle n-3 PUFA levels, as a finishing strategy, after the utilization of vegetable ingredients or other alternative protein and oil sources during the fish growth out phase (Chapter 6).
- Evaluate the effect of culinary heat treatments on the stability of functional nutrients and estimate its nutritional contribution and availability to human diets (within Chapters 3, 4, 5 and 6).

A multidisciplinary approach was applied to establish the efficacy of the nutrient fortification concept from an ingredient level, at the farm level (feeding strategy, influence on fish physiology), along the production chain (storage and processing losses) and consumers' perception.

This PhD project aimed to generate new knowledge on the fortification of seabream and trout muscle using dietary supplements and assess their beneficial contribution for an improved diet and human state of health. The development of such novel foods, with functional properties, targets new market opportunities for the Mediterranean aquaculture industry.



# CONSUMERS PREFERENCES AND PERCEPTION OF FISH AS A FUNCTIONAL FOOD

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## Chapter 2



# Farmed fish as a functional food: perception of fish fortification and the influence of origin – Insights from Portugal

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## Farmed fish as a functional food: perception of fish fortification and the influence of origin – Insights from Portugal

### Abstract

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Being a rich source of important nutrients, including highly digestible proteins, vitamins (A, D3), trace minerals (iodine, selenium) and n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), fish consumption is generally regarded as part of a healthy dietary pattern. Exogenous feeding in aquaculture unlocks the possibility to tailor fish composition with healthy valuable nutrients. However, the use of supplements in the fish feed during fish production may undermine consumers' perception opinion of these fortified products. The effectiveness success of a functional food is a combination of its efficacy and meeting consumers' expectations. A self-administered questionnaire was designed to assess consumers' preferences regarding fish consumption, and their perception of farmed seabream as a functional food. A high consumption rate of fish, (between daily, and a minimum of three times a week), was reported by 47% of the respondents. Freshness, flavour, quality and price were the four most valued attributes. Good acceptance of the fish fortification concept was observed (53%), as well as positive receptiveness to its consumption (50%). Anti-oxidants and omega-3 fatty acids were the most accepted compounds for fish fortification. Additionally, two consumer groups were established based on their high (HIG) or low (LIG) interest in fish origin (wild vs aquaculture). The LIG was more receptive to all aspects of fish fortification and showed willingness to buy and consume it. This suggests that fortified fish could be targeted to the LIG profile group, which represents 42% of the studied population. With appropriate communication, farmed fish may be a good candidate for functional food.

Keywords: Consumers' questionnaire; Fish consumption; Aquaculture; Functional-food; Portugal

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## Introduction

The strong move towards healthier eating has promoted the consumption of seafood products (Pieniak et al., 2008). The world per capita fish as food supply increased from 9.9 kg in the 1960s to 20 kg in 2014 (FAO, 2016). Portuguese consumers record the highest fish consumption in the EU, around 56.8 kg/capita/year, while the European average is less than half of that (24.9 kg/capita/year) (PCP, 2016). World demand for seafood products for human consumption is projected to keep rising in the next decades (FAO, 2016). With capture fisheries stagnated, aquaculture production is expected to counterbalance this supply issue. Under farming conditions, fillet quality traits such as fatty acid profiles and concentration of trace nutrients, may be influenced by the diet composition. The International Life Sciences Institute defines functional foods as “foods that, by virtue of the presence of physiologically-active components, provide a health benefit beyond basic nutrition” (Diplock et al., 1999). In 1999 the American Dietetic Association defined functional foods as foods that are “whole, fortified, enriched, or enhanced,” but more importantly, states that such foods must be consumed as “... part of a varied diet on a regular basis, at effective levels” for consumers to reap their potential health benefits (American Dietetic Association, 1999). Moreover, food fortification has been the primary strategy to battle nutrient deficiency in populations worldwide (WHO and FAO, 2006). Under this context, and considering the high nutritional value of fish, there is a significant potential to develop fish as a functional food.

Consumers' acceptance of new products is crucial for their market success, but their acceptance or rejection is of a multi-factorial nature. Food choice behaviour is a complex process, influenced by several factors and their interaction. Determinants like past behaviour, habits and hedonic appreciation are usually good predictors of food choice behaviour (Koster, 2007). Would a fortified aquaculture fish be well accepted by consumers with a high fish consumption level? Indeed, beliefs about the characteristics of a certain product and the way it is produced can have a relevant influence on consumer perception, such as in the case of farmed fish. Several studies on the behaviour of fish consumers often describe farmed fish as being less healthy and with lower quality when compared to wild fish

(Claret et al., 2014; Verbeke et al., 2007). Comparatively lower costs, perception of artificial-like product, and lack of information on sustainable farming practices are among the key elements conditioning the image and acceptance of aquaculture fish (Altintzoglou et al., 2010; Claret et al., 2014; Vanhonacker et al., 2011). Furthermore, it has been shown that consumers are willing to buy and to pay higher prices for healthy attributes in fish products when information is made available (Kole et al., 2010). It has thus been suggested that the aquaculture sector should evolve to cope with market demands by differentiating their offer, searching for new niche markets, and combining diversification of products, species and farming methods with the development of added value products (Barazi-Yeroulanos, 2010), such as “functional fish”.

The functional foods market continuously presents new products to meet consumers' expectations and is experiencing an annual average growth rate of about 8.5% (Bogue et al., 2017). However, barriers and concerns about the use of functional foods have been highlighted in many studies. Urala and Lähteenmäki (2007) described a general suspiciousness towards the functional food concept, though it may not necessarily influence the willingness to use concrete functional products. Consumers were not willing to sacrifice naturalness and taste for healthiness and convenience (Grunert, 2010; Siró et al., 2008). Also, costs, adverse or unknown effects (Vella et al., 2013) and manipulation (Bech-Larsen and Grunert, 2003) were referred as barriers to functional foods consumption. Moreover, the factors influencing decisions during purchasing do not only include sensory qualities and health beliefs, but also other intrinsic aspects, that should be considered in the formulation and characterization of functional foods, like prevention of diseases, (Pappalardo and Lusk, 2016). Some reviews on functional food products highlighted the importance of ingredient selection, and how crucial it is to find the right combination between ingredient and carrier to comply with consumer acceptance (Ares and Gámbaro, 2007; Bech-Larsen and Scholderer, 2007; Grunert, 2010). Functional foods presumably enable the consumer to lead a healthier life without changing eating behaviour. Tailoring aquaculture products with

health promoting compounds may create new opportunities in the production sector, leading to novel foods in a receptive and growing market (Siró et al., 2008).

Information about consumers' acceptance of "fortified fish" is very scarce. Cultural factors may play a significant role in the acceptance of functional foods (Siegrist et al., 2015), and particularly interesting is the perception of "fortified fish" by a population, like the Portuguese, who have a high fish consumption level. The main aim of the present study was to assess consumers' acceptance of farmed fish fortified with beneficial and healthy compounds. The study comprised also an assessment of fish consumption preferences following the segmentation of respondents according to the importance of knowing the fish origin (wild vs farmed). The first hypothesis in this study was that in general, consumers are sceptical about fish from aquaculture, particularly fish that have undergone any type of enhancement or manipulation. Moreover, considering that functional foods might not be readily accepted by consumers, a second hypothesis was formulated: within a population there is a group of consumers, segmented by the importance of knowing the fish origin, that are more receptive to new products developed from enhanced aquaculture fish, will vary depending on consumers' knowledge of fish origin.

## **Methodology**

### *Participants/ Description of recruitment*

The questionnaire was addressed to the Portuguese population aiming at a maximum number of participants, and the only prerequisite was to have internet access. The questionnaire was developed in Portuguese language and built using an on-line software platform - surveymonkey®. The recruitment was carried out by email invitations and facebook® announcements using the snowball sampling technique (Grbich, 1999). In the introduction, the enquiries' anonymity and the utilization of the answers only within the study purposes were guaranteed. Answers were collected over two months, from July until September 2011. A total of 932 answered questionnaires were collected, though only 778

respondents' answer all the proposed questions, and these were the ones considered valid (response rate 83.5%).

### *Description of questionnaire*

The questionnaire was structured in four sections assessing:

#### **Fish consumption and preferences:**

In this section three items were assessed: a) fish consumption frequency (never, less than once a month, 2-3 times per month, 1-2 times per week, 3-6 times per week, daily); b) the three most frequently consumed species, from a list of 10 given possibilities (cod, hake, mackerel, meagre, seabream, seabass, salmon, sardine, trout, tuna and a blank space to non-listed species); c) the two most valued fish attributes (freshness, taste, price, quality, nutritional value, texture, convenience of use, easy to cook, ecological impact, origin (aquaculture/fisheries) and species). The selection of enclosed species was based on several criteria with potential interest to producers and retailers: expected highly consumed species (cod, tuna, salmon, hake, sardine and mackerel), main produced species from Portuguese aquaculture (seabream, seabass and trout) and a farmed species emergent in the Mediterranean region (meagre) (Cardoso et al., 2013; Ernst and Young, 2009).

#### **Acceptance of aquaculture fish and fish fortification:**

Concerning the interest in fish origin (wild vs aquaculture) participants were asked about their consumption of farmed fish (yes or no); the importance of knowing fish origin, using a five-point importance scale (from not important: 1 to very important: 5); and also the listing of two attributes that could be improved in farmed fish from a list of seven options (colour, texture, odour, taste, fat content, nutritional value and higher diversity of products), using a five-point importance scale (from nothing to improve: 1 to very much to improve: 5). The options were selected based on described differences between the perception of wild and farmed fish, related to taste, sensory traits and nutritional value (Grigorakis et al., 2003; Verbeke et al., 2007).

Seafood fortification aims to provide nutrients that tend to be deficient in the diet. Participants were asked about their agreement on fish fortification through feeding, with beneficial nutrients for the human health, using a five-point scale (from totally disagree: 1 to completely agree: 5). Gilthead seabream (hereafter will be referred as seabream) was mentioned as target species, since it is the most cultivated species in the Mediterranean area and one of the most consumed farmed fish in Portugal. Participants were also requested to identify two nutrient types suitable for farmed fish fortification from an eight options list (antioxidants, iodine, magnesium, omega-3 fatty acids, selenium, taurine, vitamin D, none). A brief description of the nutrients beneficial effects was made available to help respondent's make an informed decision. The information provided was carefully balanced to avoid any influence in the respondents' answers.

#### **Consumption and purchasing options towards fortified fish**

Participants were asked about their willingness to consume fortified seabream (from not willing: 1 to totally willing: 5); their buying preference without a price change (selecting between common or fortified seabream); and their willingness to pay a higher price for fortified seabream (yes or no). A set of four additional questions concerning daily life aspects that could influence purchasing decision were presented: what is the importance you give to food? What is the level of association between food and health? (five-point importance scale from not important: 1 to very important: 5 was used); the presence of children in the household (yes or no); and the respondent's role in food purchasing (yes or no).

#### **Demographic characterization**

Participants' demographic characteristics included: age (date of birth); gender; education level and monthly family income (less than or equal to 1000€; between 1001 and 2000€; between 2001 and 3000€ and more than 3000€).

### *Data analysis*

Fish consumption regularity was evaluated as frequencies of consumption. Analysis regarding suggested improvements of farmed fish was performed by clustering respondents who answered categories 4 and 5, and by expressing data as percentage of total answers for each option. This data treatment aimed to include, in the outcomes, all the answers obtained for each option combining high and low scale scores. Concerning respondents age, four classes were defined: less than or equal to 25 years; between 26 and 40 years; between 41 and 65 years and more than 66 years.

### **Consumer groups**

Answers regarding the importance of knowing fish origin (wild versus aquaculture) allowed the establishment of two groups with distinct profiles and attitudes towards fish attributes and acceptance of farmed and fortified fish. Respondents with rating scores 4 and 5 were classified as high interest group (HIG), while those with lower rating scores were classified as low interest group (LIG).

### **Statistical data treatment**

Cross tabulations with Pearson's Chi-square tests were applied to test associations between categorical variables. One-way analysis of variance (ANOVA) was applied to compare means when continuous variables were used. Statistical significance was tested at 0.05 probability level. Additionally, data was subjected to an exploratory analysis using Principal Component Analysis, conducted with 12 variables with orthogonal rotation (varimax). The Kaiser-Meyer-Olkin measure was at the lower limit for sampling adequacy (KMO=0.704). Bartlett's test of sphericity (2095.55) presents a  $P < 0.001$  indicating that variables are significantly correlated. Four components accounted for 60% of the variance observed across the 12 variables. Variables were selected focusing on questions related to: a) acceptance and willingness to buy enriched fish; b) origin of fish; c) valued fish characteristics such as convenience (ready to eat, easy to cook) and sensorial properties (freshness, flavour); and d) price-related aspects. All statistical tests were performed using IBM SPSS®22 software.

## Results and Discussion

### *Demographic characterization of survey respondents*

A total of 778 complete answers were obtained through a self-administered online questionnaire. A detailed demographic characterization is presented in Table 1. By comparing the studied sample with the Portuguese population census from 2011 (INE, 2012) several differences were revealed, whereby extrapolations for the Portuguese population could not be made. The main differences found concerned the age distribution and education level. In a 2011 census, 55% of the population ranged between the ages of 25 and 64, and 19% were over 65 years old. Respondents also showed to be more highly educated than the national average, since only 12% of the population has a higher education. The gender difference is not very pronounced (48% men vs 52% women) compared to the survey. Nevertheless, although the sample of participants in this survey was not representative of the general Portuguese population a large age range (16 – 83 years) was obtained. Another recent online survey on seafood consumption patterns in the Portuguese population (1083 answers) described a very similar sample characterization: only 2.2% of individuals were over 65; 64% were women and 83% had higher education (Cardoso et al., 2013). The recruitment via internet surely reached a larger educated fraction of the Portuguese population and a lower representativeness of the older age group. Census revealed that in 2011, 93% of individuals with secondary education and 95% with higher education had internet access in Portugal (INE, 2012). Online surveys are becoming a commonly accepted method of reaching volunteers for consumer research (Evans and Mathur, 2005; Wright, 2005). A common limitation of these web-based approaches is the lack of control of sample characteristics and the possible bias for the research cohort, despite the saving of time and efforts; the access to large populations and the increased anonymity of participants give online surveys significant advantages over other formats (Campos et al., 2011; Riva et al., 2003). To maximize the information from the data obtained, two consumer groups were established, based on their high (HIG, 58%) or low (LIG, 42%) interest in fish origin (wild vs aquaculture). This clustering of respondents resulted in distinct demographic profiles ( $P < 0.05$ ) regarding age distribution and gender but did not affect ( $P > 0.05$ ) sample characteristics on education

level, monthly family income, presence of children in the household, and fish purchasing decisions (Table 2.1).

**Table 2.1.** Description of socio-demographic characteristics of respondents.

	Portuguese population <sup>1</sup>	Total respondents <sup>2</sup>	High Interest Group	Low Interest Group	<i>P</i> value <sup>3</sup>
<b>Number of participants (%)</b>	10 561 614	778 (100%)	451 (58%)	327 (42%)	
<b>Age (years)</b>					0.06
Less 25	26 (%)	76 (10%)	9%	11%	
26 – 40	55 (%)	456 (59%)	55%	63%	
41 – 65		234 (30%)	34%	25%	
Plus 65	19 (%)	12 (2%)	2%	1%	
<b>Gender</b>					0.027
Female	52 (%)	481 (62%)	(264) 59%	(217) 66%	
Male	48 (%)	297 (38%)	(187) 42%	(110) 34%	
<b>Education level</b>					0.415
Primary	74 (%)	6 (1%)	7%	9%	
Undergraduate	14 (%)	87 (11%)	10%	13%	
Graduate	12 (%)	685 (88%)	89%	86%	
<b>Family Income (€)<sup>4</sup></b>					0.424
Less 1000		143 (20%)	20%	21%	
1001 - 2000		219 (31%)	30%	33%	
2001 - 3000		190 (27%)	27%	28%	
Plus 3000		150 (21%)	24%	18%	
<b>Children in the household</b>		53%	55%	51%	0.308
<b>Responsible for buying food at home</b>		88%	89%	86%	0.142

<sup>1</sup>INE, 2012

<sup>2</sup>The column “total respondents” corresponds to the characterization of all respondents with complete answers either in numbers as in percentages by categories. The columns “low interest group” and “high interest group” correspond to the demographic characterization of these specific groups.

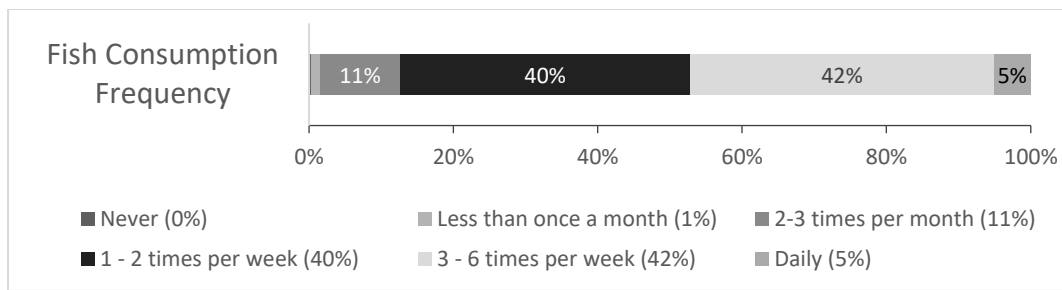
<sup>3</sup> Indicates statistical differences between low and high interest groups following Chi-square analysis.

<sup>4</sup> Calculations were performed with data from 702 respondents, 76 claimed not knowing their family income.

### **Fish consumption and health concerns**

Strong fish consumption levels were found in this study (fig. 2.1), reflecting previously reported data for Portuguese consumers (FAOSTAT, 2011). The sampled population showed high fish consumption rates, with 47% eating fish daily, or more than three times a week,

and 87% eating fish at least one or two times a week. This consumption comprises both high levels of wild and cultured fish since respondents were very positive regarding consumption of fish from aquaculture, with 79% affirmative answers. Outcomes also revealed that the frequency of fish consumption was positively influenced by children in the household ( $P = 0.021$ ), by age ( $P = 0.046$ ), and by higher family incomes ( $P < 0.001$ ). Similar conclusions were drawn in studies performed in other countries where fish consumption increased in elderly populations (Brunsø, 2003; Jahns et al., 2014; Olsen, 2003); with higher incomes (Jahns et al., 2014; Verbeke and Vackier, 2005) and with education levels (Jahns et al., 2014). In this study, and probably due to the methodology, most respondents (88%) had a higher education, whereby a correlation between education and consumption was not observed.



**Figure 2.1.** Fish consumption frequency.  
Values represent the percentage of answers for each class.

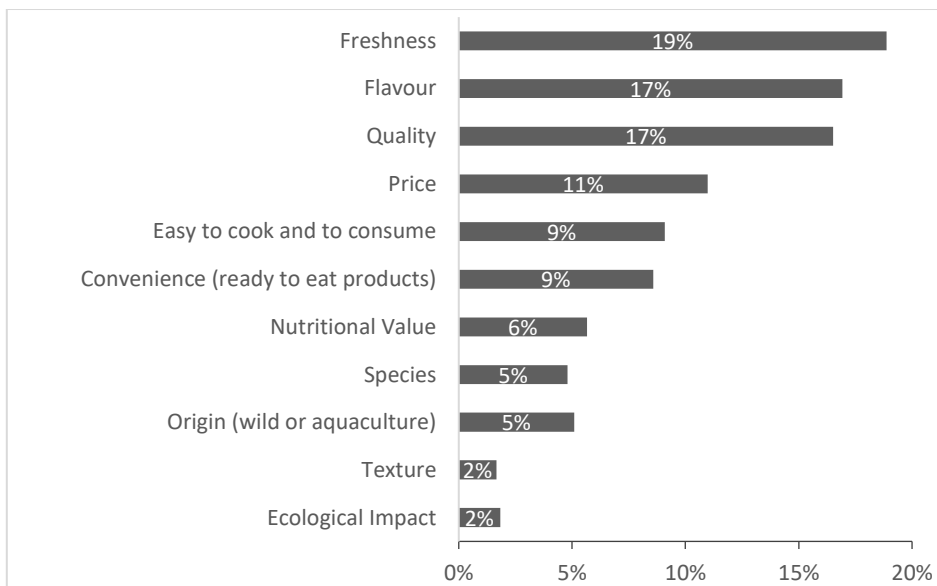
Consumers' awareness of the relationship between food and health is clearly established. Food consumption, and fish in particular, has been frequently associated with beneficial health effects, with new evidences being acknowledge every day. Pieniak et al. (2008) described European consumers as very involved with their health and very interested in healthy eating. Accordingly, Portuguese enquiries in the present study stated similar interest, where 93% of participants reported to give much importance to food concerns on daily life and 99% believed in a strong relation between food and health. Healthy eating is known to positively influence fish consumption among European consumers since fish and seafood are considered healthy dietary choices and part of a balanced diet (Altintzoglou et al., 2011; Brunsø et al., 2009; Olsen, 2004).

*Preferred selected species and preferred attributes of consumed fish*

Portuguese fish markets offer a large diversity of species which contribute for good dietary diversification. Some species, however, are seasonal, and their consumption is less representative. According to our study, the main species consumed were cod (68%), tuna (51%) and hake (45%) coming from wild fisheries, followed by salmon (41%) and seabream (33%) mainly produced in aquaculture systems. These findings are consistent among the studies. The same species were recently reported as the top five fish consumed in Portugal in a survey conducted by Cardoso et al. (2013, 2016). Ernst and Young (2009) had previously referred cod, tuna, hake, mackerel and sardine as the most consumed species in Portugal. This consistency may be supported by studies on consumer attitudes describing habits and past behaviour as main determinants associated with fish consumption patterns (Verbeke and Vackier, 2005). Also, Trondsen et al. (2004b) reported that seafood consumption is strongly related to traditional food and knowledge passed down through generations.

Besides daily habits, food choice behaviour is a complex process dependent on several factors. In general, taste and sensorial attributes are the main drivers, according to several consumer behaviour studies on food selection (Olsen, 2003). Januszewska et al. (2011) performed a study of European consumers assessing the factors that most influence daily food choice, and found as primary motivation sensory appeal, followed by health, natural content and price ahead of convenience. Published information on consumers' attitudes when buying seafood shows diverse results, with consumption varying between countries, with different habits and consumers' groups (such as fish lovers or experienced consumers) (Brunsø, 2003). Still, sensorial attributes were considered one of the main factors motivating fish consumption in numerous studies (Bredahl and Grunert, 1997; Olsen, 2001), followed by freshness and price (Brunsø, 2003). However, other works give taste a minor role (Leek et al., 2000) and point out health concerns as a major influence for seafood consumption (Olsen, 2004; Pieniak et al., 2008; Pieniak et al., 2010). In the present study, freshness, flavour and overall quality were the most important fish attributes selected by the sampled population, ahead of price (fig. 2.2). Being easy to cook and to consume, together with convenience, were referred only by 20% of the enquiries. Convenience is often considered

as an important factor in food choice (Steptoe et al., 1995) and with seafood in particular (Brunsø, 2003; Olsen, 2003; Olsen et al., 2007). Fish may be perceived as not convenient due to the time and effort required in its preparation, as well as the bones, which might be a barrier for its consumption (Brunsø, 2003). Whole fish consumption is still a main preference for Portuguese consumers and is closely associated with the freshness and quality attributes (Cardoso et al., 2013) and much less to convenience issues. Nutritional value, often the second food criteria for consumers after sensory appeal (Roininen et al., 1999), was only seventh choice, maybe because fish is already perceived as healthy and nutritious food (Verbeke et al., 2005) and is thus not a priority to consumers at the purchasing moment. Ecological impact also scored very low as observed by Pappalardo and Lusk (2016).



**Figure 2.2.** Valorised fish attributes by Portuguese consumers.

Values represent the percentage of answers for each attribute. Participants selected two attributes resulting in a sum of percentages superior to 100%.

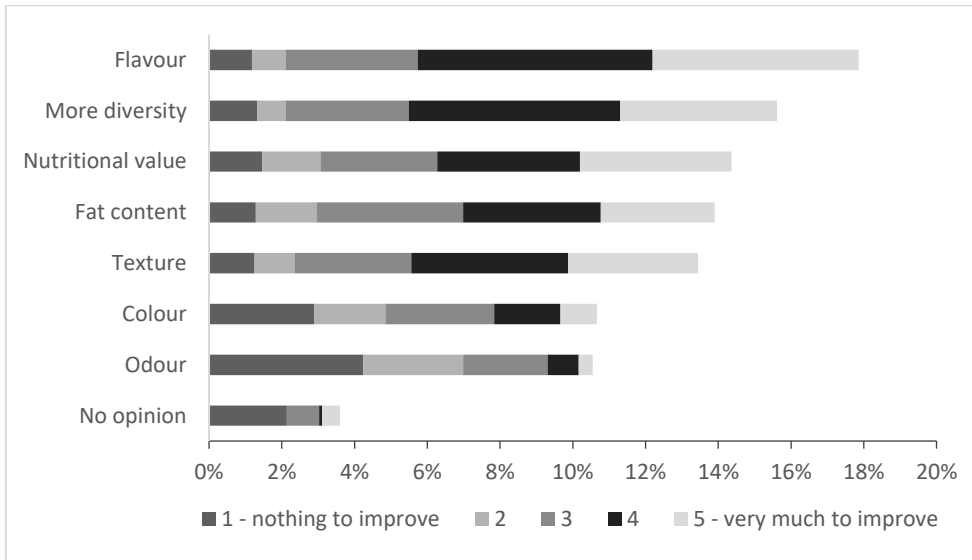
### ***Farmed fish and selected improvements***

The supply of farmed fish is increasing, along with its positive image among European consumers, as described in different research works in this field (Altintzoglou et al., 2011; Claret et al., 2014; Vanhonacker et al., 2011). The population sample assessed in the survey, where fish consumption was high, revealed that fish from aquaculture was consumed extensively (78%). Some differences were found between gender and age groups. Men were

more favourable than women (83% vs 75%  $P = 0,006$ ) to consume farmed fish. The older age groups were more receptive to aquaculture fish consumption with 83%, 79% and 80% for the three older age groups compared with 64% observed in the <25 group ( $P = 0,016$ ). Recently, Cardoso et al. (2016) described older respondents as more unwilling to consume aquaculture fish than younger ones, however this tendency was not observed in the present survey. The relative proportion between age classes is very similar between surveys, and therefore the differences may arise from having questions formulated differently or having distinct populations generating diverse preferences (results). The acceptance of aquaculture fish and its consumption are influenced by several perceptions that may be based on real events, experience, and sometimes on preconceived ideas. Some consumer opinion studies suggest that aquaculture fish is regarded as less healthy and with lower nutritional value than wild fish (Claret et al. 2014). However, in a study performed in Belgium most sampled consumers did not report differences between wild and farmed fish, although mean perception of taste, health and nutritional value still scored slightly higher for wild fish (Verbeke et al., 2007). Consumers also expect the sensory properties of farmed fish to be similar to those of wild fish (Sérot et al., 1998). Fish consumers, particularly experienced ones, are able to distinguish between farmed and wild fish, depending on the species (Grigorakis et al., 2003; Fuentes et al., 2010), although these differences may not be transversely perceptible among consumers. Verbeke et al. (2007) and Vanhonacker et al. (2011) suggested that perceived differences between farmed and wild fish were mainly driven by emotion rather than by rationality, and also that consumers' opinions on farmed fish might be a reflection of intensive livestock farming.

In the present study, respondents showed strong attitudes concerning aspects to improve in aquaculture fish. Most participants clearly selected flavour, more diversity, nutritional value, fat content and texture as the main desired improvements in farmed fish (fig. 2.3). Food choice tests revealed that participants were able to distinguish between wild and farmed fish mainly based on taste and texture descriptors (Fuentes et al., 2010; Grigorakis et al., 2003; Grigorakis, 2007). Farmed fish sensory traits, such as odour, flavour intensity and texture, were shown to be positively correlated with the fat content present in the fillet (Grigorakis

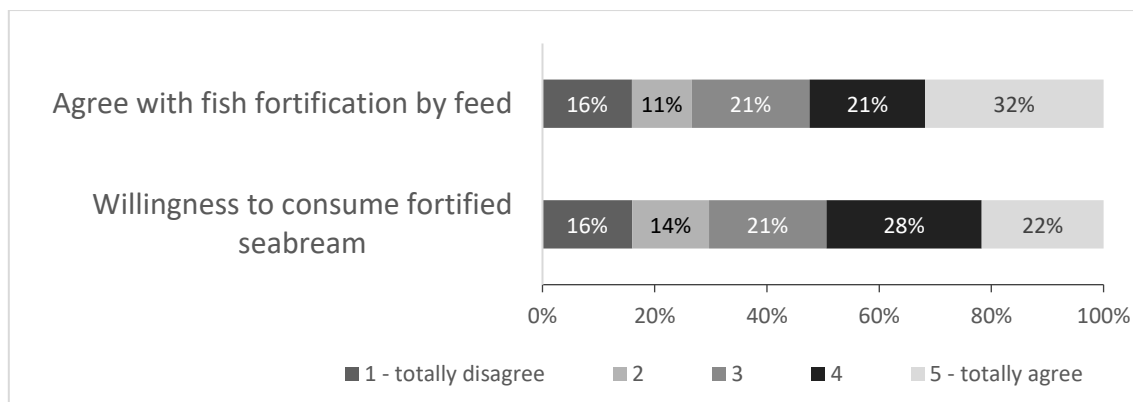
et al., 2003; Valente et al., 2011) but also quite dependent on production practices (Valente et al. 2011).



**Figure 2.3.** Attributes to improve in farmed fish chosen by inquired consumers  
Values represent the percentage of answers for each attribute categorized from nothing to improve (1) to very much to (5).

#### *Fish fortification: concept and selected nutrients*

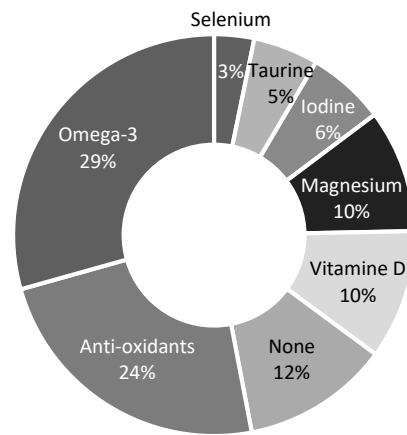
In terms of one of the main goals of this questionnaire, results revealed a good receptiveness of the fish fortification concept (fig. 2.4). More than half (52%) of the respondents agreed with fish fortification, 27% disagreed and 21% neither agreed nor disagreed, men being generally more favourable (59%) than women (49%) ( $P = 0.003$ ). Moreover, 49% of respondents showed willingness to consume fortified fish, mainly among the oldest (90%) and youngest (68%) groups ( $P = 0.001$ ), though all groups were positive towards such consumption. These results did not confirm the first established hypothesis grounded on consumers' scepticism about the enhancement of fish from aquaculture.



**Figure 2.4.** Percentage of respondents concerning: 1) agreement on fish fortification and 2) willingness to consume fortified seabream  
 Values represent the percentage of answers for each question categorized from totally disagree (1) to totally agree (5).

Of prime importance is nutrient used in fortification and the combination between food carrier and beneficial ingredient. Some studies have concluded that the carrier product may be a more important predictor of intention to purchase than the health claims related to the food item (Lyly et al., 2007), or the main health effects of an ingredient (Krutulyte et al., 2011). Moreover, it was described that food fortification with an already present compound would improve the perceived naturalness (Grunert, 2005) and healthiness (Ares and Gámbaro, 2007) of the product. It was also observed that consumers fear an off-flavour caused by a functional ingredient when there is no natural link to the carrier product (Grunert, 2010). In this study, omega-3 fatty acids and anti-oxidants were the most popular nutrients for fish fortification (fig. 2.5), followed by vitamin D and magnesium. Familiarity with omega-3 fatty acids and anti-oxidants, and knowledge of their beneficial health effects, probably owed to strong media advertisement, even if associated with other products, may have induced participants' choice. It has been referred that consumers are more likely to accept functional ingredients that they are familiar with, usually due to better knowledge of their health benefits, but also influenced by longer market permanencies (Bech-Larsen and Scholderer, 2007; Urala and Lähteenmäki, 2007). Omega-3 fatty acids were previously chosen in other studies as preferred nutrients in functional foods (Bech-Larsen and Grunert, 2003) and included in the top bioactive ingredients considered as most effective for improving health (Vella et al., 2013). Moreover, fish is already known for its high omega-3

levels, and fortification with a naturally present nutrient may be perceived as a more natural product.



*Figure 2.5. Selected nutrients for farmed fish fortification*  
Values represent the percentage of answers for each nutrient.

#### *Fish fortification: purchasing intentions*

The evaluation of consumers' purchasing intentions allows predicting, at least to some degree, future behaviour and market viability of theoretical food items. If fortified seabream was marketed with the same price as common farmed seabream, more than half the respondents (55%) would buy it. Additionally, the oldest and youngest segments (68%) were slightly more favourable to this purchasing than the other age groups (52%: 26-40 and 55%: 41-65) ( $P = 0.047$ ). In the case of a higher market price consumers were still receptive to purchasing fortified seabream, though to a lesser extent (35%).

An interesting fact is that the percentage of respondents willing to buy fortified seabream (55%) was higher if compared with the number of respondents who agreed with fortification (52%) and were willing to consume it (49%), which suggests that price accessibility may positively influence undecided consumers. In fact, about 20% of respondent's undecided about fish fortification ( $P=0.000$ ) and 22% undecided about fortified fish consumption ( $P=0.000$ ) would select fortified seabream. Only when faced with the question of paying a higher price for fortified fish the score was quite lower, but still 35% showed interest in purchasing. Recently Pereira et al. (2016) assessed the receptiveness of institutional buyers such as hotels and schools to fortified seabream, but also evaluated potential

commercialization channels for this new product. Results showed that 45.8% of respondents would probably buy fortified seabream if it was currently available in the market, while only 20.9% stated the opposite. Additionally, it was found that participants were willing to pay a premium price for this food item, on average 7.8% more than the current seabream price, though for some consumer segments the price could be 20% higher. Studies describe that price may impact consumers' choice of functional foods (Annunziata and Vecchio, 2013). Consumers are willing to pay premium prices for products that convey health benefits (Maynard and Franklin, 2003) but only up to a certain cost (Siró et al., 2008). Norwegian consumers would pay 15% extra for organic and animal welfare-labelled salmon compared with conventionally farmed salmon (Olesen et al., 2010). Whitmarsh and Palmieri (2011) also described the willingness to pay premium for salmon produced under minimized pollution methods. Price has been referred as one of the barriers for purchasing functional foods, but not the most important one. The carrier product (Ares and Gámbaro, 2007) and taste (Vella et al., 2013) were referred ahead of price as barriers to try functional foods. It was also referred that the type of product and associated health benefits would greatly influence the disposition to pay premium prices for improved products (West et al., 2002). Overall, fortified fish seems to be a promising functional food with potential niche markets.

### *Description and characterization of consumer segments*

Previous studies on consumer behaviour showed that structuring consumers into segments with similar characteristics results in a better understanding of consumption patterns (Brunsø, 2003; Pieniak et al., 2007). Differences related to functional food acceptance were reported among consumer segments, depending on age, gender (Ares and Gámbaro, 2007), education, and presence of children in the household (Gulseven and Wohlgenant, 2014). Purchasing fish and seafood products can be linked with specific criteria such as health beliefs, eating habits, convenience or production method (i.e. origin: farm vs wild) (Carlucci et al., 2015). Even if not the most important criteria, knowing the origin of fish could influence purchasing decisions (Vanhonacker et al., 2011). This criterion was found more relevant in populations with high fish consumption levels, as Spain and Portugal (Brunsø et

al., 2009; Cardoso et al., 2016; Pieniak et al., 2010). In the present study two consumer groups were identified based on their high or low interest in knowing the origin of purchased fish: wild versus aquaculture. Knowing the fish origin was claimed to be important or very important by 58% participants, corresponding to the high interest group (HIG), while 42% of participants did not prioritise information about fish origin. This result showed that the majority of the studied population was receptive to the consumption of farmed fish, although being aware or not indifferent to the differences between wild and farmed fish.

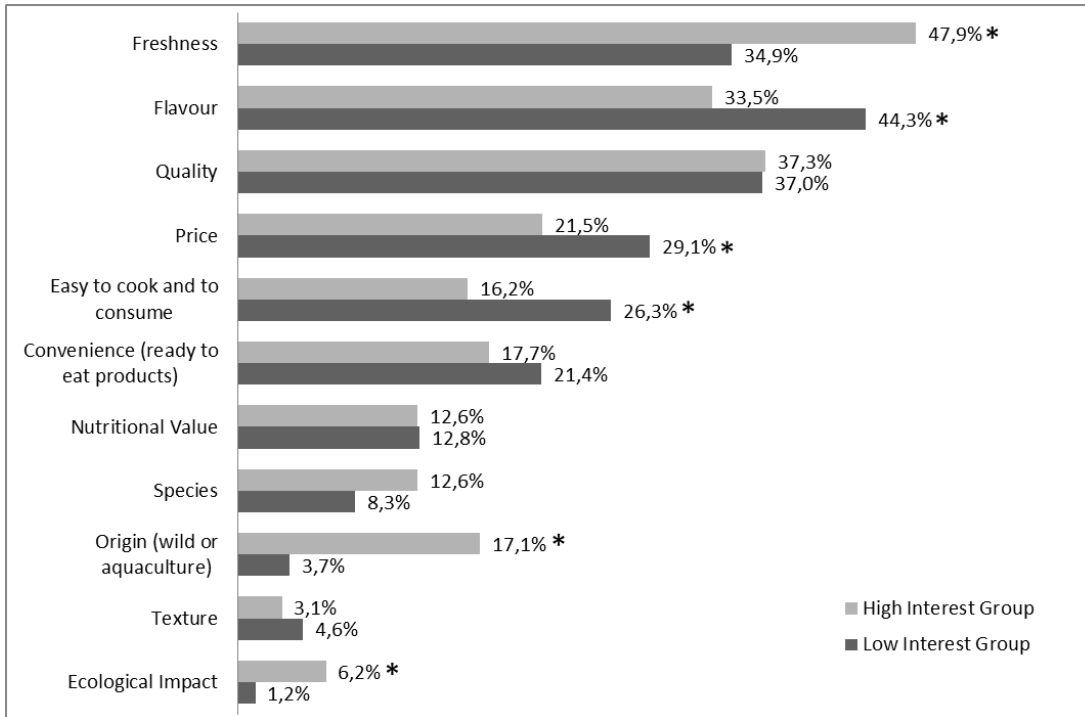
The Socio-demographic characterization of LIG and HIG is very similar (table 1), as the surveyed groups tended to reflect the sampled population. In both groups more than 86% of respondents had higher education (total sample: 87%), more than 55% were married (total sample: 56%), and 35% to 39% were single (total sample: 37%). Family income was well distributed among the group, with incomes being slightly higher for HIG. Children in the household were almost equally present in both groups (about half as in total sample: 53%). An important fact was that the participants responsible for buying foods at home were evenly distributed among interest groups (LIG: 86%; HIG: 89%; total sample: 87%). Significant differences were found only concerning gender, with more men included in the HIG (187) compared with the LIG (110), unbalancing the gender ratio. Men seem to be more aware of food issues and sharing both meal planning/preparing and food shopping activities (Flagg et al., 2014). In the present survey 82% of men and 92 % of woman reported they were responsible for buying food at home.

### *Fish consumption and valued attributes of fish*

Reflecting the total sampled population, both HIG and LIG showed to be heavy fish consumers. Nevertheless, HIG presented a higher ( $P=0.002$ ) consumption frequency, revealed a stronger belief in the relation between food and health ( $P=0.039$ ), and a greater concern with food issues on a daily basis ( $P=0.001$ ). The preferences of fish consumption by species were similarly distributed among interest groups, reflecting a cultural pattern, as previously discussed, rather than a casual option or individual attitudes. Regarding

consumption of fish from aquaculture LIG scored higher (86%) when compared to HIG (75%) ( $P < 0.001$ ), though both groups were still very positive.

One of the main differences found between groups was the relative importance given to each fish attribute (fig. 2.6). Interestingly, the four preferred attributes of consumed fish were the same as for the total group, however LIG and HIG ranked the attributes differently. For LIG, flavour ( $P = 0.002$ ) was the first most important attribute followed by quality and freshness ( $P < 0.001$ ). Price ( $P = 0.016$ ), easy cooking and consuming ( $P = 0.001$ ), and convenience were significantly more important for LIG. In contrast, LIG showed little concern regarding fish species or ecological impact. HIG selected freshness as first priority, followed by quality, and flavour. Convenience and being easy to cook and to consume were not as important as for LIG. As remarked before, this group was more concerned about fish origin ( $P < 0.001$ ) and ecological impact ( $P = 0.001$ ). Interestingly, HIG gave the same importance to fish origin as to being easy to cook and the convenience aspect. These findings suggest a preference on the part of HIG for whole and wild fish consumption rather than convenience products, and a stronger concern with environmental factors. Vanhonacker et al. (2011) also described a tendency among respondents that were more interested in fish origin being predominantly wild fish consumers. As mentioned previously, price and convenience are key purchasing factors for younger people (Krystallis et al., 2008), a tendency reflected by LIG.

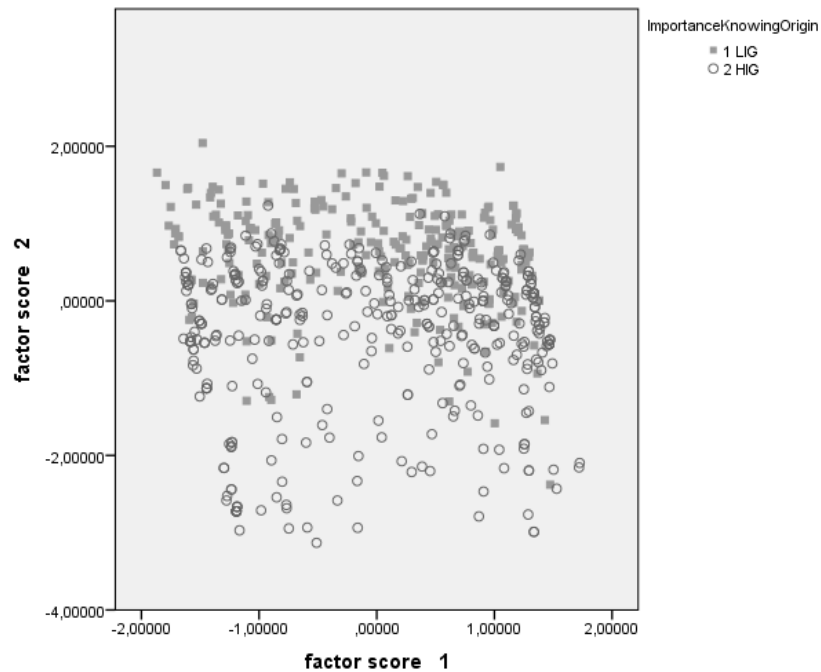


**Figure 2.6.** Fish attributes valued by consumers when buying or consuming fish, within each interest group.

Values represent the percentage of high score answers (4 and 5) in comparison with total numbers answers (categories 1 to 5) for each option and within each interest group. Each participant chose two or more characteristics; therefore, the sum of percentages is superior to 100% in both groups.

\*indicates statistical differences between groups. Dark grey colour represents LIG and light grey colour represents HIG.

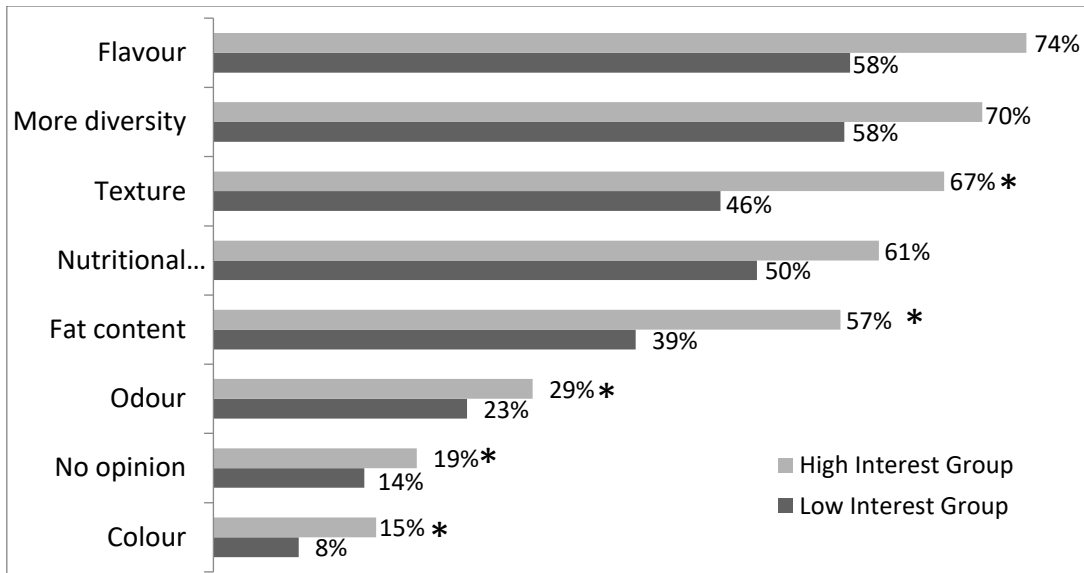
The performed PCA as complementary analysis (fig. 2.7) showed that both interest groups are more populated in the higher scores of acceptance and willingness to buy enriched fish (component 1) suggesting a positive receptiveness to fish enhancement questions. LIG showed higher scores for characteristics related to aquaculture fish consumption and very few observations on lower scores associated with fish origin issues, on the contrary HIG presents more observations on the lower scores for component 2 (fish origin issues and flavour)



**Figure 2.7.** Principal Component Analysis plot between scores 1 and 2.

These two components accounted for 39.5% of the variance observed across variables. Circles represents HIG and squares represents LIG.

The selected characteristics to improve aquaculture fish were ranked almost by the same order in both groups (fig. 2.8) and coincided with the results found for the total sample. This suggests that both groups had a similar perception of farmed fish traits. The only difference found between groups is that LIG scored nutritional value in third and texture in fourth place, while HIG ranked them in the inverse order.



**Figure 2.8.** Attributes to improve in farmed fish chosen by inquired consumers.

Values represent the percentage of high score answers (4 and 5) in comparison with total numbers answers (categories 1 to 5) for each option and within each interest group. Each participant chose two or more attributes therefore the sum of percentages is superior to 100% in both groups.

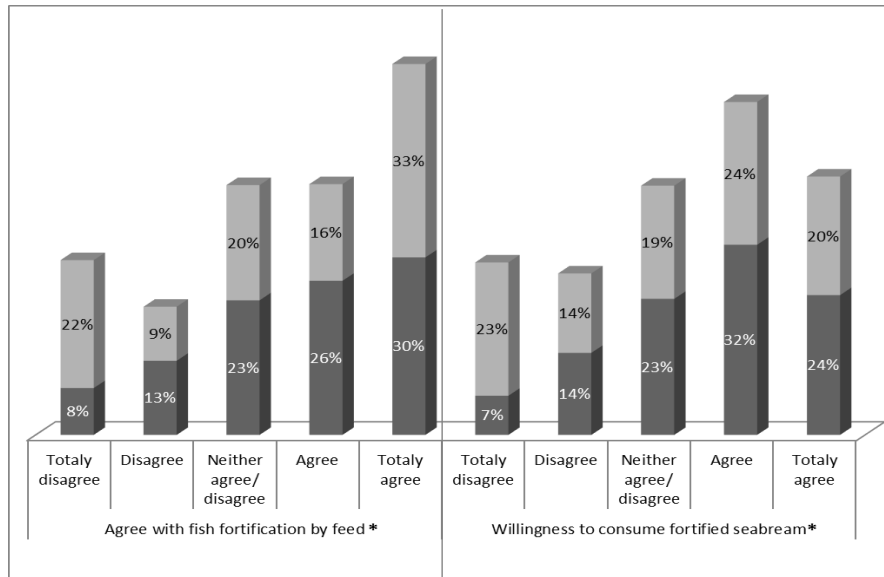
\*indicates statistical differences between groups. Dark grey colour represents LIG and light grey colour represents HIG.

### Fish fortification concept

Differences between the groups were observed regarding the concept of fish fortification through feeding. LIG showed a higher acceptance level (sum of answers scoring 4 and 5: 56% - LIG and 49% - HIG;  $P < 0,001$ ) and a stronger desire to consume fortified seabream (56% LIG vs 44% HIG;  $P < 0,001$ ) (fig. 2.9). Nevertheless, both groups showed good receptiveness to fortified fish. Almost half of HIG respondents agreed with this concept, and HIG had more participants willing to consume it (sum of answers scoring 4 and 5: 44%) than not (sum of answers scoring 1 and 2: 37%). Regarding fortification compounds, both HIG and LIG referred omega-3 fatty acids and anti-oxidants as most requested nutrients, as observed for total sample.

A very sceptic fraction was found in HIG who did not agree with fish fortification (31%), did not choose any nutrient for supplementation (23%), and showed no willingness to consume fortified fish (37%). However, it is interesting to notice that the HIG includes more participants in the scale extremes (agree totally/ disagree totally) concerning fish fortification (Fig. 2.9). Maybe owed to a greater concern about health issues observed in HIG, and

consumers with higher health concerns were also more likely to buy functional foods (Krutulyte et al. 2011).



**Figure 2.9.** Percentage of respondents by interest group regarding: 1) agreement on fish fortification and 2) willingness to consume fortified seabream.

Values represent the percentage of answers for each question categorized from totally disagree (1) to totally agree (5).

\*indicates statistical differences between groups. Dark grey colour represents LIG and light grey colour represents HIG.

Purchasing intentions reflected, as expected, the receptiveness previously showed towards the fortification concept. Both groups of respondents showed a positive intention to buy fortified seabream, 59% and 52% respectively for LIG and HIG, if accessible at the same price as commonly produced seabream ( $P = 0,042$ ). At a higher price, receptiveness was lower in both groups (LIG: 39% and HIG: 33%). As observed for the total sample, market price may influence undecided consumers in both interest groups. The results from the present study showed segmentation between groups, with consumers valuing fish attributes differently, having distinct expectations for fish as a food product and revealing different receptiveness towards fish fortification. There was a clear tendency for a higher acceptance and willingness to buy and to consume fortified seabream within the LIG.

## Conclusion

The present study showed high fish consumption frequency among all ages of the sampled population and high concern with health issues in daily life. Outcomes also revealed that fish consumption increased with children in the household, and with age and family income. Fish from aquaculture was very well accepted among consumers as was the concept of fish fortification. Based on the importance that participants gave to fish origin, two consumer segments were defined: a high, and a low interest group. The characterization of the HIG profile was more associated with older people, higher seafood consumption but less of farmed fish, and more interest in freshness, health issues and impact on sustainability. The LIG included younger people, with slightly lower seafood consumption, but of more aquaculture products, and more interested in flavour, convenience products and price. Both interest groups were consistent in their preference of fish species, attributes to improve in aquaculture fish, and selected ingredients for fish fortification. Corroborating the second formulated hypothesis, LIG was more receptive to all aspects of the fish fortification concept and showed willingness to buy and consume fortified seabream. However, the sampled population included a highly educated fraction of Portuguese consumers, not reflecting the characterization made in the Portuguese census 2011 (INE, 2012), which is a major limitation in making an extrapolation of the results for the Portuguese food market. Nevertheless, specific fortified fish products combined with an appropriate communication strategy could be designed for targeting LIG profile, which represented almost half of the population in this study.

Consumers may find enrichments as interfering with nature, therefore the perceived healthiness of the base product, the combination carrier-ingredient and the communication strategy are considered key factors for consumer acceptance of functional foods. Fish which is generally accepted as healthy and an integrant part of a balanced diet could be considered a suitable carrier product, in particular if omega-3 or anti-oxidants were the main functional compounds used. Enhanced fish may act as a novel food and may be an opportunity for the aquaculture industry to enter the fast-growing market of functional foods. However, the data produced are hypothetical since such products do not exist in the Portuguese market and

consumers' reactions in real events may be different from the considerations made during the on-line questionnaire. Nevertheless, new products will be developed in the aquaculture sector to face rising demand, and to cope with a changing consumer mentality associated with the importance of health care and prevention. Moreover, the development of new products and new technologies are also gradually eliminating consumption barriers and increasing fish products' convenience (Ernst and Young, 2009).

In the present study, as in others (Pereira et al., 2016), several participants asked for more information concerning fortification processes and specific health benefits obtained by consuming fortified fish. Effective and clear communication of farming fortification practices and health benefits were suggested to increase consumers' acceptance of both farmed fish and functional foods (Annunziata and Vechio, 2013; Siró et al., 2008). Communicating fortification in a transparent way, as well as the health benefits associated with the consumption of such products, may be an efficient approach to increasing awareness and trust in the process. Considering that the effectiveness of a functional food concept is a combination of its efficacy and consumer compliance, farmed fish has a high potential to act as functional food.

For future research it would be interesting to assess if consumers who are sceptic about farmed fish and fortification issues would become more positive and receptive after being exposed to information on these topics (leaflets and brochures). Moreover, contact with fortified fish in a real-life event would bring more evident conclusions on consumers' receptiveness.

### *Acknowledgements*

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authors are grateful to all the participants who contributed to the self-administered questionnaire.



# FORTIFICATION WITH TRACE ELEMENTS: IODINE AND SELENIUM

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Chapter 3



# Dietary macroalgae is a natural and effective tool to fortify gilthead seabream fillets with iodine: effects on growth, sensory quality and nutritional value

## Chapter 3.1

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## Dietary macroalgae is a natural and effective tool to fortify gilthead seabream fillets with iodine: effects on growth, sensory quality and nutritional value

### Abstract

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Marine fish are a rich source of iodine in the human diet. Exogenous feeding under captivity opens the possibility to tailor fish composition with health valuable nutrients, such as iodine, and establish it as a functional food. A study was undertaken to test the efficacy of various dietary iodine supplemental forms on the growth performance of gilthead seabream and assess the effects on sensory attributes and nutritional value of fillets. Duplicate groups of 35 seabream (IBW: 252 g) were fed over 118 days: a) control diet (CTRL) with 3 mg I·kg<sup>-1</sup>, supplied as potassium iodide; b) this same control diet supplemented with potassium iodide (diet KI: 26 mg I·kg<sup>-1</sup>) or with ethylenediamine dihydroiodide (diet EDDI; 22 mg I·kg<sup>-1</sup>); c) a fourth diet with 10% of *Laminaria digitata*, an iodine-rich macroalgae (diet LAM). At the end of the trial, fish doubled their initial weight, but irrespective of the iodine form used, the elevation of dietary iodine level had no effect ( $P>0.05$ ) on feed intake, growth rate, feed conversion, nutrient utilization or whole-body composition. Dietary iodine supplementation led to a significant increase ( $P<0.05$ ) of the iodine content in seabream fillets. In comparison to CTRL treatment (0.13 mg·kg<sup>-1</sup>), seabream fed KI and EDDI supplemented diets showed a significant enhancement ( $P<0.05$ ) of their fillet iodine content (0.17 mg·kg<sup>-1</sup>). Feeding seabream with the iodine-rich brown algae *Laminaria digitata* resulted in a significant ( $P<0.05$ ) 6.5-fold increase (0.84 mg·kg<sup>-1</sup> fillet) of fillet iodine content over the levels found in the CTRL treatment. Overall sensory difference between CTRL and fish fed supplemented diets was significant ( $P<0.05$ ) only for fish fed the EDDI diet. Steam-cooking elicited 13-20% losses of the iodine content of seabream fillets. Iodine supplementation had no effect ( $P>0.05$ ) on the fatty acid profile of seabream muscle. Dietary iodine-rich macroalgae was an effective and natural strategy to fortify muscle with iodine, showing that a 160 g portion of steam-cooked seabream fillets could cover approximately 80% of the Daily Recommended

Intake for iodine and 370% of the Daily Adequate Intake of EPA+DHA for enhanced cardiovascular health in adults.

Keywords: Gilthead seabream; Iodine; Fortification; Macroalgae; Culinary

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## Introduction

Food and nutritional sciences have moved from identifying and correcting nutritional deficiencies to designing foods that promote optimal health and reduce the risk of disease. The Concerted Action on Functional Food Science in Europe (1999) defined as functional, a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of disease, also it should be consumed as part of a normal food pattern (Diplock et al., 1999). Being a rich source of important nutrients, including highly digestible proteins, vitamins (A, D, niacin and B12), trace minerals (iodine, selenium) and n-3 PUFAs, fish is generally regarded as healthy food. In particular, marine fish lipids have been unequivocally associated to a protective role against a number of human diseases (FAO and WHO, 2011; Levitan et al., 2009; Raatz et al., 2013; Saravan el al., 2010). Moreover the American Heart Association recommends an intake of at least two portions of fish per week with cardio-protective effects (Gidding, 2009; Lichtenstein, 2006). Globally, in 2009 fish represented about 16.6% of animal protein supply for human consumption. In 2011 the annual per capita fish consumption was 18.8 kg (FAO, 2012) and by 2022 is estimated to rise up to 20.6 kg per capita. With the stagnation of capture fisheries aquaculture is expected to respond to this increasing demand (OECD-FAO, 2013).

Recent studies estimate that almost 44% of school-age children in Europe have inadequate iodine intake and extrapolations to the general population reveal 1.88 billion individuals worldwide with insufficient iodine intake (Andersson et al., 2012). Additionally, studies performed in pregnant women found critical levels of iodine status in several European countries (Aguayo et al., 2013; Bath et al., 2013; Hiéronimus et al., 2009; Limbert et al., 2010).

Iodine deficiency disorders (IDD) results in several metabolic changes including goiter and hypothyroidism which can occur at any stage of life, but the most devastating consequences take place during fetal development and childhood, with stillbirth, miscarriages, poor growth, and cognitive impairment (Andersson et al., 2012). Although cretinism is the most extreme manifestation, of considerably greater significance are the more subtle degrees of mental impairment that lead to poor school performance, reduced intellectual ability, and impaired work capacity (Bath et al., 2013). Iodine deficiency is the world's greatest single cause of preventable brain damage, and this fact is the primary motivation behind the current worldwide drive to eliminate iodine deficiency (WHO et al., 2007).

The primary strategy for iodine supplementation relies on salt iodization, however with the implementation of salt intake reduction programs, prevention of iodine deficiency may be less effective, and alternatives are required (Land et al., 2013). Several foods, including fish, have been suggested as suitable vehicles for iodine fortification as one of the alternatives to salt (WHO, 2014). A previous WHO and FAO (2006) report defined a suitable food to serve as vehicle for extra nutrients as being consumed in constant quantities, affordable and available all year long. Fish and seafood are the richest natural sources of iodine in food (Haldimann et al., 2005; Institute of Medicine, 2001). Since fish also meets the defined criteria for a food vehicle, fish fortification with iodine could be an excellent tool in the strategy of diminishing iodine deficient in world population.

Exogenous feeding in aquaculture unlocks the possibility to tailor fish composition in terms of its content of health valuable nutrients. Under farming conditions, fish quality traits such as fatty acid composition and concentration of several trace nutrients are known to be influenced by diet composition. Several studies have demonstrated that feed supplements can effectively tailor the composition of fish fillets in terms of bioactive fatty acids (Kennedy et al., 2007b; Ramos et al., 2008; Rosa et al., 2010) or selenium (Lorentzen et al., 1994; Schram et al., 2010). Iodine has also been successfully used in fish fortification either as an

organic form with macroalgae (Schmid et al., 2003) or an inorganic salt, potassium iodide (Julshamn et al., 2006).

The objective of this study was to test the effect of various dietary iodine supplemental forms on the growth performance of gilthead seabream, assess iodine deposition efficacy in fish muscle and evaluate its effects on the nutritional value and sensory quality of fish fillets.

## Material and methods

### *Experimental diets*

Four experimental diets were formulated to be isonitrogenous (48% crude protein), isolipidic (19% crude fat) and isoenergetic (23 MJ·kg<sup>-1</sup> gross energy). A control diet (CTRL), similar to a commercial seabream feed was formulated to contain 3 mg iodine·kg<sup>-1</sup>, supplied as potassium iodide through the mineral premix and the endogenous content of the various ingredients. Based on this control formulation, two other diets were manufactured with a target level of 20 mg iodine·kg<sup>-1</sup>, supplied either as the inorganic form potassium iodide (KI) (diet KI) and organic form ethylenediamine dihydroiodide (EDDI) (diet EDDI). Potassium iodide (KI) is currently used as iodine source in fish feeds, while the organic iodine salt (EDDI) is frequently used in terrestrial animals, but to our knowledge never tested in fish. Both products were purchased from Sigma-Aldrich (ref. 30315: potassium iodide with 68.8% iodine; ref. S573019: ethylenediamine dihydroiodide with 79.5% iodine). The level of 20 mg iodine·kg<sup>-1</sup> was adopted since it is the currently authorized maximum content of total iodine in complete feeds for fish in Europe. These iodine sources were applied post-extrusion by vacuum coating with the oils. A fourth diet was formulated with 10% of *Laminaria digitata*, an iodine-rich macroalgae (Holdt and Kraan, 2011) (diet LAM), with 426.2 mg iodine·kg<sup>-1</sup>. Given its low protein content and to maintain isonitrogenous conditions among the feeds, macroalgae was incorporated mainly at the expense of carbohydrates (wheat and peas), with small adjustments on vegetable protein sources (wheat gluten and corn gluten). Diets were also supplemented with selected crystalline indispensable amino acids and dicalcium phosphate to avoid essential amino acid or phosphorus imbalance.

**Table 3.1.** Ingredient and proximate composition of experimental diets.

Ingredients, g·kg <sup>-1</sup>	CTRL	KI	EDDI	LAM
Fishmeal LT 70 <sup>1</sup>	100	100	100	100
Fishmeal FAQ <sup>2</sup>	200	200	200	200
Wheat gluten <sup>3</sup>	40	40	40	55
Corn gluten <sup>4</sup>	116	116	116	120
Soybean meal <sup>5</sup>	140	140	140	140
Whole wheat	139	139	139	80
Dehulled pea grits <sup>6</sup>	90	90	90	30
Fish oil <sup>7</sup>	140	140	140	140
Vitamin and mineral premix <sup>8</sup>	10	10	10	10
Guar gum <sup>9</sup>	5	5	5	5
Dicalcium phosphate <sup>10</sup>	10	10	10	10
L-Lysine <sup>11</sup>	5	5	5	5
DL-Methionine <sup>12</sup>	5	5	5	5
KI (mg·kg <sup>-1</sup> ) <sup>13</sup>		29.1		
EDDI (mg·kg <sup>-1</sup> ) <sup>13</sup>			25.2	
Macroalgae meal <sup>14</sup>				100
Dry matter (DM), %	94.8 ± 0.1	94.3 ± 0.1	93.7 ± 0.1	91.7 ± 0.0
Crude protein, %DM	42.4 ± 0.0	42.1 ± 0.3	42.8 ± 0.1	42.3 ± 0.5
Crude fat, % DM	17.0 ± 0.2	16.7 ± 0.0	17.0 ± 0.1	16.8 ± 0.5
Ash, %DM	9.6 ± 0.0	9.5 ± 0.0	9.6 ± 0.0	11.5 ± 0.1
Total phosphorus, % DM	1.4 ± 0.0	1.5 ± 0.1	1.4 ± 0.0	1.4 ± 0.0
Iodine, mg·kg <sup>-1</sup> DM	2.4 ± 0.4	26.1 ± 1.7	22.9 ± 1.7	426.2 ± 24
Gross energy, kJ·g <sup>-1</sup> DM	21.7 ± 0.1	21.7 ± 0.0	21.8 ± 0.2	21.5 ± 0.1

<sup>1</sup> Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru. <sup>2</sup> Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal. <sup>3</sup> VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France. <sup>4</sup> Corn gluten feed: 61% CP, 6% CF, COPAM, Portugal. <sup>5</sup> Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal. <sup>6</sup> Aquatex G2000: 24% CP, 0.4% CF, SOTEXPRO, France. <sup>7</sup> COPPENS International, The Netherlands.

<sup>8</sup> Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate, 7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings. <sup>9</sup> Seah International, France. <sup>10</sup> Dicalcium phosphate: 18% phosphorus, 23% calcium, Fosfitalia, Italy. <sup>11</sup> Biolys 54.6%, EVONIK Nutrition & Care GmbH, Germany. <sup>12</sup> DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany <sup>13</sup> Sigma-Aldrich: KI - potassium iodide ref. 30315; EDDI - ethylenediamine dihydroiodide, ref. S573019. <sup>14</sup> Dry Laminaria digitata: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France.

Ingredients were ground (below 250 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Powdered ingredients were then mixed accordingly to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France), No oils were incorporated at this stage. Both CTRL and LAM diets were manufactured by SPAROS, at a pellet size of 5.0 mm, by means of a twin-screw extruder (model BC45, Clextal, France) with

a screw diameter of 55.5 mm and temperature ranging 115-118°C. Upon extrusion, feeds were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling of the pellets, the oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). At this stage, and using the pellets from the CTRL treatment, KI and EDDI products were blended with fish oil and applied by vacuum coating. Throughout the duration of the trial, experimental feeds were stored at room temperature, but in a cool and aerated emplacement. Samples of each diet were taken for proximate composition and total iodine analysis (Table 3.1).

### *Growth trial and sampling*

The trial was conducted at the Experimental Research Station of CCMAR (Faro, Portugal). Experiments were directed by trained scientists (following category C FELASA recommendations) and conducted according to the European guidelines on protection of animals used for scientific purposes (EC 2010 - Directive 2010/63/EU).

Eight homogenous groups of 35 seabream each, with a mean initial body weight of 252 g were stocked in 1000 L circular plastic tanks. Fish were fed to apparent satiety, by hand, three times a day (09.30, 14.30 and 16.30h) during week days, twice a day in Saturdays and unfed on Sundays. Excess feeding was minimized, and feed intake was quantified throughout the trial. Each experimental treatment was tested in duplicate tanks over 118 days. Rearing tanks were supplied with flow-through gravel-filtered, aerated seawater (salinity: 35 parts per thousand; temperature: 22-27°C; dissolved oxygen  $6.9 \pm 1.1 \text{ mg}\cdot\text{L}^{-1}$ ) and subjected to natural photoperiod changes through summer conditions. Final samplings were done 48 hours following the last meal. Fish were slaughtered by immersion in ice-saltwater slurry (4:1) until death. At the start (6 fish from the initial stock) and at the end of the trial, three fish from each tank were sampled for analysis of whole-body composition. Moreover, seabream skinless right fillets were collected at the start (n=2), after 6 and 12 weeks (n=2) and at the end of trial (n=2 pools of 3 fish each), stored at -80°C for analysis of iodine content and fatty acid composition. Left fillets collected at the end of the trial were also used for

quantitation of iodine content after being subjected to a culinary steam-cooking process. For sensory analysis, after slaughter, fish were packed in insulated polystyrene boxes, with the ventral side upward, covered with plastic and flaked ice and immediately transported to the laboratory. Fifteen fish from each treatment were weighed, scaled and filleted 24 h after death. Left and right fillets (with skin) were separately packed in low density polypropylene bags (15.2 x 33.0 cm) and kept at 4°C until sensory assessment.

#### *Culinary steam-cooking processing*

Regarding the effect of culinary processing on the iodine content of seabream fillets only two treatments were evaluated, the control and LAM groups. From each treatment, six fillets with 1.5 mm thick were divided at random in two pools of three fillets, weighted and wrapped in aluminium foil. Each pool was individually steam cooked for 15 min in a household Themomix TM31 equipped with a Varoma tray (Vorwerk International Strecker & co., Wollerau, Switzerland). Temperature was set over 100°C to generate hot steam and the mixing bowl contained 1 litre of tap water. At each cooking process the mixing bowl was refilled with 1l of tap water and fillets were only introduced in the tray when it reached the set steam temperature. After cooking, fillets were left to cool for 10 min with the aluminium foil closed to avoid steam losses. Water losses were quantified, and cooked fillets were weighed and stored frozen at -20°C until iodine and fatty acid analysis.

#### *Analytical methods*

Proximate composition analysis of the diets and whole fish was made by the following procedures: dry matter by drying at 105°C for 24 h; ash by combustion at 550°C for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428, Leco Instruments, USA); crude fat after dichloromethane extraction by the Soxhlet method; total phosphorus according to the ISO/DIS 6491 method using the vanado-molybdate reagent; and gross energy in an adiabatic bomb calorimeter (model C2000, IKA-Werke GmbH & Co. KG, Staufen, Germany). Iodine content of feeds and fillets was determined according to Mesko et al. (2010) by

inductively coupled plasma-mass spectrometry (ICP-MS) at an external analytical laboratory. Briefly, samples were digested by microwave-assisted acid digestion (Multiwave 3000, Anton Parr, Graz, Austria) with nitric acid and hydrogen peroxide. After cooling, digests were diluted with ammonium hydroxide and iodine was determined by ICP-MS (model X7, Thermo Elemental, Franklin, MA, USA), using potassium iodate (KIO<sub>3</sub>) as standard. The accuracy of the iodine quantification method was assessed in both feed and fillet samples, by spiking samples (n=3) with graded levels of KIO<sub>3</sub> standard (spike recovery ranged from 97.8 to 100.4%). Total lipids in the fillets were extracted according to the method of Folch et al. (1957) and subsequently, the fatty acid composition of fillets was determined by gas-chromatography analysis of methyl esters, according to the procedure of Lepage and Roy (1986), modified by Cohen et al. (1988) and described in further detail by Costa et al. (2013). Based on the fatty acid profile of steam-cooked seabream fillets, the atherogenic and thrombogenic index (AI and TI, respectively) were calculated accordingly to Ulbricht and Southgate (1991) for evaluation of the predisposition for incidence of coronary heart disease. The nutritional contribution (NC) of steam cooked seabream fillets was calculated as the percentage of the Daily Recommended Intake (DRI) for iodine and of the Daily Adequate Intake (DAI) for combined EPA and DHA, according to the following formula:

$$NC (\%) = 100 \times \frac{C \times M}{DRI \text{ or } DAI}$$

Where:

C: mean concentration of nutrient (iodine or EPA+DHA) in mg·kg<sup>-1</sup>

M: meal fillet portion consumed is 0.160 kg, assuming a 40% fillet yield in a commercial size seabream of 0.400 kg.

DRI for iodine is 0.150 mg·d<sup>-1</sup> for adolescents and adults (WHO and FAO, 2004).

DAI for EPA+DHA is 500 mg·d<sup>-1</sup> for cardiovascular health in adults (ISSFAL, 2004).

### *Sensory Evaluation*

Sensory evaluation was carried out in an acclimatized room equipped with individual booths. To reduce the variability within the fillets, the parts close to the head and the tail were rejected. Each fillet was individually wrapped with aluminium foil to avoid odour losses and cooked for 6 min at 100 °C in a saturated steam oven (Rational Combi-Master CM6 Cross

KuchentechnikCmbH, Landsberg, Germany). Cooked fillets were presented to the panellists sequentially in coded white dishes under normal white lighting. A difference from control test was carried out using a panel composed by eighteen panellists. The test objective was the measurement of a perceived overall sensory difference between the control (clearly labelled) and the treatments samples. A blind control was also included within the test samples and the panellists were informed about it (Meilgaard et al., 1999). Panellists rated the size of the difference between the labelled control and the test samples (control was evaluated first) on a line scale. The scale ranged from 0 cm – no differences to 12 cm – evident differences. Results express the average distance (in cm) between tested samples, including the blind-control, and the known control sample.

### *Statistical analysis*

Growth performance and iodine deposition data are expressed as means of two replicates  $\pm$  standard deviation. Data were subjected to a one-way analysis of variance. Parameters expressed as percentages were subjected to arcsin square root transformation. Sensory data were analysed by one-way analysis of variance followed by the Dunnett Test to evaluate the differences between tested samples and the blind-control. Culinary treatment data was analysed with Student's T-test. Statistical significance was tested at 0.05 probability level. All statistical tests were performed using the SPSS software (v21, IBM, USA).

### **Results**

At the end of the trial fish had doubled their initial weight, reaching a final body weight ranging from 489 to 506 g (Table 2). Specific growth rate (SGR) varied between 0.56 and 0.58  $\% \cdot \text{day}^{-1}$ , while the feed conversion ratio (FCR) ranged from 1.96 to 2.06. Irrespective of the iodine source tested, the elevation of dietary iodine level had no effect ( $P > 0.05$ ) on the feed intake, growth rate, feed efficiency or protein efficiency ratio. Similarly, dietary treatments had no effect ( $P > 0.05$ ) on the whole-body moisture, protein, fat, ash, phosphorus and energy contents (Table 3.2). Data on weight gain, feed intake and whole-body composition of fish

allowed the estimation of protein and fat retention, which were not affected ( $P>0.05$ ) by dietary treatments.

**Table 3.2.** Growth performance, whole-body composition and nutrient retention of fish.

		CTRL	KI	EDDI	LAM
IBW <sup>1</sup> , g		251.4 ± 0.8	251.7 ± 1.2	254.9 ± 0.8	254.6 ± 3.6
FBW <sup>2</sup> , g		488.9 ± 2.2	494.7 ± 3.1	505.5 ± 16.9	502.9 ± 8.6
VFI <sup>3</sup>		0.98 ± 0.01	0.95 ± 0.02	0.97 ± 0.00	0.97 ± 0.03
SGR <sup>4</sup> , %·d <sup>-1</sup>		0.56 ± 0.01	0.57 ± 0.00	0.58 ± 0.03	0.58 ± 0.00
FCR <sup>5</sup>		2.06 ± 0.05	1.96 ± 0.00	1.98 ± 0.11	2.02 ± 0.01
PER <sup>6</sup>		1.68 ± 0.07	1.52 ± 0.03	1.52 ± 0.03	1.52 ± 0.03
<b>Body composition</b>					
	<b>Initial</b>				
Moisture, % ww	66.5	61.3 ± 0.1	62.2 ± 0.3	61.1 ± 0.1	63.6 ± 2.3
Protein, % ww	17.3	17.7 ± 0.2	17.7 ± 0.0	17.8 ± 0.2	17.8 ± 0.8
Fat, % ww	12.0	16.6 ± 0.3	16.1 ± 0.4	16.3 ± 1.5	15.0 ± 2.0
Ash, % ww	4.3	3.8 ± 0.3	3.9 ± 0.2	3.9 ± 0.4	3.6 ± 0.2
Phosphorus, % ww	0.7	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
Energy, kJ·g <sup>-1</sup> ww	8.4	10.5 ± 0.2	10.2 ± 0.4	10.3 ± 0.4	9.5 ± 0.7
<b>Retention<sup>7</sup></b>					
Protein, % intake		19.7 ± 0.9	21.1 ± 0.4	19.9 ± 2.0	21.4 ± 1.8
Fat, % intake		63.4 ± 3.5	63.6 ± 1.7	61.7 ± 7.3	56.0 ± 12.6

Values are means ± standard deviation (n=2). Absence of superscript letters within a row represent no significant differences between treatments ( $P>0.05$ ).

<sup>1</sup> Initial mean body weight.

<sup>2</sup> Final mean body weight.

<sup>3</sup> Voluntary feed intake: crude feed intake / (IBW + FBW) / 2 / 118 days.

<sup>4</sup> Specific growth rate: (Ln FBW – Ln IBW) x 100 / 118 days.

<sup>5</sup> Feed conversion ratio: dry feed intake / wet weight gain.

<sup>6</sup> Protein efficiency ratio: wet weight gain / crude protein intake

<sup>7</sup> Retention: 100 x (FBW x final carcass nutrient – IBW x initial carcass nutrient) / nutrient intake.

Dietary iodine supplementation led to a significant increase ( $P<0.05$ ) of the iodine content in seabream fillets (Table 3.3; Figure 3.1). After 118 days of feeding, fish fed the CTRL diet had a fillet iodine content of 0.13 mg·kg<sup>-1</sup>. In comparison to CTRL treatment, seabream fed the ethylenediamine dihydroiodide (EDDI) and potassium iodide (KI) supplemented diets showed a significant enhancement ( $P<0.05$ ) of their fillet iodine content (0.17 mg·kg<sup>-1</sup>). Irrespective of the supplemental iodine source, this change represented a 1.3-fold increase over the iodine levels found in the CTRL treatment. Additionally, feeding seabream with the iodine-rich brown algae *Laminaria digitata* resulted in a 6.5-fold increase (0.84 mg·kg<sup>-1</sup> fillet) of fillet

iodine content over the levels found in the CTRL treatment. The fillet iodine content found in seabream fed the LAM diet was significantly higher ( $P < 0.05$ ) than values found in fish fed all other diets. Daily iodine gain at the muscle level followed a similar trend, with lower values found in fish fed the CTRL diet ( $0.65 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ), intermediate in fish fed the KI and EDDI diets ( $1 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ) and highest in those fed the LAM diet ( $6.57 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ).

**Table 3.3.** Iodine content in seabream fillets fed the various experimental diets.

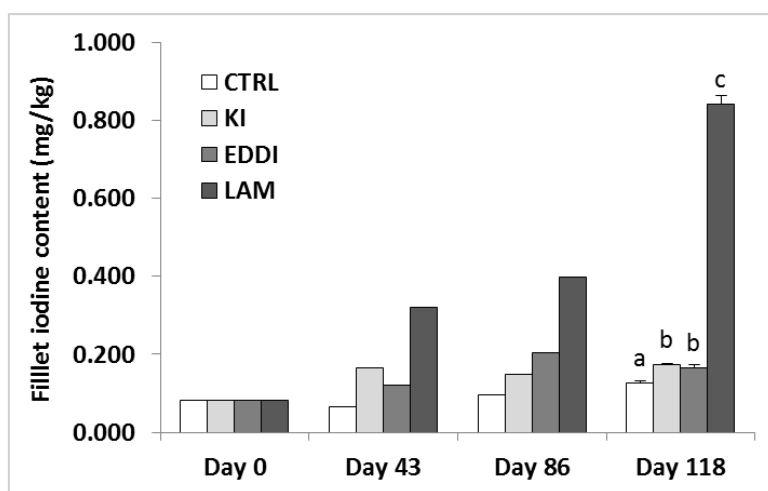
	CTRL	KI	EDDI	LAM
<b>Iodine</b>				
Initial raw fillets, $\text{mg}\cdot\text{kg}^{-1}$	$0.08 \pm 0.00$	$0.08 \pm 0.00$	$0.08 \pm 0.00$	$0.08 \pm 0.00$
Final raw fillets, $\text{mg}\cdot\text{kg}^{-1}$	$0.13 \pm 0.01^a$	$0.17 \pm 0.00^b$	$0.17 \pm 0.00^b$	$0.84 \pm 0.02^c$
Daily iodine gain, $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$	$0.65 \pm 0.05^a$	$1.04 \pm 0.02^b$	$1.00 \pm 0.02^b$	$6.57 \pm 0.21^c$
Steam-cooked fillets, $\text{mg}\cdot\text{kg}^{-1}$	$0.10 \pm 0.01^a$	–	–	$0.74 \pm 0.03^b$
Iodine losses <sup>1</sup> , %	$19.6 \pm 10.6$	–	–	$12.5 \pm 1.1$
<b>Nutritional contribution, % DRI<sup>2</sup></b>				
Iodine	10.7	–	–	78.6

Values are means  $\pm$  standard deviation ( $n=2$ ). Each replicate sample was a pool of three fillets.

Different superscripts within rows represent significant differences between treatments ( $P < 0.05$ ).

<sup>1</sup> Iodine losses:  $100 \times (\text{iodine raw sample} - \text{iodine cooked sample}) / \text{iodine raw sample}$ .

<sup>2</sup> For definition of DRI calculations please refer to section 2.4.



**Figure 3.1.** Iodine content of seabream fillets over the experimental feeding period. Day 43 and day 86 values represent one analysis of a pool sample from each treatment.

Bars with different superscripts letters indicate significant differences between treatments ( $P < 0.05$ ).

Compared to raw fillets, steam cooked fillets showed a 19.6% loss of iodine in the CTRL treatment and 12.5% loss in the LAM treatment (Table 3). However, these fillet iodine losses

were not significantly different ( $P>0.05$ ) by the dietary treatments. Water loss during steam-cook processing averaged 14% (data not showed) in both treatments. The iodine nutritional contribution associated to the consumption of a steam-cooked seabream fillet (portion of 160 g steam-cooked fillet, assuming a 40% fillet yield in a 400 g market size fish) is also presented in Table 3.3. Consumption of CTRL fillets represents 11% of the iodine Daily Recommended Intake (DRI) while consumption of LAM fillets cover almost 80% of iodine DRI.

The fillet fatty acid content of seabream fed diets CTRL and LAM is presented in Table 3.4. In comparison to the CTRL treatment, feeding seabream with the iodine-rich brown algae *Laminaria digitata* had no effect ( $P>0.05$ ) on the fatty acid content of fillet. In comparison to the raw fillets, the steam-cooking culinary treatment was associated to a general trend to decrease the fillet fatty acids content, which was statistically significant ( $P<0.05$ ) for total SFA, C18:2 n-6, C18:3 n-3, C18:4 n-3, C20:4 n-6, total PUFA n-3 and total PUFA n-6. The atherogenic index (AI) in both raw and steam cooked seabream fillets ranged from 0.545 to 0.559, while the thrombogenic index (TI) varied between 0.283 and 0.289. The nutritional contribution of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids, associated to the consumption of steam-cooked seabream fillet were also calculated (Table 3.4). Irrespective of the dietary treatment, consumption of a 160 g portion of seabream fillet contributes to 370% of the Daily Adequate Intake (DAI) of combined EPA and DHA for enhanced cardiovascular health in adults.

**Table 3.4.** Summarised fatty acids content of raw (R) and steam-cooked (SC) fillets.

Fatty acids <sup>1</sup> (mg·100 g <sup>-1</sup> fillet)	Experimental treatments				ANOVA P value <sup>2</sup>		
	CTRL - R	CTRL-SC	LAM - R	LAM - SC	D	C	D x C
Total SFA <sup>3</sup>	2387 ± 44	2320 ± 29	2350 ± 6	2286 ± 15	NS	0.025	NS
Total MUFA <sup>4</sup>	2655 ± 17	2616 ± 43	2678 ± 19	2614 ± 40	NS	NS	NS
C18:2 n-6	567 ± 3	552 ± 1	568 ± 3	556 ± 1	NS	0.001	NS
C18:3 n-3	99 ± 4	93 ± 2	99 ± 4	90 ± 4	NS	0.049	NS
C18:4 n-3	162 ± 4	148 ± 3	159 ± 3	150 ± 4	NS	0.011	NS
C20:4 n-6	63 ± 3	56 ± 3	61 ± 4	55 ± 2	NS	0.035	NS
C20:4 n-3	68 ± 2	62 ± 5	64 ± 2	59 ± 3	NS	NS	NS
C20:5 n-3 [EPA] <sup>5</sup>	683 ± 29	645 ± 20	673 ± 20	657 ± 32	NS	NS	NS
C22:5 n-3	247 ± 5	239 ± 12	244 ± 19	232 ± 8	NS	NS	NS
C22:6 n-3 [DHA] <sup>5</sup>	491 ± 9	476 ± 3	489 ± 13	478 ± 5	NS	NS	NS
Total PUFA <sup>6</sup>	2529 ± 27	2403 ± 8	2512 ± 51	2416 ± 25	NS	0.008	NS
Total PUFA n-3	1773 ± 23	1691 ± 9	1753 ± 59	1697 ± 21	NS	0.045	NS
Total PUFA n-6	669 ± 6	647 ± 5	669 ± 1	651 ± 0	NS	0.002	NS
PUFA n-3/n-6	2.65 ± 0.01	2.61 ± 0.03	2.62 ± 0.09	2.61 ± 0.03	NS	NS	NS
EPA + DHA	1174 ± 37	1121 ± 22	1161 ± 33	1135 ± 37	NS	NS	NS
AI <sup>7</sup>	0.559	0.547	0.551	0.543			
TI <sup>8</sup>	0.286	0.289	0.283	0.283			
Nutritional contribution, % MDAI <sup>9</sup>							
EPA + DHA		329		325			

Values are means ± standard deviation (n=2). Each replicate sample was a pool of three fillets.

<sup>1</sup> Despite not shown in this summarised format, all identified fatty acids were considered in the composite fractions.

<sup>2</sup> Two-way ANOVA, D: diet; C: cooking.

<sup>3</sup> SFA: Saturated fatty acids.

<sup>4</sup> MUFA: Monounsaturated fatty acids.

<sup>5</sup> EPA (C20:5n-3) and DHA (C22:6n-3).

<sup>6</sup> PUFA: Polyunsaturated fatty acids.

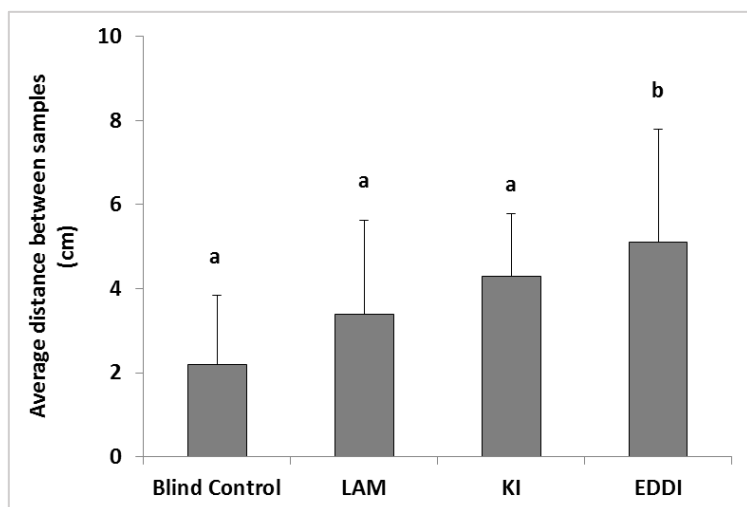
<sup>7</sup> AI: (C12:0 + (4 × C14:0) + C16:0) / (Total MUFA + Total n-3 + Total n-6)

<sup>8</sup> TI: (C14:0 + C16:0 + C18:0) / ((0.5 × Total MUFA) + (0.5 × Total n-6) + (3 × Total n-3) + ratio n-3/n-6).

<sup>9</sup> For calculation of Minimum Daily Adequate Intake please refer to section 2.4.

The potential effect of iodine supplementation on the sensory quality of fish flesh was evaluated by an overall difference test (difference from control test) applied to steam-cooked fillets (fig 3.2). As expected, the blind-control sample was considered the closest to the known control followed by LAM treatment while the fillets from KI and EDDI treatments were considered the most different from CTRL fish. Nevertheless, these differences were significant only for EDDI treatment which distanced around 42 % from CTRL in the line scale.

In this test sensory attributes were not individually evaluated, though panellists were invited to comment on what motivated their classification. The taste was the main attribute referred for the perceived difference in fillets from EDDI and KI diets.



*Figure 3.2. Overall sensory difference (difference from control test) of seabream fillets supplemented with various iodine sources.*

*Bars with different superscript letters indicate statistical differences between samples and the blind-control (Dunnet test,  $P < 0.05$ ).*

## Discussion

Iodine is an essential trace element and an integral component of thyroid hormones, with multiple biological functions in humans and animals. Fish easily absorb iodine from the diet through the digestive tract (Gregory and Eales, 1975), but can also uptake it from the surrounding water via the gills (Hunn and Fromm, 1966; Hunt and Eales, 1979). Iodine requirements are not established for most fish and may vary considerably depending on species and physiological status (Watanabe, 1997). However, dietary iodine levels of 1 to 5  $\text{mg}\cdot\text{kg}^{-1}$  have been estimated as adequate for most fish species (Lall, 2003; National Research Council, 2011). In commercial aquaculture feeds, iodine is generally derived from fishmeal and added as potassium iodide (KI) in the mineral premix. The iodine content is generally high in diets for carnivorous fish, which are based mainly on fishmeal (0.8-8  $\text{mg}\cdot\text{kg}^{-1}$ ; Underwood and Suttle, 2001). The mean iodine content in commercial fish feed was reported to be 4.6  $\text{mg}\cdot\text{kg}^{-1}$ , and ranged between 1.2-10.5  $\text{mg}\cdot\text{kg}^{-1}$  (Julshamn et al., 2001).

The trend towards a reduction of fishmeal incorporation levels and the concomitant use of glucosinolates-rich plant ingredients (e.g. rapeseed meal), may potentiate the need for a compensatory increase of commonly used dietary iodine levels in fish feeds. According to the EU FEEDAP EFSA Panel, in farmed fish, the supplementation of the diet with the maximum recommended levels ( $20 \text{ mg}\cdot\text{kg}^{-1}$ ) still results in tissue concentrations lower than those found in wild marine fish (EFSA, 2005). Potassium iodide (KI) supplementation ( $20 \text{ mg I}\cdot\text{kg}^{-1}$ ) of a commercial diet was reported to enhance weight gain and reduce stress responses of steelhead trout (Gensic et al., 2004), while iodine supplementation (as KI) in moist pellets up to  $86 \text{ mg I}\cdot\text{kg}^{-1}$  increased salmon fillet iodine concentration without impacting growth or plasma thyroid hormone status (Julshamn et al., 2006). Moreover, iodine supplemented diets were shown to improve the immune status and decrease disease susceptibility in salmonids (Gensic et al., 2004; Lall et al., 1985; Mustafa and MacKinnon, 1999). Our study shows that an increase of dietary iodine levels, from  $2.4 \text{ mg}\cdot\text{kg}^{-1}$  DM (CTRL diet) to maximum recommended levels of  $23\text{-}26 \text{ mg}\cdot\text{kg}^{-1}$  DM (diets EDDI and KI) and to a much higher level of  $426 \text{ mg}\cdot\text{kg}^{-1}$  DM (diet LAM) had no effect on feed intake, growth rate, feed efficiency or nutrient utilization of gilthead seabream. However, the impact of high iodine intake on the homeostasis of thyroid function in fish requires a detailed assessment to avoid any potential goitrogenic effects. Whole-body composition of seabream was not affected by this wide variation on dietary iodine levels. The similar performance criteria observed with the inorganic (KI) and organic (EDDI) iodine supplemental forms suggests a similar utilization of both forms. However, our data does not allow us to dismiss differences on the bioavailability or metabolic role among these two iodine forms.

Over recent years, there has been a renewed interest on the use of macroalgae in aquaculture feeds. Available literature data shows that dietary macroalgae, such as *Porphyra*, *Ascophyllum* and *Ulva*, used at 4-10% dietary levels, promoted growth performance in Nile tilapia (Ergün et al., 2009) and had no detrimental effects in rainbow trout (Dantagnan et al., 2009; Soler-Vila et al., 2009), in gilthead seabream (Emre et al., 2013) and European seabass (Valente et al., 2006). Higher inclusion levels were described by Kraan and Mair (2010),

showing that the utilization of 15% of a marine macroalgae mix in salmon feeds resulted in improvements of growth rate, feed conversion, flesh flavour and texture and resistance to sea lice infestation. Our study demonstrates that the incorporation at a 10% level of the brown algae *Laminaria digitata* had no effect on the growth performance, feed conversion or nutrient utilization in gilthead seabream.

Given its low to moderate protein content (12-30%), macroalgae are difficult to promote as a bulk ingredient in fish feeds (Shields and Lupatsch, 2012). However, marine macroalgae may have a great potential in the animal feed sector as a functional ingredient (products incorporated in the feed to convey a benefit above and beyond fulfilling the basic nutritional needs). Within a broad concept of sustainable, chemical-free and organic farming, which is highly appealing to consumers, marine macroalgae stand out as a potential source of natural mineral additives to replace inorganic mineral salts that are most commonly used in the animal feed industry. The use of macroalgae as part of a dietary bio-fortification strategy of farmed fish shows great potential in delivering tailored fish with health valuable trace minerals to consumers. Our study shows that dietary iodine supplementation is an effective strategy to increase the iodine content in seabream fillets. After 118 days of feeding, fish fed the basal dietary levels (CTRL diet) had a fillet iodine content of  $0.13 \text{ mg}\cdot\text{kg}^{-1}$ , while those fed the diets supplemented at the maximum recommended levels (diets KI and EDDI) showed a 1.3-fold increase of fillet iodine levels. However, the dietary incorporation of the iodine-rich brown macroalgae *Laminaria digitata* was the most efficient form to fortify seabream fillets, resulting in a 6.5-fold increase over the control fish. In a previous study, Schmid et al. (2003) reported that the dietary use of this same macroalgae originated a 4-fold increase of the iodine content in the fillet of freshwater char (*Salvelinus* sp). The fillet fortification with inorganic iodine sources has also been studied in fish. In Atlantic salmon fed moist diets (minced herring and saithe) supplemented with KI at 40 and  $80 \text{ mg}\cdot\text{kg}^{-1}$  over 150 days, the muscle iodine concentration was increased 1.3 and 2.4-fold respectively, relative to the non-supplemented diet (Julshamn et al., 2006). Thus, the effectiveness of iodine fortification by the incorporation of *Laminaria* in seabream diet seems very clear. In spite of actual

consumers request for healthy food they are also aware about natural sensory properties of each food item. Thus, the nutritional improvement of food should avoid negative effects on the sensory traits. In this context our sensory results confirm that fillets fortification with *Laminaria* was the most interesting choice since overall sensory quality was the closest to the CTRL.

Consumer expectations regarding seafood are closely linked to the fact that fish is often identified as one of the richest natural source of health beneficial nutrients, such as the long-chain n-3 polyunsaturated fatty acids (n-3 PUFA, namely EPA and DHA), selected vitamins (e.g. vitamin D3) and trace minerals like iodine and selenium (Larsen et al., 2011). In our study, raw gilthead seabream fillets showed average values of saturated ( $2.37 \pm 0.03 \text{ mg} \cdot 100 \text{ g}^{-1}$  fresh fillet), monounsaturated ( $2.67 \pm 0.02 \text{ mg} \cdot 100 \text{ g}^{-1}$  fresh fillet) and polyunsaturated fatty acids (PUFA) ( $2.52 \pm 0.01 \text{ mg} \cdot 100 \text{ g}^{-1}$  fresh fillet), in accordance to values previously reported for this species (Mnari et al., 2010) and extensively reviewed by Grigorakis et al. (2007). Given that in our study the main source of dietary lipids was marine fish oil and it remained unchanged among the various diets, the combined content of EPA and DHA ( $1.17 \pm 0.01 \text{ mg} \cdot 100 \text{ g}^{-1}$  fresh fillet) and PUFA n-3/n-6 ratio ( $2.63 \pm 0.02$ ) in raw fillets was relatively constant. The iodine content in seafood is extremely variable, with highest values generally found in marine fish ( $1\text{--}6 \text{ mg I} \cdot \text{kg}^{-1}$  muscle) (Julshamn et al., 2001; Erkan, 2011), and 5 to 10-fold lower values in freshwater species (Eckhoff and Maage, 1997; Haldimann et al. 2005). Available data shows that the iodine content in fish varies not only among species, but also between individuals of the same species, fish size and fillet sampling method since the iodine content in the skin can be more than ten times of the muscle tissue (Eckhoff and Maage, 1997; Erkan, 2011; Julshamn et al., 2001; Karl et al., 2001). In the present work, the iodine content in seabream raw skinless fillets ranged from  $0.13 \text{ mg} \cdot \text{kg}^{-1}$  with the non-supplemented diet, up to  $0.84 \text{ mg} \cdot \text{kg}^{-1}$  with 10% inclusion of *Laminaria digitata* in the feed. Using the maximum dietary levels allowed ( $22\text{--}26 \text{ mg} \cdot \text{kg}^{-1}$ ) either as EDDI or KI incremented raw fillet iodine content of  $0.17 \text{ mg} \cdot \text{kg}^{-1}$ , in both groups. A previous study reported a muscle iodine content of  $507 \text{ mg} \cdot \text{kg}^{-1}$  in farmed gilthead seabream (Erkan and Özden, 2007), but

given the extremely high discrepancy of this value in comparison to the maximum iodine levels found in all other fish species, we suspect an error in unit of data expression.

While assessing the nutritional value of fish to the consumer it is important to take into account the effect of culinary treatments, since these may lead to nutrient losses (Badiani et al., 2013; Mnari et al., 2010, 2012; WHO, 1996). In the present study, steam-cooking originated a mean iodine loss of 13% in seabream fillets of the *Laminaria* treatment and 20% in the control treatment, but these differences were not statistically significant. Our estimates of iodine losses in steam-cooked seabream fillets are in line with literature values. In broad terms, Winger et al. (2008) estimated that steam-cooking originated 20% average losses of iodine. Longvah et al (2012) studied the influence of cooking processes in the iodine retention from iodised salt added to food when cooking and observed that 68% iodine was retained with steaming. In fish products, a 10% iodine loss during steam-cooking of cod fish balls was reported by Karl et al. (2005), and almost all iodine lost during the steaming process was accounted in the dripping water. Depending on the species, steam-cooking resulted in both losses and concentration of iodine content in several fish species (Erkan, 2011). Regarding the fatty acid profile of fillets, our data suggests that in comparison to raw fillets, steam-cooking processing tended to decrease the overall fatty acids contents, which was statistically significant for total saturated fatty acids, linoleic acid (C18:2n-6),  $\alpha$ -linolenic acid (C18:3n-3), stearidonic acid (C18:4 n-3), arachidonic acid (C20:4 n-6), total PUFA n-3 and total PUFA n-6. Irrespective of the dietary treatment, the average fatty acid reduction in steam-cooked fillets was 5%. This trend towards a reduction of fatty acid contents in association to steam-cooking of seabream fillets was also previously reported by Mnari et al. (2010).

The World Health Organization (WHO et al., 2007) recommendations concerning daily iodine intake (RDI) are 150  $\mu\text{g}\cdot\text{day}^{-1}$  for adolescents and adults (from 13 years onwards) and higher for pregnant and breastfeeding women (250  $\mu\text{g}\cdot\text{day}^{-1}$ ). Over the last decades, salt iodization programs have been effective in preventing and controlling iodine deficiency disorders. But according to Zimmermann and Andersson (2012), these programs need to be strengthened

and extended to reach nearly one-third of the global population that still has inadequate iodine intakes. At the same time, WHO is promoting the implementation of programs to reduce population salt intake as one of the most cost-effective strategies to reduce the burden of non-communicable diseases (e.g. cardiovascular diseases). Considering the high content of iodine in seafood, its potential use in battling global iodine deficiency may have been somewhat neglected. Our data shows that the dietary use of the iodine-rich macroalgae (*Laminaria digitata*) was an effective and natural strategy to fortify seabream fillets in iodine, and a portion of 160 g of seabream fillet (edible fraction of a 400 g commercial size fish) would cover about 80% of the iodine RDI for young and adults. According to Regulation (EC 1924/2006) in the European market, a food item can be labelled as “source of iodine” if it supplies 15% of the RDI in 100 g of product and “high in iodine” in case of containing at least twice this value (30% of iodine RDI). Under this definition, the steam-cooked LAM fillets produced in our trial could be labelled as a food “high in iodine” since it supplies  $73.7 \mu\text{g iodine}\cdot 100 \text{ g}^{-1}$  (49% of RDI) and therefore could be considered as functional food (EFSA, 2009a). Moreover, the consumption of this same 160 g portion of seabream fillet would represent 370% of the Daily Adequate Intake (DAI) of combined EPA and DHA, as recommended for enhanced cardiovascular health in adults (ISSFAL, 2004). Since, fish is not generally consumed on a daily basis, calculations of a weekly intake seem more appropriate. Consumption of LAM seabream fillets twice a week would cover 105% of the adequate EPA+DHA intake for enhanced cardiovascular health and contribute to 22% of the weekly recommended iodine intake in adults.

In conclusion, the dietary incorporation of *Laminaria digitata*, an iodine-rich macroalgae, was an effective and natural strategy to fortify gilthead seabream fillets with iodine, without compromising fish performance and sensory traits. Nowadays, the successful development of novel seafood products requires a strong focus on consumer trends and nutritional needs. Regarding the fortification of fish with health-promoting nutrients, the use of natural sources, such as algae may facilitate consumer’s acceptance of such tailored fish products. But in broader terms, the success of fish as a functional food relies on a delicate balance

between the safety and quality of fish for human consumption, the environmental cost and welfare standards of production systems and its socio-economic implications.

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# Natural fortification of trout with dietary macroalgae and selenised-yeast increases the nutritional contribution in iodine and selenium

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## Chapter 3.2

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## Natural fortification of trout with dietary macroalgae and selenised-yeast increases the nutritional contribution in iodine and selenium

### Abstract

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Fish and seafood consumption are increasing worldwide and the contribution of aquaculture products to consumers' diets is significant. External feeding in aquaculture unlocks the possibility of tailoring fish products with health beneficial compounds. A study was undertaken to evaluate the feed fortification with an iodine-rich macroalgae (*Laminaria digitata*) and selenised yeast, at its maximum permitted levels, on minerals and vitamins content in rainbow trout edible part. Dietary supplementation resulted in a six-fold increase for iodine and a 2.9-fold increase for selenium contents in trout fillets without altering sensory traits. The fortified fish presented a nutritional contribution of 12.5% DRI for iodine and 78% DRI for selenium, but all produced fish could supply 80% DRI for vitamin D<sub>3</sub>. Overall, fish from this trial could be labelled as "high in selenium and high in vitamin D<sub>3</sub>" under the EFSA definition for a functional food.

Keywords: Rainbow trout; Iodine; Selenium; Vitamin D<sub>3</sub>; Fortification; Nutritional contribution

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### Introduction

Fish and shellfish are generally associated with healthy dietary patterns and improved wellbeing. Seafood is a valuable source of multiple essential nutrients, since most species provide the recommended amounts of n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA), contributing also to cover the needs of other essential nutrients, such as vitamin D, iodine or selenium (EFSA, 2014a). In recent years, the identification of functional bioactive nutrients from marine origin and their biological effects has been the object of important research efforts (Shahidi and Ambigaipalan, 2015). High fish consumption, with high n-3

LCPUFA levels, namely eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) fatty acids, has been unequivocally associated to a protective role against a number of human diseases (FAO and WHO, 2011; Raatz et al., 2013). Other essential nutrients such as selenium, iodine and vitamin D, of which fish is considered the main dietary source have also been positively correlated with disease prevention and improved health status. Additionally, recent studies suggest that some health benefits, attributed to LCPUFA, may be potentiated in the presence of selenium (Berr et al., 2009) and iodine (Bath et al., 2013). These outcomes reinforce the importance of eating fish for its holistic properties rather than the ingestion of pills or supplements. A food-based approach has also been recommended by nutritionists for achieving nutrient adequacy, preventing and treating diseases. Dietary guidelines recommend at least two fish portions per week with cardiovascular protective effects and up to four servings per week during pregnancy with better functional outcomes of neurodevelopment in children (EFSA, 2014a).

Worldwide seafood consumption is steadily expanding, with *per capita* records of 20 kg in 2014, and prospects are to continue rising in the next years (FAO, 2015). Provision of seafood from capture fisheries is declining though aquaculture is overcoming this supply issue and the actual contribution at world level already overtook that of wild fish for human consumption (FAO, 2015). However, the current trend in aquafeeds for replacing marine-derived ingredients (fish meal and fish oil) by vegetable protein and oil sources can interfere with the fish nutritional profile. For instance, vegetable ingredients are often characterized by low amounts of n-3 LCPUFAs. Although conditioned by the elemental soil content of cultivars, cereals, pulses and oilseeds are also poorer sources of iodine and selenium when compared to marine protein ingredients (Van Paemel et al., 2010). Moreover, current levels of vitamins and minerals in fish feeds target an optimal fish growth and welfare, without regarding a potential enhancement of the beneficial effects to consumers' health. Within this scenario, a new perspective towards consumers' dietary needs must be considered when designing aquafeeds. Fillets traits have already been effectively changed by modulating fish feeds in terms of bioactive fatty acids (Kennedy et al., 2007b; Ramos et al., 2008; Rosa et al.,

2010), selenium (Lorentzen et al., 1994; Schram et al., 2010) and iodine (Julshamn et al., 2006; Ramalho Ribeiro et al., 2015). However, the efficacy of muscle fortification seems to be dependent on supplemental dose, feeding strategies and, also important, the supplement product form (inorganic vs. organic). Additionally, some of these studies also lack an overall assessment regarding the nutritional contribution of fish to human consumption.

A dietary fortification trial was performed with rainbow trout owed to its importance as a major freshwater species consumed in Europe. The macroalgae *Laminaria digitata* and selenium-enriched yeast were used as iodine and selenium sources and their effects on fish fortification were evaluated, either isolated or concomitantly. Afterwards, the nutritional contribution was estimated, and sensory traits of trout fillets were also assessed.

## Material and methods

### *Experimental diets*

Four experimental diets were formulated to be isonitrogenous (42.7% crude protein), isolipidic (23.3% crude fat) and isoenergetic (23.5 MJ·kg<sup>-1</sup> gross energy) (table 3.5). A control diet (CTRL), similar to a commercial trout feed was formulated to contain 1.9 mg iodine·kg<sup>-1</sup> supplied as potassium iodide and 0.25 mg selenium·kg<sup>-1</sup>, supplied as sodium selenite through the mineral premix and the endogenous content of the various ingredients. Based on this control formulation, three other experimental diets were manufactured. The LAM diet targeted an iodine level of 20 mg·kg<sup>-1</sup>, supplied as *Laminaria digitata*, an iodine-rich macroalgae; SE diet targeted a total selenium level of 0.5 mg·kg<sup>-1</sup>, supplied partially as selenium enriched yeast and LAMSE diet with a simultaneous supplementation of 20 mg iodine·kg<sup>-1</sup>, as *Laminaria digitata*, and of 0.5 mg selenium·kg<sup>-1</sup>, supplied as selenised yeast. The levels of 20 mg iodine·kg<sup>-1</sup> and 0.5 mg selenium·kg<sup>-1</sup> were adopted since it is the currently authorized maximum content of total iodine and selenium in complete feeds for fish in the European market. Experimental extruded diets were manufactured at SPAROS Lda (Olhão, Portugal).

**Table 3. 5.** Ingredient and proximate composition of experimental diets.

Ingredients, g·kg <sup>-1</sup>	CTRL	LAM	SE	LAMSE
Fishmeal LT 70 <sup>1</sup>	125.00	125.00	125.00	125.00
Fishmeal 60 <sup>2</sup>	50.00	50.00	50.00	50.00
Soy protein concentrate <sup>3</sup>	160.00	160.00	160.00	160.00
Wheat gluten <sup>4</sup>	100.00	100.00	100.00	100.00
Corn gluten <sup>5</sup>	100.00	100.00	100.00	100.00
Soybean meal <sup>6</sup>	50.00	50.00	50.00	50.00
Rice protein concentrate <sup>7</sup>	50.00	50.00	50.00	50.00
Wheat meal <sup>8</sup>	121.30	117.65	121.20	117.55
Fish oil <sup>9</sup>	195.00	195.00	195.00	195.00
Vitamin and mineral premix <sup>10</sup>	10.00	10.00	10.00	10.00
Soy lecithin <sup>11</sup>	2.00	2.00	2.00	2.00
Guar gum <sup>12</sup>	4.00	4.00	4.00	4.00
Zeolite <sup>13</sup>	10.00	10.00	10.00	10.00
Antioxidant <sup>14</sup>	2.00	2.00	2.00	2.00
Dicalcium phosphate <sup>15</sup>	11.40	11.40	11.40	11.40
Astaxanthin	0.30	0.30	0.30	0.30
L-Lysine <sup>16</sup>	7.00	7.00	7.00	7.00
DL-Methionine <sup>17</sup>	2.00	2.00	2.00	2.00
Macroalgae ( <i>Laminaria</i> ) <sup>18</sup>		3.65		3.65
Selenised yeast <sup>19</sup>			0.10	0.10
Dry matter (DM), %	95.2 ± 0.1	95.7 ± 0.1	96.0 ± 0.2	95.8 ± 0.0
Crude protein, %DM	42.7 ± 0.1	42.6 ± 0.4	42.7 ± 0.0	42.8 ± 0.1
Crude fat, % DM	23.3 ± 0.1	23.4 ± 0.2	23.4 ± 0.1	23.4 ± 0.1
Ash, %DM	8.5 ± 0.0	8.8 ± 0.1	8.7 ± 0.1	8.8 ± 0.1
Total phosphorus, % DM	1.13 ± 0.02	1.21 ± 0.03	1.14 ± 0.02	1.20 ± 0.01
Iodine, mg·kg <sup>-1</sup> DM	1.89 ± 0.11	22.70 ± 1.49	2.09 ± 0.06	22.35 ± 3.13
Selenium, mg·kg <sup>-1</sup> DM	0.24 ± 0.02	0.26 ± 0.02	0.53 ± 0.02	0.51 ± 0.01
Gross energy, kJ·g <sup>-1</sup> DM	23.5 ± 0.1	23.6 ± 0.2	23.5 ± 0.1	23.5 ± 0.0

<sup>1</sup> Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru. <sup>2</sup> Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal. <sup>3</sup> Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands. <sup>4</sup> VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France. <sup>5</sup> Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal. <sup>6</sup> Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal. <sup>7</sup> Rice 50: 48.3%CP, 6.5% CF, SEAH International, France. <sup>8</sup> Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal. <sup>9</sup> COPPENS International, The Netherlands. <sup>10</sup> Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg; betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate, 7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings. <sup>11</sup> Lecico P700IPM, LECICO GmbH, Germany. <sup>12</sup> Seah International, France. <sup>13</sup> Kielseguhr, LIGRANA GmbH, Germany. <sup>14</sup> Paramega PX, Kemin Europe NV, Belgium. <sup>15</sup> Dicalcium phosphate: 18% phosphorus, 23% calcium, Fosfitalia, Italy. <sup>16</sup> Biolys 54.6%, EVONIK Nutrition & Care GmbH, Germany. <sup>17</sup> DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany. <sup>18</sup> Dry *Laminaria digitata*: 5.4% CP, 0.5% CF, 3700 mg iodine/kg, Agrimer, France. <sup>19</sup> ALKOSEL R397: 2200 mg selenium/kg, Lallemand, France.

### *Growth trial and sampling*

The trial was conducted at the Experimental Research Station of University of Trás-os-Montes e Alto Douro (UTAD, Portugal). Experiments were directed by trained scientists (following category C FELASA recommendations) and in compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei nº. 113/2013, de 7 de Agosto) legislation on the protection of animals for scientific purposes. UTAD facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Eight homogenous groups of 48 rainbow trout each, with a mean initial body weight of 238 g were stocked in 350 L fiberglass tanks, supplied with flow-through freshwater (water-flow rate: 7 L·min<sup>-1</sup>, temperature: 15 ± 1 °C) and exposed to natural photoperiod conditions (14 light/10 h dark). Each experimental treatment was tested in duplicate tanks over 91 days. Fish were hand-fed to apparent satiety, twice a day. Final samplings were done 48 hours following the last meal and fish were slaughtered by immersion in ice-water slurry (4:1) until death. At the start (6 fish from the initial stock) and at the end of the trial, three fish from each tank were sampled for analysis of whole-body composition. Moreover, trout skinless fillets were collected at the start (n=3) and at the end of trial (n=2 pools of 3 fish each), stored at -80 °C for analysis of minerals and vitamins content and fatty acid composition. For sensory analysis, ten fish from each treatment were weighed, scaled, filleted and kept at 4 °C until sensory assessment.

### *Analytical methods*

Proximate composition analysis of the diets and whole fish was made by the following procedures: dry matter by drying at 105°C for 24 h; ash by combustion at 550°C for 12 h; crude protein (N×6.25) through the release of nitrogen by a combustion technique followed by thermal conductivity detection (LECO FP528, Leco Instruments, USA); crude fat after dichloromethane (CID: 6344) extraction by the Soxhlet method; total phosphorus in the

feeds was quantified according to the ISO/DIS 6491 method using the vanado-molybdate reagent; and gross energy in an adiabatic bomb calorimeter (model C2000, IKA-Werke GmbH & Co. KG, Staufen, Germany). Minerals (Fe, Zn, Mg, K, I and Se) content in feeds and fillets was determined at an external certified laboratory (Silliker, Mérieux Nutrisciences, Portugal) by microwave digestion with nitric acid and hydrogen peroxide (CID: 784), followed by inductively coupled plasma mass spectrometry (ICP-MS) detection. Digested sample solutions were analysed for I, Se, Fe, Zn, K and Mg using certified external calibration standards (Inorganic Ventures Europe S.L., Spain). Standards were prepared by systematically diluting 1000  $\mu\text{g}\cdot\text{mL}^{-1}$  stock solutions of the respective elements and the calibration curves of five concentration points showed correlation coefficients (R) between 0.995 and 1. Reagent blanks (n=6) were prepared in identical conditions. Spiking was performed, for each element, in two trout fillet samples as a method validation tool. Recovery values were in the range 93.7–106.1%, which fell within the acceptance level of the certified laboratory. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by the formulas  $\text{LOD} = 3 \sigma/b$  and  $\text{LOQ} = 10 \sigma/b$ , where  $\sigma$  = standard deviation of the blank and  $b$  = slope of the calibration curve. The LOD ( $\text{mg}\cdot\text{kg}^{-1}$ ) for the various elements were: I (0.0004), Se (0.0013), Fe (0.0632), Zn (0.0034), K (0.0022) and Mg (0.0844). The LOQ ( $\text{mg}\cdot\text{kg}^{-1}$ ) for the various elements were: I (0.0011), Se (0.0227), Fe (0.1172), Zn (0.0086), K (0.0054) and Mg (0.2212). The same external laboratory determined also Vitamin D<sub>3</sub> (cholecalciferol) by reversed phase HPLC with UV detection, according to the AOAC method 2002.05 and vitamin A (all-E-retinol, 13-Z-retinol) by HPLC according to EN 12823-1:2014 method. Total lipids in the fillets were extracted according to the method of Folch, Lees and Stanley (1957). The fatty acid composition of fillets was determined by gas-chromatographic analysis of methyl esters, according to the procedure of Lepage and Roy (1986), modified by Cohen et al. (1988) and described in further detail by Costa et al. (2013). Data in mg/100 g of edible part were calculated using the peak area ratio (% of total fatty acids) and the lipid conversion factors set by Weihrauch et al. (1977). Based on the fatty acid profile of trout fillets, the atherogenic (1) and thrombogenic (2) index (AI and TI, respectively) were

calculated accordingly to Ulbricht and Southgate (1991) for evaluation of the predisposition for incidence of coronary heart disease.

$$AI = \frac{C12:0 + (4 \times C14:0) + C16:0}{\text{Total MUFA} + \text{Total n-3} + \text{Total n-6}} \quad (1)$$

$$TI = \frac{C14:0 + C16:0 + C18:0}{(0.5 \times \text{Total MUFA}) + (0.5 \times \text{Total n-6}) + (3 \times \text{Total n-3}) + \text{ratio n-3/n-6}} \quad (2)$$

### *Nutritional Contribution*

The nutritional contribution (NC) of trout fillets was calculated as the percentage of the Daily Recommended Intake (DRI)/Dietary reference values (DRVs) for selected minerals and vitamins and also of the Daily Adequate Intake (DAI) for combined EPA and DHA. When distinct recommendations exist for males and females, calculations were adapted accordingly and the following formula (3) was used:

$$NC (\%) = \frac{C \times M}{\text{DRI/DRV or DAI}} \times 100 \quad (3)$$

Where:

C: mean concentration of nutrient in g·kg<sup>-1</sup>, mg·kg<sup>-1</sup> or µg·kg<sup>-1</sup>.

M: meal fillet portion consumed is 0.160 kg (Costa et al., 2013).

DRV established as Adequate Intake (AI) for iodine is 0.150 mg·d<sup>-1</sup> for adults (EFSA, 2014b).

DRV established as Adequate Intake (AI) for selenium is 0.070 mg·d<sup>-1</sup> for adults (EFSA, 2014c)

DRI established as Recommended Dietary Allowance for iron is 8.0 mg·d<sup>-1</sup> for males and 18 mg·d<sup>-1</sup> for females (Institute of Medicine [IOM], 2001).

DRV established as Population Reference Intakes (PRIs) for zinc are 7.5 to 12.7 mg·d<sup>-1</sup> for women (mean value = 10.1 mg·d<sup>-1</sup>) and 9.4 to 16.3 mg·d<sup>-1</sup> for men (mean value = 12.9 mg·d<sup>-1</sup>) (EFSA, 2014d)

DRI established as Adequate Intake (AI) for potassium is 4.7 g·d<sup>-1</sup> for adolescents and adults (IOM, 2005b).

DRV established as Adequate Intake (AI) for magnesium is 350 mg·d<sup>-1</sup> for adult males and 300 mg·d<sup>-1</sup> for adult females (EFSA, 2015a)

DRI established as Recommended Dietary Allowance for Vitamin D is 15.0 µg·d<sup>-1</sup> for young and adults (IOM, 2011).

DRV established as Population Reference Intakes (PRIs) for Vitamin A is 750 µg retinol·d<sup>-1</sup> for men and 650 µg retinol·d<sup>-1</sup> for women (EFSA, 2015b).

DAI for EPA+DHA is 500 mg·d<sup>-1</sup> for primary prevention of cardiovascular disease in adults (International Society for the Study of Fatty Acids and Lipids [ISSFAL], 2004), corresponding to a weekly intake of 3.5g of EPA+DHA.

### *Sensory Evaluation*

Sensory evaluation was carried out in an acclimatized room equipped with individual booths. To reduce the variability within the fillets, the parts close to the head and the tail were rejected. Each skin-on fillet was individually wrapped with aluminium foil to avoid odour losses and cooked for 6 min at 100 °C in a saturated steam oven (Rational Combi-Master CM6 Cross KuchentechnikCmbH, Landsberg, Germany). A multisample difference test was conducted (Meilgaard et al., 1999) using a sensory panel composed by ten trained panellists. Cooked fillets were presented to the panellists sequentially in coded white dishes under normal white lighting. Each panellist received the four samples in a balanced random order and rated the intensity of sensory attributes on a 9-point scale ranging from absent (0) to very intense (8 points). The attributes selected were the odour, taste and texture (firmness and succulence).

### *Statistical analysis*

Growth performance and iodine deposition data are expressed as means of two replicates  $\pm$  standard deviation. Data were subjected to a one-way analysis of variance. Parameters expressed as percentages were subjected to arcsin square root transformation. Sensory data were analysed by one-way analysis of variance followed by the Dunnet Test to evaluate the differences between tested samples and the blind-control. Culinary treatment data was analysed with Student's T-test. Statistical significance was tested at 0.05 probability level. All statistical tests were performed using the SPSS software (v22, IBM, USA).

### **Results**

At the end of the trial, fish reached a final body weight ranging from 335 to 350 g and both *Laminaria* and selenised yeast supplemented feeds led to a significant increase ( $P=0.020$ ) of final body weight (table 3.6). Feed intake was not affected by the various dietary treatments ( $P=0.168$ ). FCR was highest in fish the CTRL diet and significantly reduced in those fed diets supplemented with *Laminaria* and selenised yeast ( $P=0.004$ ). Moreover, fish fed the diets supplemented with selenised yeast (SE and LAMSE) presented lower FCR than those fed diet

LAM. The whole-body composition of fish, in terms of moisture, protein, fat and ash, was not affected by the dietary treatments ( $P>0.05$ ) (table 3.6).

**Table 3.6.** Growth performance and whole-body composition of fish.

		CTRL	LAM	SE	LAMSE	P - Value
IBW <sup>1</sup> , g		237.5 ± 1.2	238.1 ± 1.5	239.6 ± 0.6	237.3 ± 2.7	
FBW <sup>2</sup> , g		334.9 ± 0.2 <sup>a</sup>	343.4 ± 0.8 <sup>b</sup>	349.0 ± 4.1 <sup>b</sup>	349.6 ± 3.9 <sup>b</sup>	0.020
VFI <sup>3</sup>		0.45 ± 0.01	0.44 ± 0.00	0.42 ± 0.01	0.44 ± 0.01	0.168
SGR <sup>4</sup> , %·d <sup>-1</sup>		0.38 ± 0.00	0.40 ± 0.00	0.41 ± 0.02	0.43 ± 0.00	0.062
FCR <sup>5</sup>		1.20 ± 0.02 <sup>a</sup>	1.12 ± 0.01 <sup>b</sup>	1.03 ± 0.02 <sup>c</sup>	1.04 ± 0.02 <sup>c</sup>	0.004
<b>Whole -body composition</b>	<b>Initial</b>					
Moisture, % ww	74.7	70.5 ± 0.2	70.9 ± 0.1	70.6 ± 0.3	70.5 ± 0.1	0.172
Protein, % ww	14.1	16.7 ± 0.1	16.8 ± 0.1	16.9 ± 0.2	17.1 ± 0.1	0.172
Fat, % ww	8.2	9.6 ± 0.2	9.4 ± 0.0	9.6 ± 0.1	9.7 ± 0.2	0.400
Ash, % ww	2.7	2.9 ± 0.0	2.9 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	0.271
Energy, kJ·g <sup>-1</sup> ww	6.18	7.89 ± 0.1	7.74 ± 0.1	7.60 ± 0.1	7.51 ± 0.0	

Values are means ± standard deviation (n=2). Absence of superscript letters within a row represent no significant differences between treatments ( $P>0.05$ ).

<sup>1</sup> Initial mean body weight.

<sup>2</sup> Final mean body weight.

<sup>3</sup> Feed intake: crude feed intake / (IBW + FBW) / 2 / 91 days.

<sup>4</sup> Specific growth rate: (Ln FBW – Ln IBW) x 100 / 91 days.

<sup>5</sup> Feed conversion ratio: dry feed intake / wet weight gain.

Dietary fortification strategies led to a significant increase ( $P<0.001$ ) of iodine and selenium content in rainbow trout fillets (table 3.7). Trout fed *Laminaria digitata* supplemented diets showed a significant increase ( $P<0.001$ ) of their fillet iodine content (0.12 mg·kg<sup>-1</sup>), representing a six-fold increase over the iodine levels found in the CTRL treatment (0.02 mg·kg<sup>-1</sup>). Similarly, trout fed with selenium-enriched yeast resulted in a significant 2.9-fold increase in muscle selenium content (0.43 mg·kg<sup>-1</sup>), when compared to the selenium level in the CTRL treatment (0.15 mg·kg<sup>-1</sup>). The concomitant supplementation of *Laminaria* macroalgae and selenium-enriched yeast (LAMSE diet) significantly increased both selenium and iodine levels in trout muscle, to similar levels as those found with individual supplementation strategies. The dietary inclusion of *Laminaria* macroalgae and selenium-enriched yeast had no effect ( $P>0.05$ ) on the fillet content of other minerals (iron, zinc, potassium and magnesium) and vitamins A and D<sub>3</sub>.

**Table 3.7.** Vitamins and minerals content in rainbow trout fillets fed the various experimental diets and nutritional contribution as % of Daily Recommended Intakes (DRV).

	CTRL		LAM		SE		LAMSE	
Iodine, mg·kg <sup>-1</sup>	0.02 ± 0.01 <sup>a</sup>		0.12 ± 0.01 <sup>b</sup>		0.02 ± 0.00 <sup>a</sup>		0.12 ± 0.00 <sup>b</sup>	
Selenium, mg·kg <sup>-1</sup>	0.15 ± 0.01 <sup>a</sup>		0.14 ± 0.01 <sup>a</sup>		0.43 ± 0.01 <sup>b</sup>		0.43 ± 0.02 <sup>b</sup>	
Iron, mg·kg <sup>-1</sup>	4.0 ± 0.1		4.0 ± 0.3		4.1 ± 0.0		4.1 ± 0.2	
Zinc, mg·kg <sup>-1</sup>	3.3 ± 0.1		3.3 ± 0.1		3.3 ± 0.0		3.3 ± 0.1	
Potassium, g·kg <sup>-1</sup>	4.9 ± 0.0		4.9 ± 0.2		4.8 ± 0.1		4.8 ± 0.1	
Magnesium, mg·kg <sup>-1</sup>	298.5 ± 6.4		298.5 ± 14.8		313.5 ± 16.3		288.5 ± 13.4	
Vitamin D <sub>3</sub> , ug·kg <sup>-1</sup>	122.8 ± 5.1		119.8 ± 7.2		121.5 ± 1.1		118.7 ± 4.9	
Vitamin A, ug·kg <sup>-1</sup>	139.0 ± 5.7		142.0 ± 2.8		137.5 ± 2.1		140.5 ± 3.5	
<b>Nutritional contribution</b>								
% DRI <sup>1,2</sup>	Female Male		Female Male		Female Male		Female Male	
Iodine	2.5		12.5		2.1		12.4	
Selenium	33.8		31.8		98.3		97.8	
Potassium	16.5		16.7		16.3		16.2	
Iron	3.6	8.0	3.6	8.0	3.6	8.2	3.6	8.1
Zinc	5.1	4.0	5.2	4.1	5.2	4.1	5.1	4.0
Magnesium	15.9	13.6	15.9	13.6	16.7	14.3	15.4	13.2
Vitamin D <sub>3</sub>	131.0		127.8		129.6		126.6	
Vitamin A	3.4	3.0	3.5	3.0	3.4	2.9	3.5	3.0

Values are means ± standard deviation (n=2). Each replicate sample was a pool of three fillets. Different superscripts within rows represent significant differences between treatments (P<0.05).

<sup>1</sup> For definition of DRV calculations refer to materials and methods section.

<sup>2</sup> DRV calculations considered gender differences on recommendations.

The nutritional contribution (NC) of trout fillets to cover the DRV of selected minerals and vitamins A and D<sub>3</sub> in adults' female and male is presented in table 3.7. The consumption of a 160 g portion of a CTRL trout fillet represented 2.5% and 33.8% of the DRV for iodine and selenium, respectively. Fillets of trout fed the iodine-rich macroalgae (LAM and LAMSE) covered 12.5% of iodine DRV, while those resulting from trout fed with selenised yeast accounted for 98% of selenium DRV. Although not affected by dietary treatments, vitamin D<sub>3</sub> content in trout fillets was high (119-123 µg·kg<sup>-1</sup>), and a 160 g meal covered 127-131% of DRV. The nutritional contribution of all other nutrients (Fe, Zn, K, Mg, vitamin A) was not affected by dietary treatments.

The summarised fatty acid contents of trout fillets are presented in table 3.8. Dietary incorporation of iodine-rich macroalgae and selenised yeast did not affect (P>0.05) the fillet profile in saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

Consequently, total levels of n-3 and n-6 fatty acids, its ratio and both thrombogenic (TI) and atherogenic (AI) indexes were also not affected by dietary treatments ( $P>0.05$ ). The consumption of a 160 g portion of trout fillet, twice a week, represented 85-91% of the Weekly Adequate Intake (WAI) of combined EPA and DHA for the primary prevention of cardiovascular disease in adults.

**Table 3.8.** Summarised fatty acids content of fillets

Fatty acids <sup>1</sup> (mg·100 g <sup>-1</sup> fillet)	Experimental treatments				P value
	CTRL	LAM	SE	LAMSE	
Total SFA <sup>2</sup>	1537 ± 73	1403 ± 78	1479 ± 151	1576 ± 92	NS
Total MUFA <sup>3</sup>	1753 ± 1	1625 ± 68	1648 ± 315	1748 ± 189	NS
C18:2 n-6	393 ± 31	375 ± 35	357 ± 59	382 ± 44	NS
C18:3 n-3	57 ± 2	56 ± 6	54 ± 10	56 ± 6	NS
C18:4 n-3	79 ± 3	80 ± 7	81 ± 17	81 ± 7	NS
C20:4 n-6	32 ± 1	32 ± 0	31 ± 5	31 ± 1	NS
C20:4 n-3	39 ± 0	35 ± 4	36 ± 6	39 ± 4	NS
C20:5 n-3 [EPA] <sup>4</sup>	275 ± 20	291 ± 5	268 ± 50	264 ± 1	NS
C22:5 n-3	80 ± 10	83 ± 9	75 ± 17	78 ± 4	NS
C22:6 n-3 [DHA] <sup>4</sup>	684 ± 54	704 ± 41	676 ± 117	670 ± 26	NS
Total PUFA <sup>5</sup>	1805 ± 66	1815 ± 4	1730 ± 324	1779 ± 98	NS
Total PUFA n-3	1259 ± 90	1294 ± 38	1235 ± 229	1235 ± 48	NS
Total PUFA n-6	463 ± 30	443 ± 34	423 ± 71	460 ± 43	NS
PUFA n-3/n-6	2.72 ± 0.37	2.93 ± 0.31	2.92 ± 0.05	2.69 ± 0.15	NS
EPA + DHA	959 ± 74	995 ± 36	944 ± 167	934 ± 27	NS
AI <sup>6</sup>	0.614	0.559	0.608	0.643	
TI <sup>7</sup>	0.291	0.263	0.291	0.304	
Nutritional contribution, % DAI <sup>8</sup>					
EPA + DHA	307	318	302	299	

Values are means ± standard deviation (n=2). Each replicate sample was a pool of three fillets.

<sup>1</sup> Despite not shown in this summarised format, all identified fatty acids were considered in the composite fractions.

<sup>2</sup> SFA: Saturated fatty acids.

<sup>3</sup> MUFA: Monounsaturated fatty acids.

<sup>4</sup> EPA (C20:5n-3) and DHA (C22:6n-3).

<sup>5</sup> PUFA: Polyunsaturated fatty acids.

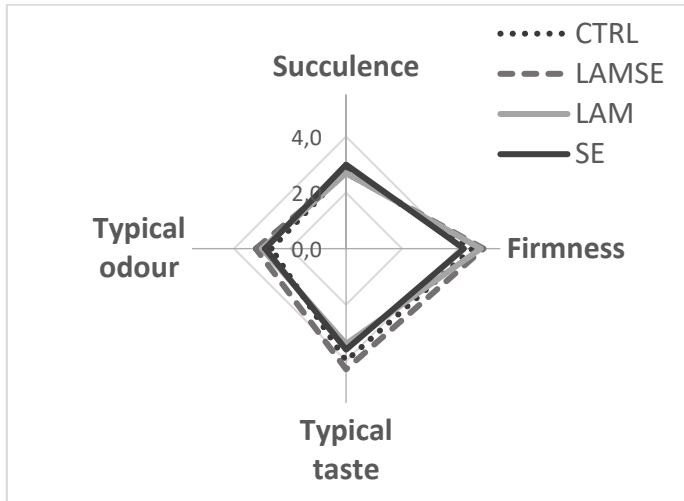
<sup>6</sup> AI: Atherogenic Index.

<sup>7</sup> TI: Thrombogenic Index.

<sup>8</sup> For calculation of Nutritional Contribution refer to materials and methods section.

Organoleptic properties of steamed cooked trout fillets were not affected by the dietary inclusion of *Laminaria* and selenised yeast ( $P>0.05$ ) (fig. 3.3). Sensory panel rated typical

odour as slight to moderate (evenly rated between treatments) and typical taste as moderate (with higher tendency in both *Laminaria* groups) to intense (with higher tendency in CTRL and SE groups). Additionally, non-typical odour and non-typical taste were mainly classified as absent. Concerning texture-related criteria, succulence was scored as slight to moderate and firmness as moderate to intense.



**Figure 3.3.** Sensory analysis of steam-cooked trout fillets supplemented with macroalgae and selenised-yeast.

Data represent average values for each measured organoleptic property.

## Discussion

Fish are a rich source of vitamin D, iodine, selenium and n-3 LCPUFA. Nevertheless, their contents in seafood are extremely variable, not only among species but also between individuals of the same species (EFSA, 2014a). Although subjected to high variability, wild fish generally present higher iodine and selenium levels than farmed counterparts (Julshamn et al., 2001; Maage et al., 1991). The current trend for replacing marine-derived ingredients by plant feedstuffs in fish diets may accentuate such differences and consequently change consumer expected intakes of health valuable nutrients. Moreover, it is known that fish feeds do not make full use of the maximum approved levels for iodine and selenium (EFSA, 2005). Therefore, dietary supplementation strategies can be used as tools to enhance the iodine

and selenium content in fish fillets, to convey benefits beyond fulfilling the basic nutritional needs of the animal, namely in terms of its nutritional value to consumers.

Selenium levels in human populations are generally influenced by geographical region, due to soil content of selenium, seasonal changes, protein content and food processing (Navarro-Alarcon and Cabrera-Vique, 2008). A recent systematic data review indicate that Se intake and status is suboptimal in most European and Middle Eastern countries (Stoffaneller and Morse, 2015). Potential approaches for enhancing human Se status comprise selenium soil fertilization; animal feed and food fortification; and intake of single supplements. In our study, an increase of current feed selenium level ( $0.25 \text{ mg selenium}\cdot\text{kg}^{-1}$ ) to maximum approved levels in the European market ( $0.5 \text{ mg selenium}\cdot\text{kg}^{-1}$ ) with a selenised-yeast led to a 2.9-fold increase for selenium contents in trout fillets. Although variable in the magnitude of selenium deposition, previous studies have also reported the successful Se fortification of fish fillets (Hunt et al., 2011; Kuçukbay et al., 2009; Pacitti et al., 2015; Rodríguez and Rojas, 2014). The utilization of organic Se sources, as selenised-yeast, is also advantageous, since organic bound selenium was found to be a higher bioavailable form of Se when compared to inorganic sources in rainbow trout fortification studies (Kuçukbay et al., 2009; Rider et al., 2009). The daily nutritional contribution of a 160 g fillet portion was raised from 34% to 98% in the case of selenium fortified trout, considering a daily adequate intake for Se of  $70 \mu\text{g}$ , as recently set by EFSA (2014c). Concerning the beneficial effects for human health, high selenium bioavailability was reported from fish and shellfish food items (Moreda-Piñeiro, Moreda-Piñeiro and Bermejo-Barrera, 2015). One main achievement of our study was to establish the dietary use of selenised-yeast as an effective and natural strategy to fortify rainbow fillets in selenium.

Iodine deficiency is the world's greatest single cause of preventable brain damage, and this fact is the primary motivation behind the current worldwide drive to eliminate iodine deficiency (WHO et al., 2007). Deficiency is largely the result of inadequate iodine intake rather than poor absorption. The primary strategy for iodine supplementation relies on salt

iodization. However, with the implementation of salt intake reduction programmes makes this strategy less effective and alternatives are required. In our study, trout fed diets supplemented with *Laminaria digitata*, showed a significant increase of their fillet iodine content, representing a six-fold increase over the iodine levels found in the regular fed trout. Dietary macroalgae have been successfully used to achieve higher iodine contents in rainbow trout (Valente et al., 2015), chars (Schmid et al., 2003) and gilthead seabream (Ramalho Ribeiro et al., 2015). Several of these studies report higher iodine fillet contents than the ones observed in the present trial. This variation is probably due to the different macroalgae used, dietary iodine levels tested, and fish species considered. As described for other minerals, iodide (I<sup>-</sup>) the predominant iodine species in macroalgae is reported to have a higher bioavailability compared to other inorganic compounds improving digestibility, absorption and retention in fish (Doucha et al., 2009; Cotter et al., 2009; Kouba et al., 2014). Trout subjected to our iodine fortification strategy, implemented within the maximum allowed levels (20 mg·kg<sup>-1</sup>) in fish feeds, covered 12.5% of the daily adequate intake for adults, with a 160g portion, considering a daily adequate intake for iodine of 150 µg. Literature data on the bioavailability of iodine from fish is limited. Nevertheless, good evidence exist that iodine accumulates in fish muscle in an inorganic and therefore a complete absorption after ingestion by humans is assumed (Hurrell, 1997). A work performed by Schmid et al. (2003) reported high bioavailability of iodine from *L. digitata* enriched chars in a trial with 14 volunteers, although potential cooking losses were not assessed stalling an accurate absorption measurement.

In most European countries, there seems to be a shortfall in achieving current vitamin D recommendations, particularly in older institutionalised adults and in some ethnic groups (Cashman et al., 2011). Vitamin D status is highly dependent on several factors like diet, sunlight exposure and lifestyle. Fish are among the richest sources of vitamin D, available as the D<sub>3</sub> chemical form (Schmid and Walther, 2013). Moreover, vitamin D<sub>3</sub> shows a higher efficacy to raise serum 25-hydroxycholecalciferol (25(OH)D) concentrations than vitamin D<sub>2</sub>

(Cashman et al., 2011). Despite not being the target of our fortification strategy, trout fillets fed the various experimental diets showed high vitamin D<sub>3</sub> levels (119-123 µg·kg<sup>-1</sup>).

In our study the main source of dietary lipids was marine fish oil and it remained unchanged among the various diets. Also, *Laminaria* and selenised yeast had no interference on muscle fatty acids profile. Therefore, the combined content of EPA and DHA ( $0.96 \pm 0.08 \text{ g} \cdot 100 \text{ g}^{-1}$  fresh fillet) and PUFA n-3/n-6 ratio ( $2.82 \pm 0.22$ ) in raw fillets was relatively constant among the various treatments. The consumption of a 160 g portion of trout fillets, twice a week, represented, on average, 88 % of EPA and DHA combined Adequate Intake, as recommended for primary prevention of cardiovascular disease in adults (ISSFAL, 2004). Similarly, the fillet content of all other measured minerals (Fe, Zn, K, and Mg) and vitamin A remained unaffected by the dietary supplementation strategies. The nutritional contribution of trout fillets evaluated according to the established recommended values for adults, and if applicable for men and women distinctively, shows that a portion of 160 g of fillet of any test group contributed to the daily adequate intake for potassium (16%), magnesium (14% men; 16% women), iron (8% men; 4% women), zinc (4% men; 5% women) and vitamin A (3%). Most dietary guidelines convey the message that health benefits associated to a given nutrient are generally stronger when derived from a whole food matrix, as part of a synergistic dietary pattern, rather than its supply as a single supplement. Under this scenario, tailoring farmed fish with health beneficial compounds is becoming particularly important owed to the role of seafood as part of a healthy food pattern (EFSA, 2014a). Also, of importance is the fact that the fortified trout fillets could be promoted in the functional foods market. According to EC 1924/2006 regulation, in the European market, a food item can be labelled as “source of selenium” if it supplies 15% of the DRV in 100 g of product and “high in selenium” in case of containing at least twice this value (30% of selenium DRV). Under this definition, the selenium supplemented fillets produced in our trial could be labelled with a nutrition claim like “high source of selenium” since it supplies  $43 \text{ µg selenium} \cdot 100 \text{ g}^{-1}$  (61% of DRV). Similarly, all produced fillets could be labelled as “high source of vitamin D<sub>3</sub>”, since

on average they provide 80.5% of RDI in 100 g of food item. Therefore, these food items could be considered as functional foods (EFSA, 2009b; EFSA, 2015c).

In spite of actual consumer request for healthy food, there is also awareness about the natural sensory properties of food items (Verbeke, 2005). Thus, a nutritional fortification of food should avoid any negative effects on the sensory traits. In the current trial, dietary supplementation with selenised yeast and/or iodine-rich macroalgae did not alter organoleptic characteristics of steamed cooked trout fillets. Typical taste and typical odour, usually determinant traits for consumers' acceptance, were found similar between all dietary treatments. Also, fillets texture, assessed as firmness and succulence, was not affected by experimental diets. Previous studies also showed that dietary fortification with macroalgae did not alter sensorial traits in trout (Valente et al., 2015) and seabream (Ramalho Ribeiro et al., 2015). However, channel catfish fed a diet supplemented with Se-enriched garlic, flavour traits were significantly altered, although the effect was attributed to the garlic odour and flavour (Schram et al., 2010).

Acceptance of a novel or enriched food product is largely dependent on its positive health and natural image (Dickson-Spillmann et al., 2011). The use of synthetic or non-naturally occurring supplements may undermine consumer perception of enriched food products. Consumers often perceive natural additives as inherently better and healthy, while artificial chemicals in food are associated with higher risk perception (Dickson-Spillmann et al., 2011). A consumer survey performed in Germany, UK and Italy showed a general high acceptance for foods with seaweed ingredients (Buehrlen et al., 2005). Brown macroalgae, such as *Laminaria digitata*, although perceived as a natural product and a rich source of health valuable nutrients (e.g. iodine), may also contain elevated levels of some toxic metals such as arsenic (As) and cadmium (Cd), which may limit their use as food and feed ingredients. In order to protect animals, the consumer and the environment, the European feed legislation (Commission Directive 2002/32/EC and amended by Commission Regulation EU 1275/2013), has set maximum permitted levels for a range of undesirable substances in feed materials

and complete compound feeds. Currently, the maximum total arsenic level in feed materials from seaweed and products thereof is  $40 \text{ mg}\cdot\text{kg}^{-1}$  (or  $2 \text{ mg}\cdot\text{kg}^{-1}$  of inorganic arsenic) and the maximum total arsenic level in complete fish feeds is  $10 \text{ mg}\cdot\text{kg}^{-1}$ . For cadmium, the current maximum permitted level in both feed materials of vegetable origin and complete fish feed is  $1 \text{ mg}\cdot\text{kg}^{-1}$ . A recent study assessed the potential risks of macroalgae harvested in Norwegian waters when used as feed materials and food for animals and humans (Duinker, et al., 2016). Their data showed that several macroalgae species, and particularly brown algae, exceeded the current maximum permitted levels of Cd and As, clearly limiting their use as ingredients in animals' feeds, including fish. However, the high variability on As and Cd levels associated to the geographical origin of the macroalgae and the lower toxicity of organic As forms generally occurring in these marine-derived products are aspects that require further studies to fully elucidate the potential risks associated to their use.

In conclusion, rainbow trout fillets may be successfully tailored to improve its nutritional value conveying an added value for consumers' diets. The supplementation of a traditional trout feed with macroalgae (*Laminaria digitata*) and selenised yeast significantly enhanced the daily nutritional contribution in iodine and selenium, while vitamin D<sub>3</sub> and n-3 LCPUFA were also present at high levels. Moreover, the use of natural sources of trace elements, replacing inorganic mineral salts in fish feeds, fits a broader concept of sustainable, chemical-free farming, which is highly appealing to consumers.

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# FORTIFICATION WITH VITAMIN D<sub>3</sub>

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Chapter 4



# Biofortification of trout and seabream fillets with vitamin D<sub>3</sub> and 25-hydroxyvitamin D enhances the nutritional value of farmed fish

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## Chapter 4.1

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*In preparation to be submitted*

## Biofortification of trout and seabream fillets with vitamin D<sub>3</sub> and 25-hydroxyvitamin D enhances the nutritional value of farmed fish

### Abstract

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Oily fish are regarded as one of richest dietary sources of vitamin D, mainly available in the form of cholecalciferol (vitamin D<sub>3</sub>). Exogenous feeding in aquaculture opens the possibility to tailor fish composition in terms of its content of health valuable nutrients, such as liposoluble vitamin D<sub>3</sub>. Two trials were performed with rainbow trout (IBW: 240 g) and gilthead seabream (191 g) to assess the effects of the dietary biofortification of gilthead seabream and rainbow trout with vitamin D<sub>3</sub> and 25-hydroxyvitamin D on the growth performance, vitamin D<sub>3</sub> deposition in fillets, sensory quality traits and nutritional contribution. Each trial comprised three dietary treatments: a control diet (CTRL), mimicking a commercial formulation for the species and containing vitamin D<sub>3</sub> supplied as cholecalciferol, at levels of 42 µg·kg<sup>-1</sup> and 31 µg·kg<sup>-1</sup> for trout and seabream, respectively. Based on this control formulation, two other diets were supplemented to a target level of 75 µg·kg<sup>-1</sup> of either cholecalciferol (diet D3) or 25-hydroxyvitamin D (diet HyD). At the end of the experimental feeding period, the increase of dietary vitamin D<sub>3</sub> and 25-hydroxyvitamin D levels had no effect (P>0.05) on weight gain, feed intake, feed conversion ratio and whole-body composition of both trout and seabream. Fillet content on vitamin D<sub>3</sub> was not affected by dietary treatments (P>0.05). Despite not statistically significant, the absolute vitamin D<sub>3</sub> content in the fillets of seabream fed diets D3 and HyD was higher than that of fish fed the CTRL diet. For seabream, a meal portion of 160 g presented a nutritional contribution for vitamin D<sub>3</sub> of 46, 55 and 60% of dietary reference value (DRV) for the CTRL, HyD and D3, respectively. In trout, a meal portion of 160 g presented a nutritional contribution for vitamin D<sub>3</sub> of 109, 98 and 129% of DRV for the CTRL, D3 and HyD, respectively. Vitamin D<sub>3</sub> and 25-hydroxyvitamin D supplementation did not affect the typical taste of steamed cooked trout and seabream fillets, as assessed by a trained panel (P>0.05). Overall data shows that feed

supplementation with vitamin D<sub>3</sub>, within the maximum permitted levels in fish feeds, enhances the nutritional contribution of farmed fish and contributes towards an increase of the vitamin D<sub>3</sub> intake in general populations.

Keywords: Rainbow trout; Gilthead seabream; vitamin D<sub>3</sub>; 25-hydroxyvitamin D; Biofortification

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## Introduction

Recent studies confirm the prevalence of low vitamin D intakes and vitamin D deficiency or inadequate vitamin D status in several segments of the European population (Spiro and Buttriss, 2014; Cashman et al., 2016). Although there are some areas of debate in relation to vitamin D requirements and the impact of vitamin D intake and status on many aspects of human health, there is a general agreement that prevention of vitamin D deficiency is a public health priority (WHO, 2015). Food fortification represents an opportunity to increase the vitamin D supply on a population-wide basis (Spiro and Buttriss, 2014; Cashman, 2015). In traditional fortification, exogenous vitamin D is added to the foodstuffs when they are being processed (milk and dairy products, margarines and spreads, some breakfast cereals and fruit juices). However, the concept of biofortified foods with vitamin D is gaining momentum, since it may increase other metabolites of vitamin D which would boost the overall relative effectiveness of these foods in raising vitamin D status (Cashman, 2015). The selection of foods suitable for fortification and biofortification must comply with several conditions such as widely available; accessible to all age ranges within a population; affordable and be part of a healthy dietary pattern (WHO and FAO, 2006). This vitamin D biofortification approach relies on the fact that by adding vitamin D or 25-hydroxyvitamin D (where permissible) to farmed animals feeds (such as fish, beef, pork, lamb, chicken, and products thereof eggs and milk) their contents on these compounds could increase. Several studies have now demonstrated the efficacy of this vitamin D biofortification approach in livestock (Cashman et al., 2015; Jakobsen et al. 2007; Mattila et al., 2003; Mattila et al., 2011; Montgomery et al., 2004; Swigert et al., 2004; Yao et al., 2013; Wilborn et al., 2004).

Exogenous feeding in aquaculture unlocks the possibility to tailor fish composition in terms of its content of health valuable nutrients. Fish quality traits such as fatty acid composition and concentration of several trace nutrients are known to be influenced by diet composition. Several biofortification studies have demonstrated that feed supplementation can effectively tailor the composition of fish fillets in terms of bioactive fatty acids (Kennedy et al., 2007b; Ramos et al., 2008), selenium (Lorentzen et al., 1994; Schram et al., 2010) and iodine (Julshamn et al., 2006; Ramalho Ribeiro et al., 2015; Valente et al., 2015). Although being one of the more relevant natural sources of vitamin D<sub>3</sub>, data on the efficacy of dietary vitamin D<sub>3</sub> biofortification in fish is extremely scarce (Horvli et al., 1998; Mattila et al., 1999; Lock et al., 2010).

Within this general context, two trials were performed with rainbow trout and gilthead seabream, two of the most consumed fish species in Europe, to assess the effect of vitamin D<sub>3</sub> (cholecalciferol) and 25-hydroxyvitamin D dietary supplementation, on the growth performance, vitamin D<sub>3</sub> deposition in muscle, sensory quality traits and fillets nutritional contribution.

## Material and methods

### *Experimental diets*

Each trial, with rainbow trout and gilthead seabream, comprised three experimental diets (Table 4.1). A control diet (CTRL), mimicking a commercial formulation contained vitamin D<sub>3</sub> (cholecalciferol), at levels of 42  $\mu\text{g}\cdot\text{kg}^{-1}$  and 31  $\mu\text{g}\cdot\text{kg}^{-1}$  for trout and seabream, respectively. Based on this control formulation, two other diets were manufactured to a target level of 75  $\mu\text{g}\cdot\text{kg}^{-1}$  of vitamin D<sub>3</sub>, supplied either as cholecalciferol (diet D3) or as 25-hydroxyvitamin D (diet HyD). The upper limit of vitamin D<sub>3</sub> supplementation was set at 75  $\mu\text{g}$  vitamin D<sub>3</sub> per kg feed (3000 IU $\cdot\text{kg}^{-1}$ ), to comply with the current maximum permitted levels in fish feeds (EC, 2017). For supplementation purposes, 1 IU (international unit) of 25-hydroxyvitamin D was assumed equivalent to 1 IU vitamin D<sub>3</sub>. Experimental diets were manufactured by extrusion (SPAROS Lda, Portugal). Vitamin D forms (cholecalciferol and 25-hydroxyvitamin D) were

blended with the oils and applied post-extrusion to the pellets by vacuum coating (PG-10VCLAB, Dinnissen, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature, but in a cool and aerated emplacement. Samples of each diet were taken for proximate composition and vitamin D<sub>3</sub> analysis (Table 4.1). Within each species, diets were isonitrogenous, isolipidic and isoenergetic.

**Table 4.1.** *Ingredients and proximate composition of experimental diets.*

Ingredients, g·kg <sup>-1</sup>	CTRL BREAM	Ingredients, g·kg <sup>-1</sup>	CTRL TROUT
Fishmeal LT 70 <sup>1</sup>	125.00	Fishmeal LT 70 <sup>1</sup>	175.00
Porcine blood meal <sup>2</sup>	50.00	Soy protein concentrate <sup>3</sup>	160.00
Soy protein concentrate <sup>3</sup>	130.00	Wheat gluten <sup>4</sup>	100.00
Wheat gluten <sup>4</sup>	55.00	Corn gluten <sup>5</sup>	100.00
Corn gluten <sup>5</sup>	60.00	Soybean meal 48 <sup>6</sup>	50.00
Soybean meal 48 <sup>6</sup>	100.00	Rice protein concentrate <sup>7</sup>	50.00
Rice protein concentrate <sup>7</sup>	60.00	Wheat meal <sup>10</sup>	128.80
Rapeseed meal <sup>8</sup>	50.00	Fish oil <sup>11</sup>	195.00
Sunflower meal <sup>9</sup>	30.00	Vitamin and mineral premix <sup>14</sup>	2.00
Wheat meal <sup>10</sup>	137.20	Vitamin C <sup>15</sup>	0.30
Fish oil <sup>11</sup>	120.00	Vitamin E <sup>16</sup>	0.50
Rapeseed oil <sup>12</sup>	35.00	Soy lecithin <sup>17</sup>	2.00
Linseed oil <sup>13</sup>	5.00	Guar gum <sup>18</sup>	4.00
Vitamin and mineral premix <sup>14</sup>	2.00	Zeolite <sup>19</sup>	10.00
Vitamin C <sup>15</sup>	0.30	Antioxidant <sup>20</sup>	2.00
Vitamin E <sup>16</sup>	0.50	Dicalcium phosphate <sup>21</sup>	11.40
Soy lecithin <sup>17</sup>	2.00	L-Lysine <sup>22</sup>	7.00
Guar gum <sup>18</sup>	4.00	DL-Methionine <sup>23</sup>	2.00
Zeolite <sup>19</sup>	10.00		
Antioxidant <sup>20</sup>	2.00		
Dicalcium phosphate <sup>21</sup>	7.00		
L-Lysine <sup>22</sup>	10.00		
DL-Methionine <sup>23</sup>	5.00		

	CTRL	D3	HyD
<b>Rainbow trout</b>			
Dry matter (DM), %	95.2 ± 0.1	95.2 ± 0.2	95.3 ± 0.2
Crude protein, %DM	42.7 ± 0.1	42.7 ± 0.2	42.7 ± 0.1
Crude fat, %DM	23.3 ± 0.1	23.3 ± 0.1	23.4 ± 0.1
Ash, %DM	8.5 ± 0.0	8.3 ± 0.0	8.4 ± 0.0
Gross energy, MJ·kg <sup>-1</sup> DM	23.5 ± 0.1	23.6 ± 0.1	23.5 ± 0.0
Total phosphorus, %DM	1.1 ± 0.0	1.1 ± 0.0	1.2 ± 0.0
Vitamin D <sub>3</sub> , µg·kg <sup>-1</sup> DM	42.0 ± 2.1	75.0 ± 0.5	74.4 ± 0.6
<b>Gilthead seabream</b>			
Dry matter (DM), %	94.5 ± 0.1	94.2 ± 0.1	94.6 ± 0.1
Crude protein, %DM	42.6 ± 0.1	42.5 ± 0.0	42.6 ± 0.1
Crude fat, %DM	18.5 ± 0.2	18.5 ± 0.1	18.6 ± 0.1

Ash, %DM	9.6 ± 0.1	9.7 ± 0.1	9.6 ± 0.0
Gross energy, MJ·kg <sup>-1</sup> DM	20.9 ± 0.0	20.9 ± 0.0	20.9 ± 0.0
Total phosphorus, %DM	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0
Vitamin D <sub>3</sub> , µg·kg <sup>-1</sup> DM	30.9 ± 0.5	77.4 ± 1.4	76.8 ± 1.2

<sup>1</sup> Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru. <sup>2</sup> Pork blood meal: 89% CP, 0.4% CF, SONAC, The Netherlands. <sup>3</sup> Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands. <sup>4</sup> VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France. <sup>5</sup> Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal. <sup>6</sup> Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal. <sup>7</sup> Rice 50: 48.3% CP, 6.5% CF, SEAH International, France. <sup>8</sup> Defatted rapeseed meal: 34% CP, 2% CF, SORGAL SA, Portugal. <sup>9</sup> Sunflower meal: 23% CP, 3% CF, PREMIX Lda, Portugal; <sup>10</sup> Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal. <sup>11</sup> COPPENS International, The Netherlands. <sup>12</sup> JC Coimbra, Portugal. <sup>13</sup> Henry Lamotte Oils GmbH, Germany. <sup>14</sup> Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg; betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate, 7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings. <sup>15</sup> Vitamin C35% (sodium calcium ascorbyl phosphate), ORFFA, The Netherlands. <sup>16</sup> Vitamin E 50% (all-rac-alpha-tocopheryl acetate), ORFFA, The Netherlands. <sup>17</sup> Lecico P700IPM, LECICO GmbH, Germany. <sup>18</sup> Seah International, France. <sup>19</sup> Kielseguhr, LIGRANA GmbH, Germany. <sup>20</sup> Paramega PX, Kemin Europe NV, Belgium. <sup>21</sup> Dicalcium phosphate: 18% phosphorus, 23% calcium, Fosfitalia, Italy. <sup>22</sup> Biolys 54.6%, EVONIK Nutrition & Care GmbH, Germany. <sup>23</sup> DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.

### Feeding trials

The trial with rainbow trout was conducted at the Experimental Research Station of University of Trás-os-Montes e Alto Douro (UTAD, Vila Real, Portugal), while the trial with gilthead seabream took place at the Experimental Research Station of the University of Algarve (UALG, Faro, Portugal). Experiments were directed by trained scientists (following category C FELASA recommendations) and in compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei nº. 113/2013, de 7 de Agosto) legislation on the protection of animals for scientific purposes. Both experimental facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

#### SEABREAM TRIAL

Nine homogenous groups of 51 seabream each, with a mean initial body weight of 191 g were stocked in 1000 L circular tanks. Each experimental treatment was tested in triplicate tanks over 84 days. Rearing tanks were supplied with flow-through gravel-filtered and

aerated seawater (salinity: 35 parts per thousand; temperature: 22–27 °C; dissolved oxygen above  $6.9 \pm 1.1 \text{ mg}\cdot\text{L}^{-1}$ ) and subjected to natural photoperiod changes through summer conditions. Fish were fed to apparent satiety, by hand, twice a day (09.30 and 16.00h) during week days, once a day in Saturdays and unfed on Sundays. Excess feeding was minimized, and feed intake was quantified throughout the trial. At the end of the feeding period, fish were bulk weighted by tank for the assessment of growth performance criteria.

#### TROUT TRIAL

Nine homogenous groups of 32 trout each, with a mean initial body weight of 240 g were stocked in 1000 L tanks. Each experimental treatment was tested in triplicate tanks over 91 days. Rearing tanks were supplied with flow-through filtered and aerated freshwater (temperature: 13-15 °C; dissolved oxygen above  $7.2 \pm 1.1 \text{ mg}\cdot\text{L}^{-1}$ ) and subjected to natural photoperiod changes during spring conditions. Feeding regime and fish weighing was identical to the one previously described for seabream.

#### *Sampling*

All samplings were done 48 h following the last meal. Fish were slaughtered by immersion in ice–water slurry (4:1) as normally done in commercial farms. At start (six fish from the initial stock) and at the end of the trial, six fish from each tank were sampled for analysis of whole-body composition. At the end of the trial, skinless right fillets were collected from 4 fish per tank, an identical section of the dorsal muscle isolated and stored at -80°C for analysis of vitamin D<sub>3</sub> and 25-hydroxyvitamin D content. Skinless left fillets from the same fish were used for analysis of proximate composition, fatty acids, vitamin A and minerals (Fe, Zn, I, Se, K, Mg) content. For sensory analysis, after slaughter, fish from each treatment were packed in insulated polystyrene boxes, with the ventral side upward, covered with flaked ice and immediately transported to the laboratory. Fish from each treatment were scaled and filleted 24 h after death. Right fillets were kept at 4°C overnight until sensory assessment.

### *Analytical methods*

Diets were grinded prior to analysis of proximate composition. Frozen whole-body samples and fillets were minced, mixed, a representative sample freeze-dried and homogenized with a laboratory mill prior to analysis. The chemical composition analysis of diets and whole-fish was made using the following procedures: dry matter after drying at 105°C for 24 h; ash by combustion at 550°C for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428); fat by dichloromethane extraction (Soxhlet); gross energy in an adiabatic bomb calorimeter (IKA). Minerals (P, Fe, Zn, Mg, K, I and Se) content in feeds and fillets was determined at an external certified laboratory (Silliker, Mérieux Nutrisciences, Portugal) according to ISO 27085:2009, implying a microwave digestion with nitric acid and hydrogen peroxide and detection by inductively coupled plasma mass spectrometry (ICP-MS). The same external laboratory determined also vitamin A (all-E-retinol, 13-Z-retinol) by HPLC according to EN 12823-1:2014 method. Vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> content in the feeds and fillets of gilthead seabream and rainbow trout was analyzed by a liquid chromatography with tandem MS detection method. Total lipids in the fillets were extracted according to the method of Folch, Lees and Stanley (1957) and subsequently, the fatty acid composition of fillets was determined by gas-chromatography analysis of methyl esters, according to the procedure of Lepage and Roy (1986), modified by Cohen et al. (1988) and described in further detail by Costa et al. (2013). Data in mg/100 g of edible part were calculated using the peak area ratio (% of total fatty acids) and the lipid conversion factors set by Weihrauch et al. (1977). Cholesterol was assayed by gas chromatography after saponification in alkaline conditions, based on the method of Naemmi et al. (1995) and modified by Oehlenschläger (2000). It was analysed in a CX3400 VARIAN gas chromatograph and the separation was carried out with helium as the carrier gas in an HP5 column (30 m × 0.5 mm id). The temperatures of the oven, injector, and detector were 280, 285, and 300 °C, respectively. The detection limit (DL) was 6.34 µg/ml.

Based on the fatty acid profile, the atherogenic (AI) and thrombogenic (TI) indexes were calculated accordingly to Ulbricht and Southgate (1991) for evaluation of the predisposition for incidence of coronary heart disease.

$$AI = \frac{C12:0 + (4 \times C14:0) + C16:0}{\text{Total MUFA} + \text{Total } n-3 + \text{Total } n-6}$$

$$TI = \frac{C14:0 + C16:0 + C18:0}{(0.5 \times \text{Total MUFA}) + (0.5 \times \text{Total } n-6) + (3 \times \text{Total } n-3) + \text{Ratio } n-3/n-6}$$

### *Nutritional Contribution*

The nutritional contribution (NC) of trout and seabream fillets was calculated as the percentage of the Daily Recommended Intake (DRI), Dietary Reference Values (DRVs) for selected minerals and vitamins and Daily Adequate Intake (DAI) for combined EPA and DHA. When distinct recommendations exist for males and females, calculations were adapted accordingly, and the following formula was used:

$$NC (\%) = 100 \times \frac{C \times M}{\text{DRI/DRV or DAI}}$$

*Where:*

C: mean concentration of nutrient in g·kg<sup>-1</sup>, mg·kg<sup>-1</sup> or µg·kg<sup>-1</sup>.

M: meal fillet portion consumed is 0.160 kg (Costa et al., 2013).

DRV established as Adequate Intake (AI) for iodine is 0.150 mg·d<sup>-1</sup> for adults (EFSA, 2014b).

DRV established as Adequate Intake for selenium is 0.070 mg·d<sup>-1</sup> for adults (EFSA, 2014c)

DRV established as Population Reference Intake (PRIs) for iron is 11.0 mg·d<sup>-1</sup> for adult males and 11.0 to 16.0 mg·d<sup>-1</sup> for adult females (mean value = 13.5 mg·d<sup>-1</sup>) (EFSA, 2015d).

DRV established as Population Reference Intakes for zinc are 9.4 to 16.3 mg·d<sup>-1</sup> for males (mean value = 12.9 mg·d<sup>-1</sup>) and (7.5 to 12.7 mg·d<sup>-1</sup> for adult females (mean value = 10.1 mg·d<sup>-1</sup>) (EFSA, 2014d)

DRV established as Adequate Intake (AI) for potassium is 3.5 g·d<sup>-1</sup> for adolescents and adults (EFSA, 2016a).

DRV established as Adequate Intake (AI) for magnesium is 350 mg·d<sup>-1</sup> for adult males and 300 mg·d<sup>-1</sup> for adult females (EFSA, 2015a)

DRV established as Adequate Intake for Vitamin D is 15.0 µg·d<sup>-1</sup> for young and adults (EFSA, 2016b).

DRV established as Population Reference Intakes (PRIs) for Vitamin A is 750 µg retinol·d<sup>-1</sup> for adult males and 650 µg retinol·d<sup>-1</sup> for adult females (EFSA, 2015b).

DAI for EPA+DHA is 500 mg·d<sup>-1</sup> for primary prevention of cardiovascular disease in adults (International Society for the Study of Fatty Acids and Lipids [ISSFAL], 2004), corresponding to a weekly intake of 3.5g of EPA+DHA.

DRV established as Population Reference Intakes (PRIs) for protein is 62 g·d<sup>-1</sup> for adult males (18-59 years) and 52 g·d<sup>-1</sup> for adult females (EFSA, 2012a).

### *Sensory Assessment*

The three dietary treatments of seabream and trout were evaluated in two independent sensory sessions. Sessions took place 48 h after death in an acclimatized test room equipped with individual booths outfitted with water and white light. Sensory panel was composed by twelve trained participants within 34-50 years old. A multisample difference test was applied to evaluate the three dietary treatments of both species focusing on typical taste (Meilgaard et al., 1999). The right-side fillets from each fish were removed, washed and drained for five minutes. Only the anterior dorsal part of each fillet was used to have pieces with uniform thickness. Each piece of fillet was individually wrapped with aluminium foil and cooked for 12 min and 8 min at 100 °C, respectively for seabream and trout, in a saturated steam oven (Rational Combi-Master CM6 Cross KuchentechnikCmbH, Landsberg, Germany). Cooked fillets from each dietary treatment were presented to the panelists sequentially, in coded white dishes, in the six possible different combinations. The subjects rated the intensity of typical taste based on an 8-point scale ranging from absent (0) to strong intensity (8) (Meilgaard et al., 1999).

### *Statistical analysis*

Data were subjected to a one-way analysis of variance, and when appropriate, means were compared by the Tukey test. Parameters expressed as percentages were subjected to arcsin square root transformation. Statistical significance was tested at 0.05 probability level. All statistical tests were performed using the SPSS V22 software.

### **Results**

In both, rainbow trout and gilthead seabream studies, the overall growth performance criteria (e.g. weight gain, feed intake and FCR – data not shown) and the proximate composition of whole-fish (moisture, protein, fat and ash) at the end of the trial were not significantly affected by the various dietary treatments ( $P>0.05$ ).

The fillet proximate composition and content on vitamins (A and D<sub>3</sub>) and minerals (iodine, selenium, iron, zinc, potassium, magnesium) were not affected by dietary treatments

( $P > 0.05$ ) (Table 4.2). Trout and seabream fillets showed similar levels of Fe, Zn, Se, K and Mg. On the other hand, in comparison to trout fillets, those of seabream showed a higher content of iodine and cholesterol and lower levels of vitamins A and D<sub>3</sub>.

**Table 4.2.** Proximate composition, vitamin and mineral contents in trout and seabream fillets.

	CTRL	D3	HyD	P-value
<b>Rainbow trout</b>				
Moisture, % ww	67.3 ± 0.1	67.5 ± 0.5	67.8 ± 0.4	0.505
Protein, % ww	18.0 ± 0.6	18.0 ± 0.2	17.9 ± 0.5	0.972
Fat, % ww	13.4 ± 0.5	12.9 ± 0.1	12.9 ± 0.1	0.262
Ash, % ww	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.395
Iodine, mg·kg <sup>-1</sup>	0.02 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.939
Selenium, mg·kg <sup>-1</sup>	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.861
Iron, mg·kg <sup>-1</sup>	4.0 ± 0.1	4.1 ± 0.5	4.0 ± 0.3	0.986
Zinc, mg·kg <sup>-1</sup>	3.3 ± 0.1	3.9 ± 0.4	3.5 ± 0.4	0.259
Magnesium, mg·kg <sup>-1</sup>	298.5 ± 6.4	296.0 ± 21.2	285.0 ± 12.7	0.662
Potassium, g·kg <sup>-1</sup>	4.9 ± 0.0	4.7 ± 0.2	4.7 ± 0.2	0.579
Cholesterol, mg·kg <sup>-1</sup>	499.5 ± 17.7	468.0 ± 29.7	471.0 ± 5.7	0.357
Vitamin A, µg·kg <sup>-1</sup>	139.0 ± 5.7	136.0 ± 17.0	140.5 ± 3.5	0.912
Vitamin D <sub>3</sub> , µg·kg <sup>-1</sup>	102.5 ± 10.6	91.5 ± 19.1	121.0 ± 28.3	0.453
<b>Gilthead seabream</b>				
Moisture, % ww	72.6 ± 0.1	72.5 ± 0.3	72.7 ± 0.1	0.505
Protein, % ww	17.2 ± 0.4	17.3 ± 0.3	17.2 ± 0.4	0.972
Fat, % ww	7.7 ± 0.2	8.0 ± 0.3	7.7 ± 0.3	0.262
Ash, % ww	2.2 ± 0.2	2.0 ± 0.1	2.2 ± 0.1	0.395
Iodine, mg·kg <sup>-1</sup>	0.18 ± 0.01	0.18 ± 0.01	0.18 ± 0.00	0.933
Selenium, mg·kg <sup>-1</sup>	0.16 ± 0.01	0.16 ± 0.00	0.15 ± 0.01	0.946
Iron, mg·kg <sup>-1</sup>	4.1 ± 0.4	4.2 ± 0.1	4.2 ± 0.4	0.897
Zinc, mg·kg <sup>-1</sup>	4.1 ± 0.3	5.3 ± 0.5	5.3 ± 0.2	0.072
Magnesium, mg·kg <sup>-1</sup>	310.0 ± 12.7	314.5 ± 12.0	314.0 ± 4.2	0.897
Potassium, g·kg <sup>-1</sup>	5.8 ± 0.9	5.6 ± 0.4	5.9 ± 0.3	0.901
Cholesterol, mg·kg <sup>-1</sup>	607.5 ± 19.1	608.5 ± 3.5	616.5 ± 10.6	0.762
Vitamin A, µg·kg <sup>-1</sup>	57.0 ± 10.4	57.3 ± 2.9	59.4 ± 6.4	0.938
Vitamin D <sub>3</sub> , µg·kg <sup>-1</sup>	43.0 ± 11.3	56.5 ± 10.6	52.0 ± 11.3	0.537

Values are means ± standard deviation ( $n=3$ ; each replicate sample is a pool of 4 fillets).

The summarized fatty acid composition of trout and seabream fillets is presented in Table 4.3. In comparison to the CTRL treatment, feeding trout and seabream with the diets supplemented with vitamin D<sub>3</sub> or 25-hydroxyvitamin D had no significant effect ( $P > 0.05$ ) on the fatty acid profile and contents of fillets. Consequently, total levels of n-3 and n-6 fatty

acids, its ratio and both thrombogenic (TI) and atherogenic (AI) indexes were also not affected by dietary treatments ( $P>0.05$ ).

**Table 4.3.** Summarised fatty acid content of raw trout and seabream fillets.

Fatty acid (mg·100 g <sup>-1</sup> fillet)	CTRL	D3	HyD	P-value
<b>Rainbow trout</b>				
Total SFA <sup>1</sup>	3973 ± 227	3719 ± 23	3774 ± 67	0.295
Total MUFA <sup>2</sup>	4586 ± 50	4537 ± 114	4473 ± 74	0.482
C18:2 n-6	1015 ± 75	997 ± 53	1010 ± 47	0.953
C18:3 n-3	148 ± 4	154 ± 2	149 ± 11	0.600
C18:4 n-3	206 ± 9	200 ± 11	194 ± 2	0.514
C20:4 n-6	84 ± 4	77 ± 1	78 ± 1	0.127
C20:5 n-3 [EPA]	710 ± 58	669 ± 44	686 ± 11	0.667
C22:6 n-3 [DHA]	1768 ± 152	1692 ± 28	1702 ± 28	0.693
Total PUFA <sup>3</sup>	4667 ± 201	4446 ± 3	4533 ± 14	0.303
Total PUFA n-3	3140 ± 242	2980 ± 60	3038 ± 29	0.591
Total PUFA n-6	1527 ± 41	1467 ± 57	1495 ± 43	0.526
PUFA n-3/n-6	2.1 ± 0.2	2.0 ± 0.1	2.0 ± 0.1	0.933
EPA + DHA	2479 ± 210	2362 ± 71	2388 ± 39	0.677
AI <sup>4</sup>	0.60 ± 0.04	0.56 ± 0.01	0.58 ± 0.00	0.414
TI <sup>5</sup>	0.30 ± 0.00	0.29 ± 0.00	0.29 ± 0.01	0.208
<b>Gilthead seabream</b>				
Total SFA	1988 ± 49	2086 ± 93	2002 ± 84	0.485
Total MUFA	3472 ± 117	3571 ± 174	3438 ± 99	0.635
C18:2 n-6	883 ± 2	898 ± 45	843 ± 36	0.369
C18:3 n-3	167 ± 10	177 ± 8	173 ± 1	0.459
C18:4 n-3	50 ± 4	60 ± 14	46 ± 9	0.465
C20:4 n-6	47 ± 1	48 ± 2	46 ± 2	0.507
C20:5 n-3 [EPA]	287 ± 19	306 ± 25	285 ± 21	0.616
C22:6 n-3 [DHA]	562 ± 10	596 ± 26	574 ± 16	0.316
Total PUFA	2275 ± 47	2377 ± 100	2241 ± 100	0.382
Total PUFA n-3	1241 ± 46	1331 ± 54	1259 ± 48	0.301
Total PUFA n-6	1034 ± 1	1046 ± 46	982 ± 52	0.363
PUFA n-3/n-6	1.2 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	0.103
EPA + DHA	849 ± 29	902 ± 51	859 ± 37	0.465
AI	0.34 ± 0.00	0.35 ± 0.00	0.37 ± 0.01	0.060
TI	0.32 ± 0.00	0.31 ± 0.00	0.32 ± 0.01	0.313

Values are means ± standard deviation ( $n=3$ ; 4 individual fillets per replicate tank). Despite not shown in this summarised format, all identified fatty acids were considered in the composite fractions.

<sup>1</sup> SFA: Saturated fatty acids.

<sup>2</sup> MUFA: Monounsaturated fatty acids.

<sup>3</sup> PUFA: Polyunsaturated fatty acids.

<sup>4</sup> AI:  $(C12:0 + (4 \times C14:0) + C16:0) / (Total\ MUFA + Total\ n-3 + Total\ n-6)$

<sup>5</sup> TI:  $(C14:0 + C16:0 + C18:0) / ((0.5 \times Total\ MUFA) + (0.5 \times Total\ n-6) + (3 \times Total\ n-3) + ratio\ n-3/n-6)$ .

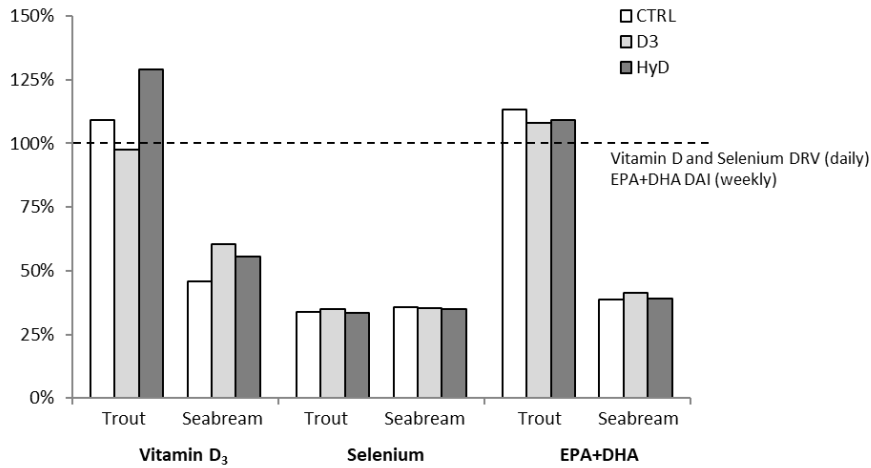
The nutritional contribution (NC) of trout and seabream fillets to cover the DRV of selected minerals, vitamins A and D<sub>3</sub> and the DAI of combined EPA and DHA in adults' female and male are presented in Table 4.4 and Figure 4.1. In both species, the nutritional contribution on Fe, Zn, Mg and vitamin A is lower than 17% of DRV. In trout the iodine represented 2.5% of DRV, while in seabream it contributed to 20% of DRV. Fillets from both species, contributed to 21-27% and 35% of DRV for potassium and selenium, respectively. Trout fillets have a high nutritional contribution 98-129% of DRV for vitamin D<sub>3</sub>. In comparison to the CTRL treatment, trout fed the HyD diet showed an increase (18%) on the nutritional contribution to vitamin D<sub>3</sub> DRV. While fillets from seabream fed the CTRL diet contributed to 46% of DRV of vitamin D<sub>3</sub>, those fed the D3 and HyD diets represented 60 and 55%, respectively. On a relative basis, this variation represented a 31% (D3) and 21% (HyD) increase of the nutritional contribution of fortified seabream fillets.

**Table 4.4** Nutritional contribution (%DRV) of raw trout and seabream fillets.

% DRV <sup>1,2</sup>	CTRL		D3		HyD	
	Female	Male	Female	Male	Female	Male
<b>Rainbow trout</b>						
Vitamin D <sub>3</sub>	109.3		97.6		129.1	
Vitamin A	3.4	3.0	3.3	2.9	3.5	3.0
EPA + DHA (1 portion/week)	113.3		108.0		109.2	
Protein	55.3	46.4	55.3	46.4	55.0	46.2
Iodine	2.5		2.7		2.7	
Selenium	33.8		35.0		33.6	
Potassium	22.2		21.7		21.5	
Iron	4.7	5.8	4.8	5.9	4.7	5.8
Zinc	5.1	4.0	6.1	4.8	5.5	4.3
Magnesium	15.9	13.6	15.8	13.5	15.2	13.0
<b>Gilthead seabream</b>						
Vitamin D <sub>3</sub>	45.9		60.3		55.5	
Vitamin A	1.4	1.2	1.4	1.2	1.5	1.3
EPA + DHA (2 portions/week)	77.6		82.5		78.5	
Protein	53.1	44.5	53.2	44.6	52.8	44.3
Iodine	19.0		19.1		18.8	
Selenium	35.9		35.4		35.1	
Potassium	26.4		25.7		27.0	
Iron	4.8	5.9	4.9	6.0	5.0	6.1
Zinc	6.5	5.1	8.3	6.5	8.3	6.5
Magnesium	16.5	14.2	16.8	14.4	16.7	14.4

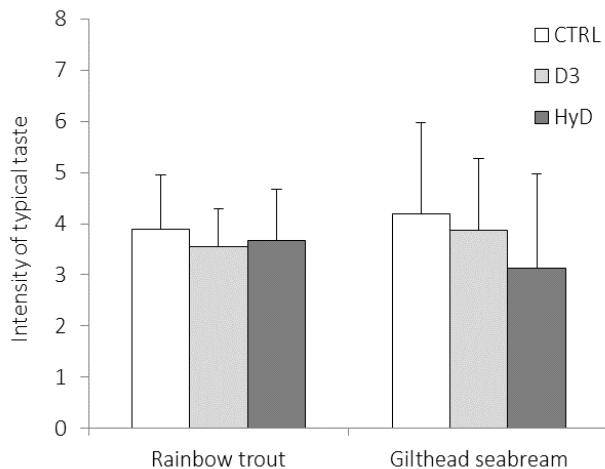
<sup>1</sup> For definition of DRV calculations please refer to section 2.4.

<sup>2</sup> DRV calculations considered gender differences on recommendations.



**Figure 4.1.** Nutritional contribution (%) of raw fillets to the daily Dietary Reference Values (DRV) of vitamin D<sub>3</sub> and selenium; and the weekly Dietary Adequate Intake (DAI) of EPA+DHA.

Typical taste of steamed cooked trout and seabream fillets was not affected by the dietary supplementation with cholecalciferol and 25-hydroxyvitamin D ( $P > 0.05$ ) (Figure 4.2). Sensory panel rated typical taste as light to moderate (ranging between 3.6 and 3.9 in trout and 3.1 and 4.2 in seabream). Overall non-typical taste was classified as absent to light in both species.



**Figure 4.2.** Sensory assessment by a trained panel of typical taste of trout and seabream on an 8-point intensity scale ranging from absence (0); light (2); moderate (4); intense (6); to highly intense (8)

## Discussion

Vitamin D deficiency is not only associated with malnutrition in developing countries, but also intrinsically linked to modern lifestyles with changed dietary patterns, lower diversity of food items and increase dependency of convenience and processed foods with low micronutrients density, in industrialized countries (Fulgoni et al., 2011; Mensink et al., 2013; Cashman and Kiely, 2014; Troesch et al., 2012). Farmed fish are undoubtedly a good option for vitamin D biofortification, since already considered part of a healthy diet and its consumption may significantly contribute to cover daily needs of n-3 LCPUFA, vitamin A and D, iodine and selenium with benefits on improved nutritional status and disease prevention (EFSA, 2014a).

### Efficacy of biofortification strategy in fish

Aquaculture feeds target mainly an optimal fish growth and welfare status, without valuing its potential as a tool to bring additional benefits to consumers' health. It is recognized that current feeds do not make full use of micronutrients at its maximum allowed levels (EFSA, 2005). Moreover, the current trend in aquafeeds for replacing marine-derived ingredients (fish meal and fish oil) by vegetable protein and oil sources can interfere with the fish nutritional profile and consequently change consumer expected intakes of health valuable nutrients, such as n-3 LCPUFAs and vitamin D<sub>3</sub>. In our study, raising dietary vitamin D<sub>3</sub> from a basal level of 42  $\mu\text{g}\cdot\text{kg}^{-1}$  and 31  $\mu\text{g}\cdot\text{kg}^{-1}$  (trout and seabream control diets, respectively) to the maximum permitted level of 75  $\mu\text{g}\cdot\text{kg}^{-1}$  (D<sub>3</sub> and HyD diets) did not significantly enhance fillet vitamin D<sub>3</sub> levels, which ranged 92-121  $\mu\text{g}\cdot\text{kg}^{-1}$  in trout fillets and 43-57  $\mu\text{g}\cdot\text{kg}^{-1}$  in seabream fillets. The large range observed is in accordance with results from several studies that evidence a large variation on the vitamin D<sub>3</sub> fillet concentrations for the same fish species (Mattila et al., 1995; Lu et al., 2007; Schmid and Walther, 2013), even when fed an identical diet (Mattila et al., 1999). Although being one of the more relevant natural sources of vitamin D<sub>3</sub>, data on the efficacy of dietary vitamin D<sub>3</sub> biofortification in fish is extremely scarce. Atlantic salmon fed over 11 weeks with graded dietary levels of vitamin D<sub>3</sub> (40, 2210 and 28680  $\mu\text{g}\cdot\text{kg}^{-1}$  feed) showed a positive correlation between the content of vitamin D<sub>3</sub>

found in tissues and dietary levels (Horvli et al., 1998). On the other hand, rainbow trout fed over four months with three dietary vitamin D<sub>3</sub> levels (89, 174 and 539  $\mu\text{g}\cdot\text{kg}^{-1}$  feed) did not show a positive relationship between muscle cholecalciferol content and dietary vitamin D<sub>3</sub> dose (Mattila et al., 1999). Literature data suggests that increased concentrations of vitamin D<sub>3</sub> in salmon muscle were only positively correlated with a feed fortification strategy, when dietary levels ranged from 32 to 800 times higher than the ones used in present study, which targeted the maximum permitted level of 75  $\mu\text{g}\cdot\text{kg}^{-1}$  in aquaculture feeds (Graff et al., 2002; Horvli et al., 1998). When dietary concentrations were only two to seven times higher it was not possible to observe a significant increase on trout and salmon muscle vitamin D<sub>3</sub> content (Graff et al., 2002; Horvli et al., 1998; Mattila et al., 1999). Absolute values of muscle vitamin D<sub>3</sub> content in trout were double of those found in seabream. Being a liposoluble vitamin, the higher dietary fat levels as well as the increased fat content in trout muscle compared to seabream may condition the magnitude of vitamin D<sub>3</sub> deposition in muscle. Previous studies with fish did not find a significant correlation between body fat content and muscle vitamin D<sub>3</sub> contents (Mattila et al., 1995; Mattila et al., 1997; Mattila et al., 1999; Schmid and Walther, 2013). However, in humans there are evidences that the presence of fat in a meal taken together with vitamin D<sub>3</sub> supplement, significantly enhances absorption of the vitamin D<sub>3</sub> (Dawson-Hughes et al., 2015). Given that vitamin D<sub>3</sub> plays a role in mineral metabolism, we have also assessed the effects of dietary vitamin D<sub>3</sub> biofortification on the muscle content of some nutritional relevant minerals. Our data showed that increasing supplementation level of vitamin D<sub>3</sub> up to 75  $\mu\text{g}\cdot\text{kg}^{-1}$ , in both trout and seabream feeds, did not affect muscle contents in iodine, selenium, iron, zinc, potassium, magnesium. However, in a previous study with juvenile catfish, a significant increase of whole-body sodium, potassium, magnesium, zinc, phosphorous and calcium retention was observed when vitamin D<sub>3</sub> dietary level was increased from 50 to 100  $\mu\text{g}\cdot\text{kg}^{-1}$  (Zhu et al., 2015).

### Nutritional contribution

Our data show that raising dietary levels of vitamin D<sub>3</sub> could improve trout and seabream fillets nutritional contribution, evaluated according to the established recommended values

for adults. A meal portion of 160 g of trout fillets showed to have a high nutritional contribution for vitamin D<sub>3</sub>, varying between 98-129% of DRV. The same 160 g of fillets from seabream fed the CTRL diet contributed to 46% of vitamin D<sub>3</sub> DRV, while those fed the D<sub>3</sub> and HyD diets represented 60 and 55%, respectively. Vitamin D<sub>3</sub> supplementation did not affect vitamin A and minerals muscle content, however it is noteworthy the nutritional contribution of several nutrients presents in the studied species. Regarding iodine levels, 160 g of seabream fillet may contribute to 20% of iodine daily adequate intake (DAI) while in trout iodine only represents 2.5% of DAI. Additionally, fillets from both species, may contribute to 21-27% and 35% of DRV for potassium and selenium, respectively. The nutritional contribution of Fe, Zn, Mg and vitamin A was minor and lower than 17% of DRV with both species. Moreover, the observed high contents in n-3 LCPUFA, particularly EPA and DHA could also make a significant contribution to cover their recommended intake in the primary prevention of cardiovascular diseases. Because trout is fattier than seabream one single meal of 160g would cover between 108-113% of the weekly recommendation of combined EPA+DHA, while same meal portion of seabream fillet would contribute to 40% of the same recommendation. Additionally, thrombogenic index was found similar between species while atherogenic index was lower in seabream compared with trout fillets.

Calculation of nutritional contribution were expressed using a 160g meal portion, though for labelling purposes it should be expressed in a 100g basis. According to EC 1924/2006 regulation, in the European market, a food item can be labelled as “source of” if it supplies 15% of the DRV in 100g of product and “high in” in case of containing at least twice this value (30% of DRV). Although not affected by the feed fortification strategy, trout fillets fed the various experimental diets showed high vitamin D<sub>3</sub> levels that provided between 61 and 81% of DRV in 100 g of fillet and could therefore potentially be labelled with the nutritional claim “high in vitamin D<sub>3</sub>” in Europe (EFSA, 2010). Likewise, an equivalent labelling could be applied to seabream supplemented fillets (HyD and D<sub>3</sub>) as they provide between 35% and 38% of DRV in 100 g of food item (corresponding to 52 and 57 µg·kg<sup>-1</sup>, respectively). Furthermore, under the same definition, the high selenium content determined in trout and seabream

fillets ranging from 0.147 to 0.157 mg·kg<sup>-1</sup>, could justify the nutritional claim “source of selenium” due to its nutritional contribution of about 21-22% DRV. Altogether these data support the rich nutritional value of fish from aquaculture and its contribution to cover several nutrients requirements within a balanced dietary pattern.

### Sensory evaluation

Despite actual consumer request for healthy food they are also aware about the natural sensory properties of each food item. Thus, the nutritional improvement of food should avoid negative effects on the sensory traits. Moreover, studies on consumers’ preferences refer the importance of the food carrier on the compliance and acceptance of fortified food items (Grunert, 2010; Siró et al., 2008). It was shown that an already present nutrient will be easily accepted in a fortified food than other ingredients that can be related to odour and taste modifications (Siró et al., 2008). A trained seafood panel found no effects of dietary vitamin D<sub>3</sub> supplementation on the typical taste of steamed cooked trout and seabream fillets. Information on sensorial traits of fish products related to vitamin D<sub>3</sub> fortification is scarce. Studies performed on other animal species found no differences on flavour, juiciness and tenderness in pork meat (Wilborn et al., 2004; Swigert et al., 2004), or chicken eggs sensory properties (Mattila et al., 2003; Yao et al., 2013) after feed supplementation with high levels of vitamin D<sub>3</sub>.

### Conclusions

An increase of vitamin D<sub>3</sub> level in fish feeds, within the maximum authorized limit in the EU market of 75 µg·kg<sup>-1</sup>, enhanced the contribution of rainbow trout and gilthead seabream fillets to the Dietary Reference Value, without affecting fish growth or sensory traits. Moreover, the fish fillets showed to have high nutritional value with significant levels of n-3 LCPUFA (EPA and DHA), selenium and iodine which may significantly contribute to cover nutrients requirement and improve consumers’ nutritional status. The biofortification of farmed fish is an efficient strategy to enhance the vitamin D<sub>3</sub> intake in general populations, adding to the already implemented fortified food items.

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# FORTIFICATION WITH MICROALGAE

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## Chapter 5



# *Phaedactylum tricornutum* in finishing diets for gilthead seabream: effects on skin pigmentation, sensory properties and nutritional value

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## Chapter 5.1

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## *Phaedactylum tricornutum* in finishing diets for gilthead seabream: effects on skin pigmentation, sensory properties and nutritional value

### Abstract

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Microalgae biomasses are known to play a major role in fish pigmentation, which is particularly important in farmed fish, since colour and external appearance are consumers' first cue when choosing seafood. A study was undertaken to assess the potential of microalgae biomass from *Phaedactylum tricornutum* as a functional ingredient for gilthead seabream (*Sparus aurata*) feeds. Three experimental diets were designed: a control diet (CTRL); this same diet supplemented with 2.5% of *P. tricornutum* wild strain (diet MA20); and a third diet with 2.5% of *P. tricornutum* biomass (diet MA37) cultivated under different temperature and light regimes that resulted in higher levels of fucoxanthin. Microalgae diets led to a reduction ( $P < 0.05$  in MA37) of whole-body fat and lower lipid retention ( $P < 0.05$  in MA20 and MA37). Microalgae did not impact odour, flavour, whiteness and fatness perception in cooked fillets. Overall, colour analysis showed that *P. tricornutum* biomass originated significant differences compared to control in specific areas: the MA37 diet induced a significantly ( $P < 0.05$ ) lighter and more vivid yellow coloration of seabream operculum ( $\Delta E^* \approx 5$ ) perceptible to the human eye; ventral skin lightness was also affected by the dietary treatments ( $P = 0.040$ ), being higher for microalgae-fed groups, though this difference was not perceptually strong ( $\Delta E^* \approx 1.7$ ). *P. tricornutum* biomass can be used as a functional ingredient, improving external pigmentation and thus contributing to meet consumer expectations in relation to farmed gilthead seabream.

Keywords: Gilthead seabream; Microalgae; *Phaedactylum tricornutum*; Skin pigmentation; Quality.

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## Introduction

Being a rich source of important nutrients, including highly digestible proteins, vitamins (A, D, niacin and B12), trace minerals (iodine, selenium) and n-3 polyunsaturated fatty acids (PUFA), fish is considered to be a healthy dietary choice (Kris-Etherton et al., 2002; Larsen et al., 2011; Lund, 2013; EFSA, 2015d). In fish markets where fish is commercialised whole, visual cues such as body shape and skin pigmentation patterns are considered important consumer purchasing criteria (Vasconcellos et al., 2013; Colihueque and Araneda, 2014).

Gilthead seabream (*Sparus aurata*) is the major farmed marine fish species in the Mediterranean region (FEAP, 2015). Wild specimens show a golden coloration of the forefront, a reddish operculum and a yellow colored lateral band, while farmed seabream registered thicker skin, which is darker in the dorsal and head areas, and the characteristic iridescent colours are much duller (Grigorakis et al., 2002; Rogdakis et al., 2011; Šimat et al., 2012). Like observed for seabream, external appearance can also vary between farmed and wild European seabass (Grigorakis, 2007; Arechavala-Lopez et al., 2013). Previous studies have shown that rearing conditions (Flos et al., 2002; Valente et al., 2011) and dietary factors (Wassef et al., 2010) may affect fish pigmentation and external appearance. Fish, like other animals, cannot synthesise carotenoids de novo and therefore depend entirely on dietary sources to achieve their natural pigmentation patterns.

Synthetic carotenoid pigments are commercially available as feed additives but increasing consumer awareness of synthetic additives has promoted interest in the use of natural carotenoid sources. Microalgae biomasses have been successfully tested and may lead to valuable ingredients for the animal feed sector (Shields and Lupatsch, 2012), as they are an excellent source of protein, vitamins, trace minerals, LCPUFAS and natural pigments (Leu and Boussiba, 2014). Microalgae carotenoids were shown to have important biological functions in various fish species (Shahidi et al., 1998), such as antioxidant properties (Pham et al. 2014; Sahin et al., 2014), acting as immune system modulators (Abdel-Tawwab and Ahmad, 2009; Cerezuela et al., 2012a, 2012b; Kim et al., 2013; Reyes-Becerril et al., 2013) and influencing

flesh and skin pigmentation (Pham et al., 2014; Sefc et al., 2014). The marine diatom, *Phaedactylum tricorutum*, is characterized by high levels of n-3 PUFAs, mainly eicosapentaenoic acid (EPA), and high contents of fucoxanthin, an orange-coloured carotenoid (Reboloso-Fuentes et al., 2001; Peng et al., 2011; Kim et al., 2012). *Phaedactylum tricorutum* has been successfully used in fish diets with beneficial effects on the seabream immune system (Cerezuela et al., 2012a). However, no literature data exists regarding the effects of *Phaedactylum tricorutum* on fish quality criteria, such as skin pigmentation and sensory traits.

The objective of this study was to evaluate the potential of two *Phaedactylum tricorutum* biomasses, differing in their fucoxanthin content, as functional ingredients in finishing diets for gilthead seabream (*Sparus aurata*). Assessment criteria comprised zootechnical growth performance and a detailed characterization of effects on the nutritional value and sensory traits of fillets and skin pigmentation.

## Material and methods

### *Experimental diets*

A control diet (CTRL) was formulated with practical ingredients to contain 45.3% crude protein, 18.6% crude fat and 22.2 MJ·kg<sup>-1</sup> gross energy (dry matter basis). Based on the CTRL formulation, two other diets (MA20 and MA37) were produced. The MA20 diet incorporated 2.5% of *Phaedactylum tricorutum* wild strain biomass, at the expenses of whole peas. The MA37 diet contained also 2.5% of *Phaedactylum tricorutum* biomass with higher levels of fucoxanthin, resulting from different temperature and light regimes used during the cultivation. The strain used was *Phaeodactylum tricorutum* UTEX 640, sourced from the University of Texas (Austin, USA). On a dry basis, the composition of the *Phaedactylum tricorutum* biomass was crude protein 34%, crude lipid 10%, ash 29% and fucoxanthin 12 mg·g<sup>-1</sup> for MA20 and 16 mg·g<sup>-1</sup> for MA37. Microalgae biomasses were produced by A4F S.A. (Lisbon, Portugal) in photobioreactors. Diets were isonitrogenous, isolipidic and isoenergetic. Ingredients were ground (below 250 µm) in a micropulverizer hammer mill (model SH1,

Hosokawa-Alpine, Augsburg, Germany). Powdered ingredients were then mixed accordingly to the targeted formulation in a double-helix mixer (model 500L, TGC Extrusion, Rouillet-Saint-Estèphe, France) to attain a basal mixture. Diets (pellet size 5.0 mm) were manufactured at SPAROS, Lda (Olhão, Portugal) by means of a twin-screw extruder (model BC45, Cleextral, Firminy, France) with a screw diameter of 55.5 mm and temperature ranging 115-120°C. Upon extrusion, extruded feeds were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, Rouillet-Saint-Estèphe, France). After cooling of the pellets, the oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, Sevenum, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature, but in a cool and aerated emplacement. Samples of each diet were taken for proximate composition analysis (Table 5.1).

**Table 5.1.** Ingredient and proximate composition of experimental diets.

Ingredients, g·kg <sup>-1</sup>	CTRL	MA20W	MA37G
Fishmeal LT 70 <sup>1</sup>	120	120	120
Fishmeal 60 <sup>2</sup>	180	180	180
Soy protein concentrate <sup>3</sup>	50	50	50
Wheat gluten <sup>4</sup>	60	60	60
Corn gluten meal <sup>5</sup>	80	80	80
Soybean meal 48 <sup>6</sup>	100	100	100
Rapeseed meal <sup>7</sup>	50	50	50
Wheat meal	70	50	50
Wheat: corn DDGS <sup>8</sup>	30	50	50
Whole peas	98	73	73
Fish oil <sup>9</sup>	105	105	105
Palm oil <sup>10</sup>	35	35	35
Vitamin & Mineral Premix <sup>11</sup>	10	10	10
Binder <sup>12</sup>	10	10	10
Antioxidant <sup>13</sup>	2	2	02
Microalgae biomass MA20 <sup>14</sup>	0	25	0
Microalgae biomass MA37 <sup>14</sup>	0	0	25
Dry matter (DM), %	96.9 ± 0.0	94.8 ± 0.1	94.6 ± 0.0
Crude protein, %DM	45.5 ± 0.3	45.4 ± 0.0	45.3 ± 0.2
Crude fat, % DM	18.6 ± 0.1	18.5 ± 0.2	18.6 ± 0.2
Ash, %DM	9.9 ± 0.2	10.9 ± 0.1	10.7 ± 0.2
Total phosphorus, % DM	1.1 ± 0.0	1.2 ± 0.1	1.2 ± 0.1
Gross energy, kJ·g <sup>-1</sup> DM	22.1 ± 0.0	22.4 ± 0.0	22.4 ± 0.0

<sup>1</sup> Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru. <sup>2</sup> Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal. <sup>3</sup> Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands; <sup>4</sup> VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France; <sup>5</sup> Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; <sup>6</sup> Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal; <sup>7</sup> Defatted rapeseed meal: 34% CP, 2% CF,

SORGAL SA, Portugal; <sup>8</sup> Wheat: Corn (80:20) dry distillers' grains with solubles: 33% CP, 2.5% CF, AB Agri, England. <sup>9</sup> COPPENS International, The Netherlands; <sup>10</sup> Crude palm oil: Gustav Heess GmbH, Germany; <sup>11</sup> Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate, 7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings. <sup>12</sup> Kielseguhr (natural zeolite): LIGRANA GmbH, Germany; <sup>13</sup> Paramega PX, Kemin Europe NV, Belgium. <sup>14</sup> Dry biomass of *Phaedactylum tricornutum*: 34% CP, 10% CF (MA20 with 12 mg·g<sup>-1</sup> fucoxanthin and MA37 with 16 mg·g<sup>-1</sup> fucoxanthin), A4F S.A., Portugal.

### **Growth trial and sampling**

The trial was conducted at the Experimental Research Station of CCMAR (Faro, Portugal). Experiments were directed by trained scientists (following category C FELASA recommendations) and in compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei nº. 113/2013, de 7 de Agosto) legislation on the protection of animals for scientific purposes. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Each diet was tested in duplicate groups of 30 seabream with a mean initial body weight of 233 g stocked in 1000 L circular plastic tanks, for 84 days. Fish were fed to apparent satiety, by hand, twice daily during week days, once a day in Saturdays and unfed on Sundays. Excess feeding was minimized, and feed intake was quantified throughout the trial. Rearing tanks were supplied with flow-through gravel-filtered, aerated seawater (salinity: 34 psu; temperature: 19-27°C; oxygen content of outlet water maintained higher than 5 mg·L<sup>-1</sup>) and subjected to natural photoperiod changes through Summer-Autumn conditions (early August till end-October). At the end of the trial, fish were slaughtered by immersion in ice-saltwater slurry (4:1) until death. All samplings were done within the 24 hours following the last meal. At the beginning of the trial, six fish from the initial stock and three fish per tank at the end of the trial were sampled for analysis of whole-body composition. For analysis of quality criteria, after slaughter, fish were packed in insulated polystyrene boxes, with the ventral side upward, covered with plastic and flaked ice and immediately transported to the

laboratory. Fifteen fish from each treatment were weighed, scaled and filleted 24 h after death. Left and right fillets (with skin) were separately packed in low density polypropylene bags (15.2 x 33.0 cm) and kept at 4°C until sensory assessment by a trained panel.

### *Biochemical analytical methods*

Proximate composition analysis of the diets, whole fish and fillets was made by the following procedures: dry matter by drying at 105°C for 24 h; ash by incineration of dry sample in a furnace at 550°C for 12 h; crude protein (N×6.25) by a combustion technique (at 850 °C) followed by thermal conductivity detection of nitrogen an using LECO FP 528 analyser; crude fat after dichloromethane extraction by the Soxhlet method and gross energy in an adiabatic bomb calorimeter (IKA). Macro minerals (S, Cl, K and Ca), trace minerals (Fe, Cu, Zn, Br and As) and metallic elements (Rb) were measured using spectroscopy X-ray energy dispersive (EDXRF) methodology according to Carvalho et al. (2005). Total lipids in the fillets were extracted according to the method of Folch et al. (1957) and subsequently, the fatty acid composition of fillets was determined by gas-chromatography analysis of methyl esters, according to the procedure of Lepage and Roy (1986), modified by Cohen et al. (1988) and described in detail by Costa et al. (2013). Lipid oxidation in fish fillets was assessed at the time of slaughter (T0) and after 25 weeks of frozen storage at -20°C, using the Polyene Index (PI) calculated as the fatty acid ratio: (EPA + DHA)/C16:0. The nutritional contribution (NC) of steam cooked seabream fillets was calculated as the percentage of the Daily Adequate Intake (DAI) for combined EPA and docosahexaenoic acid (DHA), according to the following formula:

$$NC(\%) = 100 \times \frac{C \times M}{DRI \text{ or } DAI}$$

Where:

C: EPA+DHA content (mg·kg<sup>-1</sup>)

M: typical meal portion consumed (0.160 kg, assuming a 40% fillet yield in a commercial size seabream of 0.400 kg).

DAI considered for EPA+DHA was 500 mg·d<sup>-1</sup> for primary prevention of cardiovascular disease in adults (International Society for the Study of Fatty Acids and Lipids [ISSFAL], 2004).

### *Pigmentation*

Skin colour was measured with a tristimulus colorimeter (Macbeth Color-Eye 3000) and the L\*, a\* and b\* coordinates from CIELab system were recorded. Eleven random fish from each treatment were used for this procedure. Colour was determined in triplicate measures in several predefined zones in the fish: the interorbital band (left, medium and right areas), operculum, skin [dorsal, dorsal, intermedium (close to lateral line) and ventral areas] and muscle (dorsal, medium and ventral, corresponding to the skin areas). From a\* and b\* coordinates, chroma (C\*) and hue (H<sup>0</sup>) parameters were calculated according to Schubring (2009). To estimate perceptible colour differences ( $\Delta E^*$ ) among dietary treatments the CIE 76 formula (based on the Euclidian distances between colours in CIELab space) was applied:

$$\Delta E^* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

For each evaluated zone, average values of colour coordinates were used for  $\Delta E^*$  calculation (CTRL vs MA20; CTRL vs MA37 and MA20 vs MA37). Following the definition of the CIELab (1976) colour space and Mahy et al. (1994), distances between colours were considered as being indicative of either an “irrelevant perceptual difference” ( $\Delta E^* < 1$ ), a “slight perceptual difference” ( $1 < \Delta E^* < 2.3$ ) or a “clear perceptual difference” ( $\Delta E^* > 2.3$ ).

### *Sensory Evaluation*

A sensory evaluation was carried out in an acclimatized test room equipped with individual booths. The sensory panel was composed by four trained panellists (non-smokers, 50% men, age within 40 – 57 years old) from the Portuguese Institute of Sea and Atmosphere, specifically trained in descriptive methods for sensory assessment of wild and farmed fish, according to the guidelines described in Meilgaard et al. (1999) and Martinsdóttir et al. (2009). To reduce the variability within the fillets, the parts close to the head and the tail were rejected. Each fillet was individually wrapped with aluminium foil to avoid odour losses and cooked for 10 min at 100 °C in a saturated steam oven (Rational Combi-Master CM6, Cross Kuchentechnik GmbH, Landsberg, Germany). Eight cooked fillets from each treatment

were assessed in two independent sensory sessions. In each session four fillets per treatment were presented to the panellists sequentially in coded white dishes under normal white lighting (each panellist assessed three fillets, one per treatment). The panellists rated the intensity of sensory attributes on an unstructured line scale (Meilgaard et al., 1999) ranged from 0 cm (absence of attribute) to 12 cm (extremely intense). Results were expressed as the distance (in cm) of each evaluated attribute: odour (typical and atypical), flavour (typical and atypical), white colour and fatness.

### *Statistical analysis*

Except for hue values, data were expressed as means  $\pm$  standard deviation. All data were subjected to one-way analysis of variance. For ANOVA analysis, parameters expressed as percentages were subjected to arcsine square root transformation. Following ANOVA, means were compared by the Tukey HSD multiple range test. Given that hue is an angular measure, data were treated by a one-way circular ANOVA and group comparison was done by the Watson-Williams test. Statistical significance was tested at 0.05 probability level. In order to assess the possibility of dose-dependent effects, linear models relating "fucoxanthin levels" with the colour variables affected by the experimental factors were fitted by least-squares regression. The hypotheses that fucoxanthin levels have an effect different from zero were assessed via F-test ( $P < 0.05$ ). All colour data were also subjected to a correlation analysis (for quality control purposes) and a Principal Component Analysis (with no scaling of variables) prior to the ANOVA. Given that the CIELab colour space is considered approximately perceptually uniform, the lack of variable scaling is required to ensure that the perceptual difference between colours (i.e. the untransformed Euclidian distance in CIELab space) is preserved by the PCA analysis. Statistical analysis was performed using the SPSS (v22, IBM, USA) and R (v3.2.2) statistical software.

### **Results**

At the end of the trial, fish reached a final body weight (FBW) ranging from 413 to 416 g (Table 5.2). Specific growth rate (SGR) varied between 0.68 and 0.69 %·day<sup>-1</sup>, while feed

conversion ratio (FCR) ranged from 1.69 to 1.74. Overall growth performance criteria (FBW, SGR, FCR, feed intake and protein efficiency ratio) were not affected ( $P>0.05$ ) by dietary treatments.

**Table 5.2.** Growth performance, whole-body composition and nutrient retention.

		CTRL	MA20	MA37	P-Value
IBW <sup>1</sup> , g		233 ± 2	234 ± 0	233.0 ± 1	
FBW <sup>2</sup> , g		415 ± 3	413 ± 6	416.0 ± 9	0.907
VFI <sup>3</sup>		1.13 ± 0.02	1.15 ± 0.00	1.13 ± 0.02	0.534
SGR <sup>4</sup>		0.69 ± 0.02	0.68 ± 0.02	0.69 ± 0.02	0.773
FCR <sup>5</sup>		1.69 ± 0.02	1.74 ± 0.05	1.69 ± 0.08	0.552
PER <sup>6</sup>		1.35 ± 0.01	1.33 ± 0.04	1.39 ± 0.07	0.532
<b>Body composition</b>					
	<b>Initial</b>				
Moisture, % ww	63.9	62.8 ± 0.3 <sup>a</sup>	62.5 ± 0.1 <sup>a</sup>	63.5 ± 0.2 <sup>b</sup>	0.038
Protein, % ww	18.1	17.8 ± 0.5	18.4 ± 0.0	18.5 ± 0.5	0.278
Fat, % ww	13.4	15.4 ± 0.2 <sup>b</sup>	13.9 0.3 <sup>ab</sup>	13.0 ± 0.6 <sup>a</sup>	0.020
Ash, % ww	4.5	3.9 ± 0.3 <sup>a</sup>	5.0 ± 0.2 <sup>b</sup>	4.7 ± 0.1 <sup>ab</sup>	0.029
Phosphorus, % ww	0.7	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.251
Energy, kJ·g <sup>-1</sup> ww	9.1	9.5 ± 0.1 <sup>b</sup>	9.4 ± 0.0 <sup>a</sup>	9.2 ± 0.1 <sup>a</sup>	0.028
<b>Retention<sup>7</sup></b>					
Protein, % intake		23.4 ± 1.2	25.0 ± 0.7	26.3 ± 2.6	0.365
Fat, % intake		59.3 ± 2.0 <sup>b</sup>	46.6 ± 3.1 <sup>a</sup>	42.0 ± 2.5 <sup>a</sup>	0.014
Energy, % intake		27.6 ± 0.1	26.7 ± 0.7	26.1 ± 0.7	0.173

Values are means ± standard deviation (n=2). Different superscripts within a row represent significant differences between treatments ( $P<0.05$ ).

<sup>1</sup> Initial mean body weight.

<sup>2</sup> Final mean body weight.

<sup>3</sup> Voluntary feed intake: crude feed intake / (IBW+FBW)/2 / 84 days.

<sup>4</sup> Specific growth rate: (Ln FBW – Ln IBW) x 100 / 84 days.

<sup>5</sup> Feed conversion ratio: dry feed intake / wet weight gain.

<sup>6</sup> Protein efficiency ratio: wet weight gain / crude protein intake

<sup>7</sup> Retention: 100 x (FBW x final carcass nutrient – IBW x initial carcass nutrient) / nutrient intake.

Dietary treatments did not affect ( $P>0.05$ ) the whole-body protein and phosphorus contents. In contrast, whole-body moisture, fat, ash and energy varied between treatment groups (Table 5.2). In comparison to the CTRL treatment, seabream fed both microalgae-rich diets showed an increase of whole-body ash content, significant only for MA20 ( $P=0.029$ ) and a reduction of whole-body fat, yet only significant for MA 37 ( $P=0.020$ ). Seabream fed diet MA37 presented significantly higher levels of whole-body moisture ( $P=0.038$ ) and lower

energy content ( $P=0.028$ ) in comparison with CTRL and MA20 groups. Data on weight gain, feed intake and whole-body composition of fish allowed the estimation of nutrients and energy retention. Protein and energy retention were not affected ( $P>0.05$ ) by dietary treatments. However, the incorporation of microalgae (diets MA20 and MA37) led to a significant reduction ( $P=0.014$ ) of fat retention in comparison with CTRL treatment (Table 5.2). The biochemical composition of seabream fillets is presented in Table 5.3. Dietary treatments had no effect ( $P>0.05$ ) on the protein, lipid and ash content of fish fillets. Similarly, the content of minerals (S, Cl and K), trace elements (S, Cl, K, Fe, Cu, Zn, Br, As) and metal (Rb) were not affected by dietary treatments ( $P>0.05$ ). In comparison to CTRL fish, those fed the MA37 diet showed a significant reduction ( $P<0.05$ ) of muscle calcium (Ca) content.

**Table 5.3.** Proximate composition and mineral contents of seabream fillets.

	CTRL	MA20	MA37	P-Value
Protein, % ww	20.7 ± 0.6	20.1 ± 1.2	20.4 ± 0.8	0.760
Lipids, % ww	6.7 ± 1.2	7.6 ± 1.6	8.3 ± 0.6	0.168
Ash, % ww	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.153
S, g·kg <sup>-1</sup>	1.63 ± 0.23	1.56 ± 0.22	1.61 ± 0.25	0.878
Cl, g·kg <sup>-1</sup>	0.42 ± 0.03	0.42 ± 0.08	0.43 ± 0.06	0.961
K, g·kg <sup>-1</sup>	3.64 ± 0.23	3.49 ± 0.27	3.46 ± 0.21	0.452
Ca, g·kg <sup>-1</sup>	0.13 ± 0.03 <sup>b</sup>	0.10 ± 0.02 <sup>ab</sup>	0.09 ± 0.02 <sup>a</sup>	0.039
Fe, mg·kg <sup>-1</sup>	3.44 ± 0.76	3.18 ± 0.55	3.89 ± 0.88	0.346
Cu, mg·kg <sup>-1</sup>	1.10 ± 0.06	1.08 ± 0.06	1.13 ± 0.08	0.476
Zn, mg·kg <sup>-1</sup>	4.02 ± 0.19	3.94 ± 0.36	3.97 ± 0.30	0.908
As, mg·kg <sup>-1</sup>	3.00 ± 0.08	3.06 ± 0.26	3.09 ± 0.26	0.802
Br, mg·kg <sup>-1</sup>	3.10 ± 0.14	3.36 ± 0.36	3.21 ± 0.29	0.382
Rb, mg·kg <sup>-1</sup>	0.60 ± 0.08	0.71 ± 0.06	0.68 ± 0.04	0.059

Values are means ± standard deviation (n=5). Different superscripts within a row represent significant differences between treatments ( $P<0.05$ ).

The summarised fatty acid composition of seabream fillets is presented in Table 5.4. Dietary incorporation of microalgae did not affect the muscle profile in saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Consequently, total levels of n-3 and n-6 fatty acids, its ratio and both thrombogenic (TI) and atherogenic (AI) indexes were also not affected by dietary treatments ( $P>0.05$ ). Lipid oxidation expressed as the

polyene index (PI) showed no differences ( $P>0.05$ ) between experimental groups, at time of slaughter or after 25 weeks of frozen storage at  $-20^{\circ}\text{C}$ .

**Table 5.4.** Summarised fatty acid content of raw fillets and lipid oxidation.

Fatty acids <sup>1</sup> (g·100g <sup>-1</sup> )	CTRL	MA20	MA37	P-Value
Total SFA <sup>2</sup>	1.76 ± 0.33	1.93 ± 0.48	2.14 ± 0.28	0.299
Total MUFA <sup>3</sup>	1.97 ± 0.41	2.29 ± 0.53	2.46 ± 0.21	0.179
C18:2n-6	0.46 ± 0.10	0.51 ± 0.12	0.57 ± 0.05	0.201
C18:3n-3	0.07 ± 0.01	0.07 ± 0.02	0.08 ± 0.01	0.280
C20:4n-6	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.222
C20:5n-3 (EPA)	0.46 ± 0.09	0.53 ± 0.11	0.55 ± 0.04	0.259
C22:5n-3	0.17 ± 0.03	0.21 ± 0.04	0.22 ± 0.02	0.078
C22:6n-3 (DHA)	0.73 ± 0.13	0.81 ± 0.12	0.85 ± 0.09	0.244
Total PUFA <sup>4</sup>	2.31 ± 0.44	2.65 ± 0.52	2.84 ± 0.18	0.156
Total PUFA n-3	1.63 ± 0.30	1.86 ± 0.34	1.97 ± 0.14	0.180
Total PUFA n-6	0.57 ± 0.12	0.65 ± 0.14	0.72 ± 0.06	0.167
EPA+DHA	1.19 ± 0.22	1.34 ± 0.22	1.41 ± 0.12	0.230
PUFA n-3/n-6	2.88 ± 0.24	2.88 ± 0.17	2.76 ± 0.24	0.604
Atherogenic Index (AI) <sup>5</sup>	0.55 ± 0.07	0.52 ± 0.05	0.55 ± 0.07	0.663
Thrombogenic Index (TI) <sup>6</sup>	0.27 ± 0.04	0.25 ± 0.02	0.27 ± 0.03	0.701
NC <sup>7</sup> of EPA+DHA, % DAI	381	429	451	
Polyene Index (PI) <sup>8</sup> T0	1.05 ± 0.17	1.08 ± 0.13	1.03 ± 0.17	0.870
Polyene Index (PI) T25	0.91 ± 0.08	0.87 ± 0.19	0.82 ± 0.09	0.682

Values are means ± standard deviation (n = 5).

<sup>1</sup> Despite not shown in this summarised format, all identified fatty acids were considered in the composite fractions.

<sup>2</sup> SFA: Saturated fatty acids.

<sup>3</sup> MUFA: Monounsaturated fatty acids.

<sup>4</sup> PUFA: polyunsaturated fatty acids.

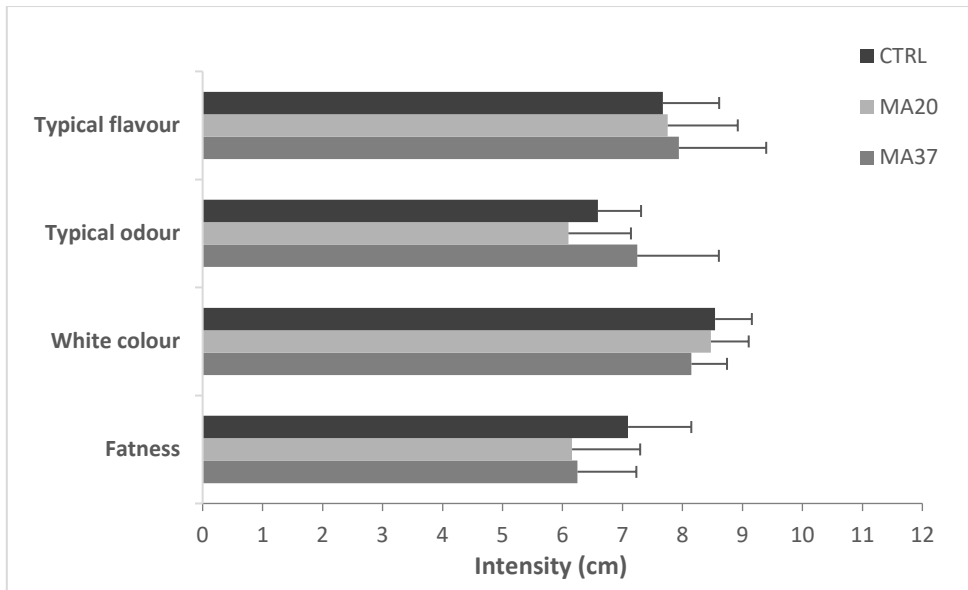
<sup>5</sup> AI:  $(\text{C12:0} + (4 \times \text{C14:0}) + \text{C16:0}) / (\text{total MUFA} + \text{total n-3} + \text{total n-6})$ .

<sup>6</sup> TI:  $(\text{C14:0} + \text{C16:0} + \text{C18:0}) / ((0.5 \times \text{total MUFA}) + (0.5 \times \text{total n-6}) + (3 \times \text{total n-3}) + \text{ratio n-3/n-6})$ .

<sup>7</sup> NC: nutritional contribution of EPA+DHA, considering a meal portion of 160g, as % of the Daily Adequate Intake (DAI) of 500 mg-d-1 for cardiovascular health in adults (ISSFAL, 2004).

<sup>8</sup> PI:  $(\text{EPA+DHA})/\text{C16:0}$ .

Sensory analysis in steam-cooked fillets found no differences ( $P<0.05$ ) between treatments concerning typical odour, typical flavour, white colour and fatness (Figure 5.1). Atypical odours and flavours were not considered relevant by the panel with mean values below 8.8% and 5.6%, respectively (data not showed), of the scale intensity.



**Figure 5.1.** Sensory scores of seabream steam-cooked fillets by a trained panel. Attributes intensity was rated in an unstructured line scale ranged from 0 (absence) to 12 cm (extremely intense). Bars represent mean values  $\pm$  standard deviation ( $n=8$ ).

The main results of colour measurements are presented in table 5.5. Considering colour variables ( $L^*$ ,  $a^*$ ,  $b^*$ ) of the 10 evaluated zones using 11 fish per group, a principal component analysis (PCA) was performed, showing a distinction between control fish and microalgae fed fish along the first two components (fig. 5.2). These two components (which separated control diet fish from microalgae diet fish) accounted for 70% of observed variance, supporting the idea that the dietary inclusion of these microalgae induced a change in fish coloration, compared to the control group. Correlation analysis (fig. 5.3) confirms the expected consistency between related measurements (e.g. all lightness measurements of the different parts of the muscle are positively correlated), suggesting no technical problems during colour measurements.

**Table 5.5.** Colour parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$ , Hue) in the skin of seabream.

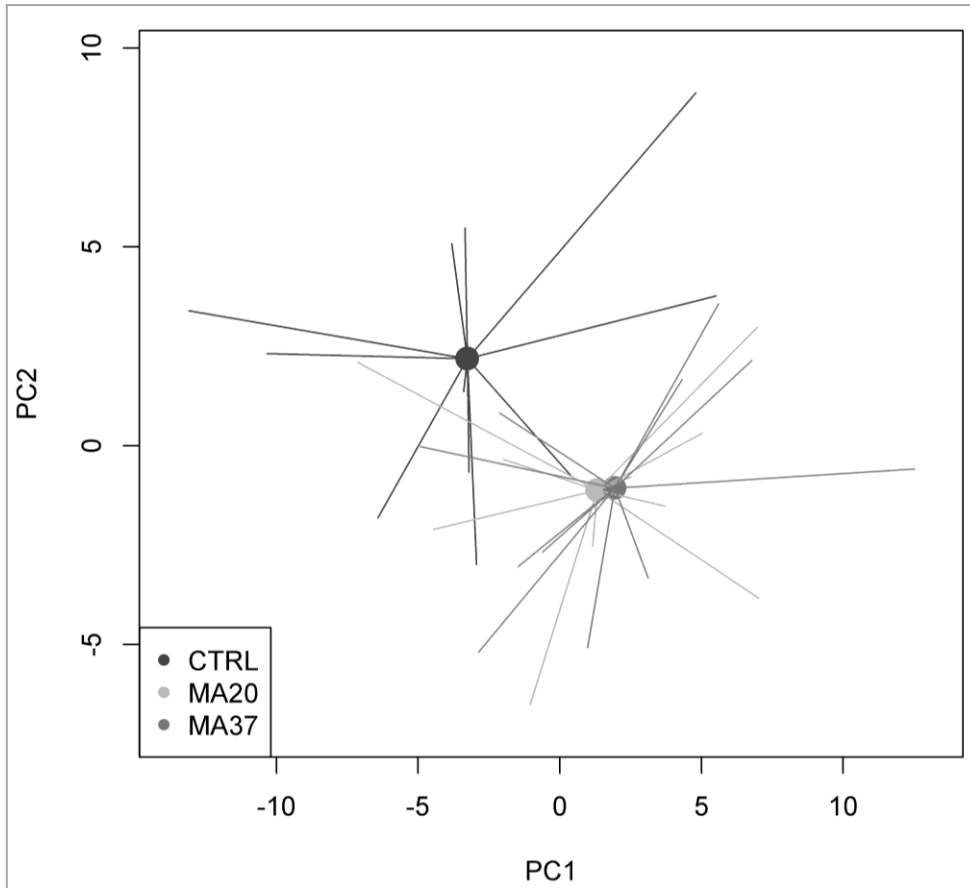
	CTRL	MA20	MA37	P-value
<b>Dorsal skin</b>				
$L^*$	64.1 ± 2.0	64.9 ± 2.0	65.4 ± 2.2	0.378
$a^*$	-1.5 ± 0.1	-1.4 ± 0.1	-1.4 ± 0.2	0.590
$b^*$	0.7 ± 0.4	0.8 ± 0.3	1.1 ± 0.6	0.152
$C^*$	1.7 ± 0.2	1.7 ± 0.2	1.9 ± 0.4	0.154
Hue	155.6 (11.4)	151.5 (11.4)	144.9 (16.0)	0.160
<b>Ventral skin</b>				
$L^*$	82.9 ± 2.3	84.4 ± 1.3	84.6 ± 1.3	0.040
$a^*$	-1.4 ± 0.1	-1.4 ± 0.1	-1.3 ± 0.2	0.200
$b^*$	1.4 ± 0.3	1.2 ± 0.6	1.7 ± 1.0	0.274
$C^*$	2.0 ± 0.2	1.9 ± 0.5	2.3 ± 0.7	0.253
Hue	135.0 (5.9)	140.5 (11.9)	132.3 (22.4)	0.403
<b>Interorbital band<sup>1</sup></b>				
$L^*$	60.8 ± 1.9	60.8 ± 2.3	61.5 ± 2.7	0.700
$a^*$	-1.7 ± 0.2	-1.5 ± 0.1	-1.5 ± 0.2	0.071
$b^*$	2.2 ± 0.9	2.1 ± 0.9	2.4 ± 0.7	0.636
$C^*$	2.8 ± 0.7	2.6 ± 0.7	2.9 ± 0.5	0.611
Hue	129.3 (11.7)	128.9 (13.2)	123.6 (8.6)	0.400
<b>Operculum</b>				
$L^*$	74.8 ± 3.7 <sup>a</sup>	79.8 ± 3.2 <sup>b</sup>	79.9 ± 2.7 <sup>b</sup>	0.001
$a^*$	0.2 ± 0.3	0.7 ± 0.7	0.8 ± 0.8	0.073
$b^*$	5.3 ± 0.9 <sup>a</sup>	6.5 ± 1.1 <sup>ab</sup>	6.8 ± 1.5 <sup>b</sup>	0.019
$C^*$	5.4 ± 1.0 <sup>a</sup>	6.5 ± 1.2 <sup>ab</sup>	6.9 ± 1.6 <sup>b</sup>	0.019
Hue	88.0 (3.0)	84.5 (5.2)	83.8 (5.21)	0.083

<sup>1</sup> Interorbital band values refer to the medium zone, left and right areas data are not shown.

Values are means ± standard deviation (n=11). For Hue values, the mean angle and the circular standard deviation are provided. Different superscripts within a row represent significant differences between treatments (P<0.05).

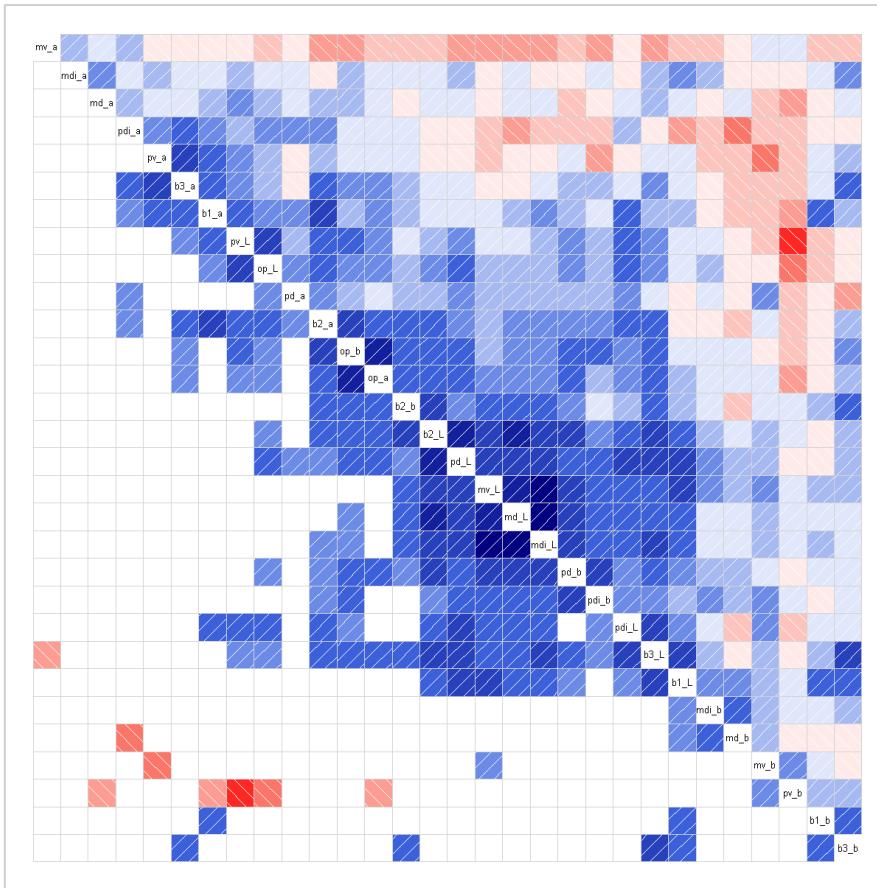
The interorbital band (left and right values not shown), dorsal intermedium skin (data not shown), dorsal skin and muscle (data not shown) were not affected by the dietary treatments (P>0.05). The skin colour measured at the operculum zone showed that fish fed the microalgae-rich diets (MA20 and MA37) had significantly higher lightness ( $L^*$ ) values than those fed the control diet (ANOVA, P=0.001). Dietary treatments had no effect on  $a^*$  values (ANOVA, P>0.05). However,  $b^*$  and  $C^*$  values were significantly higher (Tukey HSD, P=0.019 in both cases) in fish fed the MA37 diet than in those fed the control diet. The hue values

were close to 90°, confirming the predominance of a yellow coloration in the operculum. Although hue values in operculum were not significantly different between the three treatments following circular ANOVA ( $P > 0.05$ ), a trend can be perceived, since the mean hue of fish fed the MA37 diet was significantly different from that of control fish (Watson-Williams test,  $P = 0.032$ ). Additionally, fitting a linear model relating “opercular hue” and “fucoxanthin level” also supports the notion that there might be a real (F-test,  $P = 0.026$ ), though small, dose-dependent effect of microalgae inclusion on opercular hue. Overall, this study shows a clearly perceptible difference between control and microalgae groups at the operculum ( $\Delta E^* \approx 5$ ) level. Additionally, a significant effect of the dietary treatments on ventral skin lightness was also noted ( $P = 0.040$ ), showing increased values for the microalgae groups, though pairwise differences between groups (Tukey HSD) did not reach significance due to high variability in the control group. Nevertheless, fitting a linear model relating “ventral skin lightness” and “fucoxanthin level” supports the existence of a dose-dependent effect (F-test,  $P = 0.019$ ) that would explain the slight perceptual differences observed between the ventral skin colour of control and microalgae-fed groups ( $\Delta E^* \approx 1.7$ ).



**Figure 5.2.** Principal Component Analysis scores plot, showing the similarity between fish pigmentation when considering all colour measurements performed (operculum, interorbital band, dorsal, intermedium and ventral skin and muscle).

Values represent projections of the samples (i.e. fish) onto the first two principal components, connected by a line to the corresponding centroid, obtained from a PCA analysis (no scaling) of all the colour measurements ( $L^*$ ,  $a^*$  and  $b^*$ ) for all measured zones in each fish ( $n=11$  per dietary treatment). Zones considered were: operculum; interorbital band (left, medium and right); skin (dorsal, intermedium and ventral) and muscle. These two components accounted for 70% of the variance observed across the 30 variables. Samples are coloured according to the corresponding dietary treatment.



**Figure 5.3.** Correlogram plot showing the Pearson correlation between every pair of colour measurements. The upper right side shows all the correlations between variables, where dark blue: positive correlation; light colours: weak correlation; dark red: negative correlation. The down left side shows only statistically significant correlations (t-test,  $P < 0.05$ ), where dark blue: positive correlations, dark red: negative correlations and white squares are non-significant correlations.

Legend: op\_L: operculum L\*; op\_a: operculum a\*; op\_b: operculum b\*; b1\_L: interorbital band left side L\*; b1\_a: interorbital band left side a\*; b1\_b: interorbital band left side b\*; b2\_L: interorbital band medium L\*; b2\_a: interorbital band medium a\*; b2\_b: interorbital band medium b\*; b3\_L: interorbital band right side L\*; b3\_a: interorbital band right side a\*; b3\_b: interorbital band right side b\*; pd\_L: dorsal skin L\*; pd\_a: dorsal skin a\*; pd\_b: dorsal skin b\*; pdi\_L: dorsal intermedium skin L\*; pdi\_a: dorsal intermedium skin a\*; pdi\_b: dorsal intermedium skin b\*; pv\_L: ventral skin L\*; pv\_a: ventral skin a\*; pv\_b: ventral skin b\*; md\_L: dorsal muscle L\*; md\_a: dorsal muscle a\*; md\_b: dorsal muscle b\*; mdi\_L: dorsal intermedium muscle L\*; mdi\_a: dorsal intermedium muscle a\*; mdi\_b: dorsal intermedium muscle b\*; mv\_L: ventral muscle L\*; mv\_a: ventral muscle a\*; mv\_b: ventral muscle b\*

## Discussion

Alongside the well-established applications of microalgae in aquaculture hatcheries, there is currently a drive to exploit the use of algae biomasses in formulated animal feeds, both for aquaculture species and terrestrial livestock. However, high costs of algal biomass compared to commodity feedstuffs currently confine their commercial use to niche applications, in order to convey specific benefits, either nutritional or physiological, during different periods of the life cycle (Shields and Lupatsch 2012; Chauton et al., 2015). A beneficial effect on fish quality criteria is a potential application of microalgae in aquaculture feeds. Quality is a broad and dynamic concept that is also dependent on consumer's perception of a food product (Grunert, 1995). Several definitions may be drawn from the literature, but often quality is associated with intrinsic and extrinsic cues that are used by consumers to form their perception of food quality (Oude-Ophuis and Van Trijp, 1995). Intrinsic traits are linked with the food physicochemical characteristics such as nutrient content, flavour, sensory properties, food safety, among other and extrinsic traits concern the imaging, branding, packaging and cost. In the present work, several intrinsic traits, like omega-3 profile, lipid oxidation, sensory evaluation and colour, were measured to infer if a dietary supplement of *Phaeodactylum tricornutum* could improve the quality of farmed seabream.

Our data shows that 2.5% dietary supplementation of microalgae *Phaeodactylum tricornutum*, with variable levels of fucoxanthin, did not impair growth and feed efficiency of seabream. In general, the inclusion of microalgae biomass at low levels leads to no differences or even improved results when compared with commercial or control feeds. In gilthead seabream juveniles, the supplementation of 5% and 10% *Phaeodactylum tricornutum* (Cerezuela et al., 2012a) and up to 20% supplementation of *Scenedesmus almeriensis* (Viscaíno et al., 2014) showed no differences on specific growth rate with beneficial effects observed on the immune system response and intestinal function, respectively. Other studies performed in seabream showed that neither growth nor feed efficiency were affected by the dietary inclusions of 4% *Haematococcus pluvialis* (Gomes et al., 2002) and

6% of *Chlorella vulgaris* (Gouveia et al., 2002). In our study, both microalgae fed groups presented a reduction of whole-body fat content and consequently a lower dietary fat retention. This reduction of body fat content, probably occurred at the level of perivisceral fat (a preferential fat deposition site in seabream), given that no differences were found on the fillet fat content. A lipid lowering effect of microalgae-rich diets has been previously observed. Reduction of whole-body fat associated with dietary inclusion of microalgae was described in Japanese flounder (Kim et al., 2002), in common carp (Nandeesh et al. 1998; Kiron et al., 2012) and Atlantic salmon (Kiron et al., 2012). *Spirulina* supplementation was shown to increase hepatic carnitine palmitoyltransferase activity and hepatic carnitine level, inducing lipid mobilization and reducing lipid accumulation in red seabream (Nakagawa et al., 2000). Similarly, an activation of the lipid metabolism by hormonal regulation induced by dietary algae was also previously mentioned (Nematipour et al., 1987; Nematipour et al., 1990). Though some explanations have been put forth, the mechanisms underlying this effect are not completely understood. Fucoxanthin, the major carotenoid present in *P. tricornutum* biomass has been associated with lower accumulation of abdominal white adipose tissue in rodents. In these studies, fucoxanthin was linked to a depression of lipogenic enzymes activities and an increase on fatty acid oxidation (Peng et al., 2011; Ha and Kim, 2013; Maeda, 2015). Our data shows a (non-significant) trend for a higher magnitude on the whole-body lipid lowering effect with the MA37 biomass, which contained higher fucoxanthin levels, than the MA20 biomass, suggesting that the lipid lowering effect of fucoxanthin might be dose-dependent.

The nutritional value of farmed fish is largely associated with its fatty acid profile, more specifically with its content in omega-3 long chain poly unsaturated fatty acid (n-3 LCPUFAs) due to their physiological importance in human health (Kris-Etherton et al., 2009). In general, the fatty acid composition of fish fillets tends to mimic dietary composition. The microalgae *Phaedactylum tricornutum* is known to have relatively high levels of EPA (Fajardo et al., 2007). However, given the low dietary microalgae incorporation levels tested (2.5%), dietary EPA levels were similar among the various experimental diets. Consequently, microalgae-rich

diets did not affect the seabream fillet profile in saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Total levels of omega-3 (n-3) and omega-6 (n-6) fatty acids, its ratio and both thrombogenic (TI) and atherogenic (AI) indexes were also not affected by the inclusion of microalgae. In our study, raw gilthead seabream fillets showed total EPA and DHA levels ranging from 1.19 g·100 g<sup>-1</sup> in fish fed the CTRL diet, 1.34 and 1.41 g·100 g<sup>-1</sup> in those fed diets MA20 and MA37, correspondingly. The consumption of a 160 g portion of seabream fillet would represent 381, 429 and 451% (for treatments CTRL, MA20 and MA37 respectively) of combined EPA and DHA Daily Adequate Intake primary prevention of cardiovascular disease in adults (ISSFAL, 2004). Since fish is not generally consumed on a daily basis, calculations of a weekly intake seem more appropriate. Consumption of seabream fillets twice a week would cover 109-129% of the adequate EPA + DHA intake for enhanced cardiovascular health. The presence of highly unsaturated fatty acids increases the fillets' susceptibility to lipid oxidation over time. Lipid oxidation originates undesirable off-flavours and unhealthy compounds such as free radicals and reactive aldehydes, which are considered particularly unpleasant by consumers (Frankel et al., 2005). The Polyene index used to measure lipid oxidation in seabream fillets revealed no differences between treatments, at time of slaughter or after 25 weeks of frozen storage at -20°C. Microalgae antioxidant potential and its application in food preservation have been described (Rodriguez-Garcia and Guil-Guerrero, 2008). Enhanced resistance to lipid oxidation was reported in oil:water emulsions containing microalgae by Gouveia et al. (2006). The previously described antioxidant effect associated to dietary algae biomasses (Goiris et al., 2012) and in particular due to the presence of high fucoxanthin levels (Rodriguez-Garcia and Guil-Guerrero, 2008; Peng et al., 2011) was not shown to have any preventive effect of lipid oxidation during storage, in our study.

Results from sensory evaluation by a trained panel showed that *P. tricoratum* supplemented diets did not affect the organoleptic properties of steam-cooked seabream fillets. Literature refers similar results for channel catfish fed *Schizochytrium* sp. diets (Li et al., 2009), common carp fed *Spirulina* diets (Nandeeshia et al., 1998) and European seabass

fed *Isochrysis* sp. diets (Tibaldi et al., 2015). A recent study with a DHA-rich *Schizochytrium* sp. reported no effect of microalgae incorporation levels on instrumentally measured texture criteria and water holding capacity of Atlantic salmon fillets (Kousoulaki et al., 2015).

Consumer's acceptance of a food product is highly conditioned by its appearance. The influence that colour and visual image may exert on flavour perception and food acceptability is well described for several food products (Hutchings, 1999; Spence et al., 2010). Dietary carotenoids are known to exert a primary role on fish skin and muscle pigmentation, being responsible for the typical colour of many important seafood products. Though fish are unable to synthesize *de novo* carotenoids, they are capable of modifying and metabolising dietary carotenoids (Goodwin, 1984; Shahidi et al., 1998; Sefc et al., 2014). The colour of fish and shellfish products was shown to influence the market value and acceptance by consumers (Sacton, 1986; Vasconcellos et al., 2013). Carotenoid deposition is not only influenced by the fish species and carotenoid source and chemical composition, but also largely dependent on the organs and tissues considered (reviewed by Shahidi et al., 1998). There is a large body of literature describing carotenoids' metabolism, deposition and their role on skin and flesh pigmentation in salmonids (Torrissen, 1985; Storebakken et al., 1987; Bjerkgeng, 2000). Experimental studies targeting the skin pigmentation of *Sparidae* species, like red porgy or gilthead seabream, are much scarcer. In red porgy, skin pigmentation was successfully improved with the use of microalgae at a dietary inclusion of 5% *Spirulina* and 3.3% *Haematococcus* (Chatzifotis et al., 2011) and also with shrimp shell meal (Kalinowski et al., 2005). Conversely, in studies performed with gilthead seabream using dietary microalgae *Chlorella* and *H. pluvialis*, an increase in skin carotenoid deposition level was observed, although this was not reflected as an improvement in skin pigmentation (Gomes et al., 2002; Gouveia et al., 2002). Coloration is determined by the specific carotenoids used and the carotenoid composition (Bjerkgeng, 2000), and its concentration alone cannot be used as a criterion of perceived colour (Little et al., 1979). The usual dimensions of perceived colour are hue, chroma and lightness, usually instrumentally measured as L\* lightness; a\* redness and b\* yellowness (CIE, 1976). Considering colour data from all measured zones (using PCA

analysis), a clear difference was found between control and microalgae groups. However, differences found with PCA were attributed to dissimilar skin areas between groups, particularly at operculum level. For this area, higher lightness values were found in both microalgae fed groups, compared to the control treatment. The operculum hue values were close to 90°, confirming the predominance of a yellow coloration in all groups. Additionally, hue values were significantly different in fish fed the MA37 diet compared to those fed the control diet. Moreover, using diet MA37 induced a lighter and more vivid yellow coloration of seabream operculum (higher  $b^*$  values) and higher chroma ( $C^*$ ) compared to control fish, which is probably associated to the high levels of fucoxanthin, an orange-coloured pigment in MA37, since these differences were not found between CTRL and MA20 groups. On the other hand, the use of *P. tricornutum* biomass did not affect the skin pigmentation pattern in the interorbital band and dorsal zones of seabream. Using a consumer type approach to a hedonics appearance assessment, fifteen out of sixteen untrained volunteers preferred the microalgae supplemented groups, compared to the control group. This preliminary information may be used as an indication for future consumer studies. In a recent study by Tibaldi et al. (2015) in which European seabass was fed *Isochrysis*-rich diets, it was found that skin lightness ( $L^*$ ) was not affected by dietary treatments. However, there was a significant increase of greenness ( $a^*$ ) in dorsal skin of fish fed the diet including the highest level of microalgae, which was coupled with increased hue values and slightly different colour saturation (chroma). An enhanced greenish skin pigmentation was already described in European seabass juveniles fed *Tetraselmis suecica* (Tulli et al., 2012) and in Atlantic cod fed a mixture of *Nannochloropsis* sp. and *Isochrysis* sp. (Walker and Berlinsky, 2011). In red porgy, a diet containing *H. pluvialis* resulted in reddish skin coloration, while diets with *Spirulina* and alfalfa promoted a yellowish coloration (Chatzifotis et al., 2011). Altogether, these studies demonstrate that natural carotenoids from microalgae can be used as tools to tailor the skin pigmentation in fish. However, further studies are needed to establish and understand the efficiency of the various carotenoids types, since the pathways regulating skin pigmentation are species-specific.

Overall data from our study show that *Phaedactylum tricornutum*, a microalgae rich in fucoxanthin, when incorporated at 2.5% in finishing diets for gilthead seabream resulted in a reduction of whole-body fat and originated a lighter and more vivid yellow coloration of seabream operculum and a higher lightness of ventral skin. Farmed fish require adequate pigmentation patterns to respond to consumer's demands. Colour is the first quality attribute used by consumers, impacting on visual assessment and freshness perception of fish, which are key purchase determinants. Incorporation of microalgae pigments in aquafeeds also gives aquaculture products a more natural-like image, alleviating the manipulation impression that supplementation may suggest. Consumers often associate synthetic additives in foods with higher risks, while natural additives are generally perceived as better and healthier (Devcich et al., 2007; Dickson-Spillmann et al., 2011). The dietary use of microalgae biomasses may convey benefits beyond fulfilling the basic nutritional needs of the animal, and aspects such as an improvement of external pigmentation can contribute to meet consumer expectations towards farmed fish.

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# IMPROVING LCPUFA RETENTION – AN EXPLORATORY STUDY

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Chapter 6



# Exploratory approach to enhance the dietary retention of EPA (eicosapentaenoic acid; 20:5n-3) and DHA (docosahexaenoic acid, 22:6n-3) in gilthead seabream fillets

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## Chapter 6.1

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## Exploratory approach to enhance the dietary retention of EPA (eicosapentaenoic acid, 20:5n-3) and DHA (docosahexaenoic acid, 22:6n-3) in gilthead seabream fillets

### Abstract

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Fish and seafood are considered excellent sources of n-3 LCPUFA (EPA and DHA), known to have an essential role against several human diseases. Over the last two decades, aquafeeds showed a significant reduction of fish oil levels and a concomitant increase of vegetable oils. As a result, the content of n-3 LCPUFA in fish feed and hence in farmed seafood has been gradually reduced, which has potential implications on the value of farmed fish to consumers health. One of the strategies to counteract this potential loss of nutritional value in fish relies on a higher retention of dietary n-3 LCPUFA in fish fillets. A study was undertaken with gilthead seabream (IBW: 255 g) to assess the effect of two dietary oil sources (100% fish oil or a blend of 75% fish oil:25% poultry fat) on the retention and deposition of EPA and DHA in seabream fillets. Moreover, given the large variations that exist in the formulation of commercial seabream feeds, the efficacy of the two oil blends was assessed in the fish previously fed with distinct formulation scenarios (FM, PAP and PP) and therefore with variable initial compositions. At the end of the trial, fish had a final body weight ranging from 300 to 306 g. Feed intake, specific growth rate and feed conversion ratio remained unaffected by the dietary treatments ( $P>0.05$ ). The whole-body composition of fish, in terms of moisture, protein, fat, ash and energy was not affected by the dietary treatments ( $P>0.05$ ). In the overall, our data demonstrates that retention of combined EPA and DHA in seabream fillets is relatively low (17-25% of intake), with DHA showing higher retention values than EPA. Moreover, it shows that the initial fatty acid background of fish seems to play a significant role on the EPA and DHA sparing efficiency. Changes on the dietary oil supply had no effect on the combined EPA and DHA retention for FM and PAP backgrounds ( $P>0.05$ ), while in the PP background, the use of a blend of 75% fish oil and 25% poultry fat led to a

significantly higher EPA and DHA retention than the use of a 100% fish oil ( $P < 0.05$ ). The nutritional contribution of seabream fillets from fish fed a 75% fish oil and 25% poultry fat diets were suitable to cover weekly recommended intakes in terms of combined EPA and DHA. Although exploratory, this work demonstrates that an alternative oil blend with high levels of MUFA and ALA allowed the enhancement of the efficiency of deposition of n-3 LCPUFA in seabream.

Keywords: Fish oil replacement; EPA and DHA retention; nutritional contribution; Gilthead seabream;

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## Introduction

Being a rich source of important nutrients, including highly digestible proteins, vitamins (A, D3), trace minerals (iodine, selenium) and n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), fish consumption is generally regarded as part of a healthy dietary pattern (EFSA 2014a). A vast body of literature supports the relevancy and protective role of n-3 LCPUFA, particularly of EPA (eicosapentaenoic acid; 20:5n-3) and DHA (docosahexaenoic acid, 22:6n-3), against a number of human diseases (FAO and WHO, 2011; Chowdhury et al., 2012; EFSA, 2014). The European Food Safety Authority (EFSA) set the value of 250 of combined EPA and DHA per day as the Adequate Intake (AI) for the prevention of cardiovascular disease in adults (EFSA, 2012b), whereas the International Society for the Study of Fatty Acids and Lipids recommends a minimum intake of EPA and DHA combined, of 500 mg per day (ISSFAL, 2004).

With stagnating global capture fisheries production; aquaculture is expected to cater the protein supply of a growing population. Marine-derived raw materials traditionally used in aquafeeds (e.g. fishmeal and fish oil) are rich sources of n-3 LCPUFA, and therefore contribute significantly to the high levels of EPA and DHA generally found in farmed fish. However, due to environmental, economic and social constraints, the aquaculture industry has made a successful shift towards the use of feeds based on alternative ingredients of vegetable origin (Turchini et al., 2009; Torstensen and Tocher, 2010; Waite et al., 2014;

Ytrestøyl et al., 2015). To some extent, the fatty acid profile of fish muscle will mimic the fatty acid composition of the feed. Therefore, the trend towards a replacement of fish oil by alternative lipid sources, such as vegetable oils and animal fats, can interfere with the fish nutritional profile and consequently change consumer expected intakes of health valuable nutrients, such as n-3 LCPUFAs. To maintain consumer expectations of fish being a rich source of EPA and DHA, current industrial aquafeeds generally contain a blend of vegetable oils, fish oils (and in some cases animal fats). Although variable with species, growth stage and season, it is estimated that fish oil is currently maintained at approximately 30% of the total dietary oil(s) supply. Several studies have compared datasets of the fatty acid composition of farmed fish over time. The content of EPA and DHA in fillets of Norwegian farmed salmon has decreased from 27.4 mg·g<sup>-1</sup> to 11.5 mg·g<sup>-1</sup> (a 58% reduction) between 2005 and 2015 (NIFES, 2016). A similar scenario has also been observed in the Scottish farmed salmon (Sprague et al., 2016), Australian farmed salmon and barrmundi (Nichols et al., 2014). Despite this reduction one portion of farmed salmon (130-150 grams) will provide on average 1.5 grams of EPA and DHA, a dose which still covers the weekly recommendation (EFSA, 2012b) for healthy adults. This trend towards a reduction of fish oil inclusion levels is also a reality in several Mediterranean species like gilthead seabream. Data from Vasconi et al. (2017) showed that the content of EPA and DHA in fillets of farmed seabream has decreased by about 30% between 2005 and 2014.

Given the finite-nature of fish oils, further reductions of inclusion levels on fish feeds are needed. However, from a consumer perspective, caution should be taken to guarantee an adequate EPA and DHA content in the fillets. New sources of EPA and DHA are currently being evaluated (e.g. oils from microalgae, microbial biomasses, transgenic plants, mesopelagic fisheries) (Turchini et al., 2010; Betancor et al., 2016; Sprague et al., 2017). However, as part of a sustainable solution, we should also aim to enhance the efficiency of tissue deposition of n-3 LCPUFA. According to Ytrestøyl et al. (2015) in Atlantic salmon, only 46% of EPA+DHA intake was retained in the whole-body. In fillets, the direct edible part of salmon, retention of EPA+DHA was only 26% of intake. In the same study, it is also reported

that the retention of DHA was higher than that of EPA (Ytrestøyl et al., 2015). To tackle this low retention of dietary EPA and DHA in fish, several recent studies have focused on nutritional strategies to foster the metabolic sparing of n-3 LCPUFA. Tissue fatty acid composition is not solely dependent on the quantitative fatty composition of feeds but is also affected by their relative ratios, with consequences on the metabolic fate as energy yielding substrates, bioconversion or *de novo* production. In Atlantic salmon, Codabaccus et al. (2012) showed that equally important to the absolute dietary supply of n-3 LCPUFA is an increase in the DHA:EPA ratio that promotes enhanced tissue deposition efficiency of n-3 LCPUFA. Additionally, the efficiency of tissue deposition of n-3 LCPUFA in salmonids was promoted by increasing the dietary levels of the alpha-linolenic acid (ALA, 18:3n-3), which can be preferentially oxidized over n-3 LCPUFA if present in high amounts (Turchini and Francis, 2009; Nunez-Ortíz et al., 2016). Previous studies showed also that feeds rich in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) yielded a better efficiency on the retention of n-3 LCPUFA, since both FA classes are described as preferential sources for beta-oxidation and energy production in fish compared to LCPUFA (reviewed by Tocher, 2015).

Gilthead seabream is the most important finfish species cultured in the Mediterranean region. However, little information exists on the efficiency of n-3 LCPUFA retention in seabream. Therefore, a trial was undertaken to evaluate the effect of two dietary oil sources (100% fish oil or a blend of 75% fish oil:25% poultry fat) on the retention and deposition of EPA and DHA in seabream fillets. Moreover, given the large variations that exist in the formulation of commercial seabream feeds, the efficacy of the two oil blends was assessed in fish previously fed with distinct formulation scenarios and therefore with variable initial compositions.

### Material and methods

To generate variable nutritional backgrounds at an initial stage, fish were fed for 84 days with three distinct diets, which can be characterized as being rich in fishmeal (FM) and fish oil

(FO), rich in non-ruminant animal processed proteins and with low levels of fish oil (PAP) and rich in plant-protein sources and low levels of fish oil (PP). The second phase, focused on n-3 LCPUFA sparing efficiency, fish from the distinct nutritional backgrounds were sub-divided and fed diets, in which the dietary oil source was either 100% fish oil or a blend of 75% fish oil and 25% poultry fat.

### *Experimental diets*

The trial comprised six isonitrogenous (42% CP) and isolipidic (17.5% CF) diets. The FM100FO diet contained 30% fishmeal and 100% FO. In comparison to FM100FO, two other diets comprised a 50% replacement of fishmeal by hemoglobin powder and hydrolyzed feather meal (diet PAP100FO) or vegetable protein sources (soy protein concentrate, pea protein concentrate and wheat gluten; diet PP100FO). In diets FM100FO, PAP100FO and PP100FO fish oil was used as the sole added oil source, while in the three additional diets FM75FO, PAP75FO and PP75FO, a blend of 75% fish oil and 25% poultry fat was used as added oil sources. All other ingredients were kept relatively constant among the various diets, at the exception of some supplemental crystalline amino acids and inorganic phosphate to avoid any nutritional deficiencies (Table 6.1).

All diets were manufactured by SPAROS LDA (Olhão, Portugal). Ingredients were ground (below 250  $\mu\text{m}$ ) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Augsburg, Germany). Powder ingredients were then mixed accordingly to the three target formulations in a double-helix mixer (model 500L, TGC Extrusion, Rouillet-Saint-Estèphe, France) to attain a basal mixture. Diets (pellet size 5.0 mm) were produced by means of a twin-screw extruder (model BC45, Clextal, Firminy, France) with a screw diameter of 55.5 mm and temperature ranging 115-120°C. Upon extrusion, feeds were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, Rouillet-Saint-Estèphe, France). After cooling the pellets, the various oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, Sevenum, The Netherlands). Throughout the duration of the trials, experimental feeds were stored at room

temperature, but in a cool and aerated emplacement. Samples of diets were taken for proximate composition (Table 6.1) and fatty acid analysis (Table 6.2).

**Table 6.1.** Formulation and proximate composition of the experimental diets.

Ingredients, %	FM	FM	PAP	PAP	PP	PP
	100FO	75FO	100FO	75FO	100FO	75FO
Fishmeal LT 70 <sup>1</sup>	10.00	10.00	5.00	5.00	5.00	5.00
Fishmeal 60 <sup>2</sup>	20.00	20.00	10.00	10.00	10.00	10.00
Hydrolyzed feathermeal <sup>3</sup>			7.40	7.40		
Haemoblobin powder <sup>4</sup>			5.00	5.00		
Soy protein concentrate <sup>5</sup>					5.00	5.00
Pea protein concentrate <sup>6</sup>					3.85	3.85
Wheat gluten <sup>7</sup>					5.00	5.00
Corn gluten <sup>8</sup>	11.00	11.00	11.00	11.00	12.00	12.00
Soybean meal 48 <sup>9</sup>	16.00	16.00	15.00	15.00	13.50	13.50
Rapeseed meal <sup>10</sup>	7.00	7.00	10.00	10.00	10.00	10.00
Sunflower meal <sup>11</sup>	5.00	5.00				
Wheat meal <sup>12</sup>	6.00	6.00	4.70	4.70	3.95	3.95
Pea starch <sup>13</sup>	6.00	6.00	5.00	5.00	4.00	4.00
Fish oil <sup>14</sup>	15.00	11.25	15.40	11.55	15.90	11.93
Poultry fat <sup>14</sup>		3.75		3.85		3.98
Soy lecithin <sup>15</sup>	0.50	0.50	0.50	0.50	0.50	0.50
Glycerol <sup>16</sup>			5.00	5.00	5.00	5.00
Brewer's yeast <sup>17</sup>	0.50	0.50	0.50	0.50	0.50	0.50
Binder <sup>18</sup>	0.50	0.50	0.50	0.50	0.50	0.50
Monocalcium phosphate <sup>19</sup>			2.20	2.20	2.20	2.20
L-Lysine <sup>20</sup>	0.50	0.50	0.80	0.80	1.00	1.00
DL-Methionine <sup>21</sup>	0.20	0.20	0.20	0.20	0.30	0.30
Vitamin and mineral premix <sup>22</sup>	1.80	1.80	1.80	1.80	1.80	1.80
Dry matter (DM), %	94.6 ± 0.0	94.8 ± 0.0	93.3 ± 0.1	94.7 ± 0.0	95.0 ± 0.1	95.1 ± 0.0
Crude protein, %DM	41.5 ± 0.1	42.0 ± 0.1	42.2 ± 0.0	42.0 ± 0.1	42.2 ± 0.1	42.2 ± 0.1
Crude fat, %DM	17.9 ± 0.1	17.8 ± 0.3	17.7 ± 0.4	17.5 ± 0.1	17.5 ± 0.0	17.5 ± 0.0
Ash, %DM	8.9 ± 0.0	8.7 ± 0.1	7.6 ± 0.1	7.6 ± 0.1	6.8 ± 0.1	6.8 ± 0.0
Total phosphorus, % DM	1.3 ± 0.0	1.3 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	0.9 ± 0.1	0.9 ± 0.1
Gross energy, kJ·g <sup>-1</sup> DM	21.2 ± 0.1	21.9 ± 0.2	22.1 ± 0.2	22.2 ± 0.1	22.2 ± 0.1	22.2 ± 0.1

<sup>1</sup> Fishmeal NORVIK 70: 70.3% crude protein (CP) 5.8% crude fat (CF), Sopropêche, France; <sup>2</sup> CONRESA 60: 61.2% crude protein (CP), 8.4% crude fat (CF), Conserveros Reunidos S.A., Spain; <sup>3</sup> Kerapro: 82% CP, 11% CF, SONAC, The Netherlands; <sup>4</sup> PP94: 93% CP, 1% CF, Daka Sarval A/S, Denmark; <sup>5</sup> Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; <sup>6</sup> Lysamin GPS: 78% CP, 0.9% CF; Roquette Frères, France; <sup>7</sup> VITAL: 80% CP, 7.5% CF, Roquette Frères, France; <sup>8</sup> Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; <sup>9</sup> Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, CARGILL, Spain; <sup>10</sup> Defatted rapeseed meal: 34% CP, 2% CF, Premix Lda, Portugal; <sup>11</sup> Sunflower meal: 23% CP, 3% CF, PREMIX Lda, Portugal; <sup>12</sup> Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal; <sup>13</sup> NASTAR: 0.3% CP; 0.1% CF, COSUCRA, Belgium; <sup>14</sup> SAVINOR UTS, Portugal; <sup>15</sup> Lecico P700IPM, LECICO GmbH, Germany; <sup>16</sup> Rapeseed glycerol, Belgosuc Nv, Belgium; <sup>17</sup> Brewer's yeast: 39% CP, 4.5% CF, PREMIX Lda, Portugal; <sup>18</sup> Kieselguhr (natural zeolite), LIGRANA GmbH, Germany; <sup>19</sup> MCP: 22% P, 18% Ca, Fosfitalia, Italy; <sup>20</sup> Biolys 54.6%, EVONIK Nutrition & Care GmbH, Germany; <sup>21</sup> DL-METHIONINE FOR AQUACULTURE 99%, EVONIK

Nutrition & Care GmbH, Germany; <sup>22</sup> PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate, 7.5mg; sodium chloride, 400mg; excipient wheat middlings.

**Table 6.2.** Summarised fatty acid composition of experimental diets (expressed as % feed).

Fatty acids <sup>1</sup>	FM 100FO	FM 75FO	PAP 100FO	PAP 75FO	PP 100FO	PP 75FO
Saturated	6.2 ± 0.0	6.3 ± 0.0	6.8 ± 0.0	6.7 ± 0.0	6.3 ± 0.0	6.2 ± 0.0
Monounsaturated	4.5 ± 0.0	5.4 ± 0.1	4.8 ± 0.0	5.5 ± 0.0	4.3 ± 0.0	5.2 ± 0.1
Polyunsaturated	7.3 ± 0.0	6.1 ± 0.1	6.2 ± 0.0	5.4 ± 0.0	6.8 ± 0.0	6.2 ± 0.1
C18:2n-6	1.2 ± 0.0	1.6 ± 0.0	1.1 ± 0.0	1.7 ± 0.0	1.3 ± 0.0	1.6 ± 0.0
C18:3n-3	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
C20:5n-3 (EPA)	1.6 ± 0.0	1.2 ± 0.0	1.3 ± 0.0	1.0 ± 0.0	1.5 ± 0.0	1.2 ± 0.0
C22:6n-3 (DHA)	1.2 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	1.1 ± 0.0	0.9 ± 0.0
EPA+DHA	2.8 ± 0.0	2.1 ± 0.0	2.2 ± 0.0	1.7 ± 0.0	2.6 ± 0.0	2.1 ± 0.0

<sup>1</sup> All identified fatty acids were considered in the composite fractions.

### Experimental trial

The trial was conducted at the Experimental Research Station of CCMAR (Faro, Portugal). Experiments were directed by trained scientists (following category C FELASA recommendations) and in compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei nº. 113/2013, de 7 de Agosto) legislation on the protection of animals for scientific purposes. Experimental facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Prior to this trial, fish had been fed for 84 days with three distinct practical diets to generate variable initial muscle fatty acid backgrounds. These three diets can each be characterized as being rich in fishmeal and fish oil (FM), rich in non-ruminant animal processed proteins and with low levels of fish oil (PAP) and rich in plant-protein sources and low levels of fish oil (PP). Fish from each nutritional background were manually sorted and used to constitute duplicate groups of 16 fish, with a mean initial body weight of 255 ± 5.2 g. Fish from the nutritional background FM were fed with diets FM100FO and FM75FO, those from the nutritional

background PAP were fed with diets PAP100FO and PAP75FO, and those from the nutritional background PP were fed with diets PP100FO and PP75FO, over a period of 56 days. Fish were stocked in 1000 L circular plastic tanks exposed to natural photoperiod changes through late Summer-Autumn conditions (early October till end-November) with water parameters varying accordingly (salinity: 34 psu; temperature: 17-23 °C; oxygen content of outlet water maintained higher than 6 mg·L<sup>-1</sup>). Fish were hand-fed to apparent satiety, twice a day. Fish were individually weighed at the beginning and bulk weighed at the end of the trial, following one day of feed deprivation. Final samplings were done 48 hours following the last meal. Fish were slaughtered by immersion in ice-water slurry (4:1) until death. At the end of the trial, the dorsal muscle, without skin, from six fish per replicate tank were collected and 2 pools (3 dorsal muscle samples per pool) were used for analysis of total fat content and fatty acid composition. At the start of the trial, two pools of three dorsal muscle from fish of the three distinct nutritional backgrounds were also sampled for analysis of their total fat content and fatty acid composition.

### *Analytical methods*

Proximate composition analysis of the diets and whole-fish was made by the following procedures: dry matter by drying at 105°C for 24 h; ash by combustion at 550°C for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428, Leco Instruments, USA); crude fat after dichloromethane extraction by the Soxhlet method; total phosphorus was quantified according to the ISO/DIS 6491 method using the vanado-molybdate reagent; and gross energy in an adiabatic bomb calorimeter (model C2000, IKA-Werke GmbH & Co. KG, Staufen, Germany). Total lipids in the fillets were extracted according to the method of Folch et al. (1957) and subsequently, the fatty acid composition of fillets was determined by gas-chromatography analysis of methyl esters, according to the procedure of Lepage and Roy (1986), modified by Cohen et al. (1988) and described in further detail by Costa et al. (2013).

### *Nutritional Contribution*

The nutritional contribution (NC) of seabream fillets was based on the percentage of the Daily Adequate Intake (DAI) for combined EPA and DHA. However, calculations were performed on a weekly basis considering two recommended meals, and according to the following formula:

$$\text{NC (\%)} = 100 \times \frac{C \times M}{\text{AI}}$$

Where:

C: mean concentration of nutrient in  $\text{g}\cdot\text{kg}^{-1}$ .

M: meal fillet portion consumed is 0.160 kg (Costa et al., 2013) x 2-week meals.

AI for EPA+DHA is  $500 \text{ mg}\cdot\text{d}^{-1}$  for cardiovascular health in adults (International Society for the Study of Fatty Acids and Lipids [ISSFAL], 2004), corresponding to a weekly intake of 3.5g of EPA+DHA.

### *Statistical analysis*

Evaluation criteria data are presented as mean of two replicates  $\pm$  standard deviation. Data were subjected to a one-way analysis of variance, with lipid source as variable. When appropriate, means were compared by the Student-Newman-Keuls test. Parameters expressed as percentages were subjected to arcsin square root transformation. Statistical significance was tested at 0.05 probability level. Statistical tests were performed with the SPSSV22 software.

### **Results**

As expected, at the start of the trial, fish fillets from the three distinct nutritional backgrounds presented significant variations on their fatty acids profile (Table 6.3). Fillets from initial fish originated from the FM scenario had a significantly lower fat content than those originated from PAP and PP ( $P < 0.05$ ). Moreover, initial fillets from the PP scenario had also a significantly lower fat content than those originated from PAP ( $P < 0.05$ ). In comparison to FM and PP fillets, those originated from the PAP scenario had significantly higher levels of FA and MUFA and a lower content of PUFA ( $P < 0.05$ ). Also, initial PP fillets had a significantly higher

content of linoleic (LA) and alpha-linolenic (ALA) than FM and PAP fillets ( $P < 0.05$ ). Initial FM fillets had significantly higher levels of EPA and DHA than those from PAP and PP ( $P < 0.05$ ).

**Table 6.3.** Summarised fillet fat and fatty acid composition at the start of the trial (as g per 100 g).

Fatty acids <sup>1</sup>	FM	PAP	PP	P value
Total fat	6.23 ± 0.02 <sup>a</sup>	7.20 ± 0.02 <sup>c</sup>	6.71 ± 0.01 <sup>b</sup>	<0.001
Saturated (SFA)	1.80 ± 0.02 <sup>b</sup>	2.02 ± 0.02 <sup>c</sup>	1.70 ± 0.04 <sup>a</sup>	0.003
Monounsaturated (MUFA)	1.87 ± 0.01 <sup>a</sup>	2.96 ± 0.02 <sup>c</sup>	2.44 ± 0.02 <sup>b</sup>	<0.001
Polyunsaturated (PUFA)	2.56 ± 0.01 <sup>b</sup>	2.21 ± 0.02 <sup>a</sup>	2.57 ± 0.03 <sup>b</sup>	0.001
C18:2n-6 (LA)	0.39 ± 0.02 <sup>a</sup>	0.62 ± 0.01 <sup>b</sup>	0.82 ± 0.03 <sup>c</sup>	0.001
C18:3n-3 (ALA)	0.07 ± 0.00 <sup>a</sup>	0.08 ± 0.01 <sup>a</sup>	0.37 ± 0.03 <sup>b</sup>	0.001
C20:5n-3 (EPA)	0.58 ± 0.02 <sup>b</sup>	0.37 ± 0.01 <sup>a</sup>	0.35 ± 0.02 <sup>a</sup>	0.002
C22:6n-3 (DHA)	0.66 ± 0.02 <sup>b</sup>	0.54 ± 0.01 <sup>a</sup>	0.54 ± 0.05 <sup>a</sup>	0.036
EPA+DHA	1.24 ± 0.00 <sup>b</sup>	0.92 ± 0.02 <sup>a</sup>	0.89 ± 0.07 <sup>a</sup>	0.006

<sup>1</sup>All identified fatty acids were considered in the composite fractions. Values are means ± standard deviation (n=2). Different superscripts within a row, denotes a statistical difference (P<0.05).

At the end of the trial, fish had a final body weight ranging from 300 to 306 g. Feed intake, specific growth rate and feed conversion ratio remained unaffected by the dietary treatments (P>0.05) (Table 6.4). The whole-body composition of fish, in terms of moisture, protein, fat, ash and energy was not affected by the dietary treatments (P>0.05) (data not shown).

**Table 6.4.** Growth performance at the end of the trial.

	FM 100FO	FM 75FO	PAP 100FO	PAP 75FO	PP 100FO	PP 75FO	P value
IBW <sup>1</sup> , g	254 ± 2	252 ± 2	257 ± 1	261 ± 7	253 ± 3	251 ± 2.7	
FBW <sup>2</sup> , g	303 ± 3	302 ± 1	300 ± 4	306 ± 4	306 ± 2	302 ± 3	0.275
SGR <sup>3</sup> , %/day	0.31 ± 0.03	0.32 ± 0.01	0.28 ± 0.01	0.28 ± 0.03	0.34 ± 0.01	0.33 ± 0.04	0.212
FCR <sup>4</sup>	2.33 ± 0.20	2.26 ± 0.03	2.62 ± 0.15	2.48 ± 0.25	2.13 ± 0.02	2.09 ± 0.19	0.108
FI, % ABW/d	0.73 ± 0.00	0.73 ± 0.01	0.72 ± 0.00	0.70 ± 0.00	0.72 ± 0.01	0.69 ± 0.01	0.158

Values are means ± standard deviation (n=2).

<sup>1</sup> Initial mean body weight. <sup>2</sup> Final mean body weight. <sup>3</sup> Specific growth rate: (Ln FBW – Ln IBW) x 100 / 56 days. <sup>4</sup> Feed conversion ratio: dry feed intake / wet weight gain. <sup>5</sup> Feed intake: (crude feed intake / (IBW+FBW) / 2 / 56 days) x 100.

The fat content and fatty acid composition of the fillets at the end of the trial is presented in Table 6.5. Fish fed diet FM100FO showed a significantly lower fillet fat content than those fed diets PAP100FO, PP100FO and PP75FO ( $P < 0.05$ ). Fillets from PAP100FO showed a significantly higher level of SFA than those of all other treatments ( $P < 0.05$ ). Fillet MUFA and LA levels were the lowest in fish fed the FM100FO and FM75FO diets, intermediate in PP100FO and PP75FO diets and the highest in those fed with the PAP100FO and PAP75FO diets. Fillet PUFA levels were lowest in the PAP100FO and PAP75FO fillets. Fillets from fish fed the PP100FO and PP75FO had significantly higher ALA levels than those found in all other diets ( $P < 0.05$ ). Moreover, FM100FO and FM75FO fillets had significantly higher levels of EPA and DHA than all other treatments ( $P < 0.05$ ). Additionally, FM100FO fillets had significantly higher levels of EPA and DHA than FM75FO fillets ( $P < 0.05$ ).

**Table 6.5.** Summarised fillet fat and fatty acid composition at the end of the trial (as g per 100 g).

Fatty acids <sup>1</sup>	FM 100FO	FM 75FO	PAP 100FO	PAP 75FO	PP 100FO	PP 75FO	P value
Fat	6.66 ± 0.08 <sup>a</sup>	6.87 ± 0.06 <sup>ab</sup>	7.66 ± 0.07 <sup>b</sup>	7.09 ± 0.08 <sup>ab</sup>	7.39 ± 0.04 <sup>b</sup>	7.38 ± 0.01 <sup>b</sup>	0.012
SFA	1.80 ± 0.02 <sup>a</sup>	1.86 ± 0.01 <sup>a</sup>	2.21 ± 0.10 <sup>b</sup>	1.86 ± 0.01 <sup>a</sup>	1.85 ± 0.00 <sup>a</sup>	1.82 ± 0.00 <sup>a</sup>	<0.001
MUFA	1.96 ± 0.01 <sup>a</sup>	2.15 ± 0.01 <sup>b</sup>	3.02 ± 0.06 <sup>e</sup>	2.91 ± 0.01 <sup>d</sup>	2.67 ± 0.03 <sup>c</sup>	2.61 ± 0.02 <sup>c</sup>	<0.001
PUFA	2.87 ± 0.04 <sup>c</sup>	2.83 ± 0.06 <sup>c</sup>	2.48 ± 0.00 <sup>b</sup>	2.29 ± 0.03 <sup>a</sup>	2.90 ± 0.02 <sup>cd</sup>	2.99 ± 0.00 <sup>d</sup>	<0.001
C18:2n-6	0.38 ± 0.01 <sup>a</sup>	0.45 ± 0.00 <sup>b</sup>	0.66 ± 0.01 <sup>d</sup>	0.62 ± 0.00 <sup>c</sup>	0.83 ± 0.00 <sup>e</sup>	0.89 ± 0.02 <sup>f</sup>	<0.001
C18:3n-3	0.06 ± 0.00 <sup>a</sup>	0.07 ± 0.00 <sup>a</sup>	0.09 ± 0.00 <sup>b</sup>	0.08 ± 0.00 <sup>a</sup>	0.35 ± 0.00 <sup>c</sup>	0.35 ± 0.00 <sup>c</sup>	<0.001
C20:5n-3	0.69 ± 0.01 <sup>e</sup>	0.64 ± 0.00 <sup>d</sup>	0.46 ± 0.01 <sup>c</sup>	0.40 ± 0.01 <sup>a</sup>	0.44 ± 0.01 <sup>bc</sup>	0.41 ± 0.00 <sup>ab</sup>	<0.001
C22:6n-3	0.95 ± 0.06 <sup>c</sup>	0.86 ± 0.02 <sup>b</sup>	0.67 ± 0.01 <sup>a</sup>	0.65 ± 0.01 <sup>a</sup>	0.71 ± 0.00 <sup>a</sup>	0.71 ± 0.01 <sup>a</sup>	<0.001
EPA+DHA	1.63 ± 0.05 <sup>d</sup>	1.50 ± 0.01 <sup>c</sup>	1.12 ± 0.02 <sup>b</sup>	1.05 ± 0.02 <sup>a</sup>	1.15 ± 0.02 <sup>b</sup>	1.12 ± 0.01 <sup>b</sup>	<0.001

<sup>1</sup> All identified fatty acids were considered in the composite fractions.

Values are means ± standard deviation (n=2).

Different superscripts within a row, denotes a statistical difference ( $P < 0.05$ ).

Based on the individual fatty acid intake, fillet biomass gain considering a fillet yield of 42%, and the initial and final fillet fatty acid composition, the retention and daily deposition rate of EPA and DHA was calculated (Table 6.6 and Figure 6.1). Retention of dietary EPA ranged between 9 and 15%. Fish fed diet PP75FO showed a significantly lower fillet EPA retention ( $P < 0.05$ ), while fish fed FM100FO and FM75FO showed a significantly higher fillet EPA retention than those fed all other diets ( $P < 0.05$ ). Within FM and PP nutritional backgrounds, the retention of EPA in the fillets was not significantly affected by the two fish-oil finishing strategies (100% fish oil or a blend of 75% fish oil and 25% poultry fat). Retention of DHA in fillets varied between 24 and 37%. Fish fed AP100FO showed a significantly lower DHA retention than those fed diets FM100FO, FM75FO and PP75FO ( $P < 0.05$ ). Within the various nutritional backgrounds, changes on the quality of dietary oil supply had no effect on fillet DHA retention. When considering the combination of EPA and DHA, the retention in fillets from FM100FO, FM75FO and PP75FO was significantly higher than that of PAP100FO, PPA75FO and PP100FO ( $P < 0.05$ ). Changes on the quality of dietary oil supply had no effect on the combined EPA and DHA retention for FM and PAP backgrounds ( $P > 0.05$ ), while in the PP background, the use of a blend of 75% fish oil and 25% poultry fat led to a significantly higher EPA and DHA retention than the use of a 100% fish oil ( $P < 0.05$ ).

**Table 6.6.** Retention, daily deposition and weekly nutritional contribution of EPA and DHA in fillets.

	FM 100FO	FM 75FO	PAP 100FO	PAP 75FO	PP 100FO	PP 75FO	P value
<b>Retention<sup>1</sup> (% of intake)</b>							
C20:5n-3	14.6 ± 0.2 <sup>c</sup>	15.3 ± 0.3 <sup>c</sup>	12.1 ± 0.3 <sup>b</sup>	9.1 ± 0.8 <sup>a</sup>	10.9 ± 0.8 <sup>b</sup>	12.5 ± 0.7 <sup>b</sup>	0.001
C22:6n-3	37.0 ± 7.0 <sup>b</sup>	36.7 ± 2.2 <sup>b</sup>	24.2 ± 0.3 <sup>a</sup>	29.9 ± 0.3 <sup>ab</sup>	27.7 ± 0.4 <sup>ab</sup>	35.5 ± 2.7 <sup>b</sup>	0.027
EPA+DHA	24.2 ± 2.9 <sup>b</sup>	24.6 ± 0.8 <sup>b</sup>	17.1 ± 0.3 <sup>a</sup>	17.9 ± 0.6 <sup>a</sup>	17.9 ± 0.6 <sup>a</sup>	22.2 ± 1.5 <sup>b</sup>	0.004
<b>Deposition (mg·100 g fillet·d<sup>-1</sup>)</b>							
C20:5n-3	22.7 ± 2.3 <sup>c</sup>	17.6 ± 0.6 <sup>b</sup>	17.5 ± 1.4 <sup>b</sup>	9.5 ± 1.8 <sup>a</sup>	15.2 ± 1.0 <sup>b</sup>	13.2 ± 0.5 <sup>b</sup>	0.001

C22:6n-3	43.1 ± 4.5 <sup>c</sup>	32.7 ± 1.5 <sup>b</sup>	24.9 ± 1.8 <sup>ab</sup>	22.9 ± 2.5 <sup>a</sup>	27.3 ± 0.1 <sup>ab</sup>	27.2 ± 0.5 <sup>ab</sup>	0.001
EPA+DHA	65.8 ± 2.2 <sup>d</sup>	50.3 ± 0.9 <sup>c</sup>	42.4 ± 3.2 <sup>b</sup>	32.4 ± 4.3 <sup>a</sup>	42.5 ± 1.1 <sup>b</sup>	40.4 ± 1.0 <sup>b</sup>	<0.001
<b>Weekly Nutritional Contribution<sup>2</sup> (% Adequate Intake)</b>							
NC	149 ± 5 <sup>d</sup>	137 ± 1 <sup>c</sup>	103 ± 1 <sup>ab</sup>	96 ± 2 <sup>a</sup>	105 ± 1 <sup>b</sup>	103 ± 1 <sup>ab</sup>	<0.001

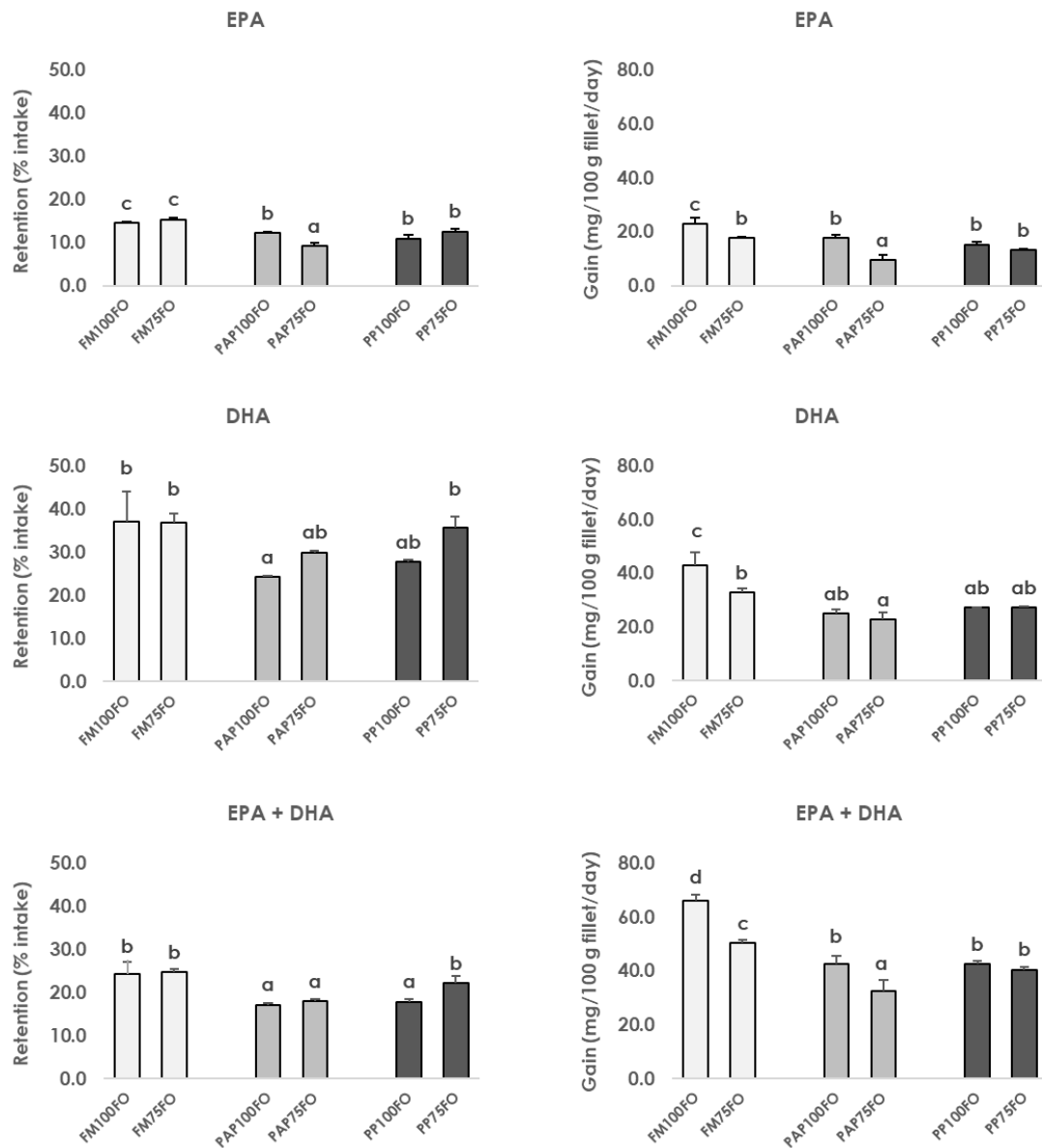
Values are means ± standard deviation (n=2).

Different superscripts within a row, denotes a statistical difference (P<0.05).

<sup>1</sup> Retention:  $100 \times (\text{FBW} \times \text{final carcass nutrient} - \text{IBW} \times \text{initial carcass nutrient}) / \text{nutrient intake}$ .

<sup>2</sup> Weekly nutritional contribution of EPA+DHA as % of the Daily Adequate Intake (DAI) of 500 mg·d<sup>-1</sup> (= 3.5 g·week<sup>-1</sup>) for cardiovascular health in adults (ISSFAL, 2004).

The daily deposition of EPA in fillets was significantly higher in FM100FO, intermediate in FM75FO, PAP100FO, PP100FO, PP75FO and lower in PAP75FO fillets (P<0.05). Within the FM and PAP nutritional backgrounds, the deposition of EPA in fish fed diets with 100% fish oil was significantly higher than in those fed with a blend of 75% fish oil and 25% poultry fat (P<0.05). Additionally, the deposition of DHA in FM100FO and FM75FO fillets was significantly higher than in PAP75FO (P<0.05). Within the FM nutritional background, the deposition of DHA in fish fed diets with 100% fish oil was significantly higher than in those fed with a blend of 75% fish oil and 25% poultry fat (P<0.05). Deposition of combined EPA and DHA was highest in FM100FO and FM75FO and significantly lowered in all other diets (P<0.05). At the exception of the PP background, the inclusion of 25% poultry fat led to a significant reduction on the fillet deposition of combined EPA and DHA in FM and PAP backgrounds (P<0.05).



**Figure 6.1.** Retention (% of intake) and daily deposition (mg·100 g·d<sup>-1</sup>) of EPA and DHA in fillets.

Values are means ± standard deviation (n=2).

Bars with different superscripts, denotes a statistical difference (P<0.05).

The fillet nutritional contribution (NC) to the recommended adequate intake of EPA and DHA is presented in Table 6.6. The weekly consumption of two meal portions of 160 g seabream fillets from the FM100FO and FM75FO diets represented 149 and 137% of the recommended Adequate Intake for EPA and DHA. In comparison to the FM background fish, those fed with

PAP and PP diets showed a significant reduction of its weekly NC to EPA and DHA adequate intake (96 to 105%) ( $P < 0.05$ ). While the 100% fish oil diet resulted in a significant increase of NC in fish fed the FM background ( $P < 0.05$ ), the reduction of fish oil with the inclusion of 25% poultry fat did not affect the NC of EPA and DHA in fillets originated from the PAP and PP backgrounds ( $P > 0.05$ ).

## Discussion

The consumption of fish and other seafood products is often associated to health benefits, particularly due to their content on n-3 LCPUFA, namely EPA and DHA. Fishmeal and fish oil traditionally used in aquafeeds are rich sources of n-3 LCPUFA and contribute significantly to the high levels of EPA and DHA generally found in farmed fish. Fish oil is currently the only practical and economically viable source of these essential fats for feed purposes. According to IFFO - Marine Ingredients Organisation, the global production of fish oil is around 1 million tonnes/year and is not expected to increase. Every year roughly 750 000 tonnes of fish oil are used in aquaculture feeds. The level of EPA+DHA in fish oil is usually between 15 and 25%, so with an average content of 20% we would expect 160 000 tonnes EPA+DHA of fish oil to be consumed in aquafeeds. Additionally, fishmeal provides 50 000 tonnes EPA+DHA for fish feed (based on 3.1 million tonnes of fishmeal containing 8% oil). At present, the aquaculture sector therefore consumes an estimated total of 210 000 tonnes of EPA+DHA, all originating from the marine environment (Shepherd and Bachis, 2014).

Over the last two decades, aquafeeds showed a significant reduction of fish oil levels and a concomitant increase of vegetable oils from rapeseed and soybean. This trend is due to uncertain supplies of fish oil coupled with increasing price compared with vegetable oils. At the same time the growth of demand for fish oil from the human nutritional supplements market (nutraceuticals) is starting to threaten the continuing supply of fish oil for aquaculture, which cannot compete on price. As a result, the content of n-3 LCPUFA in fish feed and hence in farmed seafood is gradually being reduced, which has potential implications, including for the health of farmed fish and final consumers.

A significant effort has been devoted to the development of novel sources of n-3 LCPUFAs. The first action taken was to increase the available fish oil volumes by increasing the recovery from fishery by-products. The proportion of fish meal and fish oil manufactured from trimmings by-products of fish processing was approximately 30% in 2013 and is expected to reach 40% by 2020 (Shepherd and Bachis, 2014). The exploitation of novel marine resources, such as Antarctic krill (*Euphasia superba*) is under way and krill oil is already being used for human consumption, but annual volumes are unlikely to exceed 10 000 tons. High expectations rely on the development of microalgae oils rich in EPA and DHA. Currently only small quantities of algal oil (10 000 ton/year) containing high levels of EPA and DHA are being produced from algae, mainly by fermentation and supplied to the pharmaceutical and cosmetic sectors, at prices which are higher than aquaculture could afford to pay. However, large-scale microalgae production facilities are being created and the increasing market volumes will tend to make algae oils accessible to the feed sector (Turchini et al., 2010). Another avenue that is arousing commercial interest is the production of n-3 LCPUFA rich oils from genetically modified (GM) oilseeds, such as rapeseed, soybean and *Camelina sativa*. Even if the use of oils derived from genetically modified plants is already being demonstrated experimentally (Hixson et al., 2014; Betancor et al., 2016), the large-scale GM plant production for extracting n-3 LCPUFA is still at an early stage. Moreover, the future use of GMO-derived oils in aquafeeds will surely face a strict scrutiny by regulatory authorities and the aquaculture associated supply chains before its permitted commercial use. The interest and market potential for these non-marine sources of n-3 LCPUFA rich oils is unquestionable. However, the critical question is when they will become commercially relevant in terms of volumes and costs for the aquafeed sector. Therefore, in the short-term vegetable oils and animal fats will probably continue to be the main choice when replacing fish oil in aquaculture diets.

At the end of the trial, the feed intake, specific growth rate, feed conversion ratio and the whole-body composition of seabream was not affected by the changes on the dietary oil

sources. Previous studies confirm that within reasonable limits (5-10% total oils) the use of poultry fat as an alternative dietary lipid source does not impact overall performance criteria in several fish species (Bureau and Meeker, 2010; Hatlen et al., 2013; Monteiro et al., 2018). However, it is also widely accepted that to a large extent, the fatty acid profile of fish will mimic the fatty acid composition of the feed (Izquierdo et al., 2005; Benedito-Palos et al., 2011). Therefore, the trend towards a replacement of fish oil by alternative lipid sources, such as vegetable oils and animal fats, can interfere with the fish nutritional profile and consequently lower consumer expected intakes of health valuable nutrients, such as n-3 LCPUFAs (Sprague et al., 2016; Vasconi et al., 2017; Nichols et al., 2014). According to Ytrestøyl et al. (2015), in Atlantic salmon 54% of EPA+DHA intake is lost or catabolized. A similar scenario of a relatively low whole-body retention of dietary EPA and DHA has also been demonstrated by Glencross et al. (2014). If these retention values are expressed on a fillet basis, the direct edible part of salmon, then retention of EPA+DHA is reduced to 26% of intake (Ytrestøyl et al., 2015). Our data shows that gilthead seabream fed a 100% fish oil diet retained 17 to 24% of dietary EPA+DHA intake. Moreover, similarly to what had been reported by Ytrestøyl et al. (2015) in Atlantic salmon, we observed that in seabream the retention of DHA (27-37%) was higher than that of EPA (11-15%). This low retention of scarce resources such as EPA and DHA compels the aquaculture industry to develop more efficient strategies for the utilization of dietary n-3 LCPUFA.

Tissue fatty acid composition is not solely dependent on the quantitative fatty composition of feeds, but is affected also by their relative ratios, since it affects their metabolic fate as energy substrates, bioconversion potential or de novo synthesis (Glencross et al., 2014). Studies with Atlantic salmon showed that equally important to the dietary supply of n-3 LCPUFA is an increase in the DHA:EPA ratio, which results in enhanced tissue deposition efficiency (Codabaccus et al., 2012; Glencross et al., 2014). Moreover, the whole-body deposition of n-3 LCPUFA in salmonids was enhanced by increasing the dietary levels of the alpha-linolenic acid (ALA, 18:3n-3) (Turchini and Francis, 2009; Nunez-Ortíz et al., 2016) and other fatty acid classes, particularly saturated fatty acids (SFA) and monounsaturated fatty

acids (MUFA) (Trushenski et al. 2011; Emery et al., 2014; Hixson et al., 2014; Bowzer et al., 2016; Rombenso et al., 2015; Salini et al., 2017). Additionally, other lipid classes like cholesterol have also been associated to higher DHA deposition in rainbow trout (Norambuena et al., 2013). In most of these studies, the hypothesis put forward to justify the higher retention of n-3 PUFA is the preferential peroxisomal chain shortening ( $\beta$ -oxidation) of the SFA and MUFA fractions.

In our study with seabream, we compared the EPA and DHA retention efficacy of high n-3 LCPUFA diets (100% fish oil) against MUFA and linoleic acid (LA, C18:2n-6) rich diets (a blend of 75% fish oil and 25% poultry fat), originated from distinct nutritional backgrounds, namely rich in fishmeal and fish oil rich (FM), rich in non-ruminant animal processed proteins and with low levels of fish oil (PAP) and rich in plant-protein sources and low levels of fish oil (PP). Our results show that enhanced dietary MUFA levels did not affect EPA+DHA retention on seabream fillets originated from the FM and PAP nutritional backgrounds, while it was significantly enhanced in those originated from the PP nutritional background. The daily gain of EPA and DHA in fillets of seabream fed 100% fish oil diets and subjected to FM and PAP nutritional backgrounds was significantly higher than in those fed high MUFA diets. However, fish subjected to the PP nutritional background showed a similar EPA and DHA daily fillet deposition rate. These data show for the first time, that the EPA and DHA sparing efficiency by a higher MUFA intake is also conditioned by the variable formulation scenarios to which the fish are subjected. The exact reasons for the higher EPA and DHA retention in the PP scenario and not in others are difficult to ascertain with certitude. However, the initial fatty acid composition of the fish originated from the PP background, namely their considerably higher levels of LA and alpha-linolenic acid (ALA, C18:3n-3) may have played a role. Although observed in trout and therefore potentially not valid for a marine species like seabream, Thanuthong et al. (2011) found that fish fed with an ALA-rich diet presented a 2-fold increase in fillet n-3 LCPUFA levels than fish fed a low-ALA diet, which was due to an enhanced bioconversion capacity of ALA to DHA, under conditions that ALA was not a limiting substrate. Our results with gilthead seabream tend to show that the potential to enhance the sparing

and retention of dietary n-3 LCPUFA in fish is mainly centered in DHA and less effective in EPA. Numerous studies referred a selective higher retention of DHA in muscle (Bell et al., 2002; Izquierdo et al., 2005; Mourente and Bell, 2006; Turchini et al., 2010; Glencross et al., 2014). Possibilities advanced to explain this mechanism were the specificity of fatty acyl transferases for DHA (Bell et al., 2001) and the complex system of peroxisomal oxidation required by DHA molecule (Bell et al., 2001; Tocher, 2003).

From a consumers' perspective, seafood nutritional contribution is primarily focused on n-3 LCPUFA contents. The International Society for the Study of Fatty Acids and Lipids recommends a minimum intake of combined EPA and DHA of 500 mg per day (ISSFAL, 2004) for cardiovascular preventive effects. Given that fish is generally not consumed daily, we have calculated the nutritional contribution (NC) in terms of EPA and DHA on a two-meal weekly basis and used a value of 3500 mg EPA+DHA per week as the adequate intake. As expected, changes on the dietary fatty acid profile affected the nutritional contribution of seabream fillets to the weekly adequate intake of EPA and DHA. The weekly consumption of two meal portions of 160 g seabream fillets from the FM100FO and FM75FO diets represented 149 and 137%, respectively, of the recommended Adequate Intake for EPA and DHA. In comparison to the FM background fish, those fed with PAP and PP diets showed a significant reduction of its weekly NC to EPA and DHA adequate intake (96 to 105%). While the 100% fish oil diet resulted in a significant increase of NC in fish fed the FM background, the reduction of fish oil with the inclusion of 25% poultry fat did not affect the NC of EPA and DHA in fillets originated from the PAP and PP backgrounds.

In the overall, this exploratory study demonstrates that retention of combined EPA and DHA in seabream fillets is relatively low (17-25% of intake), with DHA showing higher retention values than EPA. Moreover, it shows for the first time that the initial fatty acid background of fish seems to play a significant role on the EPA and DHA sparing efficiency by a higher MUFA intake. The nutritional contribution of seabream fillets from fish fed a 75% fish oil and 25% poultry fat diets were suitable to cover weekly recommended intakes in terms of combined

EPA and DHA. Given the limited availability of novel n-3 LCPUFA sources, and as part of a sustainable solution, the aquaculture industry needs to find new ways to enhance the efficiency of deposition of n-3 LCPUFA in fish.

### *Acknowledgements*

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# GENERAL DISCUSSION

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## Chapter 7

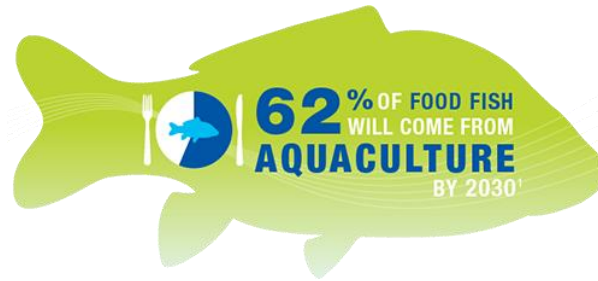


To accommodate the forecasts of population growth, the world will need to produce 70 to 100% more food by 2050

In the past, the primary solution to food shortages has been to bring more land into agriculture, intensify livestock farming and exploit new fish stocks. Yet over the past 5 decades, while grain production has more than doubled, the amount of land devoted to arable agriculture globally has increased by only ~9%. Some new land could be brought into cultivation, but the competition for land from other human activities makes this an increasingly unlikely and costly solution, particularly if protecting biodiversity and natural ecosystems (for example, carbon storage in rainforest) are given higher priority. In recent decades, agricultural land that was formerly productive has been lost to urbanization and other human uses, as well as to desertification, salinization, soil erosion, and other consequences of unsustainable land management. Moreover, there are no major new fishing grounds: Virtually all capture fisheries are fully exploited, and most are overexploited.

Thus, the most likely scenario is that more food will need to be produced from the same amount of (or even less) resources. This challenge, called “sustainable intensification” implies a more efficient use of scarce resources to meet consumer and societal expectations, to achieve high animal welfare, optimize environmental impact and deliver a proper income for those working in the animal production sector.

Aquatic products (mainly fish, aquatic molluscs, and crustaceans) play a key role in the food system, providing nearly 3 billion people with at least 15% of their animal protein intake. Aquaculture is one of the most resource-efficient ways to produce protein and this industry will be the main supplier of fish and seafood in the coming years.



**Figure 7.1.** Estimate world aquaculture food supply

Source: Global Aquaculture Alliance

The surge of aquaculture production has triggered concerns about environmental issues, such as pollution resulting from effluent discharge, loss of valuable habitats, escape of farmed organisms affecting wild-stocks, depletion of wild-living stocks due to the use of wild-caught juveniles in aquaculture systems, and environmental costs associated to feed raw materials and feeding (Duarte et al., 2009; Naylor et al., 2009; Martinez-Porchas and Martinez-Cordova, 2012).

As with many other primary food industries, aquaculture is heavily dependent on natural resources. Besides water, feed is a primary input for aquaculture. For the main European farmed fish species, industrial feeds still comprise a significant component of fishmeal (FM) (10-20% of formulas) and fish oil (FO) (5-15% of formulas) derived from industrial capture fisheries. These are renewable but finite resources, of which aquaculture is utilizing a growing share. Over the last decades, fish nutrition research has devoted a continued effort and has made significant progress with respect to improving feeding efficiency and in replacing FM and FO by alternative sources. However, this trend is altering the nutritional value of edible fish, conditioning the expected beneficial effects for consumers. The most significant impact is being observed in the reduced levels of n-3 LCPUFA and microelements such as iodine and vitamin D<sub>3</sub>, of which fish are the main natural food sources. Some of the strategies available today to counteract these potential losses of nutritional value of fish comprise the use of novel sustainable feed ingredients (e.g. animal processed by-products, microalgae, macroalgae, and microbial biomasses), the implementation of natural genetic

selection programs to raise fish with higher nutrient retention efficiencies and feed fortification with selected health-valuable nutrients.

Food fortification is an efficient and cost-effective approach to raise the intake of shortfall nutrients and mitigate associated deficiencies

Data on the efficacy of nutrient fortification strategies in farmed fish is limited. Throughout our work we contributed towards the generation of new data on the efficacy of several feed fortification strategies as tools to modulate several traits of farmed fish. Targets were the improvement of fish characteristics for consumer benefit and/or better acceptance, namely in terms of nutritional value, external appearance, sensory properties and overall quality.

### 7.1 Consumers' acceptance of fortified fish

The assessment of consumers' perception and receptiveness of a new food item is an essential step of the product development process. In our case the use of dietary fortification could undermine consumers' opinion if it was perceived as an unnatural manipulation of fish. Within our work, two approaches were used to infer consumers' acceptance of fortified fish, one performed in theoretical terms using an on-line questionnaire and the second based in real-time events with sensorial evaluation of fish after the fortification trial.

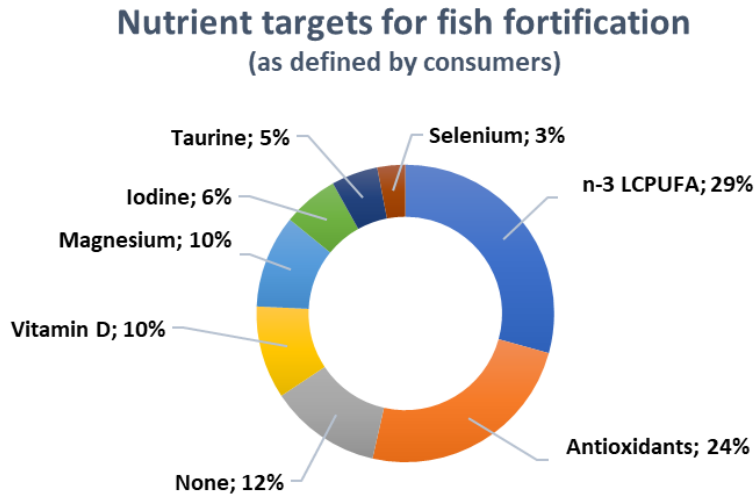
On-line questionnaires are nowadays the simpler and most cost-effective way to implement consumers' survey with high participation rates (Chapter 2). The main objectives of this survey were to assess consumers' receptiveness to the concept of fortified fish and identify relevant health beneficial nutrients worth for a target fortification.

52% of respondents agreed with fish fortification and 50% showed willingness to consume fortified fish

Respondents with low interest on fish origin were more receptive to fortification

A similar positive opinion concerning fortified seabream was also obtained in a recent survey targeting professional users of aquaculture fish (i.e. hotels, schools, etc.), in which it was stated a high purchasing intention if the product was currently available in the market (Pereira et al., 2016). Our study showed also that undecided consumers seem to shift into a positive opinion depending on the availability of additional contextual knowledge. Several participants asked for more information concerning fortification processes and specific health benefits obtained by consuming fortified fish. An effective and clear communication of farming fortification practices and health benefits were suggested to increase consumers' acceptance of both farmed fish and functional foods (Annunziata and Vechio, 2013; Siró et al., 2008). Communicating fortification in a transparent way, as well as the health benefits associated with the consumption of such products, may be an efficient approach to increase awareness and trust in the process.

The selection of the fortification compounds seems to be highly relevant. The most elected compounds for fortification, omega-3 and anti-oxidants, correspond to a natural pre-existing association of fish with omega-3 (Krutulyte et al., 2011). Several studies show the importance for consumers, and the higher success of a functional food when enriched with an already present nutrient. These factors would dismiss the fear of off-flavours and increase the perception of naturalness of final product (Grunert, 2005; 2010). Within the same context, the supplementation of fish feeds with algae biomasses, either micro or seaweeds (macroalgae), could contribute to a more natural-like image of aquaculture products alleviating the manipulation impression that fortification may suggest.



*Figure 7.2. Consumers' selected nutrients for fish fortification*

Price also acted as an important determinant regarding purchasing intentions. Consumers willing to buy fortified seabream tended to decrease if the product was presented at a higher price than traditionally farmed seabream. Several studies describe that consumers are increasingly valuing aspects related to enhanced animal-welfare, environmentally sustainable farming practices, nutritional and health benefits of farmed fish (Maynard and Franklin, 2003; Olesen et al., 2010; Whitmarsh and Palmieri, 2011).

Overall data from our survey, indicates that fortification is a promising tool to diversify the commercialization of farmed seabream into niche market segments

However, attention should be paid to consumer expectations towards an increase of price and the image of unnatural manipulation

The positive receptiveness towards fortified fish prompt us to conduct a set of experimental feeding trials targeting the fillet fortification of gilthead seabream and rainbow trout with several health beneficial nutrients. Therefore, the second approach used to evaluate consumer's acceptance of fortified fish comprised the sensory evaluation of fish by a trained panel after every experimental fortification trial. In a broad sense, the trials comprised the

fortification of gilthead seabream and rainbow trout fillets with various sources, doses and forms of iodine and selenium (Chapter 3); various forms and doses of vitamin D3 (Chapter 4); and various doses of fucoxanthin, a natural antioxidant carotenoid supplied by the microalgae (*Phaeodactylum tricornutum*) (Chapter 6). Despite some distinctions among the methods used for the various sensorial assessment tests, the main evaluated attributes were typical odour, flavour, white colour and texture.

Overall results showed that trained panels found no differences on organoleptic properties of fortified fillets in comparison with respective control groups

Fortification strategies used along our studies did not jeopardize consumers' expectations towards seabream and trout sensory traits

## 7.2 Strategies to fortify seabream and trout muscle

The fortification strategies were planned based on relevant aquaculture species and pertinent fortification nutrients. Two major cultivated species in Europe: a freshwater species – rainbow trout and a marine species – gilthead seabream, were chosen by their distinct physiologic characteristics, which allowed comparing and observing differences on muscle deposition as response to increased dietary levels of several nutrients.

The fortification nutrients were selected based on two criteria:

- Identified by consumer survey: **antioxidants (fucoxanthin)**;
- Nutrients with relevant health beneficial effects: **vitamin D<sub>3</sub>, iodine and selenium**, whose concentrations in fish might be reduced due to a lower use of fishmeal and fish oil in aquafeeds; and are currently associated to an inadequate status in several segments of the human population.

Moreover, it was considered important to whenever possible evaluate the use of natural rich-sources of each nutrient (e.g. macroalgae for iodine, yeast for selenium and microalgae for fucoxanthin). Not only because of its potential higher bio-efficacy in fish but also to promote a broader concept of sustainable and natural-like products, highly appealing to consumers.

#### IODINE FORTIFICATION

In comparison to the control diet, with an iodine level usually found in commercial feeds (2.4 mg/kg feed), both potassium iodide and the organic salt ethylenediamine dihydroiodide supplemented at the maximum authorised level (20 mg/kg feed), resulted in a 1.3-fold increase of iodine content in seabream fillets (Chapter 3).

The dietary use of an iodine-rich brown algae *Laminaria digitata* was a highly effective strategy to significantly enhance the iodine content in seabream and trout fillets



Feeding **seabream** with a diet containing 10% of *Laminaria digitata* led to a 6.5-fold increase of fillet iodine content

In **trout**, a diet containing 0.4% of *Laminaria digitata* resulted in a 6-fold increase of fillets iodine content in relation to control fish

At similar supplementation levels, the maximum fillet iodine content found in seabream (0.17 mg/kg) was higher than that found in trout (0.12 mg/kg), which is probably associated to the physiological needs for homeostasis of freshwater and marine fish.

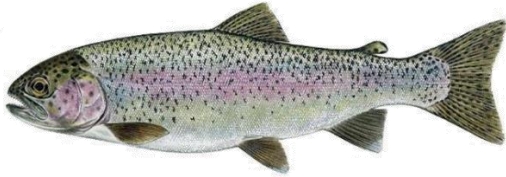
No saturation on iodine muscle levels is apparently observed, at least with the tested doses, even after supplementing a mega dietary dose of 10% *Laminaria digitata* in seabream. In

adult Atlantic salmon fed with graded dietary levels of potassium iodide (KI) a threshold was also not displayed (Julshamn et al., 2006). In the same trial, and as observed in seabream experiment, feed fortification did not result in proportional increased concentrations in muscle. Based on the described experimental studies, it seems that fish do accumulate iodine in the muscle with increased dietary levels, but deposition rate does not proportionally respond to the intake levels, independently of the iodine source used. Moreover, even when exceeding the maximum authorised level in fish feeds, it was not possible to reach the muscle iodine values found in several wild species (Julshamn et al., 2001; Maage et al., 1991). Differences on the bioavailability or metabolic role between iodine forms, species, size, duration of feeding and surrounding water may also influence iodine deposition in fish. Efficacy of dietary iodine fortification strategies may be more dependent on the targeted fish species than on iodine source used.

Consumers often perceive natural raw materials and additives as inherently better and healthy, while artificial chemicals in food are associated with higher risk perception (Dickson-Spillmann et al., 2011). A consumer survey performed in Germany, UK and Italy showed a general high acceptance for foods with seaweed ingredients (Buehrlen et al., 2005). However, it is worth mentioning that brown macroalgae, such as *Laminaria digitata*, although perceived as a natural product and a rich source of health valuable nutrients (e.g. iodine), may also contain elevated levels of some toxic metals such as arsenic (As) and cadmium (Cd), which may limit their use as feed ingredients. A recent study assessed the potential risks of macroalgae harvested in Norwegian waters when used as feed materials and food for animals and humans (Duinker et al., 2016). Their data showed that several macroalgae species, and particularly brown algae, exceeded the current maximum permitted levels of Cd and As, clearly limiting their use as ingredients in animal feeds, including fish. However, the high variability on As and Cd levels associated to the geographical origin of the macroalgae and the lower toxicity of organic As forms generally occurring in these marine-derived products are aspects that require further studies to fully elucidate the potential risks associated to their use.

### SELENIUM FORTIFICATION

In comparison to the control diet, with a total selenium level usually found in commercial feeds (0.25 mg/kg feed), the use of a selenium-rich yeast supplemented at the maximum authorised total selenium level in the European market (0.5 mg/kg feed) was an effective strategy to enhance the deposition of selenium in the fillets of rainbow trout (Chapter 3).



In trout, a diet supplemented with 0.1% of selenised-yeast resulted in a 2.9-fold increase of fillets selenium content in relation to control fish

Available literature data from land-based animals (e.g. poultry) suggested a potential synergistic effect of iodine on selenium deposition in the muscle (Bargellini et al., 2008). We also tested this hypothesis, but a concomitant supplementation with iodine and selenium did not result in additional deposition of iodine and/or selenium in rainbow trout fillets.

### VITAMIN D<sub>3</sub> FORTIFICATION

Fortification of fish muscle with vitamin D<sub>3</sub> dietary supplementation is, apparently, a more complex process presenting variable results (Chapter 4). In our studies, raising dietary vitamin D<sub>3</sub> from a basal level of 42 µg/kg and 31 µg/kg (trout and seabream control diets, respectively) to the maximum permitted level of 75 µg/kg did not significantly enhance fillet vitamin D<sub>3</sub> contents, which ranged 92-121 µg/kg in trout fillets and 43-57 µg/kg in seabream fillets.



Within the maximum authorised level in fish feeds, an increase of dietary vitamin D<sub>3</sub> was not an effective strategy to significantly fortify trout and seabream fillets with vitamin D<sub>3</sub>

Several studies evidence a large variation on the vitamin D<sub>3</sub> fillet concentrations within the same fish species (Mattila et al., 1995; Lu et al., 2007; Schmid and Walther, 2013), even when fed an identical diet (Mattila et al., 1999). Literature data suggests that increased concentrations of vitamin D<sub>3</sub> in salmon muscle were only positively correlated with a feed fortification strategy, when dietary levels ranged from 32 to 800 times higher than the ones used in our studies (Graff et al., 2002; Horvli, Lie and Aksnes, 1998). Absolute values of muscle vitamin D<sub>3</sub> content in trout were double of those found in seabream. Being a liposoluble vitamin, the higher dietary fat levels as well as the increased fat content in trout muscle compared to seabream may condition the magnitude of vitamin D<sub>3</sub> deposition in fish muscle.

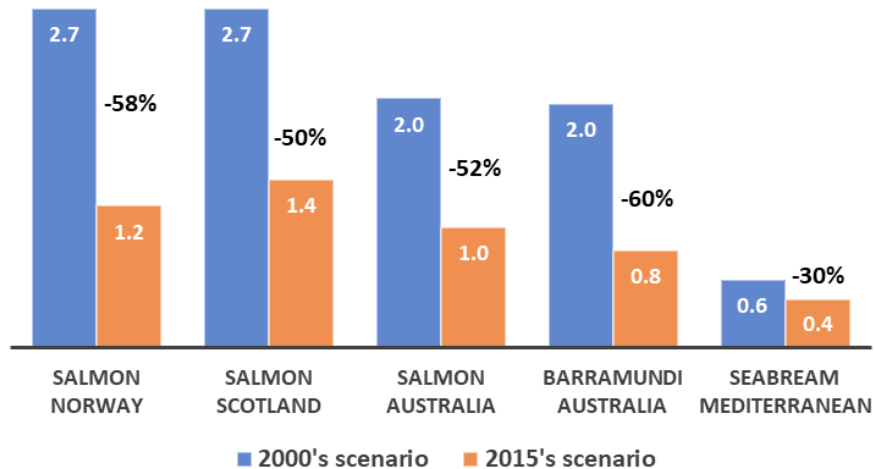
Micronutrients are part of countless molecules, including enzymes and hormones, and play determinant roles in numerous physiological processes. Micronutrients are responsible for the adequate minerals flow, such as calcium and phosphorus that regulates bone health. Therefore, its deposition in muscle are dependent on a delicate balance between the organism needs and its excess, sometimes toxic at very high levels. When considering muscle fortification with dietary micronutrients, it is essential to regard the requirement of the individual element, potential interactions with other inorganic elements and nutrients, the metabolic level in various tissues, and minerals present in the surrounding environment, as well as the species, age, and sex of the fish (Lall, 2003).

Regarding the consumers' perspective of fortification trials, the natural/organic sources used were equally or more efficacious than the synthetic ones and are then considerate as an adequate alternative. Knowing that European consumers showed a general high acceptance for foods with seaweed ingredients (Buehrle et al., 2005), the use of algae as part of a dietary bio-fortification strategy of farmed fish shows great potential in delivering tailored fish with health valuable nutrients to consumers.

### 7.3 Improving the retention of n-3 LCPUFA

Marine-derived raw materials traditionally used in aquafeeds (e.g. fishmeal and fish oil) are rich sources of n-3 LCPUFA, and therefore contribute significantly to the high levels of EPA and DHA generally found in farmed fish. However, due to environmental and economic constraints, the aquaculture industry has made a successful shift towards the use of feeds based on alternative ingredients of vegetable origin (Turchini et al., 2009; Torstensen and Tocher, 2010; Waite et al., 2014). Current industrial aquafeeds generally contain a blend of vegetable oils, fish oils (and in some cases animal fats). Although variable with species, growth stage and season, it is estimated that fish oil is currently maintained at approximately 30% of the total dietary oil(s) supply. New sources of EPA and DHA are currently being evaluated (Turchini et al., 2010; Betancor et al., 2016; Spargue et al., 2017), being the interest and market potential for these non-marine sources of n-3 LCPUFA rich oils unquestionable. However, the critical question is when will they become commercially relevant in terms of volumes and costs for the aquafeed sector. In the short-term fish oil will continue to be the sole source of n-3 LCPUFA in commercial fish feeds and vegetable oils will probably be the main choice for replacing fish oil.

To a large extent, the fatty acid profile of fish muscle mimics the fatty acid composition of the feed. Therefore, the trend towards a replacement of fish oil by alternative lipid sources, such as vegetable oils and animal fats, is interfering with the fish nutritional profile and consequently lowering consumer expected intakes of health valuable nutrients, such as n-3 LCPUFAs.



**Figure 7.3.** Changes on the EPA and DHA content (g/100g) in fish fillets between the years 2000' and 2015.

Source: Nifes (2016), Sprague et al. (2016), Nichols et al. (2014), Vasconi et al. (2017)

An important aspect is the relatively low retention of dietary EPA and DHA found in fish. In Atlantic salmon, 46% of EPA+DHA intake is retained in the whole-body, which conversely represents a 54% loss or catabolism of the dietary supply (Glencross et al., 2014; Ytrestøyl et al., 2015). If these retention values are expressed on a fillet basis, the direct edible part of salmon, then retention of EPA+DHA is reduced to 26% of intake (Ytrestøyl et al., 2015). This low retention of scarce resources such as EPA and DHA compels the aquaculture industry to search for more efficient strategies for the utilization of dietary n-3 LCPUFA.

To tackle this low retention of dietary EPA and DHA in fish, our work was not directly focused on a fortification concept, but rather focused on a nutritional strategy to foster the metabolic sparing of n-3 LCPUFA in gilthead seabream fillets (Chapter 5). The fatty acid composition of tissues is not solely dependent on the quantitative fatty composition of feeds, but is affected also by their relative ratios, since it affects their metabolic fate as energy substrates, bioconversion potential or *de novo* synthesis. Previous studies, mainly with salmonids, show also that feeds rich in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), alpha-linolenic acid (ALA, 18:3n-3), or even cholesterol yielded a better efficiency on the retention of n-3 LCPUFA (Codabaccus et al., 2012; Turchini and Francis, 2009; Nunez-Ortiz et

al., 2016; Rombenso et al., 2015; Salini et al., 2017; Tocher, 2015). In most of these studies, the hypothesis used to justify the higher retention of n-3 PUFA is the preferential peroxisomal chain shortening ( $\beta$ -oxidation) of the SFA and MUFA fractions.

In our study with seabream, we compared the EPA and DHA retention efficiency of high n-3 LCPUFA diets (100% fish oil) against MUFA and linoleic acid (LA, C18:2n-6) rich diets (a blend of 75% fish oil and 25% poultry fat). Moreover, given the large variations that exist in the formulation of commercial seabream feeds, the efficacy of these two oil blends was assessed in fish previously fed with distinct formulation scenarios (namely rich in fishmeal and rich in fish oil (FM), rich in non-ruminant animal processed proteins and with low levels of fish oil (PAP) and rich in plant-protein sources and low levels of fish oil (PP)), and therefore with variable initial fillet fatty acid compositions.



Retention of combined EPA and DHA in seabream fillets is relatively low (17-25% of intake)

Retention of DHA is generally more than double that of EPA

The initial fatty acid composition of fish seems to play a significant role on the EPA and DHA sparing efficiency by a higher MUFA intake, since a significant increase on EPA and DHA retention was only observed in fish originated from the PP nutritional background

#### 7.4 Improving skin pigmentation

In markets where fish is commercialized as whole-fish, visual cues such as body shape, external pigmentation patterns and perception of freshness are considered important consumer purchasing determinants (Vasconcellos et al. 2013; Colihueque and Araneda 2014). As observed in our questionnaire (Chapter 2), freshness, quality and flavour were the most valorised attributes of fish. By improving the external pigmentation of aquaculture fish, it is possible to positively impact on consumers' receptiveness and product market value (Sacton, 1986; Vasconcellos et al., 2013).

A study was undertaken to assess the potential of microalgae biomass from the diatom *Phaeodactylum tricornutum* as a functional ingredient in seabream feeds (Chapter 5). This marine diatom is characterized by high levels of EPA and fucoxanthin, an orange-coloured antioxidant carotenoid (Reboloso-Fuentes et al. 2001; Peng et al. 2011; Kim et al. 2012). *Phaeodactylum tricornutum* incorporation at 2.5% had no effect on growth performance and no impact on odour, flavour, whiteness, and fatness perception in cooked seabream fillets. However, microalgae-fed fish showed a reduction of whole-body fat, probably occurring at the level of perivisceral fat (a preferential fat deposition site in seabream), since no differences were found on the fillet fat content. Fucoxanthin, the major carotenoid present in *Phaeodactylum tricornutum* has been associated with lower accumulation of abdominal white adipose tissue in rodents (Ha and Kim, 2013; Maeda, 2015; Peng et al., 2011). In these studies, fucoxanthin was linked to a depression of lipogenic enzyme activity and an increase in fatty acid oxidation. Whether the lipid-lowering effect of microalgae feeds in seabream is associated to the dietary fucoxanthin content requires further studies.



Colour analysis showed that algae-fed seabream had a more vivid yellow pigmentation of the operculum and a lighter colouration of ventral skin

An untrained consumer panel showed a clear preference for algae-fed fish when compared with those fed a commercial

Being an emergent industry, microalgae biomasses are not yet cost-competitive as bulk ingredients in current aquafeeds (Chauton et al., 2015; Williams and Laurens, 2010). However, the future presents new consumer-driven challenges that are shifting the focus away from cost minimization production strategies, towards enhanced consumer confidence and product value. Therefore, microalgae biomasses may have a great potential in the animal feed sector as a functional ingredient (products incorporated in the feed to convey a benefit

above and beyond fulfilling the basic nutritional needs). The feed industry is constantly seeking innovative nutritional ingredients that could add value (nutritional or other) to their traditional feeds during sensitive periods of the production cycle or specific situations resulting from farming practices. Microalgae can serve these needs, since they show a high nutritional value, but are also perceived as part of the natural feeding habits of fish and therefore contribute to the sustainable and nature-like image of farmed fish.

### 7.5 Modulating the nutritional contribution of fish

Being a rich source of important nutrients, including highly digestible proteins, vitamins (A, D, niacin and B12), trace minerals (iodine, selenium) and n-3 LCPUFA (EPA and DHA), eating fish is considered to be a healthy dietary choice (EFSA, 2015d). The Dietary Reference Intakes (DRIs) are nutrient reference values that serve as guidelines for a given nutrient which must be consumed on a regular basis (generally daily) to maintain a healthy condition. The nutritional value of a given food item is often presented as its contribution, on a typical meal portion, to fulfil the DRI of a specific nutrient. Moreover, in the European market, a food item can be labelled as “source of” if it supplies 15% of the DRI in 100g of product and “high in” if it supplies more than 30% of DRI.

Along the various trials with seabream and trout, the nutritional contribution of the traditional and fortified fish was calculated for several health-valuable nutrients such as iodine, selenium, vitamin D<sub>3</sub> and n-3 LCPUFA (EPA and DHA). In all calculations and supported by literature data, we have considered an average meal portion size of 160 grams fillets (assuming a 40% fillet yield in a market size fish of 400 grams).

A 160 g meal of fortified **trout fillets** contributes to the Daily Recommended Intake of:

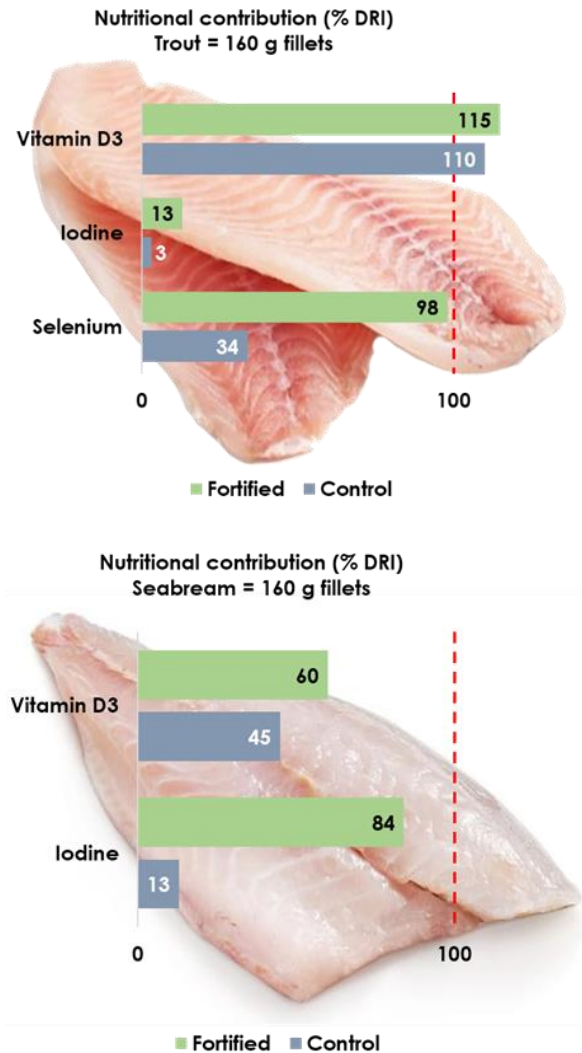
- selenium (98%)
- vitamin D<sub>3</sub> (>100%)
- iodine (13%)

Fortified trout could be labelled as “high in selenium and high in vitamin D<sub>3</sub>” under the EFSA definition for a functional food

A 160 g meal of fortified **seabream fillets** contributes to the Daily Recommended Intake of:

- vitamin D<sub>3</sub> (60%)
- iodine (84%)

When fed the 10% Laminaria diet, fortified seabream could be labelled as “high in iodine and high in vitamin D<sub>3</sub>” under the EFSA definition for a functional food



*Figure 7.4. Nutritional contribution of trout and seabream fortified fillets (160g) to Daily Recommended Intake.*

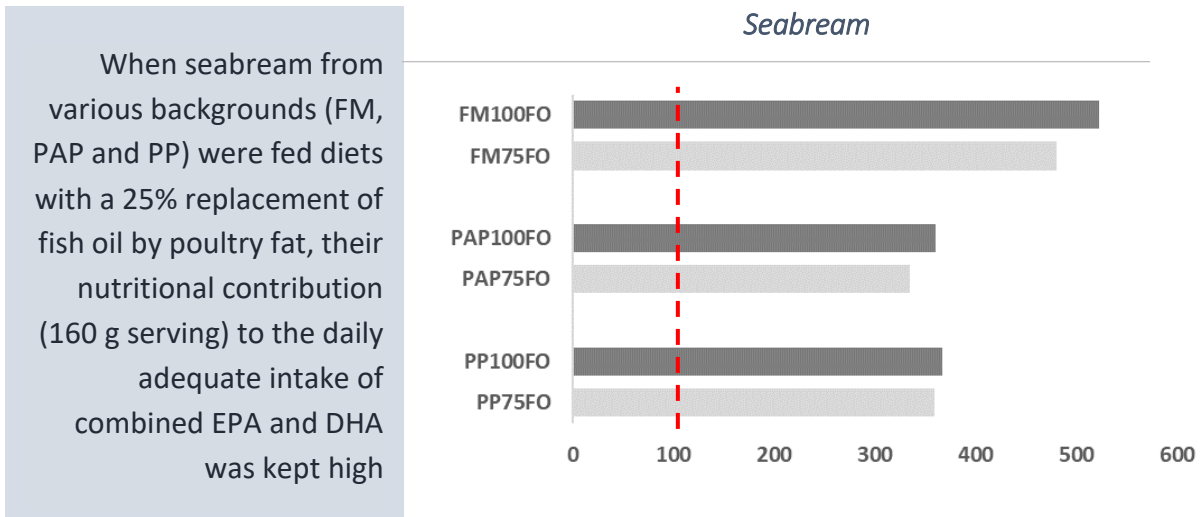
However, when inferring the nutritional value of a food item, we should not forget that the exact intake values of a given nutrient can be affected by culinary methods. Although only assessed in the iodine fortification trial with seabream, we observed that steam-cooking caused an average iodine loss of 13% in fillets of the Laminaria-fed fish and 20% in the control treatment. Moreover, an important message to consumers is that the consumption of fish fillets with skin may increase the ingested amount of iodine and therefore improve its nutritional contribution to an adequate intake level.

The fortification strategies enhanced the nutritional contribution of seabream and trout fillets in health beneficial compounds, such as iodine and selenium, resulting in novel products, candidates to the functional foods market

From a consumers' perspective, seafood nutritional contribution is primarily focused on n-3 LCPUFA contents. The International Society for the Study of Fatty Acids and Lipids recommends a minimum intake of combined EPA and DHA of 500 mg per day (ISSFAL, 2004) for cardiovascular preventive effects.

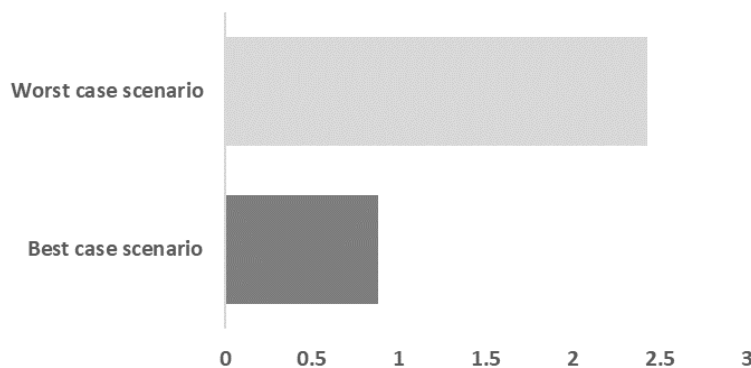


Figure 7.5. Nutritional contribution of trout and seabream fillets (160 g serving) to EPA and DHA Daily Recommended Intake



*Figure 7.6. (continuation) Nutritional contribution of trout and seabream fillets (160g serving) to EPA and DHA Daily Recommended Intake*

This data on the nutritional contribution of farmed trout and seabream to the daily adequate intake of combined EPA and DHA, should be put into a consumer perspective, since fish is not usually part of a daily consumption pattern. If we recalculate the same data on a weekly basis, we can see that consuming one serving per week of fish from the best-case scenario would be enough to guarantee the weekly adequate EPA and DHA intake, while with fish from the worst-case scenario, the adequate intake would only be covered with 2.5 meals per week.



*Figure 7.7. Number of fish servings (160 g) required to obtain a weekly intake of 3.5g EPA+DHA (ISSFAL, 2004)*

To guarantee the feasibility and success of a fish fortification strategy by changing the composition of the feeds, special attention should also be paid to a set of criteria associated to its implementation at the industrial level. The supplemental raw materials or additives should be easily available in the market, minimize the increase in formulation costs, comply with all legal framework and should not implicate radical changes on traditional manufacturing process. Within the various scenarios tested, the potential for industrial development of the selenium and iodine fortification strategies is high and without any clear barriers. However, the use of 2.5% *Phaeodactylum*, the fucoxanthin-rich microalgae, is clearly not feasible at this moment given the strong impact on formulation costs and the anticipated difficulties on a reliable quantitative supply of this raw material.

**Table 7.1.** Fortification strategies scenarios for industrial application

Fortification scenario	Selenium Yeast	Iodine Macroalgae	Fucoxanthin Microalgae
Traditional feed formulation cost, €/ton	1000.00	1000.00	1000.00
Functional additive, €/kg	14.70	8.12	20.00
Incorporation rate, %	0.01	0.40	2.50
Supplementation cost, €/ton	1.47	32.91	500.00
Fortified feed formulation cost, €/ton	1001.47	1032.91	1500.00
<b>Risk associated to cost increase</b>	<b>LOW</b>	<b>LOW</b>	<b>HIGH</b>
Effects on stability of feed components	NO	NO	NO
Need to alter manufacture process	NO	NO	NO
Market availability of functional additive	YES	YES	Unknown
Legal compliance guaranteed	YES	YES	YES
<b>Potential for industrial development</b>	<b>HIGH</b>	<b>HIGH</b>	<b>LOW</b>
<b>GO/NO GO decision on development</b>	<b>GO</b>	<b>GO</b>	<b>NO GO</b>

Although some trade-offs must be made while pursuing the sustainable intensification of aquaculture, achieving a continuously evolving balance on the cost-effective production of high quality and safe fish, with high environmental standards is a stimulating challenge for the aquafeed industry.



# CONCLUSIONS AND FUTURE PERSPECTIVES

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## Chapter 8



## Conclusions

Due to the rising demand for nutritious, safe and sustainable seafood products (fish and shellfish), aquaculture is predicted to be a major contributor in fulfilling the nutritional needs of future generations.

The dietary modulation of fish feeds directly impacts the nutritional quality of fish fillets and available nutrients for consumers. Simultaneously, care must be taken about consumers' perception of fortification strategies in farmed fish.

The work undertaken in this thesis highlights the following conclusions:

- Consumers seem to be positively receptive to the concept of fortified fish. 50 % of the enquiries would buy and consume seabream fortified with health beneficial compounds.
- The farmed fortified fish should be aimed to younger consumers with a profile more receptive to farmed fish and that also valorise flavour and convenience aspects of food products.
- The efficacy of fortification strategies is a multifactorial process, shown to be dependent on the nutrient selected, dose supplied and species. Feed fortification, at the levels applied, did not impaired animal growth performance and feed utilization.
- The fortification strategies used successfully increased seabream and trout muscle levels in iodine and selenium, strongly improving farmed fish nutritional value. The daily recommended values for these nutrients would be totally or at least to a large extent covered with the consumption of a 160 g fillet dose.
- Although economically not yet feasible in economical terms, seabream fed a diet supplemented with 2.5 % of *Phaeodactylum tricornutum* microalgae, presented a more vivid operculum pigmentation and their external appearance was positively scored by an untrained consumer panel.
- We have shown for the first time in seabream, that the retention of combined EPA and DHA in fillets is relatively low (17-25% of intake), with DHA being retained at much higher levels than EPA. Moreover, it was demonstrated that the initial fatty acid composition of fish seems to play a significant role on the EPA and DHA sparing efficiency by a higher MUFA intake.

- Using a combination of 75% fish oil with 25% poultry fat did not jeopardise the nutritional value of seabream fillets was still high and could cover the daily recommended intake of combined EPA and DHA for prevention of cardiovascular diseases.

This thesis generated relevant knowledge on fish fortification strategies that may be readily applicable on current production practices in aquaculture, originating novel products with high nutritional value.

## Future Perspectives

The world will be very different in 2050: the global population will likely increase to nine billion, and about 50% more food will be needed to sustain the quality of human life. As one of the most efficient food production systems, aquaculture is undoubtedly part of the solution, not only from a nutritional point of view, but also from economic and social standpoints.

Minimizing the health burden of specific nutrient deficiencies, such as those of minerals and some vitamins is technically and economically feasible through the fortification of fish feeds and consequently of the fish. However, current levels of selected micronutrients in fish feeds are defined based on their adequate coverage of the nutritional and welfare requirements of fish and in some cases limited by the potential detrimental impact to the environment.

**We believe that consumers dietary needs and expectations must gain relevancy when formulating tomorrow's aquafeeds**

The total seafood supplied by aquaculture will increase from 66.6 million tons in 2015 to approximately 100 million tons in 2030. The global aquafeed market was estimated at 39.9 million tons in 2017 and is forecasted to double by 2025. However, the basis for this growth of the aquaculture sector are not yet guaranteed. Future limitations on the access and scarcity of freshwater compels aquaculture to grow in a marine environment. In this context, we should not forget that both EPA and DHA are essential fatty acids for marine fish species and therefore need to be supplied by the feed. We urgently need to identify new sustainable sources of proteins and n-3 LCPUFA-rich oils to sustain the predicted growth of aquaculture.

Several novel ingredients are emerging as alternative protein sources (e.g. microalgae biomasses, insect meals, methanotrophic bacteria, lignocellulosic fermentation bacteria, yeasts biomasses, etc.). **The impact of these emerging raw materials on the various aspects of fish quality and specially those associated to the health of consumers needs to be assessed.**

Additionally, some of these novel protein sources are easily tailored in specific nutrients (mainly minerals and vitamins) during their production process. Therefore, **the development of these new ingredients should integrate the aquafeed industry needs, to jointly define the specifications for selected health valuable nutrients.**

From a consumers' perspective, seafood nutritional contribution is primarily focused on n-3 LCPUFA contents. Fish oil is currently the only practical and economically viable source of these essential fats for feed purposes. Over the last two decades, aquaculture has grown due to a significant reduction of fish oil levels in feeds and a concomitant increase of vegetable oils. Future growth under this scenario will surely lower the content of n-3 LCPUFA in farmed seafood, with detrimental implications for consumers health. The scarcity of EPA and DHA is probably the most critical challenge that aquaculture will have to face.

To tackle this challenge, both academia and industry are devoting a significant research effort on the development and evaluation of new sources of EPA and DHA (e.g. oils from microalgae, microbial biomasses, transgenic plants, mesopelagic fisheries). Although not economically viable at the moment, **the market potential for these novel sources of n-3 LCPUFA rich oils is unquestionable and knowledge generation that supports the growth of these products is needed.**

The low retention dietary EPA and DHA in fish tissues (around 45% at the whole-body level, and 20-25% in edible fillets) is a topic that deserves further studies. **Optimization of the dietary balance between n-3 LCPUFA and the fractions of saturated and monounsaturated fatty acids in order to enhance the sparing of EPA and DHA by fish** is an approach that has the potential to significantly reduce the need for fish oil, while minimizing the losses of EPA and DHA content in fish. Additionally, **the genetic selection of strains of farmed marine fish with enhanced capacities for n-3 LCPUFA biosynthesis and retention: or the metabolic programming of fish by epigenetic mechanisms, to lower their n-3 LCPUFA requirements, are interesting avenues for future research.**

Today, there is still a clear separation between studies conducted by fish nutritionists, in which a set of biochemical criteria tend to define the nutritional value of fish in the context of consumers health, and those conducted by human nutritionists that separately assess the potential effects of fish consumption in several aspects of human health. Although both experts in their fields of action, they probably only grasp the full implications and constraints of each other work. **A closer interaction between fish and human nutritionists could lead to better definition of the ideal thresholds for specific nutrients with relevancy to human health and help jointly define feed specifications and farming practices to attain it.**

As shown in our work, feed fortification can be a useful tool to increase fish nutritional value and deliver a food item with improved quality and increased beneficial properties to consumer health and well-being. If done with a minimal impact on production costs, such solutions could be implemented in mainstream markets without major risks. However, if properly managed in terms of a clear and effective communication strategy, these **fortified fish could also enter the growing market of functional foods, contributing to the differentiation and innovation of aquaculture products.**



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Chapter 9



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