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Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
Recursos Biológicos Aquáticos

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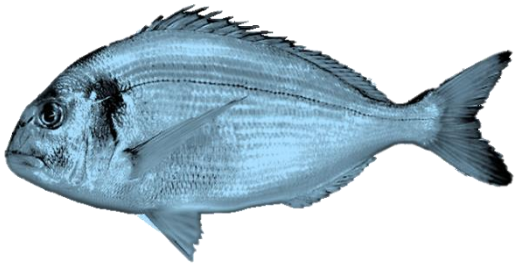

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Mestrado em Recursos Biológicos Aquáticos
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2016

Orientador
Ana Isabel Santos Couto, Investigadora Pós-Doutoramento, CIIMAR

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O Presidente do Júri,
Porto, _____/_____/_____

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O Presidente do Júri,

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Abstract

The world`s fish stocks are fully or overexplored, so the investment in aquaculture was inevitable. The witnessed rise of global human population, associated with the economic crisis experienced, lead to the decline of the principal ingredient resources utilized in aquafeeds: fishmeal and fish oil. This lead to the pursuit of alternative ingredients, such as plant feedstuffs that, although not ideal for carnivorous fish species cultivated, are already used to replace a large percentage of marine derived feedstuffs in aquafeeds.

Nevertheless, the need to find new alternative nutrient sources to formulate aquafeeds that allow the development of a sustainable and profitable aquaculture while maintaining fish health is still a reality. Microalgae present themselves as good alternative ingredients to provide macro nutrients but are also interesting sources of bioactive compounds. Anti-inflammatory, antioxidant, antitumoral are some of the effects ascribed to microalgae bioactive molecules. Microalgae biomass is not yet produced in quantities suitable to be used as bulk ingredients in aquafeeds but can be supplemented to fish diets as functional ingredients capable of improving fish health and welfare.

This study aimed at screening some of the effects of dietary microalgae in gilthead seabream (*Sparus aurata*), by evaluating fish growth, feed utilization, intestinal morphology and function as well as microbiota. The microalgae chosen to test as a functional ingredient on Gilthead seabream diets was *Nannochloropsis sp*., due to its availability and stage of production technology development.

To do so a 37 days short-term trial was performed in a thermoregulated seawater system with 60g *Sparus aurata* juveniles. A control diet (45% C.P., and 18 % C.L.) with low fish meal (20%) inclusion was formulated along with three experimental diets produced with similar composition to control diet but supplemented with *Nannochloropsis sp.*: 0.5% (Nanno 0.5), 0.75% (Nanno 0.75) and 1.5% (Nanno 1.5).

Although not statistically significant, an increase in fish daily growth index and final body weight was verified with the inclusion of the microalgae in the diets. No significant differences were found in the histomorphological evaluation. Digestive function was also statistically similar among experimental groups. In general, the Control

group showed a tendency towards higher digestive enzyme activity, immediately followed by Nanno 0.75.

Microbiota was modulated by microalgae supplementation, with Nanno 0.75 showing higher indices for richness, diversity and OTUs. Bacterial communities for each dietary treatment, demonstrated that fish fed the Nanno 0.5 and Nanno 0.75 diet were more closely related (similarity higher than 90%), while those from Nanno 1.5 and control diet diverge more (similarity bellow 90%).

In fish offered the Nanno 1.5 diet the simper similarity was higher and the replicates clustered more, revealing a homogeneous response from bacterial communities to the dietary treatment.

Nannochloropsis supplementation to a low FM diet for sea bream did not significantly improved the parameters analyzed and had no deleterious effects either. The trend towards improved growth performance should be evaluated in a growth trial and microbiota modulation should be further investigated to understand if it could it be advantageous for the animals. Nevertheless, the results achieved in this study are promising, supporting the use of microalgae as a functional ingredient for marine carnivorous fish aquafeeds.

Keywords: Aquaculture; Gilthead seabream; Aquafeeds; Microalgae; Functional feeds

Resumo

Os recursos pesqueiros a nível mundial encontram-se completamente ou sobre-explorados. O aumento da população humana, associado à crise económica vivenciada, levou ao declínio dos principais recursos de ingredientes utilizados nas dietas de organismos aquáticos: farinha e óleo de peixe. Isto conduziu à procura de ingredientes alternativos, como os ingredientes vegetais que, apesar de não serem ideais para as espécies de peixes carnívoros cultivadas, já são usados para substituir uma percentagem elevada de ingredientes marinhos nas dietas de organismos aquáticos.

No entanto, a necessidade em encontrar novas fontes alternativas de nutrientes para formular as dietas de organismos aquáticos que permitam o desenvolvimento de

uma aquacultura sustentável e rentável enquanto se mantém a saúde do peixe é ainda uma realidade. As microalgas apresentam-se como bons ingredientes alternativos para fornecer macronutrientes mas também são fontes interessantes de compostos bioativos. Anti-inflamatório, antioxidante, antitumoral são alguns dos efeitos atribuídos às moléculas bioativas das microalgas. A biomassa de microalgas ainda não é produzida em quantidades adequadas para ser utilizada como ingrediente principal nas dietas de organismos aquáticos mas podem ser suplementadas às dietas dos peixes como ingredientes funcionais capazes de melhorar a saúde e o bem-estar do peixe.

Este estudo destinou-se a encontrar alguns dos efeitos das microalgas incluídas em dietas de dourada (*Sparus aurata*), através da avaliação do crescimento, utilização do alimento, morfologia e função intestinal, bem como na modulação da microbiota intestinal. A microalga selecionada para ser testada como ingrediente funcional nas dietas de dourada foi *Nannochloropsis sp.*, devido à sua disponibilidade e ao estado do desenvolvimento tecnológico da produção.

Um ensaio de curta duração (37 dias) foi realizado num sistema termorregulado de água salgada com juvenis de 60g de *Sparus aurata*. Uma dieta controlo (45% proteína bruta e 18% gordura bruta) com inclusão de quantidades reduzidas de farinha de peixe (15%) foi formulada juntamente com três dietas experimentais produzidas com composição semelhante à dieta controlo, mas suplementadas com *Nannochloropsis sp.*: 0.5% (Nanno 0.5), 0.75% (Nanno 0.75) e 1.5% (Nanno 1.5).

Apesar de não ser estatisticamente significativo, foi verificado um aumento na taxa diária de crescimento e no peso corporal final dos peixes com a inclusão da microalga nas dietas. Não foram encontradas diferenças significativas na avaliação histomorfológica. A função digestiva também foi estatisticamente semelhante entre os grupos experimentais. Em geral, o grupo Controlo demonstrou uma tendência para uma atividade mais elevada das enzimas digestivas, imediatamente seguido por Nanno 0.75.

A microbiota foi modulada pela suplementação com microalga, com Nanno 0.75 a demonstrar índices mais elevados para a riqueza, diversidade e OTUs. As comunidades bacterianas demonstraram que os peixes alimentados com as dietas Nanno 0.5 e Nanno 1.5 estavam mais fortemente relacionados (similaridade acima de 90%), enquanto os das dietas Nanno 1.5 e controlo divergiram mais (similaridade inferior a 90%).

Nos peixes alimentados com Nanno 1.5 a análise de similaridade de percentagens (SIMPER) foi superior e os replicados agruparam-se mais, demonstrando uma reposta homogénea da comunidade bacteriana à dieta.

A suplementação de uma dieta com reduzida farinha de peixe com *Nannochloropsis* não melhorou significativamente os parâmetros analisados, mas também não provocou efeitos adversos. A tendência para melhor crescimento deve ser avaliada num ensaio de crescimento mais extenso e a modulação da microbiota deve ser futuramente investigada para perceber se poderá trazer vantagens para os animais. No entanto, os resultados obtidos neste estudo são promissores, apoiando a utilização das microalgas como ingredientes funcionais em dietas de peixes marinhos carnívoros.

Palavras – chave: Aquacultura; Dourada; Dietas de organismos aquáticos; Microalgas; Dietas funcionais

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Abbreviations

ABP: Animals by products

CL: Crude Lipids

CP: Crude Protein

CO_2: Carbon Dioxide

DHA: Docosahexaenoic acid

DGGE: Denaturing Gradient Gel Electrophoresis

DI: Distal intestine

DM: Dry matter

EAA: Essential amino acids

EGCs: Eosinophilic granular cells

EPA: Eicosapentaenoic acid

FBW: Final body weight

Fig: Figure

FM: Fish meal

FO: Fish oil

g: Grams

Kg: Kilograms

HUFA: Highly unsaturated fatty acids

IBW: Initial body weight

IELs: Intraepithelial leucocytes

LC – PUFA: Long-chain polyunsaturated fatty acids

mg: Milligrams

O_2: Oxygen

OTUs: Operation taxonomic units

PCR: Polymerase Chain Reaction

SCPs: Single-cell proteins

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Chapter 1: Introduction

1.1 World Aquaculture Today

The traditional activity of Aquaculture is lasting for over a millennia. In this last decades its value for the food production sector was recognized, mostly due to the awareness for the limited ocean resources, and the human needs for supplies with an ever growing population (Holmer, Black *et al.* 2007, Turchini, Ng *et al.* 2010).

The increase of world population and the current global economic recession associated with the over exploitation of marine resources, increased focus on sustainability, which led to a sharp investment in aquaculture (Intiative 2010).

Since the late 1980s, world capture fisheries have been static and aquaculture was responsible for the growth in fish supply for human consumption (Fig 1) (FAO 2016).

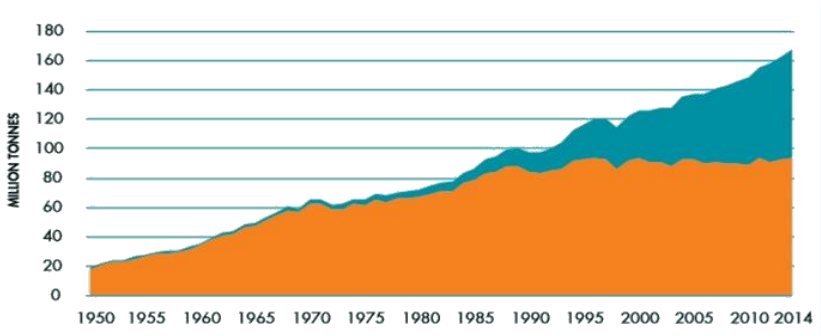

Fig 1: World capture fisheries (orange) and aquaculture production (blue) (FAO 2016).

Over the last decades, aquaculture production of fish and shellfish was the main contributor to the fastest growing food production sector, in response to the high demand for marine products all over the world (Tacon, Hasan *et al.* 2011).

In 2014, globally aquaculture produced 73.8 million tonnes of aquatic animals, while capture fisheries production amounted 93.4 million tonnes (FAO 2016).

In 2012, from the 66.6 million tonnes of farmed fish that were produced, 5.6 million tonnes were mariculture fish, and even though this value seems very insignificant when compared to the total production, these fish are mainly carnivorous, being more economic relevant than most freshwater species (FAO 2014).

The increase of world fish consumption in the last years was very clear, varying between 9.9 kg in the 1960s and 19.7 kg in 2013 (FAO 2016). Aquaculture provides half of all fish for human consumption (Boyd 2015, FAO 2016), and soon will supply more than half of this product (Klinger and Naylor 2012).

For example, it is estimated that by 2030, the world population will exceed 8 billion people and the consumption of seafood reach values between 150 to 160 million tonnes (Borgeson 2005), obligating aquaculture industry to produce more than 65% of the world´s total fish supply (Welch, Hoenig *et al*. 2010).

In the future, aquaculture it's expected to supply the demand for fisheries products because capture fisheries don´t show a tendency to increase and possibly will diminished (Boyd 2015).

Accompanying aquaculture is the industry of aquafeeds (Tacon, Hasan *et al.* 2011). Due to aquaculture intensification, aquafeeds industry possess potential for future growth (Boyd 2015).

Fish feeds formulation is becoming more complex and sustainable with time. It´s expected that aquafeeds ingredients can promote growth, quality and welfare of culture organisms, while reducing production costs and the pressure on ecosystems from which the ingredients are harvested (IUCN 2007).

1.2 Aquafeeds – Status and Prospects

Aquafeeds for carnivorous fish, can use up to five times more fish protein than that produced (Huntington and Hasan 2009) and, traditionally contain high proportions of ingredients derived from marine resources, such as fishmeal (FM) and fish oil (FO). These resources derive from reduction fisheries: small, pelagic, marine fish, like anchovies, menhaden and sardines, or trimmings of fish processing (Intiative 2010). Due to their nutritional quality, FM and FO have been used as the main protein and lipid sources in carnivorous fish aquafeeds (Oliva-Teles, Enes *et al.* 2015).

- FM is characterized by high protein content, a good balance of amino acids, high nutrient digestibility, high palatability, lack of antinutrients, functioning also as a source of high quality lipids and minerals (Oliva-Teles 2012). FO on the other

hand is an optimal source of essential fatty acids, particularly of the important highly unsaturated fatty acids of the n-3 series. Thus feeds containing these ingredients are nutritionally balanced, palatable and readily accepted by a large range of carnivorous fish species (Bendiksen, Johnsen *et al*. 2011).

Although FM production is mainly destined to the production of high - protein feed (FAO 2014), FO is used principally for human consumption for health purposes and as a feed additive (FAO 2014).

- There are some limitations to the production of FM and FO like the fact that their manufacture is subject to environmental influences, the existence of contaminants, and moreover these ingredients are from finite marine resources, having for this reason their production restricted with severe regulation of fishing and catch quotas (Tocher 2015). However, production of FM and FO used in aquaculture diets remained constant in the last 20 years (Intiative 2010), varying their worldwide production around 6 and 1 million tons during this period (Izquierdo, Turkmen *et al*. 2015). The utilization of these ingredients in aquaculture feed formulations in the last decade decreased due to higher prices, which tripled between 2002 and 2010 (Intiative 2010).

Therefore, they are utilized selectively as strategic ingredients at lower concentrations in compound feeds and for specific stages of production like hatchery, broodstock and finishing diets (FAO 2016).

Aquaculture producers are now trying to reduce costs, since feeds typically represent the largest cost in their budgets (Naylor, Hardy *et al.* 2009), symbolizing 50 to 80 % of the total production costs (Turchini, Ng *et al.* 2010). Thus, substantial research efforts are being dedicated to the identification and characterization of alternative ingredients capable of producing fish in a cost effective manner and without decreasing its beneficial effects for human health (Domínguez and Dominguez 2013).

The potential alternative ingredients are divided in three categories: animal rendered by products, plant feedstuffs and single-cell organisms (Oliva-Teles 2012).

Terrestrial animal by-products such as meat and bone meal, and poultry by-products are feed ingredients of good nutritional quality and are available in higher quantity than FM (Olsen and Hasan 2012). Although their usage is less than 1 percent

of total global compound aquafeed production, these feedstuffs are very effective in feeds for a large number of aquatic species (Tacon, Hasan *et al.* 2011). For a long time in many countries the use of by-products from warm-blooded animal in fish feed was forbidden due to the fear of transmissible spongiform encephalopathies disease transmission. Nowadays, this is unlikely to occur because feeds are adequately processed and aren´t used in feeds of the same species. The main problem with these by-products is the relatively high content of bones or exoskeleton resulting in a high ash content, which may interfere with the absorption of trace elements (Olsen and Hasan 2012). The Category 3 ABP law imposed by European Commission delayed research on use of these ingredients to replace FM and FO in aquafeeds, while promoted the look for alternatives within the terrestrial crops available.

During the last decade a great deal of research has successfully increased the use of plant ingredients as FM and FO replacers in aquafeeds. Plant ingredients continue to be the main choice to replace FM and FO in aquaculture diets because of abundance, potential for increased production, and relatively constant low cost (Intiative 2010). However, they have several nutritional drawbacks when compared to marine products, particularly in diets for carnivorous fish (which are not adapted to plant feedstuffs), like low nutrient densities and protein content, high carbohydrate content, imbalanced amino acid and fatty acid profiles, low palatability, low digestibility and the presence of anti-nutritional components that may reduce digestion or absorption of nutrients, neutralize the function of vitamins and may even induce toxicity (CELEUMANS, Coutteau *et al.* 2003, Oliva-Teles 2012, Olsen and Hasan 2012).

Antinutrients compounds can decrease fishes' growth performance, feed efficiency and enzymatic activity (Santigosa, Sánchez *et al.* 2008). Additionally, the high variety of antinutrients in plant feedstuffs make difficult to discover the element responsible for the adverse effects observed in fish fed plant-based diets (Couto, Kortner *et al.* 2014).

When included in carnivorous fish diets, plant feedstuffs cause several negative effects, like changes in gut morphology that could prejudice intestinal absorption (Santigosa, Sánchez *et al*. 2008) as increased vacuolization in enterocytes, hypertrophic intestinal submucosa (Oliva-Teles 2012), enteritis of distal intestine or decrease of immune defense mechanisms and modification of amino acids balance, fillet quality, microbiota and lipids profiles (Oliva-Teles, Enes *et al.* 2015). Nevertheless, plant

feedstuffs are, at the moment, largely used in aquafeeds due to their advantageous prices and availability.

Single-cell proteins (SCPs) are a variety of unicellular and filamentous algae, fungi and bacteria that can be produced by controlled fermentation processes (Perumal, Thirunavukkarasu *et al.* 2015). These feedstuffs possess nutraceutical properties, high palatability, devoid anti-nutritional factors, function as a vitamin B sources and usually are included in diets as probiotics. Nevertheless, SCPs contain high levels of nucleotides (12 – 20% of total N) and methionine generally is the limiting amino acid of these ingredients (Davis 2015). Comparatively to plant and animal feeds, SCPs hold innumerous advantages, since these microorganisms function as a rich protein source (<50%), have short generation time and can be produced continuously in a limited land spaced with good control. Additionally, SCPs can be cultivated based on raw carbon subtracts from industries wastes, which otherwise caused an environmental impact (Davis 2015, Perumal, Thirunavukkarasu *et al.* 2015). Microalgae are the newest SCPs source, with potential for incorporation in fish aquafeeds (Oliva-Teles, Enes *et al.* 2015).

1.2.1 Microalgae

Microalgae are unicellular or colonial photosynthetic organisms, consisting of the most primitive and simple members of the plant kingdom. Found in benthic and litoral waters, algae are the primary producers of the marine food chain (Conde, Balboa *et al.* 2013).

Although the use of microalgae by humans dates back two thousand years ago by the Chinese, the microalgae biotechnology has only been developed in the last century (Spolaore, Joannis-Cassan *et al.* 2006).

Nowadays, it is estimated that in the world there are more than 30,000 species of microalgae, but only some hundred have been studied and cultivated in industrial scale (Christaki, Florou-Paneri *et al.* 2011). Annually, 8000 to 10000 tonnes microalgae are produced to be used in animal feed or as a supplement in human food, and it is estimated that this value continues to rise in the future (Burnell and Allan 2009).

During the last decades, microalgal technology received a lot of attention comparatively to conventional agriculture, since they can achieve higher productivities

than traditional crops and can be used in areas or climates unusable by agriculture (Sousa, Gouveia *et al.* 2008). They can also be produced using non – potable water, like seawater (Das, Thaher *et al.* 2015), and have the capacity to hold the excess atmospheric CO_2 and produce O_2, helping to reduce the greenhouse effect (Christaki, Florou-Paneri *et al*. 2011).

However, microalgae production still holds high production costs associated to the low biomass concentration obtained and a limited production capacity, underlining the need of technological development (Cheirsilp and Torpee 2012, Kousoulaki, Mørkøre *et al.* 2016).

Although there is a gap between the actual microalgae production costs and the target production costs, this is likely to be resolved over the next five to eight years (Reitan 2013).

Currently there are numerous commercial applications for microalgae. In aquaculture, they are seen as an alternative to FM or FO in aquafeeds (Reitan 2013) and used to sophisticate products by improving coloration of some organisms, such as salmon or trout, thus increasing their economic value (Spolaore, Joannis-Cassan *et al*. 2006).

Furthermore, microalgae supply value added products for pharmaceutical products (Mata, Martins *et al.* 2010), cosmetics and food dyes (Spolaore, Joannis-Cassan *et al*. 2006).

They can also be applied as an energy source for methane, biodiesel and biohydrogen production (Mata, Martins *et al*. 2010) or in the wastewater treatment and bioremediation (Roy and Pal 2014).

The complex composition of these algae associated to a rational and integral utilization of them as biomass feedstock, and not forgetting the existent possibility in obtaining a range of value added products from their biomass, involving environmental friendly technologies follows a sustainable approach fitting into the concept of biorefinery (Domínguez and Dominguez 2013).

To be included in fish diets, microalgae need to possess certain attributes such as displaying appropriate nutritional qualities (protein and lipid levels, balanced amino

acid and fatty acids profiles), be highly digestible, promote fast animal growth rates and be possible to mass cultivation (Brown 2002).

The nutritional value of microalgae is related to their ability to provide essential macro and micronutrients for the target animal that will consume them, varying according with the species and being dependent of the culture conditions. Usually, microalgae have approximately 13 – 33% DM total lipids (rich in highly unsaturated fatty acids (HUFA)) and 30 – 50% DM total protein (Vizcaíno, López *et al.* 2014).

These organisms possess a high essential amino acids and protein content, being able to synthetize all type of amino acids (Conde, Balboa *et al*. 2013, Reyes-Becerril, Guardiola *et al.* 2013).

Microalgae contain high-valuable molecules such as long-chain polyunsaturated fatty acids (LC-PUFA), specifically omega-3 and omega-6 (Conde, Balboa *et al*. 2013, Reyes-Becerril, Guardiola *et al.* 2013) and other components with immunostimulant and/or probiotic effects, possessing also high pigments content (Vizcaíno, López *et al.* 2014), being their pigments capable to act as antioxidants and add market value by enhancing fish pigmentation (Gatlin 2007, Burnell and Allan 2009). Microalgae have other nutritional properties extremely relevant as is the case of essential vitamins and minerals content (Reyes-Becerril, Guardiola *et a*l. 2013).

Despite the inherent advantages at the utilization of microalgae in aquaculture, the actual tendency is to avoid them due to their high economic cost and difficulties in production, collection and storage (Spolaore, Joannis-Cassan *et al.* 2006).

Due to their chemical composition, microalgae are running as a renewable reservoir of nutraceutical compounds (Kumari, Kumar *et al.* 2013) and providing essential biomolecules such as polyunsaturated fatty acids (Hemaiswarya, Raja *et al.* 2011). The addition of a small amount of microalgal biomass in fish aquafeeds can compensate for nutritional imbalance and promote fish health and welfare.

Therefore, the appropriate microalgae can be used as ingredient in carnivorous fish diet formulations, not only to provide macro- and micro-nutrients but also to convey value-added molecules capable of acting as nutraceuticals. This may reduce the dependency on the terrestrial ingredients to a lesser extent but improve the quality of the diet to a larger extent (Kumari, Kumar *et al.* 2013).

Novel aquafeeds may influence the development of aquaculture to the extent that dietary nutrients and additives can stimulate the immune system of the fish and attenuate the consequences of pathogenic invasion and thus contributing to welfare of the recipient fish, favoring their performance and helping to avoid diseases (Kiron 2012). Additionally, the well described adverse effects of plant ingredients inclusion in aquafeeds, may be attenuated by the protective effect of microalgae bio-active molecules.

1.2.2 Microalgae in fish nutrition

Inclusion of microalgae in aquafeeds have been studied for different purposes in fish with various feeding habits.

In Atlantic salmon, the replacement of 5 or 10% FM protein by *Nanofrustulum* and *Tetraselmis* was achieved without negative repercussions in growth or feed performance (Kiron, Phromkunthong *et al.* 2012). Also for salmon, Kousoulaki, Mørkøre *et al.* (2016) tested a DHA rich microalgae biomass (*Schizochytrium sp*.) and verified no significant differences, up to 5% microalgae inclusion, in growth, feed conversion ratio, dietary protein and energy digestibility. Additionally, retention efficiency of EPA, DHA, monounsaturated fatty acids and fillet quality improved.

Pacific red snapper diets fed diets including *Navicula sp.* combined with *Lactobacillus sakei* (10^6 CFU g^{-1}) improved fish growth rate, immune status and antioxidant capabilities (Reyes-Becerril, Angulo *et al.* 2014).

Tulli, Chini Zittelli *et al.* (2012) observed that in European sea bass diets *Tetraselmis suecica* can replace fish protein up to 20% without affecting growth performance and quality traits of fish fillet. For this specie, Tibaldi, Chini Zittelli *et al*. (2015) stated that replacement of 36% FO and up to 20% FM protein by *Isochrysis sp.* biomass, did not adversely affect feed intake and growth performance, but slightly reduced n-3 LC-PUFA content in edible muscle tissue.

In *Epinephelus lanceolatus*, a blend of soybean meal, soy protein concentrate and algae meal (*Schizochytrium limacinum*) up to 40% showed no negative effects in fish performance and condition. The algae meal utilized in the mixture can be also used as the diets main lipid source without adverse consequences for the fish (García-Ortega, Kissinger *et al.* 2016).

Rainbow trout presented minor changes in intestinal microbiome and positive effects on growth, when FO was replaced in their diets by 5% *Schizochytrium limacinum* (Lyons, Turnbull *et al.* 2016).

In common carp diets, FM protein replacement by *Spirulina platensis* at 25, 50, 75 and 100% improved protein digestibility and did not negatively affect growth, carcass composition or organoleptic quality (Nandeesha, Gangadhar *et a*l. 1998). Also for common carp, Kiron, Phromkunthong *et al.* (2012) used *Nanofrustulum* and *Tetraselmis* at 25 and 40% levels to substitute FM protein and verified positive effects in growth performance. Nevertheless, an inclusion of 40% *Nanofrustulum* significantly decreased the lipid content in fish body.

In Tilapia, 40% of FM protein can be replaced by *Spirula maxima* without negative effects on growth and feeding performance (Olvera-Novoa, Dominguez-Cen *et al.* 1998). In Nile Tilapia, a mixture of *Chlorella spp.* and *Scenedesmus spp*. can be incorporated up to 50% without adverse effects on growth performance, feed utilization and body composition (Badwy, Ibrahim *et al.* 2008).

Atalah, Cruz *et al*. (2007), reported that if 2 or 4% FO in seabream juveniles diets was replaced by *Cryptecodinum cohnii,* survival and growth were improved, but with 5% *Phaeodactilum tricornutum* survival decreased and damaged intestine epithelia was observed. Vizcaíno, López *et al.* (2014) recommended a 20% *Scenedesmus almeriensis* inclusion in their diets, and noticed that microalgae were responsible for increased alkaline protease and L-aminopeptidase activities and intestinal absorptive surface, and did not modify fish nutrient utilization, growth or proximate composition. The same author Vizcaíno, Saéz *et al.* (2016) recommended a 5% *Tetraselmis suecica* meal as dietary ingredient for *Sparus aurata* fry.

Few studies have been performed to assess microalgae effects in seabream immunity. Cerezuela, Fumanal *et al*. (2012) noticed that using *Tetraselmis chuii and Phaeodactylum tricornutum* (100g/kg) single or combined with *Bacillus subtilis* (10^7 cfu/g) increased fish immune parameters, but did not result in higher infection resistance. Similar results were observed by Reyes-Becerril, Guardiola *et al.* (2013) in seabream but with *Navicula sp.* (100g/kg) and *Lactobacillus sakei* (10^6 cfu/g) containing diets.

At the present study, the elected microalgae to test in *Sparus aurata* diets was *Nannochloropsis sp.*. Even though is among the most microalgae used in marine

aquaculture nutrition (Pal, Khozin-Goldberg *et al.* 2011, Cerezuela, Guardiola *et al.* 2012), data available about the effects of *Nannochloropsis sp.* in carnivorous fish diets is very limited. Haas, Bauer *et al.* (2015) stated that a 50% FO replacement by *Nannochloropsis sp.* meal and a total replacement by *Pavlova viridis* can be made without adverse effects on growth performance and nutrient utilization of juvenile sea bass. Using *Gadus morhua*, Walker and Berlinsky (2011) verified an initial reduction in feed intake and growth with a mixture of *Nannochloropis sp.* and *Isochrysis sp.*, but these parameters seemed to improve as the study advanced.

Cerezuela, Guardiola *et al.* (2012), fed *Sparus aurata* with 50 or 100 g kg^{-1} *Nannochloropsis gaditana*, *Tetraselmis chuii* or *Phaeodactylum tricornutum* and observed enhanced immune defense activity in fish offered all microalgae diets, supporting the concept of microalgae as immunostimulant in aquafeeds.

1.3 Gilthead seabream (*Sparus aurata*)

Gilthead sea bream (Fig 2) belongs to the Sparidae family, Perciforms order and genus *Sparus* (Morretti 1999, Pavlidis and Mylonas 2011). Anatomically possess an oval body, slightly deep and compressed. Their head is curved with small eyes. The body coloration is silver-grey, possessing a huge black spot in the origin of the lateral line that extends on the upper margin of the operculum, where it is edged below by a reddish area. On the sides of the fish body, generally it´s possible to visualize dark longitudinal lines. The borders of the fork and caudal fin are edged with black (FAO 2005 - 2016, Pavlidis and Mylonas 2011). The mandible is shorter than maxilla, and both jaws present canine and molariform teeth (Morretti 1999). They have one single dorsal fin, with 11 spines and 13 to 14 soft rays. The caudal fin is grey – greenish white (FAO 2005 - 2016, Pavlidis and Mylonas 2011). The anal fin possess 11 to 12 soft rays and 3 spines (FAO 2005 - 2016). The digestive tract is reasonably small and the intestine runs straight, without loops, through the body cavity (Pickett and Pawson 1994).

Fig 2: Gilthead seabream (*Sparus aurata*) Source: Poisson Flotté

1.3.1 Habitat and Biology

Sparus aurata can be found in marine and brackish waters, inhabiting coastal lagoons and estuarine areas (FAO 2005 - 2016). This specie can reach depths of 150 meters (Arabaci, Yilmaz *et al.* 2010), especially in the beginning of the spawning season. Inhabits the Eastern Atlantic costs of Europe, from Senegal to the UK (Webster and Lim 2002), Mediterranean and occasionally the Black Sea, being frequently found in rocky, seaweed, sandy bottoms (Arabaci, Yilmaz *et al.* 2010, Froese and Pauly 2015), and at the surf zone in earlier life stages, which occurs approximately at 30 m (Froese and Pauly 2015). They are benthopelagic, euryhaline and eurythermal (Morretti 1999, Pavlidis and Mylonas 2011).

Seabream is mostly carnivorous, feeding at the basis of shellfish, like mussels or oysters, crustaceans or fish (Arabaci, Yilmaz *et al.* 2010, Pavlidis and Mylonas 2011), and accessorily herbivorous (Arabaci, Yilmaz *et al*. 2010). They are non-selective predators, adapting their diet to the food offered in the habitat (Andrade, Erzini *et al.* 1996).

Gilthead seabream is a protandrous hermaphrodite, that presents a high fecundity and a development of the ovary asynchronous, being male at the first two years and turning into a female when they reach about 30 centimeters (Arabaci, Yilmaz *et al.* 2010), at the second or third age year (Froese and Pauly 2015).

The breeding season occurs from October to December (Arabaci, Yilmaz *et al.* 2010). The spawning season starts at December and lasts up to the beginning of June (Almansa, Martian *et al.* 2001). During this time the specie possess sequenced spawning of spherical and transparent eggs with dimensions of 0.9 to 11 millimeters (Arabaci, Yilmaz *et al.* 2010, Froese and Pauly 2015). The vitellogenic period of *Sparus aurata* is very short, and the larvae usually deplete the yolk-sac 3 to 4 days later after the eggs hatch (Webster and Lim 2002).

1.3.2 Production

This specie is one of the most important marine finfish species reared in Europe, especially in the Mediterranean region (Oliva-Teles 2000), possessing a high economic

value (Andrade, Erzini *et al.* 1996). The main producers are: Greece, Turkey, Spain and Italy (FAO 2005 - 2016).

World production reach the value of 173 062 tonnes in 2013 (FAO 2005 - 2016), and is increasing annually (Martin-Perez, Fernandez-Borras *et al*. 2013) due to reduction of natural harvests, resistance to environmental conditions, species high growth rates and possibility of control the fish entire life cycle (Andrade, Erzini *et al*. 1996).

Knowledge of *Sparus aurata* nutritional requirements is very limited comparatively to other species (Martin-Perez, Fernandez-Borras *et al*. 2013). Proportion of ingredients in their commercial diets vary along life cycle, but usually include 48 – 53 % protein for larger fish, 55% protein for juveniles and 20 - 23 % lipids for both (Martin-Perez, Fernandez-Borras *et al.* 2013).

The substitution of dietary protein with nonprotein energy sources like lipids or carbohydrates, in order to reduce a diet protein content, can improve the efficacy of protein utilization and produces a protein – sparing effect, without affect fish growth (Martin-Perez, Fernandez-Borras *et al*. 2013).

In 2011, Enes, Panserat *et al.* (2011) revealed that even though carnivorous species like *Sparus aurata* possess a limited capacity to use carbohydrates as an energy source, their diets can contain until 20 % digestible carbohydrates without affecting feed utilization and fish growth.

Kalogeropoulos, Alexis *et al.* (1992) and Ibeas, Izquierdo *et al.* (1994) estimated that the n-3 HUFA (highly unsaturated fatty acids) requirement for *Sparus aurata* were 0.9% and 1.9% in the diets, respectively.

Although fish don´t have an absolute protein requirement (Oliva-Teles 2000), they need a balanced mixture of essential and nonessential amino acids in their diets (Halver and Hardy 2002). Like other marine finfish Gilthead seabream require 10 essential amino acids (EAA), that are known as arginine, isoleucine, lysine, histidine, leucine, methionine, phenylalanine, valine, tryptophan and threonine (Trushenski, Kasper *et al.* 2006). Peres and Oliva-Teles (2009), estimated the correct amino acids profile, expressing the results in ratios relative to lysine (=100): 108.3 to arginine, 58.1 threonine, 36.8 histidine, 49.7 isoleucine, 92.7 leucine, 50.8 methionine, 112.3 phenylalanine + tyrosine, 62.6 valine and 14.6 tryptophan.

Available data of seabream vitamins requirements is very limited (Oliva-Teles 2000). Morris, Davies *et al.* (1995) set the qualitative requirement of thiamin, niacin, riboflavin, pyridoxine and pantothenic. The thiamin requirement was also established by Morris and Davies (1995) as 10 mg kg^{-1} diet in given oil-rich diets and 0.5 mg kg^{-1} diet in carbohydrate rich aquafeeds. Kissil, Cowey *et al.* (1981) reported a pyridoxine requirement of 1.97 mg kg^{-1} diet. The nicotinic acid requirement was found to be 63 – 83 mg kg^{-1} diet by Morris and Davies (1995). Alexis, Karanikolas *et a*l. (1997) recommended a 50 – 3200 mg kg-1 diet incorporation of Vitamin C in their diets.

Assessment of mineral requirements is complicated, since fish absorb some from water or diet. Furthermore, several are necessary in low quantities, which makes difficult to maintain environmental conditions or formulate diets with those amounts (Pavlidis and Mylonas 2011). Among all minerals, only phosphorus has received attention in fish, since feeds are the main source of this element and is poorly available in the water, and in adition to nitrogen these elements are the principal responsible for aquaculture water pollution (Pavlidis and Mylonas 2011). Pimentel-Rodrigues and Oliva-Teles (2001) established sea bream´s mineral phosphorus requirements at 0.75% dietary phosphorous to achieve maximum growth.

1.4 *Nannochloropsis sp.*

Nannochloropsis sp. (Fig 3), belongs to the class Eustigmatophyceae, and is a eukaryotic unicellular algae world-widely distributed (Li, Pan *et al.* 2011). The genus is formed by six species morphologically similar, which makes their identification challenging (Li, Pan *et al*. 2011). Each species possess several isolates or strains (Taleb, Pruvost *et al.* 2015). They measure between 2 to 5 µm (Li, Pan *et al.* 2011).

Fig 3: *Nannochloropsis sp.* Source: CSIRO

1.4.1 Habitat and Biology

Species are frequently found offshore (Li, Pan *et al*. 2011). Five of them (*Nannochloropsis oculata*, *Nannochloropsis salina*, *Nannochloropsis granulata*,

Nannochloropsis oceanica, and *Nannochloropsis gaditana*) are found in marine ecosystems, and only one (*Nannochloropsis limnetica*) inhabits freshwater (Taleb, Pruvost *et al.* 2015).The algae is picoplanktonic (Pal, Khozin-Goldberg *et al*. 2011).

1.4.2 Application in aquaculture

Nannochloropsis sp. is among the most used microalgae in marine aquaculture nutrition (Pal, Khozin-Goldberg *et al.* 2011, Cerezuela, Guardiola *et al.* 2012). This microalgae is utilized in the sector as a source of lipid, protein and carbohydrate (Khatoon, Abdu Rahman *et al.* 2014). The ease of culturing, lack of toxicity, correct cell size and digestible cell wall makes them appealing to aquaculture sector (Khatoon, Abdu Rahman *et al.* 2014). It´s produced for marine finfish and crustaceans hatcheries, rotifers growth and feed filter feeders in reef tanks (Hemaiswarya, Raja *et al.* 2011, El Nabris 2012).

The high EPA content made this genus of microalgae a feedstuff of excellent quality for aquaculture (Cohen 1999). *Nannochloropsis* is a known source of high-quality protein (Kilian, Benemann *et al*. 2011), vitamin E and valuable pigments as chlorophyll, astaxanthin, zeaxanthin and canthaxanthin (El Nabris 2012).

Nannochloropsis is formed by valuable nutritional compounds such as 38% carbohydrates, 29% crude proteins, 18% lipids, 3% microelements, 17.4% fatty acids, 0.29% chlorophylls and 0.06 % carotenoids (Grimi, Dubois *et al.* 2014).

The algae can also be applied in biodiesel production (Taleb, Pruvost *et al.* 2015), wastewater treatment (Polishchuk, Valev *et al.* 2015) and aid reduce global warming (Mitra, Patidar *et al.* 2015).

One of the main limitations to large scale production of this microalgae is the high cost of the culture medium that support the *Nannochloropsis sp.* growth, which is very labor-intensive (El Nabris 2012).

1.5 Digestive tract

In aquaculture, fish health maintenance is a definition in which individuals must be produced under conditions that optimize the growth rate, survival and feed conversion

efficiency while decreasing complications related to infectious, environmental and nutritional diseases. The effect produced by these factors actions and interactions between them is reflected by the histological status of the organs (Saraiva, Costa *et al.* 2015).

The gut consist in a long muscular-walled tube beginning at mouth, passing through pharynx, esophagus, stomach, intestines and ending at anus (Genten, Terwinghe *et al.* 2009). The physiological processes of gut are in charge of digestion, which is a process where the ingested materials are hydrolyzed by specific enzymes into smaller units (Caruso, Denaro *et al.* 2009), and absorption of nutrients from fish diets (Chikwati, Sahlmann *et al.* 2013).

The variation in organization of fish gastrointestinal tract differs with the species, matching the diverse feeding habits and environments exploited by them. The structure and functional characteristics of the alimentary canal follows the basic features as in other vertebrates groups with small differences in phylogeny, ontogeny, feeding habits, physiological conditions, nutrition and some special functions presented in some cases by the gut. These variations insure the ideal utilization of dietary nutrients like an intestinal absorptive surface area with large dimensions and an efficient digestion of the food that is ingested by the animals (Merrifield and Ringo 2014).

In fish, there is no distinction between small and large intestines unlike what happens in mammals organisms (Genten, Terwinghe *et al*. 2009).

Intestine is an unique organ, being the primary location of food digestion and nutrient intake (Caballero, Izquierdo *et al.* 2003). Fish intestine represent 3 to 4% of fish body mass and have evolved to process specific diets. In the case of carnivorous fish, their intestine can only process a nutrient dense and high digestible diet, rich in protein and low in carbohydrate (Buddington, Krogdahl *et al.* 1996). Therefore comparatively to herbivorous fish, their intestine possess high levels of proteases and low of amylases and lipases (Caruso, Denaro *et al*. 2009).

This organ have innumerous functions like digestion and absorption of feedstuffs, endocrine regulation of digestion, metabolism and immunity, and control of water and electrolyte balance. Additionally, the intestine establish a delicate balance between efficient and rapid absorption of nutrients, while prevent invasion by pathogens organisms and harmful components existent in diet and environment, like toxins

(Buddington, Krogdahl *et al*. 1996). The best utilization of dietary nutrients depends on the effectiveness of intestine physiological functions (Caballero, Izquierdo *et al*. 2003).

Interference in homeostasis of intestine microbiota, mucosal barrier and immune system, conduct to a deficiency in mucosal layer with increased permeability that can bring inflammation and injury of intestinal mucosal cells (Cerezuela, Fumanal *et al*. 2012).

Intestine is the first line of defense against an increasingly toxic environmental, since it's constantly bombarded by antigens from diet and microorganisms (Cerezuela, Fumanal *et al*. 2012). Therefore, maintaining intestine stability and integrity is a key factor for fish defense, growth and adequate nutrition (Cerezuela, Fumanal *et al.* 2012).

1.5.1 Histology

Generally, digestive tract and organs are formed by four histological layers: a mucosa, that is the most internal layer of the tube, consisting of an inner epithelium, a middle lamina propria (an cellular connective tissue) and an muscular mucosae; a submucosa, that is connective tissue layer with blood vessels, lymphatic tissue and nerve plexi, that supports the mucosa; a muscular that is divided into an inner circular and an external longitudinal layer, being responsible for movement of gut contents; a serosa formed by connective tissue delimited by a simple squamous peritoneal epithelium (Evans and Claiborne 2006, Genten, Terwinghe *et al.* 2009)

Typically, in fish intestinal epithelium it´s possible to find several types of cells, like leucocytes, goblet cells, eosinophilic granular cells, enterocytes and muscular cells.

The immune cells of intestine epithelium are divided in two groups or populations, lamina propria leucocytes and intraepithelial leucocytes. The first population comprise macrophages, granulocytes, lymphocytes and plasma cells. The second group include T cells and B cells placed between epithelial cells (Beck and Peatman 2015).

Goblet cells appear between enterocytes, along the intestinal folds. These are mucous secreting cells, and possess an absorptive and a secretory function (Genten, Terwinghe *et al.* 2009). Their nucleus is positioned in the basal portion and in their middle

area cells expand and then contract forming an apical pore where mucus is discharged to protection against pathogens (Beck and Peatman 2015).

Eosinophilic granular cells contain antimicrobial peptides and are involved in innate immunity and inflammation. Their degranulation increase vascular permeability and promote neutrophil adhesion (Genten, Terwinghe *et al*. 2009).

Enterocytes are columnar epithelial cells with a protuberant brush border in their apical region formed by microvilli, and are responsible by almost 90% of the total intestine area. These cells are tall and narrow, with a stretched nucleus in the basal region and aggregations of lipid droplets in high quantity responsible for the lipid digestion and macromolecule intake on their cytoplasm at the apical zone. Supranuclear vacuoles of enterocytes are important structures since they can also enhance the start of an immune response by collecting antigen (Beck and Peatman 2015).

In the enterocytes brush border enzymatic activity exists, due enzymes produced by those cells (Beck and Peatman 2015). These membrane-bound enzymes pick up metabolites resulting from the hydrolyze of protein, carbohydrates and lipids by luminal digestive enzymes (García-Meilán, Ordóñez-Grande *et al.* 2016).

1.5.2 Function

Nutrient utilization by fish depends directly of the accessibility of appropriate digestive enzymes (carbohydrases, proteases and lipases) that are present along the gut (Chong, Hashim *et al.* 2002, Caruso, Denaro *et al.* 2009). Nutrient uptake happens along the intestine by diffusion, facilitated or active transport (García-Meilán, Ordóñez-Grande *et al*. 2016).

Enzymes activity can be modulated by intestinal transit time and diet composition, which in turn can influence the digestive and absorptive processes (Castro, Couto *et al.* 2015). A properly digestive process require that food contact with digestive enzymes for a certain length of time, being also subject to grinding, mixing and advance movements characteristics of the digestive tract (Furne, Hidalgo *et al*. 2005).

Studying the digestive enzymes profile reveals diverse benefits like a better elucidation of fish nutritional physiology, and help resolve nutritional problems associated

to artificial diets (Xiong, Xie *et al*. 2011). The activity of these enzymes is an important parameter that define the efficiency of a diet, food utilization and a way to enhance individuals growth (García-Meilán, Ordóñez-Grande *et al.* 2014), providing important information about the specie digestive capacity and efficacy in use feeding components of the diet (Caruso, Denaro *et al.* 2009).

Amylase is the carbohydrase responsible for hydrolysing complex polysaccharides such as glycogen and starch into oligosaccharides and maltose. Even though it´s a key enzyme in fish carbohydrates digestion (Papoutsoglou and Lyndon 2005), the enzyme activity is not fully understood, varying between species (Grosell, Farrell *et al.* 2010). The main amylase producers are pancreas and liver, but production was already confirmed in pancreatic juice, stomach and intestine (Klahan, Areechon *et al.* 2009). Amylase is mostly adsorbed at intestine mucosa and pyloric ceca (Munilla-Morán and Saborido-Rey 1996). The enzyme activity is related to fish feeding habits, being higher in herbivorous and omnivorous comparatively to carnivorous species (Hidalgo, Urea *et al.* 1999, Fernandez, Moyano *et al.* 2001, De Almeida, Lundstedt *et al.* 2006, Al-Tameemi, Aldubaikul *et al.* 2010).

In carnivorous fish, food is primary degraded in the stomach by the action of muscular contractions of stomach wall and enzymes in acid medium, like acid protease pepsin. Then products previously fragmented are expelled through pyloric sphincter into the intestine, and alkaline enzymes, like trypsin and lipases, originated by the liver and pancreas allow the progression of the food in the midgut (McClintock and Baker 2001, Caruso, Denaro *et al*. 2009).

Proteases are polyfunctional enzymes that catalyze the hydrolytic degradation of proteins (Garcia-Carrenno and Hernández-Cortés 2000). The main sites of proteases secretion in teleosts are pancreas, stomach and intestine (Ray, Ghosh *et al.* 2012). The proteases distribution varies between organs and species. A superior activity in the acid pH region of the stomach, and in the alkaline pH region of the intestine was verified in previous studies (Alarcón, Díaz *et al*. 1998). The proteolytic activity level is associated with fish species growth rate (Hidalgo, Urea *et al.* 1999). Even though carnivorous fish possess shorter intestines, the protease activity is higher comparatively to herbivorous (Lazzari, Radünz Neto *et al.* 2010) due to their dietary requirements in protein.

Trypsin is a intestine alkaline protease (Caruso, Denaro *et al.* 2009), that hydrolyses the carboxyl side of the basic amino acids, arginine and lysine, which possess

higher digestibility comparatively to other amino acids (Klomklao, Benjakul *et al.* 2006, Chan, Lee *et al.* 2008). This enzyme is extremely important in carnivorous fish digestion once assists 40 – 50% of the overall digestion activity (Klomklao, Benjakul *et al*. 2006). Trypsin is secreted in response to food digestion with pancreas enzymes (Klomklao, Benjakul *et al.* 2006, Savona, Tramati *et al.* 2011), and finishes protein hydrolysis when food reaches the intestine (Savona, Tramati *et al.* 2011). Trypsin activity is superior in carnivorous fish than omnivorous and herbivorous species due to the dietary requirements of carnivorous species (Jónás, Rágyanszki *et al.* 1983, Chong, Hashim *et al.* 2002). In fish, trypsin is the key enzyme responsible for activation of other pancreatic proteases like chymotrypsin (Rungruangsak-Torrissen, Moss *et al*. 2006, Chan, Lee *et al.* 2008).

Chymotrypsin hydrolyses proteins at the carboxyl side of phenylalanine, tryptophan, tyrosine and methionine (Chan, Lee *et al.* 2008). Generally, chymotrypsin activity is higher in omnivorous and herbivorous, than carnivorous fish species (Chong, Hashim et al. 2002). Chymotrypsin and trypsin play a cooperative role in protein digestion at an intestinal level (Chong, Hashim *et al.* 2002). Both proteases relative activities are influenced by similar factors (Rungruangsak-Torrissen, Moss *et al*. 2006), and have been proposed as indicators of the fish nutritional status (Uscanga, Moyano *et al*. 2010).

Lipase digests dietary lipids that are an important energy source for fish (Evans, Claiborne *et al.* 2014). The enzyme is responsible for the chemical breakdown of triacylglycerols into diacylglycerol and monoacylglycerol (Savona, Tramati *et al.* 2011), but to digest them needs an emulsifier, like bile salts or bile alcohols, to solubilize the lipids. A colipase is also required to defend lipase function against bile salts (Evans, Claiborne *et al*. 2014). Lipase activity was already verified in pancreas extracts, pyloric ceca and upper intestine, but is known to be extended until the distal intestine, decreasing progressively its activity (Klahan, Areechon *et al.* 2009). Due to the fact that carnivorous fish ingest fat-rich food the lipase activity is higher than in herbivorous and omnivorous fish (Tengjaroenkul, Smith *et al.* 2000, Furne, Hidalgo *et al.* 2005).

1.5.3 Microbiota

Fish gastrointestinal tract is characterized by a complex microbial community formed by viruses, yeast, archaeans, protozoans and a unique bacterial microflora that

inhabits with the host in the surfaces and cavities exposed to the environment (Cerezuela, Fumanal *et al.* 2013, Merrifield and Ringo 2014).

These organisms influence fish nutrition and development (Merrifield and Ringo 2014), aid the digestive function (Dimitroglou, Merrifield *et al.* 2011), and protect fish against pathogenic invasion, while maintain their gut integrity and enhance disease resistance and immunity (de Paula Silva, Nicoli *et al.* 2011).

The microbial community is divided in two groups, the allochthonous (group that moves with the digesta through the host´s lumen) or autochthonous (resident group strongly associated with ´s tissues) (Merrifield and Ringo 2014).

The establishment, composition, diversity and colonization in the gastrointestinal tract by the microbes is a reflection of the microbial composition existent in the rearing water, diet ingested by the host and their surrounding conditions (Nayak 2010).

The importance of studies with manipulation of fish gastrointestinal microbiota reside in allowing the development of strategies which promote host health and improve productivity (Merrifield and Ringo 2014).

Studies of fish gut microbiota have been concentrating on bacterial communities, so information about other populations of microbial community is very scarce (Merrifield and Ringo 2014). Gastrointestinal bacteria can metabolize several subtracts, suppling important components like vitamins, minerals, essential fatty acids or exogenous digestive enzymes (Floris 2011). Bacteria quantity and quality in the gastrointestinal microflora is dependent on variation of several factors like fish development stage, aqueous environment, distinctive regions, diet, gastrointestinal tract anatomy, seasonal variation, type of rearing conditions, and fish species (Floris 2011).

1.6 Aims

The aim of this study was to understand the effects of supplementing *Nannochloropsis*, already being produced in a refinery-like industry, in the diets of Mediterranean carnivorous species gilthead seabream (*Sparus aurata*). Additionally, it´s intended to evaluate if microalgae incorporation attenuated adverse effects of plant based diets offered to seabream in this trial.

Short-term effects of *Nannochloropsis* supplementation was evaluated on fish growth, feed utilization, intestinal function and morphology, and microbiota modulation to understand potential of microalgae as functional ingredient in marine fish aquafeeds, and its putative beneficial effect in fish health and welfare.

Chapter 2: Material and Methods

2.1 Microalgae characterization

The microalgae *Nannochloropsis sp.* was provided by BuggyPower, S. L. (Murcia, Spain) and was produced industrially under a biorefinery concept.

Nannochloropsis (Table 1) was evaluated in terms of dry matter(%), ash, protein and lipids as described in section of chemical analyses performed in ingredients and diets.

Table 1. Proximate analysis of the microalgae *Nannochloropsis sp.*

Proximate analyses (% dry weight basis)	
Dry matter	87.51
Crude Protein	53.07
Crude fat	2.17
Ash	18.46

2.2 Experimental diets

Four isoproteic and isolipidic diets (45% C.P., 18% C.L) were formulated, a control diet and 3 experimental diets with increasing levels of *Nannochloropsis sp.*. Lipids were provided by FO, while protein was provided by 85% plant ingredients and 20% FM. The experimental diets were supplemented with 0.5% (Nanno 0.5), 0.75% (Nanno 0.75) and 1.5% (Nanno 1.5) *Nannochloropsis*, respectively, by replacing cellulose in the formulation.

All dietary ingredients were weighed, finely ground, well mixed and dry – pelleted in a laboratory pellet mill (CPM, California Pellet Mill, Crawfordsville, IN, USA; Fig 4), through a 2 – mm die. Then pellets were dried in an oven for 24 h at 50 ºC and properly stored in plastic bags (after cooling) at the facilities. Ingredients composition and proximate analyses of experimental diets are presented in Table 2.

Fig 4: California Pellet Mill

Table 2. Composition and proximate analysis of the experimental diets

	Experimental diets			
	Control	**Nanno 0.5**	**Nanno 0.75**	**Nanno 1.5**
Ingredients (% dry weight basis)				
Wheat [a]	7.1	7.1	7.1	7.1
Wheat gluten [a]	7.5	7.5	7.5	7.5
Corn gluten [a]	15	15	15	15
Fish meal [b]	20	20	20	20
Soybean meal [a]	28.2	28.2	28.2	28.2
CLO [a]	14.5	14.5	14.5	14.5
Choline chloride (50%) [c]	0.5	0.5	0.5	0.5
Mineral premix [d]	1	1	1	1
Vitamin premix [e]	1	1	1	1
Binder [f]	1	1	1	1
Cellulose [g]	1.5	1	0.75	0
Dibasic calcium phosphate [a]	2.7	2.7	2.7	2.7
Algae (*Nannochloropsis sp*.) [h]	0	0.5	0.75	1.5
Proximate analysis (% dry weight basis)				
Dry matter	93.88	94.09	94.46	94.50
Crude protein	48.36	49.55	48.94	48.89
Crude fat	18.16	18.01	17.82	17.59
Ash	7.90	8.74	9.02	8.72
Gross energy (kJ g^{-1} DM)	22.44	22.44	22.28	22.70

DM, dry matter; CLO, Cod liver oil;

[a] Sorgal – Sociedade de Óleos e Rações Sa. São João OVR, Portugal.

[b] Vacuum Dried LT (CP: 77% DM; GL: 16% DM). Pesqueira Diamante, S.A., Lima, Peru.

[c] Premix, Lda.,Viana do Castelo, Portugal.

[d] Minerals (mg kg−1 diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phsophate, 5.9 (g kg−1 diet); potassium chloride, 1.15 (g kg−1 diet); sodium chloride, 0.4 (g kg−1 diet). Premix, Lda., Viana do Castelo, Portugal.

[e] Vitamins (mg kg−1 diet): retinol, 18,000 (IU kg−1 diet); calciferol, 2000 (IU kg−1 diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400. Premix, Lda., Viana do Castelo, Portugal.

[f] Binder (80% Lignosulfonates (E-565), 20% flavoring mixture), Lípidos Toledo S.A. (LIPTOSA), Spain.

[g] Sigma-Aldrich,St. Louis,Missouri,USA.

[h] BuggyPower, S. L., Murcia, Spain.

2.3 Experimental animals

The trial with *Sparus aurata* was performed at the Marine Zoological Station, of the Faculty of Sciences at Porto University. Fish were obtained from IPIMAR/CRIPSul, located at Olhão. Upon arrival, fish were submitted to a quarantine period in a proper system, after which fish were transferred to the experimental system (Fig 5), where the trial took place.

Fig 5: Thermo – regulated recirculation water system

2.4 Short-term trial: Experimental design

A short-term trial of 37 days was performed in a thermoregulated recirculating seawater system equipped with eighteen 100L tanks. The system was supplied by a continuous flow (2.5-3.5 L min^{-1}) of filtered seawater. During the trial salinity was maintained at 35.0 ± 1.0 g L^{-1}. Water temperature averaged 23.0 ± 0.5ºC, and dissolved oxygen was kept near saturation (7 mg L^{-1}). The water in the system was sterilized by UV and the photoperiod regime (12h light/12 h dark) in the tanks was made by artificial illumination.

Each diet was randomly assigned to three tanks of the thermoregulated recirculating seawater system. Fish were fed to apparent visual satiation twice a day, 6 days a week. Homogenous groups of 13 *Sparus aurata* juveniles with a mean body weight of 60g were established and randomly distributed to each tank.

2.5 Sampling

Fish were bulk-weighted at the beginning and by the end of the trial after 1 day of feed deprivation. At the end of the trial, a total of five individuals were killed by lethal anesthesia (phenoxyethanol) and weighed in trays. Intestinal samples of three fish were collected for histology and digestive enzymes activity analysis. Fish were dissected, the digestive tract excised and freed from adjacent adipose and connective tissues. For histology purposes distal intestine (distinguishable by an enlarged diameter and darker mucosa) samples were collected, rinsed in phosphate buffered saline (PBS), carefully blotted dry with a paper towel, fixed in phosphate buffered formalin (4%, pH 7.4) for 24

h and subsequently transferred to ethanol (70%) until further processing. The remaining gut was instantly frozen in liquid nitrogen and stored at -80ºC for the measurement of digestive enzymes activities. For microbiota characterization the gastrointestinal content (digesta and mucosa) of two fish per tank was collected, pooled in the same tubes under aseptic conditions and frozen in liquid nitrogen. Digesta was collected by squeezing the gut. Mucosa collection into Eppendorf tubes was done by scraping the gut with a microscope slide. Microbiota samples were frozen at – 80ºC, until processing.

2.6 Chemical analyses performed in ingredients and diets

Previous to analysis of diets composition, diets were grounded to obtain a homogenous sample.

2.6.1 Dry matter

To determine the dry mater, 500 mg of sample in duplicate was placed in pre-weighed and identified crucibles. Next, samples were dried in an oven at 100 ºC until constant weight. The moisture content was then determined through weight loss of the samples plus crucibles.

2.6.2 Ash

Ash content was determined in the same crucibles by incineration of the samples in a muffle furnace (Fig 6) at 450 ºC for 16h. After this process, the inorganic residue obtained was weighted.

Fig 6: Muffle Furnace

2.6.3 Protein

The protein content (Nitrogen (N) x 6.25) of *Nannochloropsis sp.* and of the experimental diets was evaluated by the Kjeldahl method after acid digestion using a Kjedahl digestor and distillation units (models 1015 and 1026, respectively; Tecator Systems, Höganäs, Sweden; Fig 7 and 8). Crude protein was determined by multiplying the total nitrogen content by the factor 6.25 (16gN/100protein). The crude protein calculation was made by estimating total nitrogen content of the sample, considering that

all nitrogen is of proteinaceous origin. Approximately, 150 mg of each sample was weighted in duplicate, and added to the distillation tubes. Two Kjeldahl tablets containing selenium (Se) were added to each samples as a catalyst, plus 5 ml of sulfuric acid. Then, samples were digested 1 h at 450 ºC in the digestor unit. At the end of digestion, the organic N was converted in ammonium sulfate. After digestion and cooling, water and sodium hydroxide (NaOH, 40%) were added to each tube and samples were distilled in the Kjeltec distillation unit, using saturated boric acid for ammonium sequestration. Finally, N was quantified by titration (Fig 9) with hydrochloric acid (HCl, 0.5 N), in the presence of a methyl orange pH indicator.

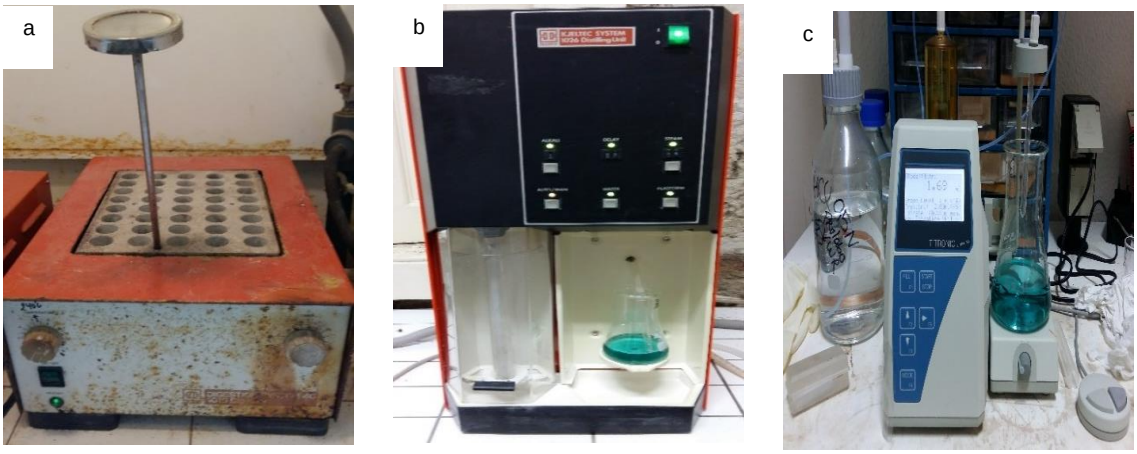

Fig 7, 8 and 9: Digestion units (a), distillation unit (b) and titration units (c) used in the experiment

2.6.4 Gross lipid

Lipid of the diets and microalgae were established by the Soxtec method, by petroleum ether extraction utilizing a SoxTec extraction system (extraction unit model 1043 and a service unit model 1046, Tecator Systems, Höganäs, Sweden; Fig 10 and 11). Approximately, 500 mg of each sample in duplicate was added in a cartridge and positioned in the extraction unit. Afterwards, 50 ml of petroleum ether was added to the extraction cups, earlier identified and weighted, and placed in the extraction unit. Samples were boiled for 1h in petroleum ether, rinsed for 2h and the extracted lipids were entirely collected in the extraction cups. After extraction, the solvent was evaporated and the cup were dried in an oven at 105 ºC. The lipid content was estimated by the difference in weight of extraction cups before and after the extraction process.

a

b

Fig 10 and 11: Lipids extraction unit (a; b)

2.6.5 Gross energy

Gross energy was calculated in an adiabatic bomb calorimeter (PARR model 6200; PARR instruments, Moline, IL, USA; Fig 12) by direct combustion. The gross content in a sample is defined by -Δ Uc, which is the combustion energy at constant volume (kJ / g) (Hubbard, Scott *et al*. 1956). About 500 mg of sample, was weighed, pelletized and combusted under a pressurized (2.53x106 Pa) oxygen atmosphere in the bomb. After combustion, the temperature in the 2 L water bucket surrounding the stainless steel bomb raised and it was measured and used to calculate the energy content in the sample. The apparatus was calibrated with benzoic acid, the conversion factor of 1 cal = 4.1814 Joule was applied.

Fig 12: Adiabatic Bomb Calorimeter

2.7 Calculations

To calculate de fish growth performance and feed utilization efficiency parameters the following formulae were utilized:

$$\text{Average Final weight (g)} = \frac{\text{Total Final weight}}{\text{fishes number}}$$

$$\text{Mortality} = 100\times\left(\frac{\text{number of dead fish}}{\text{number of initial fish}}\right)$$

$$\text{Daily Growth Increment (DGI)} = 100\times\left(\frac{IBW^{1/3} - FBW^{1/3}}{\text{days}}\right)$$

$$\text{Feed intake (g DM per fish)} = \frac{\text{Feed intake (g DM)}}{\text{fisher number}}$$

$$\text{Feed efficiency (FE)} = \frac{\text{Weight gain (g)}}{\text{Feed intake (g DM per fish)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Weight gain (\% Initial Body Weight)}}{\text{Protein Feed Intake (g DM)}\times\text{Crude Protein \%}}$$

2.8 Histomorphology

Alterations in histological intestinal structure related to diets were assessed by light microscopy. Images of the alterations in the distal intestine were obtained by the Zen software (Blue edition; Zeiss, Jena, Germany). A part of the intestine sample (c.a. 5 x 5 mm) was placed in individual cassettes properly identified and maintained in ethanol (70%) until tissue processing. A carousel-type tissue processor was used (Citadel 2000 Tissue Processor, Thermo Fisher Scientific Inc, Massachusetts, USA; Fig 13), in which samples were dehydrated in increasing concentrations of ethanol (70%; 90%; 100%), cleared with xylene and impregnated in paraffin. Then samples were embedded in paraffin to form paraffin blocks utilizing an embedding workstation (HistoStar™ Embedding Workstation, Thermo Fisher Scientific Inc, Massachusetts, USA; Fig 14). From each paraffin block sections of 5 µm were cut in a microtome (Fig 15), mounted on glass slides and dried in an oven at 40ºC, overnight. Slides were stained with haematoxylin-eosin (H-E) in an automatic slide stainer (Varistain™ 24-4 Automatic Slide Stainer, Thermo Fisher Scientific Inc, Massachusetts, USA; Fig 16). Finally, slides were mounted with Entellan (Merck Millipore, Darmstadt, Germany).

Fig 13: Citadel 2000 Tissue Processor

Fig 14: HistoStar™ Embedding Workstation

Fig 15: Microtome

Fig 16: Varistain™ 24-4 Automatic Slide Stainer

A blinded evaluation was performed giving particular attention to the following parameters suggested by Krogdahl *et al.* (2003): (1) widening and shortening of the intestinal folds, (2) widening of the lamina propria within the intestinal folds, (3) infiltration of a mixed leucocyte population in the lamina propria and submucosa, (4) alterations in supranuclear vacuolization in the absorptive cells (enterocytes) in the intestinal epithelium.

A semi-quantitative scoring system ranging from 1 to 5 was used to evaluate the extent of structure alterations. If the score of the intestinal sections was 1, that meant tissue presented normal appearance. Following scores were accounted for increasing alterations of normal tissue histomorphology (Castro, Couto *et al.* 2015). The value of histomorphological alterations was measured by averaging scores of the different parameters mentioned before.

2.9 Digestive Enzymes

Intestinal samples were homogenized (1:0.05 v/w) in ice-cold buffer (100 mM Trizma Base, 0.1 mM EDTA and 0.1% (v/v) TritonX-100, pH 7.8) with an Ultra Turrax.

Homogenates were centrifuged at 33000 g for 30 min at 4°C and the resultant supernatants were kept in aliquots and stored at -80 °C until analyses. Enzymes activity measurements were performed with a microplate spectrophotometer reader (MultiskanTM GO; Thermo Scientific, Lisboa, Portugal).

Dilution tests were performed before α-amylase, lipase, proteases, trypsin and chymotrypsin activity measurements, to ensure optimal substrate and protein concentration for measurement of maximal activity. The samples utilized for the dilution corresponded to the control diet and the higher microalgae dietary incorporation (Nanno 1.5). The enzyme activities were measured at 37ºC in a microplate reader (ELx808; Bio-Tek Instruments, Fig 17).

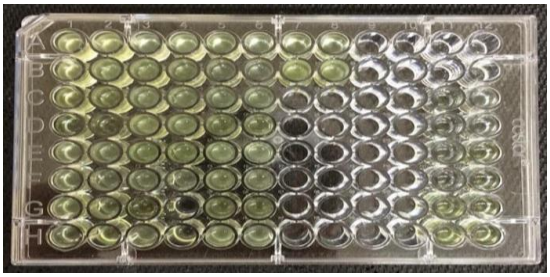

Fig 17: Measurement of enzymatic activity in a Microplate reader (ELx808; Bio-Tek Instruments). Photo by Tiago Luz.

2.9.1 Amylase activity

A Spinreact kit (Girona, Spain, ref. 41201) was utilized to measure the α-amylase (E.C.3.2.1.1) activity. This enzyme hydrolyzes 2-chloro-4-nitrophenyl-α-D-maltotrioside to release 2-chloro-4-nitrophenol and forms 2-chloro-4-nitrophenyl-α-D-maltoside, maltotriose and glucose. The rate of 2-chloro-4-nitrophenol formation (molar extinction coefficient, 12.9 $mM^{-1}cm^{-1}$), measured photometrically, is proportional to the catalytic concentration of α-amylase present in the sample. The reaction mix contained 200 µL of amylase reagent (2-chloro-4-nitrophenyl-α-D- maltotrioside) and 10 µL of diluted sample homogenate (dilution 1:1). Reagent was incubated at 37ºC before use. Absorbance (ΔDO/min) was read at 4s intervals during 3 min at 405 nm and at 37ºC.

2.9.2 Lipase activity

A Spinreact Kit (Girona, Spain, ref. 1001275) was used to measure lipase (EC 3.1.1.3) activity. This pancreatic enzyme in the presence of colipase, desoxycholate and calcium ions, hydrolyses the substrate 1-2-O-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester. The rate of methylresorufin formation (molar extinction coefficient, 60.65 $mM^{-1}cm^{-1}$) it's proportional to the concentration of catalytic lipase present in the sample homogenate. The reaction mix consisted in 200µL of reagent 1 (40 mM TRIS pH 8.3, ≥ 1 mg L-1 colipase, 1.8 mM desoxycholate and 7.2 mM taurodesoxycholate), 40 µL of reagent 2 (15 mM tartrate pH 4,0, ≥ 0.7 mM lipase substrate and 0.1 mM calcium chloride ($CaCl_2$)) and 10 µL of sample homogenate (without dilution). Reagent 1 was incubated at 37ºC before being used. The sample absorbance (ΔDO/min) was read at 10 s intervals, during 12 min, at 580 nm and 37ºC.

2.9.3 Total alkaline protein activity (TAP)

Total alkaline protease activity was measured by casein-hydrolysis method according to Walter (1984) and adapted by Hidalgo, Urea *et al.* (1999). The reaction mixture contained casein (1% w/v; 0.125 mL), buffer (0.1 M Tris-HCl, pH 9.0; 0.125 mL) and homogenate supernatant (0.05 mL) was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 0.3 mL trichloroacetic acid (TCA) (8% w/v) solution. After being kept for 1 h at 2°C, samples were centrifuged at 1800 g for 10 min. The supernatant absorbance was read at 280 nm against blanks. A control blank for each sample was prepared adding supernatant from the homogenates after incubation. Tyrosine solution was utilized to establish a calibration curve. Activity measurement was based on the extinction coefficient for tyrosine (0.008 mL μg^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme necessary to catalyze the formation of 1.0 µmol of tyrosine per min.

2.9.4 Trypsin activity

Trypsin activity was determined according to Faulk, Benninghoff *et al.* (2007) using Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (1mM BAPNA) as substrate and 50 mM Tris-base and 20 mM $CaCl_2$, pH 8.2 as buffer. The reaction mix was formed by 10 µL of diluted sample (1:9) and 70 µL of the solution previously prepared. Production

of 4-nitroanaline (molar extinction coefficient, 8.8 $mM^{-1}cm^{-1}$) was monitored at 37 °C and followed at 410 nm every 20 s during 15 min.

2.9.5 Chymotrypsin activity

Chymotrypsin was also determined by the Faulk, Benninghoff *et al.* (2007) method, but N-Benzoyl-L-tyrosine ethyl ester (0.566 mM BTEE) was used as subtract and a mixture of 0.1 mM Trizma and 25 mM $CaCl_2.H_2O$, pH 7.8 as buffer. The reaction mix was formed by 10 µL of diluted sample (1:99) and 990 µL of the solution previously prepared. Hydrolysis of BTEE (molar extinction coefficient, 0.964 $mM^{-1}cm^{-1}$) was monitored at 37°C and 256 nm during 20 min.

2.9.6 Specific enzymatic activity

All enzyme activities were as specific activity (U mg^{-1} of soluble protein for TAP and mU mg^{-1} for a-amylase and lipase). Protein concentration was determined according to Bradford (1976) utilizing a Sigma-Aldrich protein assay kit with bovine serum albumin as standard. One unit of enzyme activity was defined as the enzyme amount that catalysed the hydrolysis of 1 µmol of substrate per min at assay temperature.

2.10 Microbiota analysis

2.10.1 DNA extraction

Bacterial Genomic DNA extraction of the gastrointestinal content of two fish per tank was done according to Pitcher, Saunders *et al.* (1989) by glass bead beating, adding 500 µl of STE buffer (0.1M NaCl, 10 mM Tris-HCl, 1mM EDTA, pH 8.0), 0.4 g of glass beads (Sigma G8772) and 300 mg of digesta or mucosa samples in a 2 mL bead-beater tube. Samples were then homogenized twice for 30 s in the bead-beater (BeadBug™, Benchmark Scientific, Edison, USA) at 2500 speed with an interval of at least 30 s on ice. After a 15 min incubation at 75ºC, with gentle agitation each 5 min and 1 min centrifugation at 13500 rpm, 500 µl of supernatant was transferred to new 2 ml Eppendorf tubes. After that step, 100 µL of lysozyme (10 mg mL^{-1}) and RNAse (10 mg mL^{-1}) were added, and tubes went to incubation at 37ºC. Next, 50 µl of 10% SDS and 3 µL of proteinase K (20 mg mL^{-1}) were added and tubes were incubated at 55ºC by 30 min. After being cooled on ice for 10 min, in the presence of 500 µl of GES solution (60 g

guanidine thyicyanate, 20 ml EDTA 0.5M, pH 8.0, 120 ml ddH_20, 5ml of 10% N-lauroylsarcosine solution) and 250 µl of ammonium acetate 7.5 M, was added 500 µl of phenol: chloroform: isoamyl - alcohol (25:24:1) to perform a phenol-chloroform extraction. Then, tubes were centrifuged at 13000 g by 10 min, the upper aqueous phase was transferred to a new 2 ml tube and 500 µL of chloroform:isoamyl-alcohol (24:1) was added. A new 10 min centrifugation at 13000 g was performed, and the aqueous phase was transferred to a new 2ml tube and 600 µL of isopropanol was added. The supernatant was carefully discarded after a 15 min incubation on ice and a 15 min centrifugation at 13000 g, the DNA pellet was washed twice with 50 µL of 70% ethanol and a 10 min centrifugation at 13000 g was performed after each wash. Afterwards, DNA pellet was dried at room temperature and ressuspended in 50 µL of ultrapure water, being then stored at 4ºC.

2.10.2 Polymerase chain reaction (PCR)

A PCR technique to amplify the Bacterial 16S rRNA gene fragments was performed on a T100™ Thermal Cycler (Bio-Rad), using primers 16S-358F (which has a GC clamp at the 5´end) and 16S-517R (Muyzer, De Waal *et al.* 1993), yielding a 233bp DNA fragment. PCR mixtures of 50 µl final volume, were prepared including the following components: 24.75 µl of MiliQH_2O (Sigma), 10 µl of 5x Green GoTaq® Reaction Buffer (PROMEGA), 5 µl of DNTP´s (2 mM, PROMEGA), 2.5 µl of each primer (10 µM Forward and Reverse), 0.25 µl of GoTaq® DNA Polymerase (PROMEGA) and 5 µl of the DNA template.

In the PCR thermocycler (Fig 18) were established the following conditions: 94ºC incubation for 5 min was followed by 10 cycles of 64ºC, 1 min, 65ºC, 1 min and 72ºC, 3 min. The annealing temperature was decreased at every cycle 1ºC, until reach 55ºC. Thus, final 20 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 3 min. Final extension was at 72ºC, 10 min. After this process PCR products were resolved by electrophoresis on a 1.0% agarose gel stained with GelRed (4µl/100ml) at 120V during 45 min in 100 ml of 1xTAE buffer and visualized on a Gell Doc Ez System (Bio-Rad) with the Image Lab software v4.0.1 (Bio-Rad).

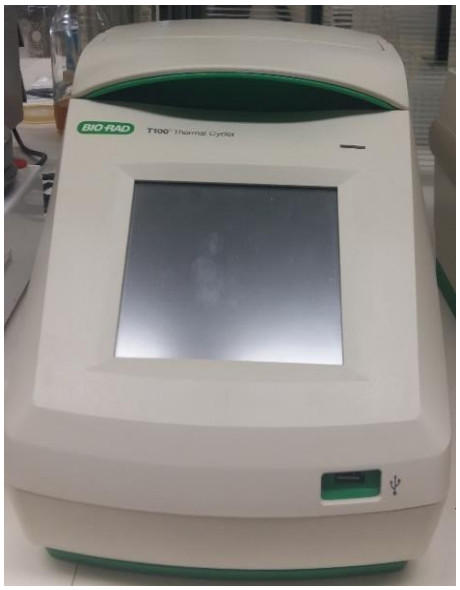

Fig 18: PCR thermocycler (T100TM Thermal Cycler (Bio-Rad)).

2.10.3 Denaturing gradient gel electrophoresis (DGGE)

Polymorphism analyses of 16S rRNA genes were analyzed by DGGE. Only digesta samples were loaded in the gel, once the quantity of DNA extracted from fish mucosa was minimal.

Electrophoresis was performed on a DCode™ universal mutation detection system (Bio-Rad), during 16h at 60ºC with 65V in 1xTAE buffer. An 8% acrylamide gel was prepared with a denaturing gradient of 30 to 70% 7M urea/40% formamide, and 10 µl of each PCR product were loaded on the gel. Gel was stained by 1 h in 200 mL of 1XTAE buffer with SYBR-Gold Nucleic Acid Gel Stain, then were put on a Gel Doc EZ System (Bio-Rad; Fig 19). Results were observed utilizing the Image Lab Software v4.0.1 (Bio-Rad).

Fig 19: Gel Doc EZ System (Bio-Rad)

DGGE banding patterns were converted into presence/absence matrices and band intensities were measured by Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad). Relative similarities between dietary treatments were calculated by Primer Software v7.0.5 (PRIMER-E Ltd, Ivybridge, UK9), and represented by Similarity percentages (SIMPER). The Margalef's index was utilized to determine species richness, while Shannon-Weaver index was used to assess the species diversity. Clustering of DGGE patterns was accomplished by construction of dendrograms utilizing the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA).

Chapter 3: Statistical Analysis

Data was statistically analyzed using the IBM SPSS Statistics v23.0 software package for Windows. The experimental unit considered was the tank (n=3). Data was tested for normality and homogeneity of variances (Shapiro-Wilk and Levene tests, respectively). Normally distributed data was subjected to a one-way analysis of variance (ANOVA), followed by Tukey's multiple range test whenever statistical differences were found. Histology data was neither normal nor homogenous thus the Kruskal-Wallis non parametric test was utilized to examine this data. Differences were considered statistically significant at $P < 0.05$.

Chapter 4: Results

4.1 Zootechnical performance

The mortality observed during the growth trial was not significant, since only one individual died during the experiment.

Fish growth performance, feed and protein utilization was similar across experimental groups (Table 3).

Table 3. Growth performance and feed utilization of seabream juveniles fed the experimental diets for 37 days.

	Experimental diets				SEM[a]	P value
	Control	**Nanno 0.5**	**Nanno 0.75**	**Nanno 1.5**		
Final body weight (g)	99.2	99.8	100.6	101.6	3.72	0.969
Daily growth increment (DGI) [b]	2.13	2.16	2.19	2.24	0.15	0.960
Feed intake (g DM per fish)	73.9	63.7	68.6	64.3	7.44	0.754
Feed efficiency (FE) [c]	0.56	0.68	0.66	0.71	0.04	0.184
PER [d]	1.16	1.37	1.34	1.44	0.09	0.229

[a] Pooled standard error of the mean (n=3)

[b] Daily growth increment: [(IBW1/3 - FBW1/3)/days] x 100.

[c] FE: wet mass gain/dry feed intake.

[d] PER: wet mass gain/crude protein intake

4.2 Histomorphological evaluation

Distal intestine histological evaluation (Table 4) revealed no significant differences between dietary treatments (P>0.05). Overall, distal intestine had a regular morphology, with an intact epithelium barrier, independently of the feed ingested by fish. Enterocytes presented well-organized brush borders, aligned nucleus, homogenous supranuclear vacuolization and an adequate cell form (columnar and high). Intercellular spaces weren´t visible between enterocytes, and goblet cells were commonly dispersed throughout the epithelium. However, slight alterations from the normal morphology were observed. Mucosal intestinal folds thickness increased with microalgae inclusion. The increase of the eosinophilic granular cells in fish fed with Nanno 1.5, points towards an immune reaction.

Table 4. Results of the score-based evaluation of the distal intestine (DI) of sea bream juveniles fed the experimental diets for 37 days, based on alterations in the mucosal fold heights[1], width and cellularity of the lamina propria[2] and submucosa[3], the number of intraepithelial leucocytes[4] (IELs) and eosinophilic granular cells[5] (EGCs), nucleus position within the enterocytes and variation of vacuolization and enterocytes shape[6] (Enterocytes). Mean scores (n=3) were calculated by averaging the scores of the separate parameters evaluated. Scores from 0 to 5, with 5 indicating major alterations. P-values of Kruskal-Wallis non parametric test are given.

	Experimental diets				SEM[a]	P –value
	Control	Nanno 0.5	Nanno 0.75	Nanno 1.5		
Mucosal folds[1]	1.67	1.78	1.89	2.56	0.44	0.159
Lamina propria[2]	1.44	1.33	1.67	1.56	0.29	0.540
Submucosa[3]	1.00	1.00	1.00	1.11	0.10	0.392
IELs[4]	2.11	2.00	2.22	2.33	0.33	0.715
EGCs[5]	1.78	1.89	1.56	2.33	0.36	0.102
Enterocytes[6]	2.22	2.33	2.33	2.67	0.11	0.425
Mean score	1.70	1.72	1.78	2.09	0.27	0.388

[a] Pooled standard error of the mean (n=3)

Figure 20, presents the intestinal histology of the sampled fish, with distinctive layers and cell components.

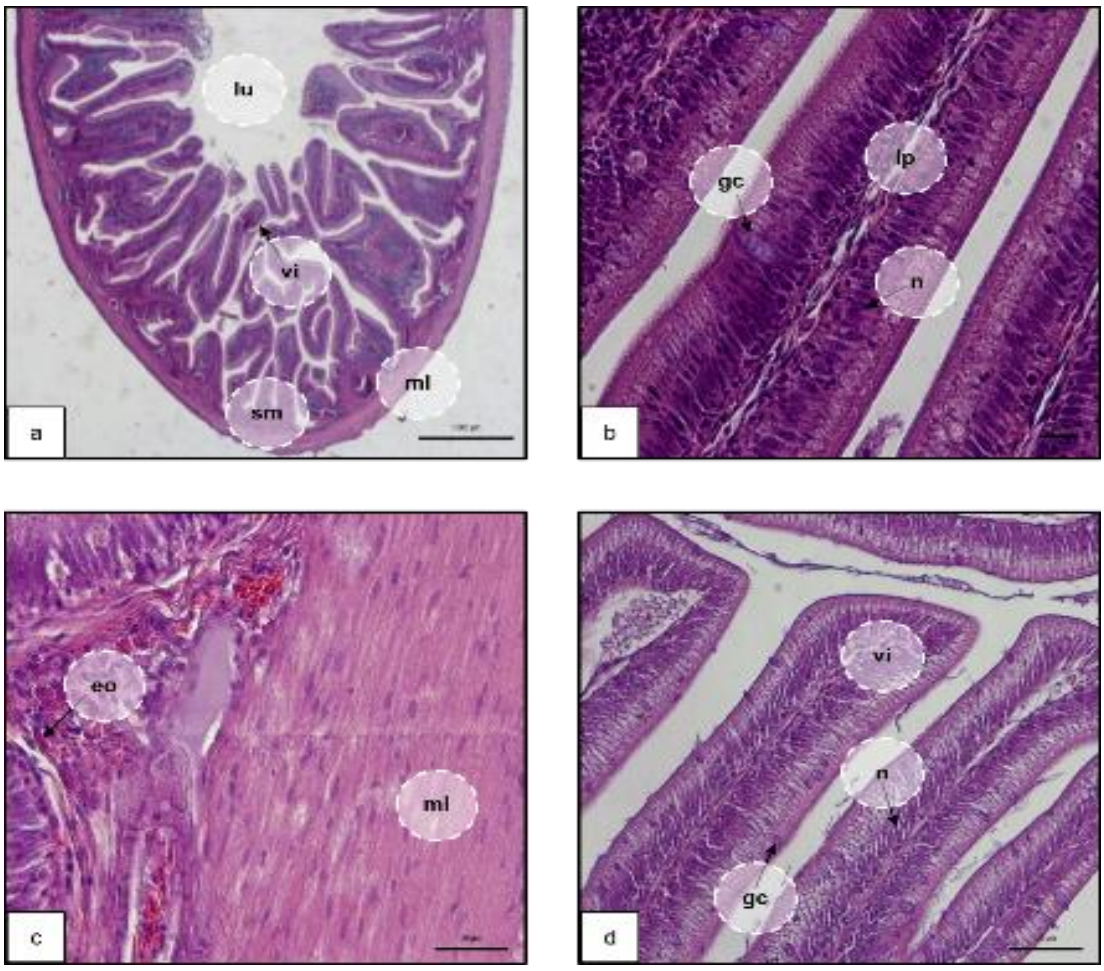

Fig 20: Histology of the distal intestine of gilthead seabream (*Sparus aurata*) juveniles fed a control diet and different microalgae meals with *Nannochloropsis sp.*. **(a)** General image of the intestine in an individual fed Nanno 0.75. **(b)** Detail of a well-organized fold from an individual fed Control diet**. (c)** Detail of eosinophilic granular cells (eo) distribution in the intestine of a fish fed Nanno 1.5. **(d)** General view of villi (vi), depicting goblet cells (gc) between enterocytes and enterocytes nucleous (n) in an individual fed Nanno 0.5. Muscular layer (ml), submucosa (sm), intestinal lumen (lu), lamina propria (lp).

4.3 Digestive Enzymes

Total alkaline proteases (pH=9), trypsin, chymotrypsin, amylase and lipase specific activity in the intestine of seabream juveniles fed the experimental diets are exhibited in Table 5. Feeding a microalgae supplemented diet for 37 days had no statistically significant effect on the activity of digestive enzymes. However, some

interesting results were noticed: excluding total alkaline proteases activity, all other enzymes activity was lower in microalgae fed fish.

Table 5. Digestive enzymes specific activity (U/mg protein) in seabream juveniles fed the experimental diets.

	Experimental diets				SEM[a]	P –value
	Control	Nanno 0.5	Nanno 0.75	Nanno 1.5		
Alkaline proteases (pH 9)	393	313	410	369	50	0.570
Trypsin	357	240	315	217	49	0.229
Chymotrypsin	37747	23783	36025	25789	5136	0.209
Amylase	272	207	252	224	29	0.458
Lipase	9	6	7	5	1	0.236

[a] Pooled standard error of the mean (n=3)

4.4 Microbiota evaluation

DGGE fingerprints of bacterial communities recovered from seabream fed different experimental diets are presented on Table 6. The polymorphism analyses of the variable V3 region of the 16S rRNA gene, revealed highest indices (richness, diversity and OTUs – Operation Taxonomical Units) for Nanno 0.75 diet, with exception of simper similarity index, which was superior in fish offered the Nanno 1.5 diet. The microalgae inclusion didn´t significantly affect shannons diversity (P=0.055) or simper similarity (P=0.727). Nevertheless, variations with significant differences in species richness (P=0.047) and at OTUs (P=0.048) were observed.

Table 6. Ecological parameters obtained from PCR-DGGE fingerprints of seabream juveniles intestinal microbiota fed the experimental diets. Different lowercase letters represent significant differences between dietary treatments (P < 0.05). The values are means of the scores (n = 3) per diet.

	Experimental diets				SEM[a]	P –value
	Control	Nanno 0.5	Nanno 0.75	Nanno 1.5		
Richness [b]	0.802^{ab}	0.747^{b}	1.008^{a}	0.840^{ab}	0.055	0.047
Diversity [c]	2.620	2.552	2.837	2.645	0.063	0.055
Simper Similarity [d]	84.143	84.558	80.563	86.843	3.889	0.727
OTUs [e]	14.000^{ab}	13.000^{b}	17.667^{a}	14.667^{ab}	0.986	0.048

[a] Pooled standard error of the mean (n=3)
[b] Margalef species richness: d=(S-1)/log (N)
[c] Shannons diversity index: H'=-∑(pi(lnpi))
[d] SIMPER, similarity percentage within group replicates
[e] OTUs: Average number of operational taxonomic unit.

Overall, the Bray-Curtis dendogram (Fig 21) showed that samples from fish fed the Nanno 1.5 diet, clustered together, revealing a homogeneous response from bacterial communities to this particular treatment. On contrary, one of three replicates for each of the remaining dietary treatments didn´t clustered with the other two. Nevertheless, when analyzing the replicates clustered together it seems that bacterial communities from fish fed Nanno 0.75 and Nanno 0.5 diets are more closely related, with percentages of similarity higher than 90%, while in individuals offered the Nanno 1.5 and control diet bacterial communities diverge more, presenting similarities below 90%.

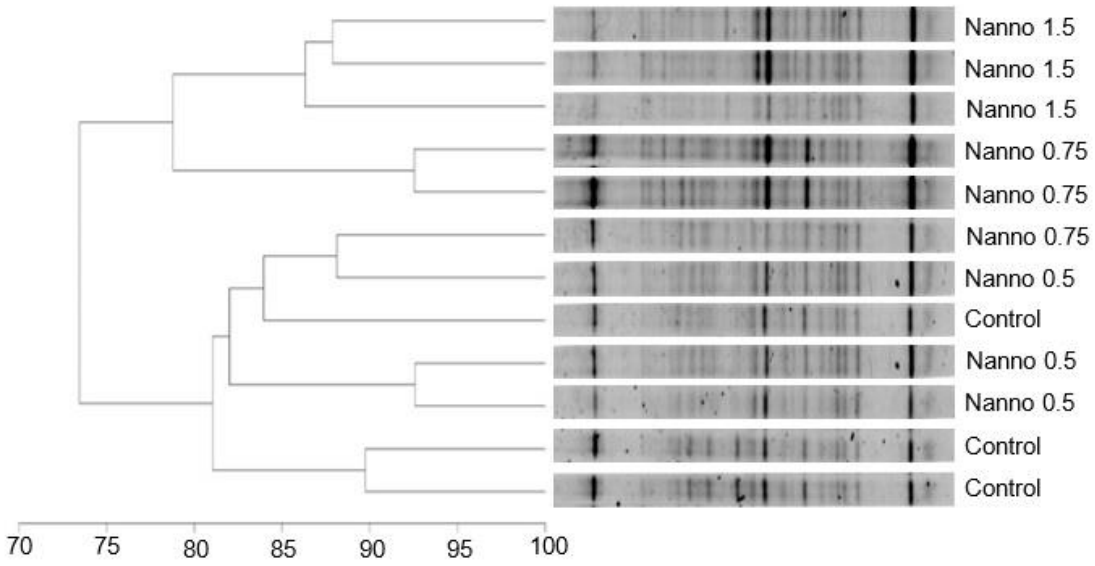

Fig 21. Dendogram and PCR-DGGE fingerprints of the intestinal microbiota of seabream juveniles, fed the experimental diets for 37 days (Control, Nanno 0.5, Nanno 0.75 and Nanno 1.5 diet).

Chapter 5: Discussion

Studies in fish have shown that there are several types of microalgae that promote the growth of individuals, while improving feed utilization, physiological activity, stress response, tolerance to hunger, disease resistance and quality of the carcass (Roy and Pal 2014).

Although not statistically significant, the inclusion of *Nannochloropsis* in *Sparus aurata* diets appeared to affect positively fish zootechnical performance with increase of final body weight and daily growth index. Using the same fish species but in a 45 day-trial, Vizcaíno, López *et al.* (2014), tested a *Scenedesmus almeriensis* meal with different inclusions levels (0%, 12%, 20%, 25% and 39%) and also did not verified any adverse effects in *Sparus aurata* growth, nutrient utilization efficiency or body composition.

Compared to this study, experiments with various carnivorous fish such as *Oncorhynchus mykiss* (Teimouri, Amirkolaie *et al.* 2013), European sea bass (Tibaldi, Chini Zittelli *et al.* 2015) and seabream (Atalah, Cruz *et al.* 2007, Vizcaíno, Saéz *et al.* 2016) with longer duration and various microalgae species, have already shown positive effects in fish zootechnical performance. Nevertheless, El-Sayed (1994) reported that

response of fish to algae in their diets is species-specific, so different fish can show distinct responses to the same microalgae.

Impaired fish growth and feed intake with algae containing diets has also been reported and was attributed to the presence of antinutrients (Vizcaíno, López *et al.* 2014) or palatability issues (Walker and Berlinsky 2011). In this study this not verified, since feed intake and feed utilization were not affected by the inclusion of microalgae in the diets. Similar results were found by Haas, Bauer *et al.* (2015) when *Pavlova viridis* and *Nannochloropsis sp*. were included in *Dicentrarchus labrax* L. juveniles aquafeeds and no substantial differences in body composition, daily feed intake and protein efficiency ratio were observed. The fact that protein efficiency ratio was not significantly different between dietary treatments, suggested that all diets had good protein quality and palatability (El-Saidy and Gaber 2003).

Diets used in the present trial were formulated to include high amounts of plant feedstuffs at the expense of fishmeal. Since carnivorous species such as seabream, do not tolerate high amounts of plant feedstuffs (Olsen and Hasan 2012), preliminary results are promising. Nevertheless, further work is needed to see if the improved zootechnical performance is also seen with a longer trial or higher microalgae amounts.

Information available about dietary microalgae effect in *Sparus aurata* digestive enzymes is reduced.

Amylase and lipase activity varies according to developmental stage, dietary composition or rearing conditions (García-Meilán, Ordóñez-Grande *et al.* 2016). In fact, a positive correlation between intestinal amylase activity with the dietary carbohydrate level and feeding intensity exists (Krogdahl, HEMRE *et al.* 2005). In this study, amylase activity was higher in fish fed the control diet than in those who have ingested the microalgae meals, which can be explained by the higher feed intake recorded in fish fed the control diet.

Generally, in carnivorous fish alkaline protease activity is higher than α-amylase or lipase (García-Meilán, Ordóñez-Grande *et al.* 2016). This is in concordance with the results obtained in the present study. Alkaline proteases activity (pH=9) was higher in fish fed Nanno 0.75 diet, even though no significant differences were found comparatively to the control diet. Vizcaíno, Saéz *et al.* (2016) witnessed a rise of total alkaline protease activity in seabream fed two distinct microalgae meals. The increased

enzyme activity associated with an adequate final body weight in fish fed 10% *Tetraselmis* meal was related to an improvement of fish digestive function. However, higher protease levels in individuals offered diets containing 10% *Tisochrysis* meal combined with lower growth and higher mortality, were justified as a compensation mechanism that occurred due to the dietary change. Different microalgae inclusion in seabream diets appeared to influence protease activity. In the present study, microalgae did not modulate proteases activity.

At the moment, few studies have been performed on fish digestive lipase, chymotrypsin and trypsin activity, especially those regarding microalgae as functional ingredients. In our study, no significant differences were found in the lipase, chymotrypsin and trypsin activities between dietary treatments, but the enzymes specific activity was higher in the control diet when compared to microalgae meals.

Regarding trypsin, Vizcaíno, Saéz e*t al.* (2016) verified that trypsin activity wasn´t affected by microalgae inclusion, as it happened in the present study with the *Nannochloropsis* diets. These results, contrast with Vizcaíno, López *et al.* (2014) which fed *Sparus aurata* juveniles with a 12% *S. almeriensis* diet, obtained a higher trypsin activity than in fish offered the control diet. Nevertheless, differences between studies can be possibly justified by the use of different microalgae in distinct amounts in seabream diets.

The histomorphological analyses revealed that no significant differences were found in the intestinal tissue between dietary treatments. Haas, Bauer *et al.* (2015) and Vizcaíno, Saéz *et al.* (2016), also did not observe any adverse histomorphological effects in seabass and seabream intestine offered different microalgae meals. At the current study, intestine intraepithelial leucocytes quantity, enterocytes structure, thickness submucosal layer and lamina propria remained unaltered in fish offered all dietary treatments.

However, the increase of the eosinophilic granular cells (EGCs) in fish fed Nanno 1.5 denoted an immune reaction. The recruitment of these cells to sites of persistent inflammatory reactions is a frequent event that was already seen in several studies with teleost fish (Reite and Evensen 2006). Fish fed with diets mainly constituted by plant ingredients, can experience adverse effects in gut morphology. In seabream fed plant protein diets, enteritis features such as granular infiltration in distal submucosa, were related to the presence of antinutritional factors, since these can interfere with the brush

border intestinal membrane, changing intestinal histology (Santigosa, Sánchez *et al.* 2008). Nevertheless, this did not appeared to be the case in the present study since EGCs infiltration alone cannot be considered an overt inflammatory reaction. Also, an immune stimulatory effect by microalgae supplementation cannot be ruled out. Further studies should be undertaken to properly understand the alterations observed at the histomorphological level.

At the moment, information regarding the effects of microalgae in fish intestinal microbiota is still very limited. The findings that species diversity increased with higher microalgae incorporation, although not significantly, suggested an adaptive response to the diets. These results are in concordance with Lyons, Turnbull *et al.* (2016), that included 5% *Schizochtrium limacinum* meal in rainbow trout (*Oncorhynchus mykiss*) diets, and obtained a higher level of microbial diversity in fish fed the microalgae diet compared to those in the control group. The authors stated that the higher level of microbial diversity in fish intestine suggests an adaptive response from fish microbiota to the breakdown and digestion of the new dietary ingredient. Additionally, the higher diversity was seen as beneficial to fish health, since it offers an extensive variety of responses to homeostatic perturbations, and can improve the digestion of numerous dietary ingredients. *Nannochloropsis* inclusion in *Sparus aurata* diets clearly affected species richness and the OTUs in the gastrointestinal tract. Nevertheless, results of Cerezuela, Fumanal *et al*. (2012) with *Sparus aurata* contrast with the results of the present study, since a decrease in species richness and diversity of the intestinal microbiota on fish offered different microalgae meals was verified. This contradictory results warrant further investigation.

Chapter 6: Conclusion

The results obtained at the microbiota level are promising, and even though no statistically significant differences were found in the other parameters analyzed, short-term administration of diets including *Nannochloropsis sp.* levels up to 1.5%, microalgae appeared to improve fish zootechnical performance, supporting the putative effect of microalgae as functional ingredient in carnivorous fish diets.

Due to the trial´s short duration, and microalgae low inclusion levels, further studies with longer trials or experiments in which microalgae are included at higher levels in aquafeeds of marine carnivorous fish, are necessary to support data obtained. Longer

trials would also allow to verify, if for a higher microalgae inclusion, the fish´s growth was maintained. Additionally, microbiota characterization of the various dietary treatments should be done to see if the modulation of Nanno 1.5 diet could benefit the host. In the near future, oxidative status at intestinal and hepatic level of the fish will be analyzed in order to evaluate the microalgae antioxidant potential. The histomorphological results obtained, made necessary to perform a gene expression study correlated to an immune response, in order to clarify if the findings are consistent with an inflammatory status or otherwise, with an immune stimulating effect.

Chapter 7: References

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