U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

MSc

MSc
2.º CICLO
FCUP
2019/2020

U.PORTO

Functional feeds to tackle meagre (Argyrosomus regius) stress: physiological responses under chronic and acute stressful conditions

Carla Daniela Mota de Sousa

FC

U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Functional feeds to tackle meagre (*Argyrosomus regius*) stress: physiological responses under chronic and acute stressful conditions

Carla Daniela Mota de Sousa

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

2019/2020

MSc

Functional feeds to tackle meagre (*Argyrosomus regius*) stress: physiological responses under chronic and acute stressful conditions

Carla Daniela Mota de Sousa

Mestrado de Recursos Biológicos Aquáticos

Departamento de Biologia

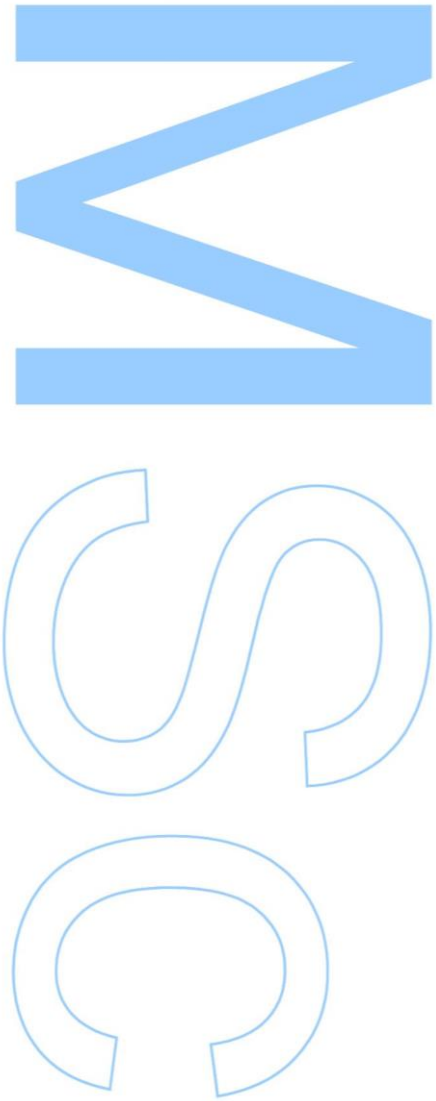
2019/2020

Orientador

Ana Couto, Researcher at Ciimar, FCUP

Coorientador

Paula Enes, Researcher at Ciimar, FCUP



Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

To my grandma. Might your light always shine upon me and guide me even in the darkest days.

Agradecimentos

A escrita desta dissertação foi com certeza um grande desafio, e por isso queria aproveitar para agradecer a todas as pessoas que de alguma forma me ajudaram nesta fase da minha vida académica.

Em primeiro lugar gostaria de agradecer à Dr. Ana Couto por me ter aceite como sua estudante de mestrado neste projeto e por toda a confiança que depositou em mim ao longo deste processo. Trabalhar neste projeto foi de facto uma experiência extremamente enriquecedora tanto em termos pessoais como profissionais, de onde certamente levarei muitas ferramentas que me ajudaram na minha vida profissional. À Dr. Paula Enes queria agradecer todos os conhecimentos científicos dispensados e toda a ajuda prestada ao longo deste projeto. Também ao Prof. Aires Oliva-Teles, por me dar a oportunidade de conhecer o trabalho que faziam neste grupo e me acolher nesta equipa.

A toda a equipa do NUTRIMU, que proporcionaram um excelente ambiente de trabalho, grande companheirismo e se mostraram sempre prestáveis para ajudar, um sincero obrigado. Um agradecimento muito especial à Marta Monteiro por toda a paciência, pela ajuda incansável e estar sempre disponível para auxiliar e ensinar. O seu apoio e motivação foram essenciais na realização deste trabalho. Também à Maria Serrano, não só pela amizade como pela grande entreajuda. Não me posso esquecer de todos os que estiveram comigo na Foz. Teremos sempre boas histórias para contar das peripécias que aí aconteceram.

Não menos importante, um agradecimento muito grande à Soja de Portugal, Fundação Amadeu Dias e toda a equipa do Projeto IJUP-Empresas, por tornarem este projeto possível.

Às minhas coabitantes preferidas, Susana e Joana, obrigada por acreditarem sempre nas minhas capacidades e por me motivarem sempre a fazer melhor. Sinto-me extremamente sortuda de terem aparecido na minha vida. A vossa amizade e companheirismo foram muito importantes durante este ano.

Às minhas batatinhas – Artur, Carina, Catarina, Diogo, Marisa, Matilde, Pedro, Sara e Xutos – que estão lá desde o primeiro dia. Obrigado por todo o apoio e amizade que me deram ao longo destes anos, e pelas memórias incríveis que me proporcionaram. No decorrer destes anos tornaram-se verdadeiramente na minha segunda família.

Por último, não posso deixar de agradecer à minha família - pais, irmã, avós, tia Inês e tio Jorge - pelo apoio incondicional que me deram durante a minha formação, por sempre acreditarem em mim e me motivarem a lutar por melhor. Sem vocês não seria o que sou hoje.

Um sincero obrigado a todos, do fundo do meu coração!

Resumo

Peixes de aquacultura estão sujeitos a múltiplos fatores de stress crónicos e agudos, como densidade de peixes por tanque, qualidade da água, flutuações de temperatura, transporte ou confinamento. Estes stresses alteram as funções endócrinas, metabólicas, celulares, antioxidantes e imunes, tornando os organismos mais vulneráveis a doenças, o que acaba por afetar o rendimento de produção através da redução do crescimento e reprodução, e consequentemente levando a perdas económicas. De forma a mitigar estes efeitos, a resposta antioxidante aos stresses pode ser modulada nutricionalmente, uma vez que fatores nutricionais foram reportados afetar o estado oxidativo de peixes.

Atualmente o mercado mediterrânico de peixes de aquacultura está saturado com robalo e dourada, criando a necessidade de domesticar novas espécies para diversificar as espécies produzidas na Europa. A corvina parece ser um bom candidato para a produção em larga escala, apresentando elevadas taxas de crescimento, forma apelativa e bom valor nutricional. No entanto, esta espécie é bastante suscetível a stress.

Este estudo tem como objetivo aliviar os efeitos negativos do stress através da utilização de dietas funcionais. Para isso, extratos de *N. gaditana* e *F. vesiculosus,* pré-selecionados no nosso laboratório pelo seu potencial antioxidante foram incorporados em dietas para corvina. Quatro dietas foram testadas – uma dieta controlo (N0F0), uma dieta suplementada com *F. vesiculosus* (N0F1), uma suplementada com *N. gaditana* (N1F0) e uma com os dois extratos (N1F1) – em dois grupos de peixes, um sujeito a um stress crónico (flutuação de temperatura) e outro a um stress agudo (manuseamento/confinamento).

No geral, a inclusão de extratos de *F. vesiculosus* e *N. gaditana* parece ter um efeito positivo na mitigação dos efeitos sentidos pelo stress. Os nossos resultados mostram que estes extratos apresentam poder anti-inflamatório e reduzem metabolitos do plasma. O uso de *F. vesiculosus* também mostra melhorias na eficiência alimentar, e melhora a habilidade de combater agentes patogénicos, oferecendo a peixes proteção a curto prazo. Este extrato mostrou um aumento da atividade antioxidante no músculo. Isto mostra que o extrato de *F. vesiculosus* reduz o desequilíbrio fisiológico resultante de stress agudo e crónico, sendo capaz de fornecer melhorias na saúde de peixes de aquacultura.

Palavras chave: corvina; dietas funcionais; stress; aquacultura; extratos de algas; *Nannochloropsis gaditana*; *Fucus vesiculosus*

Abstract

Fish in aquaculture are under a number of chronic and acute stressors, such as crowding, water quality, temperature fluctuation, handling, transportation or confinement. These stresses alter endocrine, metabolic, cellular, hematological, antioxidant and immune functions, making organisms more vulnerable to disease and affecting the production yield by reducing growth and reproduction, which leads to economic losses. To mitigate such effects, fish antioxidant response to stressors can be modulated nutritionally, since nutritional factors were reported to differently affect fish oxidative status.

Nowadays the Mediterranean market of farmed fish is saturated with European seabass and gilthead seabream, creating the need to domesticate new species to diversify the species cultured in Europe. In response to this, meagre seems to be a good candidate for large scale farming, having high growth rates, appealing shape and a good nutritional value. However, this species is very susceptible to stress.

This study aimed to alleviate negative stress effects through the use of functional feeds. For this purpose, extracts from *N. gaditana* and *F. vesiculosus,* pre-selected in our lab for their antioxidant potential were incorporated in meagre diets. Four diets were tested - a control diet (N0F0), a *F. vesiculosus* supplemented diet (N0F1), a *N. gaditana* supplemented diet (N1F0) and a mix diet (N1F1) - in two groups of fish, one subjected to a chronic stressor (temperature fluctuation) and other to an acute stressor (handling/confinement).

Overall, the inclusion of extracts from *F. vesiculosus* and *N. gaditana* seemed to have positive effects in the mitigation of the effects felt by stress. Our results show that these extracts have anti-inflammatory action and reduce plasma metabolites. The use of *F. vesiculosus* also showed improvements on feed efficiency, as well as enhanced ability to fight pathogens, which offers fish fed with this inclusion short-term protection. This extract showed an increase of antioxidant activity in the muscle. This shows that *F. vesiculosus* extracts reduce physiological imbalance resulting from chronic and acute stresses, being able to improve aquaculture fish's health and welfare.

Keywords: meagre; functional diet; stress; aquaculture; algal extracts; *Nannochloropsis gaditana*; *Fucus vesiculosus*

Table of contents

Table index

Figure index

Abbreviations

ANOVA - analysis of variance

CAT - catalase

cDNA - complementary DNA

CS - chronic stress

Ct - quantification cycle

DNA - deoxyribonucleic acid

EPA - eicosapentaenoic acid

FAO - United Nations Food and Agriculture Organization

FBW - final body weight

FCR - feed conversion ratio

FE - feed efficiency

FFCW - final feed container weight

FI - feed intake

G6PDH - glucose 6-phosphate dehydrogenase

GPX - glutathione peroxidase

GR - glutathione reductase

GSH - reduced glutathione

GSSG - oxidised glutathione

H2SO4 - sulfuric acid

HBSS - Hanks' Balanced Salt Solution

HCl - hydrochloric acid

HPI - hypothalamic-pituitary-interrenal

HSP - heat shock protein

Ht - haematocrit

IBW - initial body weight

IFCW - initial feed container weight

LPO - lipid peroxidation

LPS - lipopolysaccharides

MAFFT - Multiple Alignment using Fast Fourier Transform

MCV - mean corpuscular volume

MDA - malondialdehyde

N - nitrogen

NaOH - sodium hydroxide

NCBI - National Center for Biotechnology Information

NS - no stress

ORF - open reading frame

OSI - oxidative stress index

PCR - polymerase chain reaction

PUFA - polyunsaturated fatty acid

qPCR - quantitative Polymerase Chain Reaction

RBC - red blood cells

RNA - ribonucleic acid

ROS - reactive oxygen species

S - acute stress

SOD - superoxide dismutase

TAG - triacylglycerides

TBARS - thiobarbituric acid–reacting substances

tGSH - total glutathione

WBC - white blood cells

WG - weight gain

1. Introduction

1.1. The state of aquaculture

The production of aquatic organisms is a well-established industry with a long history of over 4000 years (Beveridge & Little, 2002), being traditionally performed as a craft, and the knowledge passed on from generation to generation (Silva & Anderson, 1994). Despite being around for such a long time, only in recent decades aquaculture had a real impact in the world seafood production (Beveridge, 2008; Silva & Anderson, 1994). This was possible through improvements on the scientific knowledge about the biology of aquatic species and the aquatic environment, that started to be developed around the 19th century (Beveridge, 2008), as well as the technology needed to support these production systems.

Aquaculture is now the fastest growing food production sector (Conte, 2004; FAO, 2020). According to the most recent data from FAO, the global production from capture fisheries and aquaculture in 2018 was about 179 million tonnes, 46% of which representing aquaculture production (FAO, 2020). The increasing numbers in aquaculture production are needed in order to supply resources for the ever-growing world population (Diana, 2009; Naylor et al., 2000; Ottinger et al., 2016).

In the last decades, aquaculture production has suffered a great spurt while capture fisheries remained static (Figure 1), making aquaculture the main responsible for the growing supply for fish consumption (FAO, 2020; Naylor et al., 2000). In parallel, it was recorded a large annual increase of human consumption of fish products, of about 3.2%, which surpassed the consumption increase of all meats, with the exception of poultry (FAO, 2018).

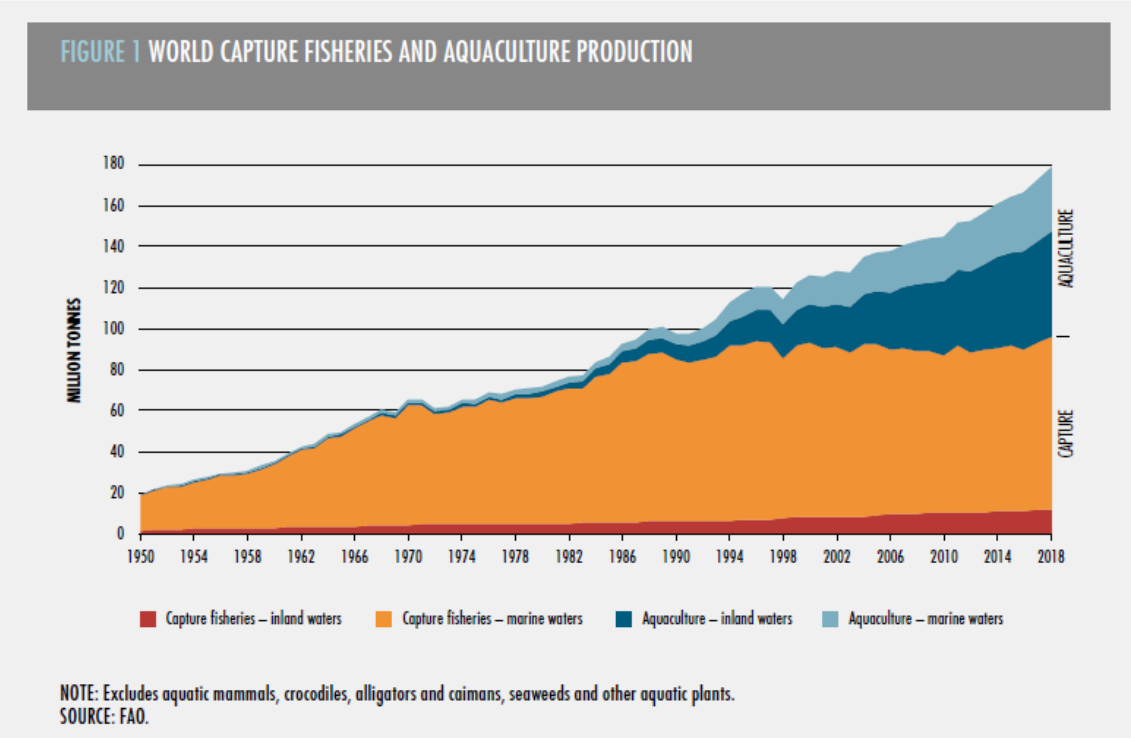


Figure 1 - Evolution of aquaculture and capture production. Retrieved from FAO, 2020.

Aquaculture production in the Mediterranean countries is mainly dominated by two species: European seabass (*Dicentrarchus labrax*) and gilhead seabream (*Sparus aurata*) (Quéméner et al., 2002). The excessive production of these species lead to market saturation, and thus to a decrease in prices and a consequent diminish of profits (Cardia & Lovatelli, 2007). This creates the need for the introduction of new species, in order to diversify the market supply (Cardia & Lovatelli, 2007). Meagre (*Argyrosomus regius)* presents itself as a great candidate for aquaculture diversification, and thus several studies have been dedicated to the optimization of its culture (Asencio-Alcudia et al., 2019). Based on its aquaculture characteristics, meagre ranks as number 8 in the top of novel species for marine Mediterranean aquaculture (Quéméner et al., 2002), having the potential to become a mass market species (Monfort, 2010).

1.2. Meagre (*Argyrosomus regius*, Asso 1801)

Meagre (*Argyrosomus regius,* Asso 1801) is one of the largest carnivorous fish from the Sciaenidae family (Quéméner et al., 2002), that feeds mostly on smaller fish species from Cupleidae and Mugilidae families and crustaceans (Jiménez et al., 2005). It is a gregarious species (Millán-Cubillo et al., 2016) that can be found across the

Mediterranean and the Black Sea, and throughout the Atlantic cost (Gonzalez-Silvera et al., 2018).

This species is very recent in aquaculture, having its culture only started around the late 90s in France and Italy (Monfort, 2010), and since then have expanded (Figure 2) (Bagni, 2005) jumping from 30 tonnes to close to 35000 tonnes in the last two decades (FishStat, 2020).

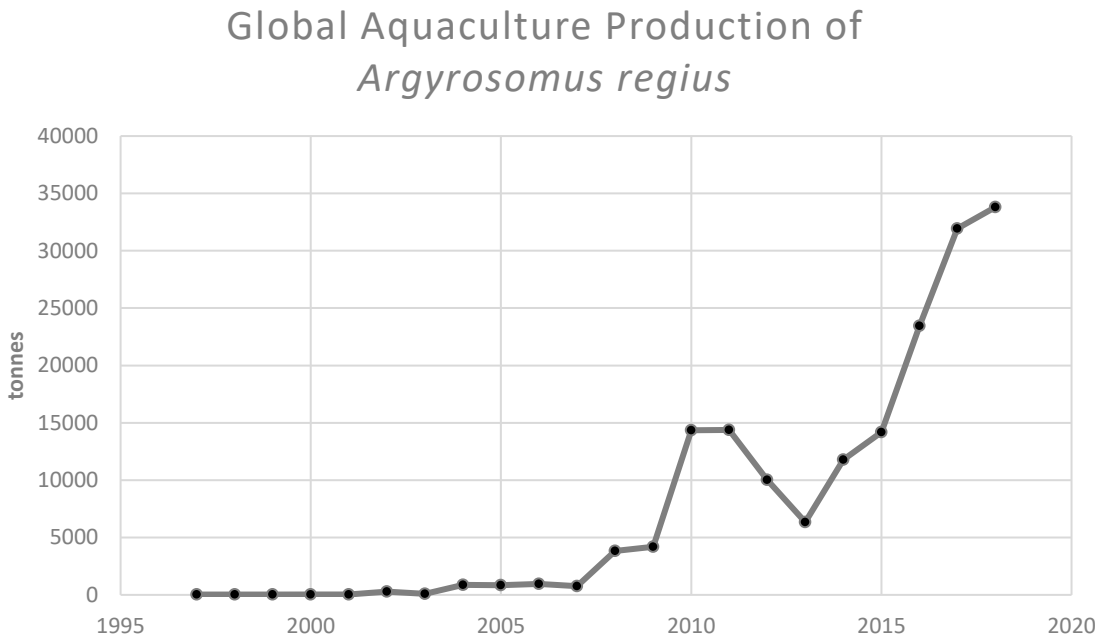


Figure 2 - Meagre production throughout the years. Data retrieved from FAO FishStat (2020).

Meagre is a marine species that shows great qualities for intensive production and commercialization. It has a high growth rate, being able to reach 2.5 kg in 24 months (Jiménez et al., 2005; Monfort, 2010), as well as a high feed conversion rate (FCR) which varies from 0.9 to 1.2 (Monfort, 2010). The previously mentioned characteristics are more similar to those found in Atlantic salmon than in the top Mediterranean aquaculture species (European sea bass and gilthead sea bream), reason why *A. regius* has been considered to be “the Mediterranean salmon” and its production is projected to grow accordingly (Duncan et al., 2013). The fact that meagre is able to reach a large size is an advantage to the aquaculture industry, since it offers many processing options (e.g. filleting, preserved foods) (Monfort, 2010). In terms of nutritional value, meagre has high protein and low lipid contents, with an interesting profile of polyunsaturated fatty acids (PUFA) (high proportion of n-3 PUFA and low n-3/n-6 ratio values) (Monfort, 2010). Studies have shown that meagre reared intensively has low intramuscular fat level, as well as long post-mortem preservation (Giogios et al., 2013; Grigorakis et al., 2011; Poli

et al., 2003). In terms of attractiveness to the consumers, meagre presents an appealing shape and a firm texture, as well as an attractive colour and good taste (Monfort, 2010).

Meagre adapted easily to captivity and its life cycle is dominated, however, due to its recent cultivation history, specimens are still prone to stress (Quéméner et al., 2002).

1.3. Stress in fish

Stress is one of the major issues in aquaculture, which impacts negatively fish welfare and overall production yield. During their production cycle, particularly in intensive conditions, fish are under several acute and chronic stressors, such as crowding, water quality, temperature fluctuation, handling, transportation or confinement (Bricknell & Dalmo, 2005). Acute stressors are the most commonly observed in nature and are characterized for its short duration and high intensity, inducing a fight-or-flight response (Tort, 2011). On the other hand, chronic stressors are characteristic for their low intensity but long duration, being a persistent stimulus, and are mostly associated to anthropogenic activities such as aquaculture (Tort, 2011). The intensity and duration of a stressor will influence the type of response the organism will develop to fight it (Yada & Tort, 2016).

When exposed to a threat, animals develop a series of neural, endocrine and autonomic response mechanisms, leading to immune, metabolic, energetic, and gene and protein expression changes, in order to maintain or re-establish homeostasis (Barton, 2002; Gonzalez-Silvera et al., 2018; Tort, 2011). Stress response can be divided in three different levels, depending on the duration of the stimulus. A primary response occurs when subjected to a short-term stressor, corresponding to an acute stress, which leads to an increase of hormonal levels and enhancement of the immune functions. A secondary response corresponds to a transition phase where alteration of metabolism and the immune system occur, as well as osmoregulatory disturbances. Finally the tertiary response is linked to a chronic stress, which causes whole-animal changes, suppressed immune response, increased disease susceptibility and behavioural alterations (Nardocci et al., 2014).

Fish under stress release the stress-hormone cortisol into the blood, due to the activation of the hypothalamic-pituitary-interrenal (HPI) axis (Gonzalez-Silvera et al., 2018). The activation of the HPI axis, and the increase of cortisol levels drives to an energy source mobilization, and thus to a reduction of glycogen stores, as well as to an increase in

plasma glucose and lactate levels, with high muscle activity and anaerobic glycolysis (Ashley, 2007; Laiz-Carrión et al., 2003).

Driven by the high cortisol levels, stress induces metabolic processes that increase oxygen consumption, leading to the production of reactive oxygen species (ROS) (Vinagre et al., 2012). Under most physiological states, ROS production is closely matched by antioxidant responses. However, when ROS levels are beyond the organism capacity to cope with, oxidative stress occurs inside the cell leading to lipids, proteins and DNA damage (Sopinka et al., 2016; Vinagre et al., 2012).

Moreover, high cortisol levels have a negative effect on fish immune response, leading to immune suppression and reduced immunocompetence (Richard et al., 2009; Vazzana et al., 2002). Cortisol is involved in ending inflammation (Yada & Tort, 2016), as it supresses most genes related to the inflammatory response, and the expression and production of proinflammatory cytokines (IL-1, IL-6, and TNF) (Castillo et al., 2009; MacKenzie et al., 2006; Sternberg, 2006; Tort, 2011; Kemenade et al., 2009). It also has a silencing effect on the production of antibodies (decreasing the antibody response), and on phagocytosis and leukocyte mitosis (Balm, 1997; Tort, 2011; Verburg-van Kemenade et al., 2009; Bonga et al., 1997; Weyts et al., 1999; Yada & Nakanishi, 2002). Thus, cortisol promotes a significant increase of granulocytes and neutrophils, as well as a decrease of lymphocytes and monocytes (Esteban et al., 2004; Harris & Bird, 2000; Weyts et al., 1998; Wojtaszek et al., 2002). All these alterations of the immune function compromise fish immune defence and resistance to pathogens, making them more vulnerable and susceptible to diseases (Asencio-Alcudia et al., 2019; Barton, 2002; Khansari et al., 2018; Kumar et al., 2012). This is in correlation with the fact that many diseases occur in fish that previously underwent stress (Tort, 2011).

Overall, exposure to stress mobilises resources that would normaly be solely focused on the normal function of the organism (such as growth) to overcome the adverse condition, leading to reduced growth, higher susceptibility to diasease, higher mortality rates and ultimaly afecting fish quality. Therefore, trying to diminish stress is a priority in aquaculture, in order to reduce economic losses.

1.4. Functional feeds

Functional feeds can be defined as diets that, not only satisfy the basic nutritional species requirements, but also aim to improve fish health and welfare, as well as production yield

and sustainability by reducing stress inherent to aquaculture production and modulating fish immune functions, through the inclusion of determined compounds (Encarnação, 2016; Soto et al., 2015). This aquafeed optimization can be implemented through dietary supplementation with several bioactive compounds, which serve as functional ingredients such as carotenoids, fatty acids, proteins, polysaccharides, phenolic compounds and vitamins that improve key metabolic pathways in organisms, therefore improving growth, health, survival, reproduction, welfare and development (Li et al., 2009; Plaza et al., 2009). These bioactive compounds possess antioxidant, antiviral, antidiabetic, antibacterial, immunomodulatory and antitumoral bioactivities (Agregán et al., 2017; Catarino et al., 2019; Imbs et al., 2015; Huifang Li et al., 2017; Onofrejová et al., 2010; Singh & Sidana, 2013; Singh et al., 2005; Veena et al., 2007; Wang et al., 2008; Wang et al., 2012), and may help to promote better health conditions.

The potential of functional feeds has been demonstrated in several studies in which the aquafeeds were supplemented with essential amino acids (Andersen et al., 2016; Asencio-Alcudia et al., 2019; Gonzalez-Silvera et al., 2018; Höglund et al., 2005; Nelson & Cox, 2013), and minerals (Hepher & Sandbank, 1984), carotenoids such as fucoidan (Sony et al., 2019), and other bioactive compounds, which resulted in improvements in growth, reproduction, disease resistance and immune response, as well as behavioural changes like reduction of aggression and cannibalism.

1.4.1. Algae as a functional ingredient

Algae are primary aquatic producers, exhibiting lipid and protein profiles that ranges from 1-45% and from 6-71%, respectively, depending on algae species and growing conditions (Chen et al., 2015; Neveux et al., 2015; Roy & Pal, 2015), as well as a rich content of bioactive compounds (Yaakob et al., 2014). The production of bioactive compounds and secondary metabolites is part of algae protection mechanism, that allows these organisms to survive in a wide range of environments (Batista et al., 2009; Gupta et al., 2012; Ibañez et al., 2012; Jiménez-Escrig et al., 2001). Algal biomass is considered to be a renewable and sustainable source of high quality substances (Michalak & Chojnacka, 2018). Algae's properties and composition are of great interest for utilization in a wide variety of industries such as the food, cosmetic, pharmaceutic, nutraceutical as well as the feed industry (Mendis, 2011; Pangestuti & Kim, 2011; Yaakob et al., 2014). The antioxidant, antiviral and antibacterial properties of algae

bioactive compounds make them of a great interest for inclusion in new functional feeds (Onofrejová et al., 2010; Singh et al., 2005).

There are several ways to include algae in diets. The most common consist on the utilization of the whole-algae and algae extracts. Extracts may be more advantageous then the use of whole-algae, as algae have rigid cell walls which make them hard to be digest by carnivorous fish, and gives them low nutrient availability (Gong et al., 2020). Extracted bioactive compounds can be used in a wide variety of products, which are useful in several industries (Michalak & Chojnacka, 2018). One advantage of using algae extracts is the ability to have a higher control on the final product characteristics, such as colour, flavour, consistency and stability, allowing the standardization required for marketed products (Kim & Chojnacka, 2015; Michalak & Chojnacka, 2014). The use of extracts also offers the possibility to avoid the incorporation of harmful compounds and extract only the compound of interest, which cannot be done when using the whole-algae (Barkia et al., 2019). Depending on the extraction techniques applied, algae extracts concentrate bioactive compounds without compromising or reducing their bioactivity (Michalak & Chojnacka, 2015). Different extraction methods allow to obtain different bioactive compounds with different properties and activities (Kantachumpoo & Chirapart, 2010), thus the type of extraction used can be chosen to meet the bioactivity desired to be incorporated in the final product.

Phenolic compounds comprise a group of bioactive components present in photosynthetic organisms and its implications in the antioxidant activity of both macro and microalgae has already been described (Bulut et al., 2019; Ganesan et al., 2008; Heffernan et al., 2014; Li et al., 2007; Yuan et al., 2018). In macroalgae, phenolic compounds detain a more significative role in the antioxidant activity (Onofrejová et al., 2010) when compared with microalgae, since the latter produce a much wider range of antioxidant compounds, such as carotenoids, PUFAs and polysaccharides (Singh et al., 2005). These compounds have an active role in cell protection against stress by providing protection against oxidative damage (Dai & Mumper, 2010; Onofrejová et al., 2010).

Some studies have investigated the topic of dietary supplementation with different algae species and algae extracted compounds in fish feeds, showing improvements not only in growth performance, feed conversion, protein efficiency (Emre et al., 2013; Sony et al., 2019) and survival rate (Magnoni et al., 2017), but also enhancing fish immune (Peixoto et al., 2016; Reyes-Becerril et al., 2013) and antioxidant responses (Peixoto et al., 2016), and reducing oxidative stress (Magnoni et al., 2017; Sony et al., 2019).

1.5. *Nannochloropsis gaditana*

Nannochloropsis gaditana (Figure 3) is a microalgae belonging to the class Eustigmatophyceae, isolated for the first time in Bay of Cádiz, Spain (Lubian, 1982). It can normally be found in marine environments (Rocha et al., 2003; Taleb et al., 2015), and performs asexual reproduction through simple division (Lubian, 1982; Murakami & Hashimoto, 2009). Under culture, its able to grow in a broad spectrum of temperatures, pH and salinities, and thrive in high densities (Radakovits et al., 2012).

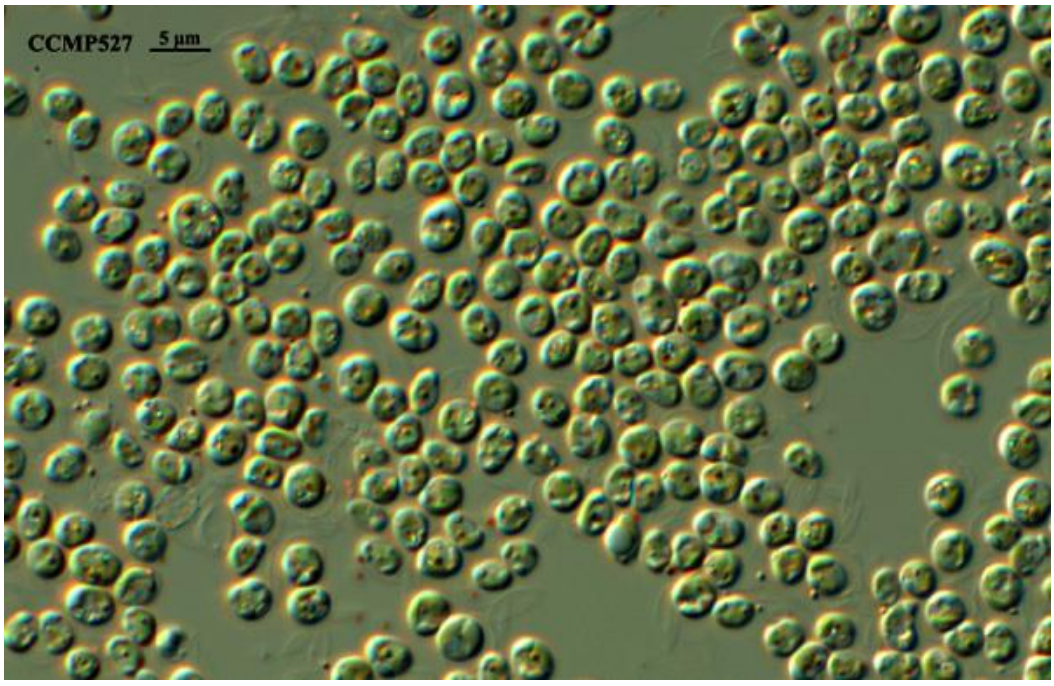


Figure 3 - Nannochloropsis gaditana, strain CCMP527. Source: Provasoli-Guillard National Center for Marine Algae and Microbiota.

This species detains a lot of commercial value. Its high biomass accumulation rates and lipid content, stored as triacylglycerides (TAG), are of great interest for the production of biofuel (Bondioli et al., 2012; Jinkerson et al., 2013; Moazami et al., 2012; Quinn et al., 2012; Radakovits et al., 2012). Moreover, it presents good nutritional value (Camacho-Rodríguez et al., 2013; Rocha et al., 2003) due to its biochemical composition, being able to produce high levels of valuable pigments such as chlorophyll a, beta-carotene, violaxanthin and vaucheriaxanthin (Macías-Sánchez et al., 2005) along with being an important source of PUFAs, especially EPA (eicosapentaenoic acid) (20:5 n-3) (Rocha et al., 2003). Its high content in lipids (about 45% of dry weight) (Ma et al., 2014) and carotenoids makes *N. gaditana* a natural source of antioxidants (Millao & Uquiche, 2016),

having some studies on its extracts revealed to possess exceptional antioxidant activity (Haoujar et al., 2019; Mekdade et al., 2016). It is a species very attractive to use in aquaculture due to its fast grow, lack of toxicity, adequate cell size and digestibility (Khatoon et al., 2014), and due to the production of high valuable compounds and respective bioactivity, thus being commonly used in aquafeeds (Camacho-Rodríguez et al., 2013; Ferreira et al., 2009; Macías-Sánchez et al., 2005).

A study performed by Cerezuela (2012) on the supplementation of gilthead seabream's diets with lyophilized *N. gaditana* at concentrations of 50 and 100 g kg^{-1} showed that this microalgae has an enhancing effect on fish immune system. The enhancement of the immune response was also demonstrated by Carballo (2020) when using whole-cell crude extract of *N. gaditana*, as well as higher growth performance of Senegalese sole larvae. These results are not consensual, as showed by a study developed by Walker (2011) where a mixed diet of *Nannochloropsis sp.* and *Isochrysis sp.* inclusion of 15% and 30% had negative effects on growth and feed intake of Atlantic cod.

1.6. Fucus vesiculosus

Fucus vesiculosus (Figure 4) is a brown macroalgae from the class Phaeophyceae, which distribution commonly occurs across the Atlantic Ocean and the Mediterranean Sea, as well as throughout the Baltic Sea, White Sea, Barents Sea and Kara Sea (Krivoruchko *et al.*, 2017). It is a marine species who is able to grow in high salinity waters and depths from 0.5 to 4 m (Catarino et al. 2017).

Figure 4 - Fucus vesiculosus. Source: Nature Picture Library.

F. vesiculosus detains a balanced content in bioactive nutrients and phytochemicals, such as phlorotannins, fucoidans, fucoxanthin, and are recognized for their high accumulation of essential minerals, particularly iodine (Catarino et al., 2018; Circuncisão et al., 2018; Ferreira et al., 2019). Fucoidans in particular are the most studied compounds of brown algae and are characteristic for their antioxidant, antibacterial, antiviral, immunomodulatory and antitumoral effects (Agregán et al., 2017; Imbs et al., 2015; Huifang Li et al., 2017; Veena et al., 2007; Wang et al., 2008). They are also rich in phlorotannins, which represent the major phenolic compounds in *F. vesiculosus* (Catarino et al., 2017) with strong antioxidant, antibacterial and antidiabetic activities (Catarino et al., 2019; Singh & Sidana, 2013; Wang et al., 2012), playing a key role in the protection against external factors (Singh & Sidana, 2013).

Studies regarding diet supplementation with *F. vesiculosus* (as whole-algae and extracted compounds) showed improvements on immune and antioxidant responses in fish fed with this diets, enhancing resistance against external agents (Peixoto et al., 2016; Traifalgar et al., 2013) as well as exhibiting anti-genotoxic and genoprotective properties (Marques et al., 2020; Pereira et al., 2019), which improve fish welfare and output.

1.7. Aims

This study aimed to improve fish health and welfare in culture conditions, through the use of dietary formulations capable of diminishing the negative effects of the stressful conditions fish undergo at certain events of their production cycle.

For this purpose, extracts from *N. gaditana* and *F. vesiculosus,* pre-selected in our lab for their antioxidant potential were incorporated in balanced diets for meagre with the major goal of contributing to reduce physiological effects resulting from sustained (temperature fluctuation) and acute (netting/confinement) stressors, and consequently improving fish health and welfare.

2. Material and methods

2.1. Experimental diets

Four experimental diets were formulated to contain 48% protein and 18% lipids (Table 1). A plant feedstuff-based diet with only 25% fish meal, the control diet (N0F0), and the control diet supplemented with three algae extracts (1% *N. gaditana* inclusion, diet N1F0; 1% *F. vesiculosus* inclusion, diet N0F1; and 0.5% *N. gaditana* + 0.5% *F. vesiculosus* inclusion, diet N1F1). These algae extracts were pre-selected in our lab for their antioxidant potential, and the best combination and concentration determined by tests previously carried out in our lab (ZEBRALGRE-PTDC/CVT-WEL/5207/2014). Algal extracts were prepared from freeze-dried biomass from *F. vesiculosus* obtained from Alga+ (Ílhavo, Portugal) and *N. gaditana* obtained from Buggy Power S.L. (San Pedro del Pinatar, Murcia, Spain), which were milled and then mixed with 1.3 L of ethanol/water or methanol/water solvent mixtures (E80:20 for *N. gaditana* and M50:50 for *F. vesiculosus*, respectively). The mixtures were then vortexed and incubated for 30 min with continuous orbital agitation, in the dark, at room temperature. Each mixture was centrifuged for 15 min at 10,000×g at 4ºC and the supernatant was collected. This procedure was repeated three times and supernatants from successive extractions were pooled. The extracts were then filtered through Whatman 1 filter paper and subsequently subjected to solvent evaporation, in a Rotavapor (BUCHI R-100, Switzerland) at < 40ºC, to remove organic solvent, and freeze-dried to remove remaining water.

All dietary ingredients were finely grounded, well mixed, and dry pelleted using a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA). Pellets were then dried in an oven at 40ºC, and then stored in closed plastic containers in a freezer until used. Formulation and proximate analysis of the experimental diets is shown in Table 1.

Table 1 - Composition and proximate analysis of the experimental diets.

	Diets			
	N0F0	**N1F0**	**N0F1**	**N1F1**
Ingredients (% dry weight)				
Fish meal [a]	25.0	25.0	25.0	25.0
Wheat gluten [b]	5.0	5.0	5.0	5.0
Corn gluten [c]	15.0	15.0	15.0	15.0
Soybean meal [d]	32.4	32.4	32.4	32.4
Wheat meal [e]	0.9	0.9	0.9	0.9
Cellulose	1.5	0.5	0.5	0.5
Ammonium phosphate	1.3	1.3	1.3	1.3
Fish oil	5.2	5.2	5.2	5.2
Soybean oil	5.0	5.0	5.0	5.0
Rapeseed oil	5.0	5.0	5.0	5.0
Taurine	0.2	0.2	0.2	0.2
Vitamin mix [f]	1.0	1.0	1.0	1.0
Mineral mix [g]	1.0	1.0	1.0	1.0
Binder	1.0	1.0	1.0	1.0
Choline	0.5	0.5	0.5	0.5
***N. gaditana* extract (E80:20)**	0.0	1.0	0.0	0.5
***F. vesiculosus* extract (M50:50)**	0.0	0.0	1.0	0.5
Proximate analyses (% dry weight)				
Dry matter	92.4	93.6	92.7	93.7
Crude protein	48.1	48.4	49.6	49.7
Crude fat	15.2	16.0	15.7	16.0
Ash	11.4	11.3	11.6	11.4

N0F0 - control diet; N1F0 - diet supplemented with 1% N. gaditana, N0F1 - diet supplemented with 1% F. vesiculosus; N1F1 - diet supplemented with both algae (0.5% inclusion each)
CP – crude protein; CF – crude fat
[a] *Sorgal, S.A. Ovar, Portugal (CP – 65.8%; CF – 8.0%)*
[b] *Sorgal, S.A. Ovar, Portugal (CP – 83.0%; CF – 2.3%)*
[c] *Sorgal, S.A. Ovar, Portugal (CP – 69.9%; CF – 3.3%)*
[d] *Sorgal, S.A. Ovar, Portugal (CP – 51.3%; CF – 1.1%)*
[e] *Sorgal, S.A. Ovar, Portugal (CP – 12.2%; CF – 1.2%)*
[f] *Vitamins (mg kg^{-1} diet): retinol, 18 000 (IU kg^{-1} diet); cholecalciferol, 2 000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bisulphate, 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.*
[g] *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; potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.44 (g kg^{-1} diet).*

2.2. Trials and sampling procedure

The experiments were directed by accredited scientists with category C following Federation of European Laboratory Animal Science Associations (FELASA) recommendations and conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

Meagre juveniles were obtained from IPMA, Olhão, Portugal. After transportation to the experimental facilities, fish were kept in quarantine for 3 weeks during which they were fed with a commercial diet (18% lipids and 44% protein, Aquasoja Sustainable Feed, Sorgal, Ovar, Portugal). After adaptation to the experimental conditions, 13 fish with an average weight of 28.8 g ± 0.1 g were randomly distributed to each tank. The trials were conducted at the Marine Zoology Station, Porto University, Portugal, in two thermo-regulated recirculating water systems with 12 tanks of 100 L each (Figure 5), supplied with a continuous flow of filtered seawater. During the one-month trial, a 12h:12h light:dark photoperiod was adopted and oxygen maintained near saturation, 7 mg L^{-1}. As for temperature, one system was set for the species optimum temperature: 22ºC with 1ºC of fluctuation allowed – no stress group - and the other system was set for the same temperature (22ºC) with induced weekly fluctuations of ± 4ºC – chronic stress group. Diets were randomly assigned to triplicate tanks and fish hand fed to apparent visual satiation, twice a day, 6 days per week, for one month. Feed consumption was recorded weekly. Mortality was daily recorded.

Figure 5 - Thermo-regulated recirculating water systems. The right side corresponds to the system subjected to thermal chronic stress and the left side to no thermal stress.

Fish from the chronic stress group exhibited a high mortality rate, thus the sampling and acute stress procedure described below was only applied to the no stress group.

By the end of the trial, 3 fish were randomly selected from each tank, gently netted and blood samples immediately collected from the caudal vein using heparinized syringes and centrifuged for 10 min at 10,000×g to obtain plasma, which was stored at −20ºC until analysis. Then fish were euthanized by anaesthetic overdose (ethylene glycolmonophenyl ether, ref.: 8.07291, Merck, Whitehouse Station, USA) and fractions of liver and muscle collected, snap frozen in liquid nitrogen and stored at -80ºC until enzymatic analysis. Another fraction of head kidney and muscle was collected, placed in RNA later at 4ºC for 24 h and subsequently stored at -20ºC until gene expression analysis. The remaining fish were subjected to an acute stress through handling. In each tank, fish were chased for 1 min every 10 min, during 1 h and then caught with a nylon net, confined in a bucket for 10 min and sampled as described above.

2.3. Fish performance parameters

To evaluate fish performance, weight gain (WG), feed intake (FI), feed efficiency (FE) and mortality (%) were determined through the following equations, where IBW stands for initial body weight, FBW for final body weight, IFCW for initial feed container weight and FFCW for final feed container weight.

$$Weight\ gain\ (g) = FBW\ (g) - IBW(g)$$

$$Weight\ Gain\ (\%\ IBW) = \frac{FBW\ (g) - IBW(g)}{IBW\ (g)} \times 100$$

$$Feed\ Intake\ (g) = IFCW(g) - FFCW(g)$$

$$Feed\ Efficiency = \frac{wet\ weight\ gain\ (g)}{dry\ feed\ intake\ (g)}$$

$$Mortality\ (\%) = \frac{nº\ dead\ fish}{nº\ initial\ fish} \times 100$$

2.4. Chemical analysis

Chemical analysis of ingredients and diets were performed according to standard methods:

2.4.1. Dry matter

Dry matter from both ingredients and diets was determined by weighing the samples in crucibles, and then drying them in an oven at 105ºC until constant weight. The crucibles

with dried samples were then weighed and the dry matter percentage calculated through the following formula:

$$Dry\ matter\ (\%) = \frac{Sample's\ weight\ before\ drying\ -Dried\ sample's\ weight}{Sample's\ weight\ before\ drying}\ X\ 100$$

2.4.2. Ash

The inorganic matter (ash) was determined by incineration of the samples previously dried in the process of dry matter determination, in a muffle furnace at 450ºC for 16 h. The crucibles were weighed before and after incineration and the weight difference indicates the percentage of inorganic matter.

2.4.3. Protein content

The protein content of diets and ingredients was determined through the quantification of nitrogen (N) in the sample by the Kjedahl method (N × 6.25), assuming proteins contain an average of 16% of N (100/16 = 6.25), following acid digestion, using Kjeltec digestion and distillation units (Tecator systems, Höganäs, Sweden, models 1015 and 1026, respectively) (Figure 6). Triplicates of each sample were weighed into distillation tubes (approximately 150 mg each), and then to each tube 1 Kjeldahl tablet was added, acting as a catalytic, with 5 mL of concentrated H_2SO_4. Samples were digested at 450ºC in the digestion unit for 1 h, converting the sample nitrogen in ammonium sulphate. Thereafter, samples were distilled in the distillation unit. To each digestion tube, water and sodium hydroxide (NaOH, 40%) was added, and saturated boric acid was used for ammonium sequestration in the distillation. Finally, the N content was quantified through titration with HCl (0.5N) in the presence of methyl orange pH indicator.

Figure 6 - Kjeltec distillation unit, used in the determination of protein content.

2.4.4. Lipid content

The lipid content of ingredients and diets was determined by continuous extraction with petroleum ether using a SoxTec system (Tecator systems, Höganäs, Sweden, extraction unit model 1043 and service unit model 1046) (Figure 7). Approximately 1 g of each sample was added, in duplicate, in a cartridge and then placed in the extraction unit. Temperature was regulated to 120ºC. Next, 50 mL of petroleum ether was added to the extraction cups and placed at the extraction unit, where they were boiled for 1 h. Samples were then rinsed for 2 h, and the lipids were extracted to a cup. After solvent evaporation, the cups were dried in the oven at 100ºC for 15 min. Finally, the cups were weighed, and the lipid content estimated through the weight difference between cups before and after extraction.

Figure 7 - SoxTec system, used to determine lipid content.

2.5. Plasma metabolites

Commercial kits were used for determination of cortisol (EIAHCOR Invitrogen), lactate and glucose (Ref. 1001330 and 1001191, respectively, Spinreact). All plasmatic parameters were analysed according to manufacturers' instructions, using a colorimetric reaction, and absorbance was measured in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

2.6. Haematological analysis

Fresh heparinized blood was used for haematocrit (Ht) determination and blood cells counts. To determine Ht, microcentrifugation was performed at 10,000×g for 10 min at room temperature. Total red blood cells (RBC) and white blood cells (WBC) counts were done in blood dilutions with HBSS (Hanks' Balanced Salt solution) using a Neubauer improved counting chamber. Blood smears were then performed, air dried and fixated with formolethanol (10% of 37% formaldehyde in absolute ethanol). Detection of peroxidase activity was used for neutrophils identification following Afonso et al. (1998). In order to identify and count neutrophils, thrombocytes, monocytes and lymphocytes, slides were stained with hematoxylin-eosin and observed in an amplification of 1000x.

2.7. Gene expression

Total RNA was isolated from muscle and head kidney samples, previously stored in RNA later, following the manufacturer's specifications. RNA was extracted and purified using Direct-zol™ RNA MiniPrep kit (Zymo Research). Samples were added to 600 µL of Trizol (Zymo Research) and then homogenised (Precellys Evolution, Bertin Technologies) and centrifuged at 13000 rpm for 2 min at 4ºC. Then, 400 µL of the supernatant was transferred to Zymo-Spin™ IIC Column collection tube, where it was added 400 µL of ethanol (95-100%) and centrifuged at 13000 rpm for 1 min at 4ºC. Samples were then treated with DNase I following Direct-zol™ RNA MiniPrep kit protocol. Finally, to elude RNA, samples were treated with 30 µL of DNase/RNase-Free water (Sigma-Aldrich). The RNA quality was then checked in 1% agarose gel. Total RNA was quantified spectrophotometrically (µDrop™ plate, ThermoScientific). Purified RNA was then converted to cDNA using NZY First-Strand cDNA Synthesis kit (MB12501, Nzytech), as described by the manufacturer.

Gene expression of heat shock proteins 70 (HSP70) and 90 (HSP90) in the muscle, and immune-related genes IL-1β (proinflammatory), TNF-α (proinflammatory), IL-10 (anti-inflammatory), and cytokines in head kidney were determined using real-time quantitative PCR (CFX Connect™ Real-Time System, Bio-Rad, California, USA). cDNA amplification was performed using specific PCR primers (Table 2). To assess heat shock proteins gene expression, and since no nucleotide sequences were available for meagre in the literature, identification and primer design for the genes of interest were performed. First, the genes of interest nucleotide sequences from species closely related to *A. regius* were blasted using NCBI database. Then, to identify conserved regions (≥80% similarity between nucleotide sequence of the studied fish and of similar species), the nucleotide sequence was aligned using MAFFT alignment software (https://www.ebi.ac.uk/Tools/msa/mafft/). Next, based on the open reading frame (ORF), primers were designed on the identified conserved regions (primer length 20-23 bp; product size 100-250 bp; Tm 60±1 °C; G/C ≤50 %) and their quality determined using ThermoFisher Scientific Multiple Primer Analyser software (https://www.thermofisher.com/pt/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). In order to determine primer efficiency, seven serial twofold dilutions of cDNA were carried out and calculated from the slope of the regression line of the quantification cycle (*Ct*) versus the log 10 of six

different dilutions of a cDNA mix solution of all samples analysed (Pfaffl, 2001). Real-time qPCR reactions were performed using 3.5 µL of ultrapure water (Sigma-Aldrich), 5 µL of SsoAdvanced Universal SYBR ® Green supermix (Bio-Rad), 0.5 µL of each primer and 1 µL cDNA from each sample, adding up to a final volume reaction of 10 µL. The different transcripts were amplified using technical triplicates per sample, under the following conditions: 95°C for 30 s for denaturation, followed by 40 cycles of 95°C for 15 s, and 58°C for 30 s. A melting curve analysis was performed to verify that only specific amplification occurred, and no primer dimers were amplified. A standard curve calculated with the equation $E=10^{-1}$/slope was used to determine Real-time PCR efficiency (E). Relative expression of each transcript was normalized to the selected housekeeping gene (EF-1α) and calculated using the Pfaffl method (Pfaffl, 2001).

Table 2 - Primers used for real-time PCR

Gene	Sequence	Efficiency	Amplicon (bp)	Reference
EF-1α	F: TACGGTTCCGATACCGCCG R: AACATGCTTGAGGGCAGTGACAA	2.0	189	Milne, 2018
IL-1β	F: GATTGCCTGGATTTTCCACTGTCTCCA R: GTGGCTCTGGGCATCAAGGG	2.3	103	Milne, 2018
IL-10	F: ACTCCTCGGTCTCTCCTCGTATCCGC R: CTGTGTCGAGATCATCGTTGGCTTCATAAAAGTC	2.1	187	Milne, 2018
TNF-2α	F: CACAAGAGCGGCCATTCATTTACAAGGAG R: GGAAAGACGCTTGGCTGTAGATGG	1.8	173	Milne, 2018
HSP70	F: ATCACAGTTCCGGCGTATTT R: ATGGACACGTCAAAGGTGCC	1.9	197	Present study
HSP90	F: ATCGTGGAGACTCTCAGGCA R: CTGTAGATGCGGTTGGAGTG	1.9	146	Present study

2.8. Enzyme activity

Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), and glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activities were determined in liver and muscle as described by Guerreiro et al. (2014). Liver and muscle samples were homogenized (1:9 and 1:3 dilution, respectively) in ice-cold buffer (100 mM-Tris-HCl, 0.1 mM-EDTA, 0.1% triton X-100 (v/v), pH 7.8; Sigma). Protein concentration in homogenates were determined by the Bradford method (Bradford, 1976) using BioRad Protein Assay Dye Reagent (Ref. 5000006, BioRad) with bovine albumin as standard.

All the analyses of enzymatic activity were carried out at 37°C. A Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China) was used to monitor the changes in absorbance.

Enzyme activities were expressed as units (CAT) or milliunits (G6PD, GPX and GR) per mg of soluble protein. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate/min under the above assay conditions.

2.9. Lipid peroxidation (LPO)

Liver and muscle samples were homogenized (1:9 and 1:3 dilution, respectively) in ice-cold buffer (100 mM-Tris-HCl, 0.1 mM-EDTA, 0.1%triton X-100 (v/v), pH 7.8; Sigma). Malondialdehyde (MDA) concentration was used as a marker of LPO level in the liver and muscle. In the presence of thiobarbituric acid, MDA reacts producing coloured thiobarbituric acid-reacting substances (TBARS) that was measured as described in Guerreiro et al. (2014). Results were expressed as nanomoles MDA per gram of wet tissue, calculated from a calibration curve.

2.10. Total and oxidised glutathione

Liver and muscle samples were homogenized (1:9 and 1:3 dilution, respectively) in an ice-cold solution of 1.3% 5-sulfosalicylic acid (w/v) (Sigma) and 10 mM HCl (Sigma). All procedures were carried out on ice in order to avoid glutathione oxidation. Homogenates were centrifuged at 14,000×g for 10 min at 4ºC and the resulting supernatants stored at -80ºC. Total glutathione (tGSH) and oxidised glutathione (GSSG) were measured as described by Griffith (1980) and Vandeputte et al. (1994) with some modifications (Pérez-Jiménez et al., 2012). Standard curves of reduced glutathione (GSH) and GSSG were used for tGSH and GSSG calculations, respectively. GSH level were calculated by subtracting GSSG from tGSH values. The oxidative stress index (OSI) was calculated through the following equation:

$$OSI = 100 \times \frac{2 \times GSSG}{tGSH}$$

2.11. Statistical analysis

Results were analysed using the IBM SPSS Statistics v26.0 software package for Windows. Data was tested for normality and homogeneity using Shapiro-Wilk and Levene tests, respectively. Results were then analysed using a three-way analysis of variance (ANOVA) with factors ‘Stress’, ‘Nanno’ and ‘Fucus’. When normality was not observed, data was analysed using Mann-Whitney’s U test. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Growth performance

Growth performance and feed utilization data of fish subjected to temperature fluctuation of 22 ± 4ºC (Chronic Stress - CS) and set to a temperature of 22 ± 1ºC (No stress - NS) are presented in Table 3. Chronic thermal stress (CS) led to significantly lower (P<0.05) feed intake, weight gain and feed efficiency (FE). Fish fed diets supplemented with *F. vesiculosus* (N0F1) showed a higher FE when compared to other diets, under NS or CS conditions. Supplementation with *N. gaditana* led to the lowest FE between all experimental diets under NS conditions. Under chronic stress, fish fed with both N1F0 and N1F1 diets showed higher FE than N0F0 diets. No differences were found in weight gain and feed intake, amongst the dietary treatments. No interactions between factors were observed.

Table 3 - Growth performance and feed utilization efficiency of meagre reared at constant temperature (22 ± 1ºC; NS) or submitted to a chronic thermal stress (22 ± 4ºC; CS) and fed the experimental diets.

Treatment	Diets	WG (g)	WG (%IBW)	Feed Intake (g)	FE
No stress (NS)	N0F0	180 ± 16	48.0 ± 4.2	287 ± 27	0.6 ± 0.1
	N0F1	223 ± 42	59.4 ± 11.4	299 ± 39	0.7 ± 0.1
	N1F0	149 ± 44	39.8 ± 11.8	327 ± 44	0.5 ± 0.1
	N1F1	170 ± 28	45.4 ± 7.3	290 ± 37	0.6 ± 0.0
Chronic stress (CS)	N0F0	26 ± 10	7.0 ± 2.6	242 ± 33	0.1 ± 0.0
	N0F1	10 ± 21	16.3 ± 5.5	187 ± 25	0.3 ± 0.1
	N1F0	53 ± 20	14.0 ± 5.4	275 ± 17	0.2 ± 0.0
	N1F1	43 ± 20	11.5 ± 5.3	228 ± 20	0.2 ± 0.1

Three-way ANOVA	WG	WG (%IBW)	Feed Intake	FE
N	0.191	0.191	0.114	**0.014**
F	0.129	0.127	0.062	**0.006**
Stress	**0.000**	**0.000**	**0.001**	**0.000**
N * F	0.252	0.250	0.521	0.165
N * Stress	0.116	0.117	0.516	0.064
F * Stress	0.500	0.499	0.234	0.840
N * F * Stress	0.683	0.688	0.375	0.111

WG - weight gain; FE - feed efficiency.
Mean initial body weight (IBW) of 28.8 g.
Data are represented as the mean ± SD.

3.1.2. Cumulative mortality under chronic stress

Results have shown that fish under chronic stress (CS) of temperature fluctuation were more fragile and susceptible to diseases. On the last week of the trial, an opportunistic pathogen took advantage of their fragile state and infected them, which led to mass mortality. Data from cumulative mortality during the last week of the trial is shown in Tables 4 and 5, and in Figure 8.

Data shows that fish fed with N1F0 diets had the highest mortality per day, being the only experimental diet to reach 100% mortality, in a short number of days. N0F0 treatment had the second highest mortality per day, reaching more than 90% mortality. *Fucus* supplementation promoted a mortality delay of 1 day in N1F1 and 2 days in N0F1. Fish treated with diets containing *Fucus* extract (N0F1 and N1F1) showed the lowest mortality percentage of all experimental diets at the end of 7 days (80-85%).

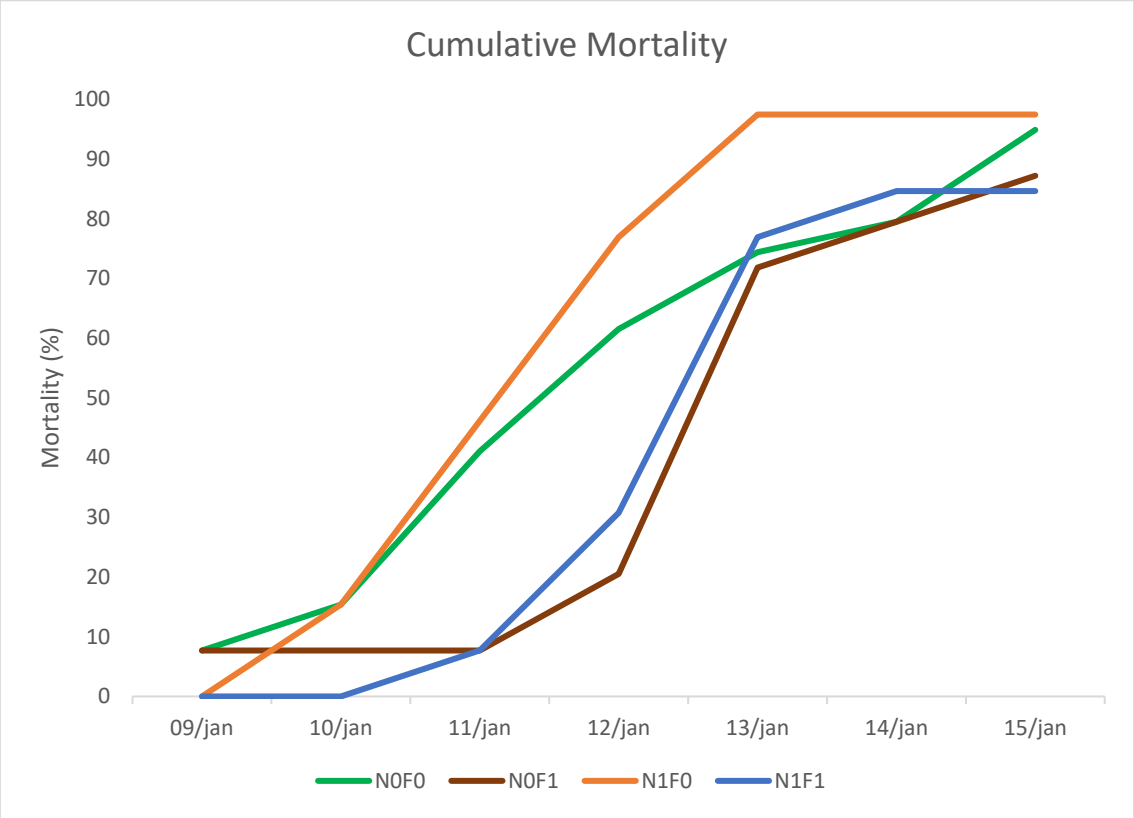


Figure 8 - Cumulative mortality of meagre under chronic stress during the last week of feeding the experimental diets.

Table 4 - Tukey tests for differences of mortality levels for each day of the last week of feeding with the experimental diets.

Date	
09/jan	A
10/jan	Ab
11/jan	B
12/jan	C
13/jan	D
14/jan	D
15/jan	D

Table 5 - Three-way ANOVA of cumulative mortality of meagre under chronic stress during the last week of feeding with the experimental diets.

Three-way ANOVA	Mortality	
N	0.000	
F	0.025	
Date	0.000	
N * F	0.079	
N * Date	0.658	
F * Date	0.000	
N * F * Date	1.000	
Interactions		**Date**
	F0	0.010
	F1	0.000

3.2. Acute stress trial

3.2.1. Plasma metabolites

Data on plasma glucose, cortisol and lactate levels in the acute stress trial are shown in Table 6. When fish under acute stress and no stress are compared, a significant increase ($P<0.05$) of plasma glucose and cortisol levels on fish under S conditions was observed. Supplementation with *F. vesiculosus* (N0F1 and N1F1 diets) lowered plasmatic glucose levels. On the other hand, supplementation with *N. gaditana* (N1F0 and N1F1 diets) only lowered glucose levels in the non-stress group (NS), while overall glucose values on the S group were higher than those of NS group, regardless of *N. gaditana* supplementation. Lactate levels were lower in stressed fish (S) regardless of dietary treatment. Moreover, considering supplementation, *F. vesiculosus* decreased lactate levels when supplemented alone (N0F1 diet), while the same lactate lowering effect was observed

when *N. gaditana* was supplemented alone (N1F0). Levels of cortisol were significantly higher under stress, regardless of dietary treatment.

Table 6 - Plasma metabolites of meagre reared under no stress (NS) and acute stress (S) and fed with the experimental diets.

Treatment	Diets	Glucose (mmol L^{-1})	Lactate (mmol L^{-1})	Cortisol (ug L^{-1})
No stress (NS)	N0F0	3.7 ± 0.9	3.9 ± 1.2	1.6 ± 0.0
	N0F1	3.3 ± 0.7	3.4 ± 1.3	1.0 ± 0.4
	N1F0	3.0 ± 0.6	3.5 ± 0.8	0.9 ± 0.5
	N1F1	3.0 ± 0.6	3.8 ± 0.9	1.4 ± 1.4
Acute stress (S)	N0F0	5.1 ± 0.5	3.5 ± 0.9	6.2 ± 3.5
	N0F1	4.6 ± 0.9	2.7 ± 1.1	12.5 ± 7.2
	N1F0	4.9 ± 0.8	2.6 ± 0.9	15.5 ± 21.8
	N1F1	4.8 ± 0.7	2.9 ± 0.8	14.6 ± 4.8

Three-way ANOVA	Glucose	Lactate
N	**0.028**	0.295
F	**0.046**	0.276
Stress	**0.000**	**0.000**
N * F	0.092	**0.010**
N * Stress	**0.036**	0.241
F * Stress	0.586	0.650
N * F * Stress	0.913	0.665
Glucose Interactions		**N**
	No stress	0.002
	Acute stress	0.942
		Stress
	N0	0.000
	N1	0.000
Lactate Interactions		**F**
	N0	0.022
	N1	0.203
		N
	F0	0.026
	F1	0.157

Mann- Whitney's U test	Cortisol
N	0.843
F	0.671
Stress	**0.000**

3-way ANOVA and Mann-Whitney's U test.
Data are represented as the mean ± SD.

3.2.2. Haematologic parameters

Haematologic parameters are presented in Table 7. From the tested parameters, only RBC was significantly affected by S conditions, where stressed fish exhibited a higher count when compared to NS fish. Among dietary treatments, fish fed diets supplemented with *F. vesiculosus* (N0F1 and N1F1 diets) exhibited a higher WBC count, under NS and S conditions.

Table 7 - Haematologic parameters of meagre reared under no stress (NS) and acute stress (S) and fed with the experimental diets.

Treatment	Diets	RBC (10^6 mm^{-3})	RBC (%)	WBC (10^4 mm^{-3})	WBC (%)	Haematocrit (%)	MCV
No stress (NS)	N0F0	1.5 ± 0.3	94.8 ± 1.1	7.8 ± 0.9	5.2 ± 1.1	22.1 ± 2.6	153 ± 18
	N0F1	1.4 ± 0.2	94.7 ± 0.8	7.8 ± 1.0	5.3 ± 0.8	21.1 ± 5.0	155 ± 64
	N1F0	1.4 ± 0.3	94.9 ± 1.3	7.2 ± 1.3	5.1 ± 1.3	19.8 ± 2.7	145 ± 26
	N1F1	1.4 ± 0.2	94.2 ± 1.4	8.6 ± 1.4	5.8 ± 1.4	19.9 ± 0.8	143 ± 18
Acute stress (S)	N0F0	1.5 ± 0.3	95.5 ± 1.7	7.0 ± 2.5	4.5 ± 1.7	23.7 ± 2.5	161 ± 34
	N0F1	1.5 ± 0.1	95.0 ± 0.4	7.9 ± 1.0	5.0 ± 0.4	19.6 ± 2.4	131 ± 18
	N1F0	1.6 ± 0.2	95.5 ± 0.6	7.4 ± 1.1	4.5 ± 0.5	21.2 ± 2.0	136 ± 20
	N1F1	1.6 ± 0.2	95.1 ± 0.8	8.2 ± 0.9	4.8 ± 0.8	23.2 ± 2.8	144 ± 23

Three-way ANOVA	**RBC (10^6 mm^{-3})**	**RBC (%)**	**WBC (10^4 mm^{-3})**	**WBC (%)**	**Haematocrit (%)**	**MCV**
N	0.690	0.516	0.578	0.893	0.081	0.846
F	0.974	0.411	**0.045**	0.121	0.149	0.327
Stress	**0.024**	0.056	0.918	0.106	0.195	0.937
N * F	0.680	0.392	0.440	0.707	0.452	0.206
N * Stress	0.671	0.773	0.535	0.776	0.783	0.898
F * Stress	0.319	0.484	0.282	0.847	**0.044**	0.682
N * F * Stress	0.870	0.529	0.946	0.911	0.270	0.738

Haematocrit Interactions		**F**
	No stress	0.369
	Acute stress	0.218
		Stress
	F0	0.299
	F1	0.453

3-way ANOVA. (RBC – red blood cells; WBC – white blood cells; MCV - mean corpuscular volume).
Data are represented as the mean ± SD.

3.2.3. Gene expression

Expression of immune-related genes TNF-α, IL-1β, IL-10 in the kidney, and heat shock protein genes HSP70 and HSP90 in the muscle of meagre under the acute stress trial are presented at Figure 9 and Figure 10, respectively.

The expression of TNF-α showed a significant increase in S fish fed with N0F0 diets compared with NS fish (Figure 9A). Although not statistically significant, fish fed with N0F1 diet had an opposite response compared to the other groups, exhibiting lower TNF-α's expression in S fish. Under S conditions fish fed N0F0 diet showed significantly higher TNF-α expression compared with the remaining dietary treatments. N0F1 diets had the lowest TNF-α expression under S conditions.

Regarding the expression of IL-1β, only the control diet (N0F0) was affected by stress (Figure 9B). Although no statistical differences were observed, there was a greater decrease of IL-1β expression levels in *F. vesiculosus* diets then in *N. gaditana* diets compared to the control. Among all dietary treatments, N0F0 diet show higher IL-1 β expression.

N0F1 was the only experimental diet where significant differences were found regarding exposure to acute stress (Figure 9C) in the expression of the anti-inflammatory gene IL-10, exhibiting lower IL-10 expression. All other dietary treatments showed a tendency to have higher expression when under S conditions.

No differences were observed in the expression of HSP70 (Figure 10A). In HSP90, N1F0 showed significant higher expression under acute stress (Figure 10B). Although not statistically significant, all algae supplemented diets resulted in the same increasing trend between NS and S groups, which contrasts with the decrease on the expression of HSP90 on the control diet (N0F0) under S conditions.

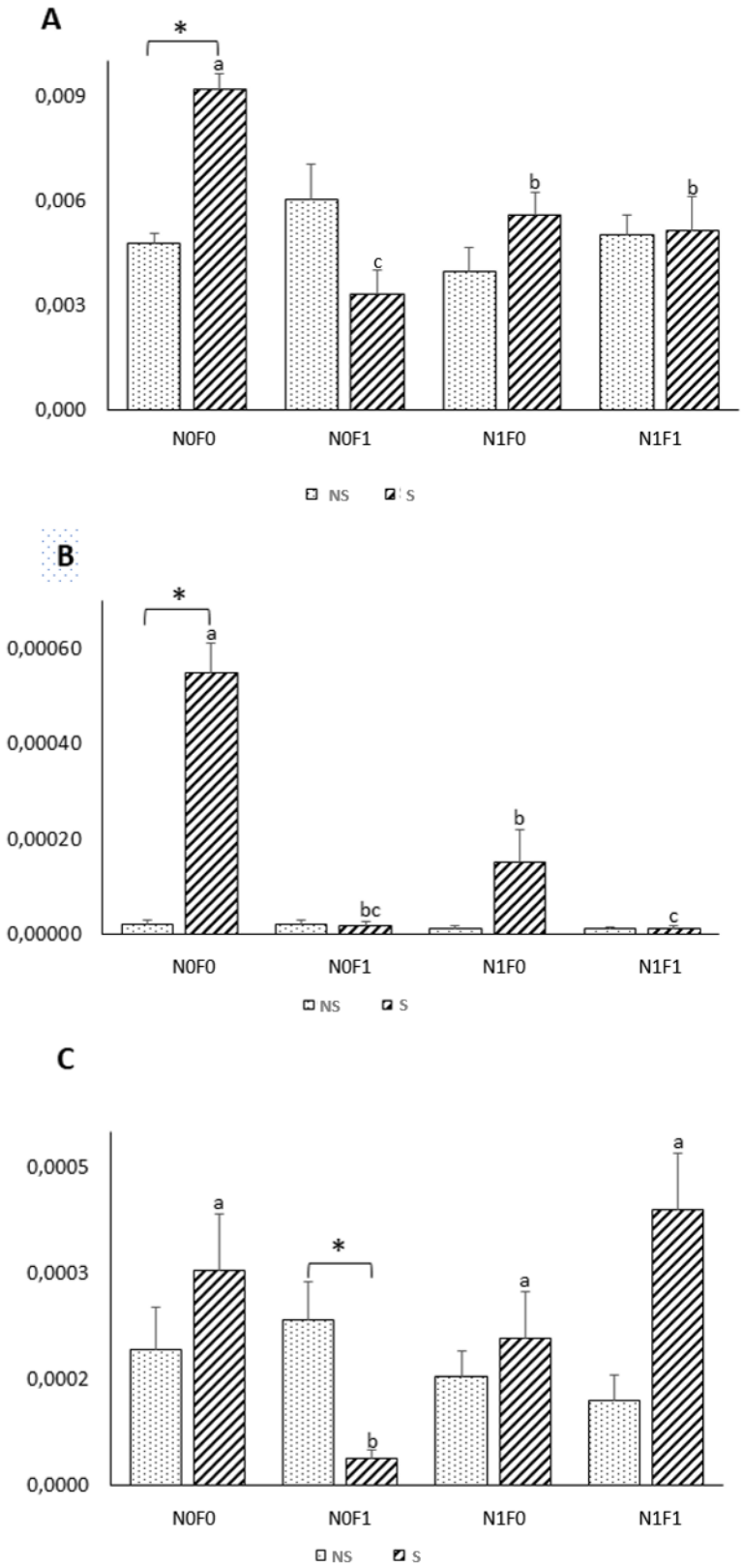


*Figure 9 - Expression of meagre immune-related genes in the head-kidney (A – TNF-α; B - IL-1β; C – IL-10), under no stress (NS) and acute stress (S) conditions, after four weeks of feeding the experimental diets. Data was analysed using Mann-Whitney's U test. Data are represented as the mean ± SE (n = 9). Different letters indicate significantly differences amongst dietary treatment (p < 0.05). * indicate differences between NS and S fish (p < 0.05).*

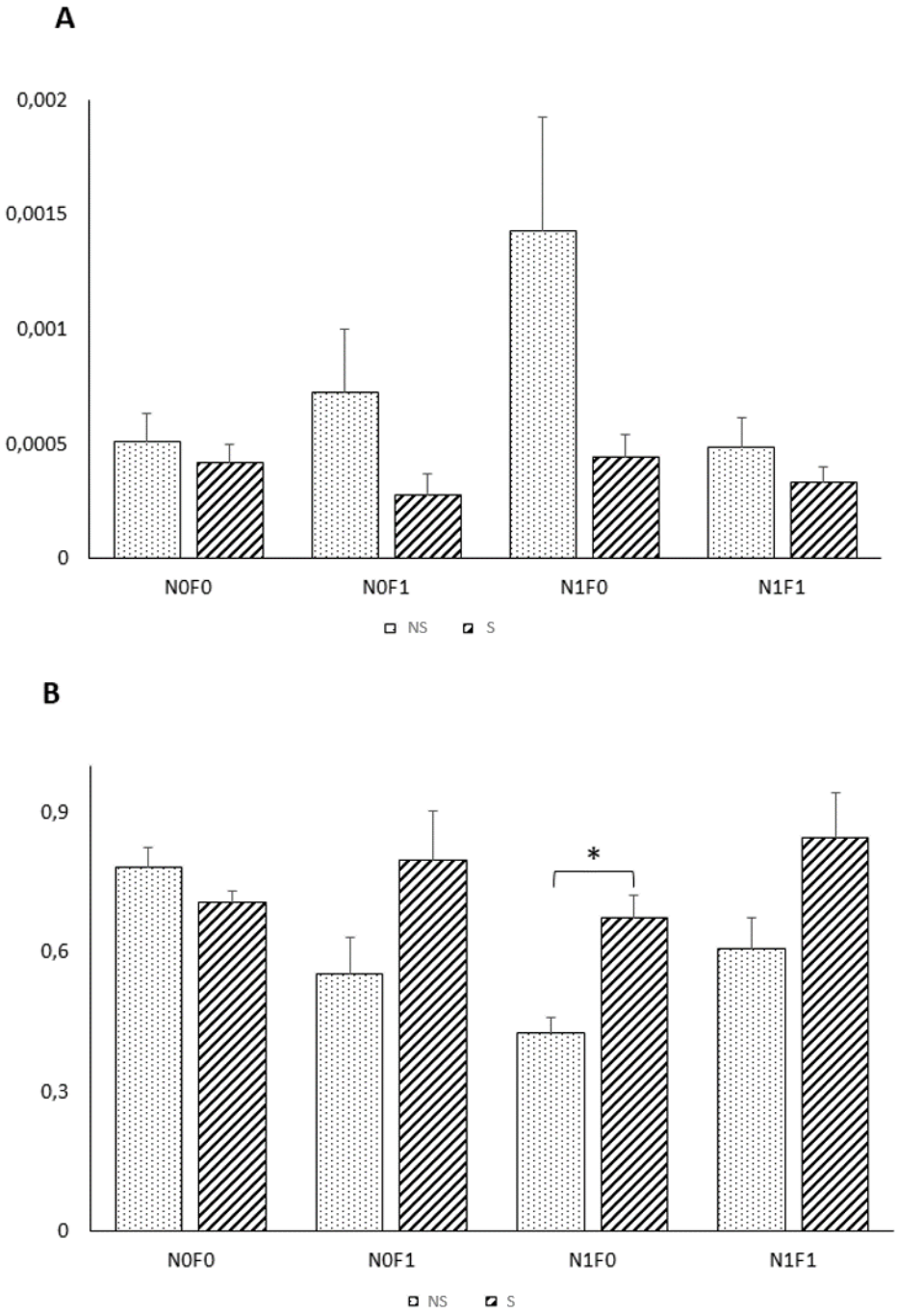


*Figure 10 - Expression of meagre heat-shock (A - HSP70; B - HSP90) genes in the muscle, under no stress (NS) and acute stress (S) conditions, after four weeks of feeding the experimental diets. Data was analysed using Mann-Whitney's U test. Data are represented as the mean ± SE (n = 9). Different letters indicate significantly differences amongst dietary treatment (p < 0.05). * indicate differences between NS and S fish (p < 0.05).*

3.2.4. Oxidative stress

The liver antioxidant enzymes activity is represented at Table 8, liver stress markers at Table 9, muscle antioxidant enzymes activity at Table 10 and muscle stress markers at Table 11.

In meagre's liver, SOD, GR and G6PDH activity (Table 8) was lower in fish fed *N. gaditana* diets with or without *F. vesiculosus* supplementation (N1F0 and N1F1 diets), regardless of the stress condition. Additionally, stressed fish showed an increase in the activity of GPX, GR and G6PDH (Table 8). SOD activity was also higher in stressed fish (S) fed *N. gaditana* diet (N1F0), while *F. vesiculosus* supplementation had no effect on SOD activity, regardless of the stress condition. GSH levels (Table 9) were higher in NS fish. tGSH levels (Table 9) were lower in diets containing *N. gaditana* (N1F0 and N1F1) compared to diets on its absence, when submitted to stress.

Table 8 - Activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) (U mg^{-1} protein), glutathione peroxidase (GPX), glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PDH) (mU mg^{-1} protein) in liver tissue of meagre reared under no stress (NS) and acute stress (S) and fed with the experimental diets.

Treatment	Diets	SOD	CAT	GPX	GR	G6PDH
No stress (NS)	**N0F0**	445 ± 52	319 ± 76	136 ± 24	13.3 ± 0.8	73 ± 19
	N0F1	462 ± 77	358 ± 58	156 ± 25	12.6 ± 1.3	88 ± 13
	N1F0	335 ± 40	317 ± 54	137 ± 24	12.3 ± 1.5	75 ± 20
	N1F1	395 ± 53	328 ± 54	138 ± 27	11.6 ± 1.4	73 ± 12
Acute stress (S)	**N0F0**	622 ± 135	308 ± 46	627 ± 101	15.1 ± 0.6	98 ± 10
	N0F1	577 ± 149	300 ± 34	592 ± 89	13.6 ± 2.3	89 ± 21
	N1F0	538 ± 125	287 ± 36	575 ± 56	13.6 ± 0.8	78 ± 13
	N1F1	402 ± 53	321 ± 25	595 ± 56	13.6 ± 2.2	86 ± 16

Three-way ANOVA	SOD	CAT	GPX	GR	G6PDH
N	**0.000**	0.625	0.277	**0.037**	**0.048**
F	0.433	0.119	0.895	0.089	0.460
Stress	**0.000**	0.083	**0.000**	**0.000**	**0.013**
N * F	0.782	0.805	0.536	0.322	0.983
N * Stress	0.824	0.590	0.598	0.770	0.601
F * Stress	**0.010**	0.667	0.541	0.945	0.272
N * F * Stress	0.131	0.162	0.216	0.394	0.052

SOD Interactions		F
	No stress	0.243
	Acute stress	0.091
		Stress
	F0	**0.000**
	F1	0.147

3-way ANOVA.
Data are represented as the mean ± SD.

Table 9 – Levels of total glutathione (tGSH), reduced glutathione (GSH), oxidised glutathione (GSSG)(nmol/ g tissue), oxidative stress index (OSI) and lipid peroxidation (LPO) (mmol malondialdehyde/g tissue) in liver tissue of meagre reared under no stress (NS) and acute stress (S) and fed with the experimental diets.

Treatment	Diets	tGSH	GSH	GSSG	OSI	LPO
No stress (NS)	**N0F0**	1925 ± 266	1820 ± 282	105 ± 58	11.2 ± 6.7	21.8 ± 6.4
	N0F1	2015 ± 310	1887 ± 314	87 ± 23	8.8 ± 3.1	24.1 ± 12.8
	N1F0	2030 ± 92	1952 ± 123	79 ± 42	7.9 ± 4.6	20.3 ± 4.5
	N1F1	1897 ± 270	1826 ± 272	71 ± 33	7.6 ± 3.6	22.6 ± 9.0
Acute Stress (S)	**N0F0**	1817 ± 117	1747 ± 124	70 ± 38	7.8 ± 4.1	25.2 ± 3.6
	N0F1	1857 ± 209	1772 ± 241	72 ± 21	7.6 ± 2.6	29.3 ± 7.2
	N1F0	1624 ± 136	1599 ± 291	92 ± 46	11.1 ± 5.5	27.8 ± 8.9
	N1F1	1574 ± 110	1531 ± 109	44 ± 21	5.4 ± 2.7	21.7 ± 5.1

Three-way ANOVA	tGSH	GSH	GSSG	OSI	LPO
N	**0.031**	0.218	0.170	0.436	0.327
F	0.694	0.692	0.278	0.150	0.759
Stress	**0.000**	**0.002**	0.114	0.451	0.067
N * F	0.178	0.267	0.140	0.379	0.210
N * Stress	**0.027**	0.077	0.636	0.325	0.801
F * Stress	0.863	0.949	0.473	0.462	0.426
N * F * Stress	0.555	0.696	0.153	0.130	0.217
tGSH Interactions		N			
	No stress	0.934			
	Acute stress	**0.001**			
		S			
	N0	0.179			
	N1	**0.000**			

3-way ANOVA.
Data are represented as the mean ± SD.

In the muscle, supplementation of *N. gaditana* (N1F0 and N1F1) to meagre diets resulted in increased CAT activity and decreased G6PDH activity (Table 10). An increased activity of CAT and decreased activity of GPX (Table 10) was observed when fish were submitted to an acute stress. Supplementation with *F. vesiculosus* (N0F1 and N1F1) resulted in higher GR activity between S and NS groups, an effect that was more evident in the NS group, regardless of *N. gaditana* supplementation. Considering the effects of *N. gaditana* and *F. vesiculosus*, regardless of stress condition, an increased GR activity (Table 10) was observed in diets with *F. vesiculosus* but devoid of *N. gaditana* (N0F1), however, when *F. vesiculosus* was combined with *N. gaditana* extract (N1F1) GR activity decreased.

In the muscle, data showed a significant increase in tGSH, GSSG and LPO levels, and OSI (Table 11) in stressed fish. Additionally, *F. vesiculosus* supplementation resulted in decreased LPO levels.

Table 10 - Activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) (U mg^{-1} protein), glutathione peroxidase (GPX), glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PDH) (mU mg^{-1} protein) in muscle tissue of meagre reared under no stress (NS) and acute stress (S) and fed with the experimental diets.

Treatment	Diets	SOD	CAT	GPX	GR	G6PDH
No stress (NS)	**N0F0**	102 ± 20	0.9 ± 0.2	18.9 ± 1.8	1.4 ± 0.5	0.5 ± 0.1
	N0F1	107 ± 33	0.9 ± 0.2	23.4 ± 3.8	1.9 ± 0.3	0.6 ± 0.2
	N1F0	114 ± 13	1.0 ± 0.2	20.8 ± 4.4	1.6 ± 0.3	0.4 ± 0.1
	N1F1	88 ± 25	1.1 ± 0.3	22.0 ± 3.5	1.9 ± 0.5	0.5 ± 0.2
Acute stress (S)	**N0F0**	102 ± 32	1.3 ± 0.4	18.1 ± 3.8	1.5 ± 0.3	0.5 ± 0.2
	N0F1	117 ± 40	1.3 ± 0.6	20.8 ± 4.7	1.8 ± 0.4	0.6 ± 0.2
	N1F0	108 ± 18	1.5 ± 0.4	16.0 ± 6.7	1.6 ± 0.4	0.5 ± 0.1
	N1F1	103 ± 24	1.3 ± 0.4	15.1 ± 3.8	1.3 ± 0.2	0.5 ± 0.1

Three-way ANOVA	SOD	CAT	GPX	GR	G6PDH
N	0.615	**0.040**	0.102	0.471	**0.034**
F	0.692	0.480	0.100	**0.029**	0.152
Stress	0.519	**0.027**	**0.001**	0.073	0.652
N * F	0.073	0.510	0.127	**0.035**	0.707
N * Stress	0.958	0.832	0.069	0.230	0.880
F * Stress	0.258	0.555	0.393	**0.024**	0.743
N * F * Stress	0.666	0.793	0.946	0.256	0.829
GR interactions		**F**			
	No stress	**0.005**			
	Acute stress	0.956			
		Stress			
	F0	0.724			
	F1	**0.010**			
		F			
	N0	**0.005**			
	N1	0.956			
		N			
	F0	0.724			
	F1	**0.010**			

3-way ANOVA.
Data are represented as the mean ± SD.

Table 11 - Levels of total glutathione (tGSH), reduced glutathione (GSH), oxidised glutathione (GSSG) (nmol/ g tissue), oxidative stress index (OSI) and lipid peroxidation (LPO) (mmol malondialdehyde/g tissue) in muscle tissue of meagre reared under no stress (NS) and acute stress (S) and fed with the experimental diets.

Treatment	Diets	tGSH	GSH	GSSG	OSI	LPO
No stress (NS)	N0F0	135 ± 17	131 ± 17	3.8 ± 0.5	5.6 ± 0.9	1.7 ± 1.1
	N0F1	142 ± 20	138 ± 20	3.5 ± 0.9	5.2 ± 1.6	0.6 ± 0.4
	N1F0	124 ± 17	120 ± 18	3.5 ± 1.2	6.1 ± 2.9	0.9 ± 0.6
	N1F1	129 ± 26	124 ± 26	3.9 ± 1.4	6.3 ± 2.1	0.6 ± 0.4
Acute stress (S)	N0F0	146 ± 18	140 ± 18	6.4 ± 3.6	8.9 ± 5.1	2.3 ± 1.1
	N0F1	142 ± 24	135 ± 24	6.9 ± 2.9	9.9 ± 4.5	1.5 ± 0.4
	N1F0	151 ± 18	143 ± 17	7.8 ± 3.3	10.3 ± 4.2	1.7 ± 0.5
	N1F1	138 ± 18	130 ± 19	8.6 ± 2.6	13.0 ± 5.1	2.2 ± 1.7

Three-way ANOVA	tGSH	GSH	GSSG	OSI	LPO
N	0.283	0.191	0.255	0.113	0.297
F	0.853	0.728	0.472	0.158	**0.023**
Stress	**0.026**	0.111	**0.000**	**0.001**	**0.000**
N * F	0.631	0.563	0.533	0.252	0.076
N * Stress	0.238	0.263	0.321	0.953	0.409
F * Stress	0.163	0.178	0.518	0.517	0.172
N * F * Stress	0.800	0.850	0.958	0.845	0.977

3-way ANOVA.
Data are represented as the mean ± SD.

3. Discussion

Stress is an inherent part of all stages of aquaculture, derived by all the maintenance processes and characteristics of fish enclosure. This leads to deterioration of fish health and welfare, as well as to economic loses. Functional feeds constitute a tool to mitigate those effects. In this study extracts from two algae, *N. gaditana* and *F. vesiculosus*, that possess strong antioxidant potential were incorporated in functional diets for meagre in order to diminish aquaculture stress effects.

In the present study, there was an evident effect of chronic stress caused by water temperature fluctuations in growth performance, which led to lower growth rates, feed efficiency and feed intake. This is in line with what is described in literature. Santos et al. (2010) performed a study with European seabass which showed that chronic stress resulting from high densities and poor water quality, led to reduced fish performance, due to lower feed intake. In terms of temperature fluctuation, a study performed by Carveth et al. (2007) shows that this stressor has a negative effect on growth of *Meda fulgida*. Lobo et al. (2018) showed that temperature and salinity oscillations led to lower weight gain and growth in European seabass. This reduced growth performance was expected when facing stress, since stress leads to energy mobilization that would, under normal circumstances, be used for growth, in order to try to re-establish homeostasis. Stressed fish also ingest less feed under stress, thus lower growth performance corresponds to a cumulative effect of these two factors.

Several authors have studied the effect of dietary algae supplementation in fish performance. Studies performed by Lobo et al. (2018) and Peixoto et al. (2016) showed that diets containing *Fucus* spp. did not affect growth performance, feed intake and feed conversion ratio (FCR) in European seabass. Works from Valente et al. (2019), Sørensen et al. (2017), and Gbadamosi et al. (2018) on the influence of *Nannochoropsis* spp. in fish diets reveal that inclusions at lower levels (up to 10%) did not affect fish growth performance, although higher FCR was observed by Valente et al. (2019) in European seabass. In the present study, no differences were also observed in terms of growth and feed intake in all algae diets. However, the presence of *F. vesiculosus* extract enhanced FE under normal rearing conditions, showing that there was a better utilization of this diet by fish. Under stressful conditions, *Fucus* extract avoids a drastic decrease on FE when compared with the control diet. On the other hand, *N. gaditana* extract supplementation has a negative effect on FE, when fish were exposed to non-stressful situations, but it can potentiate a less accentuated drop of FE when subjected to thermal

stress (although not as pronounced as *F. vesiculosus* effect), thus suggesting the possibility to mitigate negative effects of stress on FE.

Algae extracts have an immunostimulant potential (Vallejos-Vidal et al., 2016), due to their rich content in bioactive compounds (Yaakob et al., 2014). Studies showed that immunostimulants can have a role in protection against pathogens. Hang et al. (2014) observed that after a challenge with *Edwardsiella ictaluri*, fish treated with lipopolysaccharides (LPS) and levamisole (a synthetic phenylimidazolthiazole, used in medicine as an immunostimulant) showed reduced mortality. Similarly, Wang et al. (2016) showed significantly lower mortality rates on fish infected with *Aeromonas hydrophila* when fed with diets supplemented with plant polysaccharides. Dias et al. (2019) showed that *Arapaima gigas* fed with the dietary immunostimulant additive Aquate FishTM® had no mortality after challenged with *A. hydrophila*. In the present study, fish fed with diets containing only *N. gaditana* had the highest mortality per day, when exposed to an opportunist pathogen, quickly reaching 100% mortality. In contrast, fish fed with *F. vesiculosus* supplemented diets showed a delay on the start of the mortality compared to fish fed with other diets, when given alone or mixed with *N. gaditana*. However, this effect was stronger when administered alone. This may indicate that *F. vesiculosus* offers a short-term protection against an external factor. The fact that *F. vesiculosus* supplemented diets showed the highest survival rates compared with all experimental diets corroborates the potential of *F. vesiculosus* extract in the protection of the organism. *N. gaditana*, on the other hand, did not offer any kind of protection. This might be due to the much higher total phenolic content and antioxidant capacity of *F. vesiculosus* extract, as described by a study performed by Monteiro et al. (2019) on the extracts used in the current study.

In response to acute stress, a number of changes occur in metabolic activity of the organism in order to fight/adapt to the stressor. This changes can be reflected in an increase of plasma cortisol and glucose levels (Martins et al., 2006; Ruane et al., 2002; Santos et al., 2010), as well as an increase of plasma lactate levels (Wendelaar Bonga et al., 1997). In this study, the increase of plasma glucose and cortisol levels on S group confirmed that fish were exposed to a stress induced reaction resulting from netting/confinement. However, and unexpectedly, there was a slight decrease of lactate levels when exposed to stress. Lactate levels are a result of muscle anaerobic metabolism (Kieffer, 2000). Grutter et al. (2000) suggested that low plasma lactate levels paired with high cortisol levels may indicate that either the stressful event did not induce anaerobiosis or the release of lactate into the plasma was not significant.

Adding immunostimulants to fish feeds showed to have effects on modulating plasma metabolites levels. Yeganeh et al. (2015) showed that inclusion of *Spirulina platensis* in diets for rainbow trout (*Oncorhynchus mykiss*) resulted in a significant decrease of plasma cortisol and glucose levels. Similar results were obtained by Ngugi et al. (2015) with supplementation with *Urtica dioica* on *Labeo victorianus*. This is in line with results from the present study, where supplementation with all tested algae reduced plasma glucose and lactate levels in both stressful and non-stressful situations, showing the positive effects of *F. vesiculosus* and *N. gaditana* extracts.

Our results regarding the haematological parameters showed that acute stress led to an increase of RBC counts, thus indicating stress. Under stress, RBC are expected to increase due to the release of high levels of cortisol and catecholamines into the blood stream, which increases metabolism and thus, the need to transport oxygen to all tissues (Wojtaszek et al., 2002). As a result, an increment of differentiation and proliferation of erythrocytes, and their release into the blood stream is expected to occur (Wojtaszek et al., 2002). Moreover, high levels of cortisol driven by stress have a silencing effect on phagocytosis and leukocyte mitosis (Balm, 1997; Kemenade et al., 2009; Tort, 2011; Bonga, 1997; Weyts et al., 1999; Yada & Nakanishi, 2002), meaning a decrease on circulating lymphocytes and monocytes levels (Esteban et al., 2004; Harris & Bird, 2000; Weyts et al., 1998; Wojtaszek et al., 2002). WBCs release key molecules in the immune defence and inflammation processes (Sopinka et al., 2016). In this study, dietary supplementation with *F. vesiculosus* stimulated an increase of WBC. This is the opposite of what would happen under stress, indicating that this seaweed increases the ability of organisms to fight stressful events, being a potential ingredient to improve innate immunity, which was demonstrated by the lower expression IL-10 and IL-1β under acute stress in the present study. This also correlates with data from cumulative mortality under chronic stress, where the higher number of WBC protected the organism against an opportunist pathogen. Other authors demonstrated that diet supplementation with immunostimulants also had a stimulating effect on WBC (Amin et al., 2015; Dias et al., 2019).

TNF-α and IL-1β are proinflammatory cytokines involved in immune responses against pathogens. When its production is compromised, this could lead to a lack of resistance against infection (Connor et al., 2005). Under acute stress, our study showed that fish fed with the control diet - N0F0 - had higher expression of the proinflammatory genes TNF-α and IL-1β. This means that the stress imposed by handling stimulated inflammation as a response to fight the unbalance created by stress. This might have to do with the magnitude of the stressor, as studies show that stress can have

immunostimulant properties depending on the nature and intensity of the stressor (Connor et al., 2005). Acute stress derived from a short-term stressor has the effect of increasing hormonal levels and enhancing the immune functions (Nardocci et al., 2014). On the contrary, high cortisol levels, driven by chronic stress, have a suppressive effect on the expression of proinflammatory genes (TNF-α and IL-1β) (Castillo et al., 2009; MacKenzie et al., 2006; Sternberg, 2006; Tort, 2011; Kemenade et al., 2009).

In the current study, fish fed with N0F1, showed lower expression of the anti-inflammatory gene IL-10 under stress, contrary to what happened with the other diets. Compared with the control, *F. vesiculosus* supplemented diets exhibited a more pronounced effect on lowering expression of IL-1β resulting from stress. The use of *F. vesiculosus* showed to have a strong effect on the inflammatory response, thus meaning that the supplementation with this seaweed extract counteracted the effects of the acute stress. Overall, supplementation with all algae showed suppression of pro-inflammatory genes, which is in line with Robertson et al. (2015), who showed the anti-inflammatory potential of seaweed by using lipid extracts from *Porphyra dioica, Palmaria palmata, Chondrus crispus* and *Pavlova lutheri* on LPS-stimulated human THP-1 macrophages, which inhibited the production of pro-inflammatory cytokines.

The expression of HSP can be regulated by the endocrine system (Yada & Tort, 2016). Studies have shown no alterations in fish HSP70 and HSP90 when exposed to common aquaculture stressors such as handling, hypoxia, hyperoxia, capture, and feed deprivation (Vijayan et al., 1997; Zarate & Bradley, 2003). This is in agreement with our results, as no differences were observed in HSP70. However, and although only statistically significant for N1F0 diet, all algae supplemented diets showed a tendency to have higher HSP90 expression under acute stress. These proteins have the role of protecting the cell against damage as well as activate anti-inflammatory T-cells, thus leading to higher immunoregulation (Wieten et al., 2010). The upregulation of HSP90 expression with the tested algae indicated that the latter are inducing an enhancement of immunoregulation, as well as restoring and protecting cells against damage.

High levels of cortisol driven by stress conditions increase the production of reactive oxygen species (ROS) as a by-product of metabolic processes (Vinagre et al., 2012). These molecules have the capacity to damage lipids, RNA and DNA (Sopinka et al., 2016; Vinagre et al., 2012). Antioxidants enzymes have the capacity to control oxidative stress by preventing the formation of ROS, serving as primary cellular defence against oxidative stress (Hegazi et al., 2010; Sopinka et al., 2016). In this study it was observed different responses in the liver and muscle to oxidative stress under acute handling

stress. There was an overall increase on antioxidant enzyme activities when fish where exposed to the stressor, which correspond to the organism's response in order to prevent the uncontrolled formation of ROS, and an increase of LPO in the muscle indicating lipid damage. Also, the increase of GSSG levels corroborates this stress effect. The increase on activity of antioxidant enzymes was more noticeable in the liver than the muscle. This difference between tissues may be due to the fact that whole-body changes, such as muscle, correspond to a tertiary stress response, and require a longer exposure to stress - chronic stress (Nardocci et al., 2014). Our results are in line with what described by Yang et al. (2017), where Largemouth bass (*Micropterus salmoides*) exposed to short-term hypoxic stress showed higher antioxidant activity in the liver and muscle. He et al. (2015) also observed higher antioxidant activity in the liver of Nile tilapia (*Oreochromis niloticus*) subjected to short-term low temperature stress.

Few studies have been performed on the effects of dietary supplementation with *F. vesiculosus* and *N. gaditana* in fish oxidative stress. Kubiriza et al. (2019) tested supplementation of *Salvelinus alpinus* diet's with *F. vesiculosus* and found that this supplementation led to a reduction on liver activities of CAT, GPx and SOD. A work from Qiao et al. (2019) found that the supplementation with *Nannochloropsis sp.* increased antioxidant activity in both serum and muscle until a certain level (7.8% inclusion), and then decreases with higher inclusions. In this study different responses were observed in liver and muscle. Overall, it was observed an increase of antioxidant activity and decrease of LPO in the muscle in dietary supplementation with *F. vesiculosus*. The increase of antioxidant activity means that *F. vesiculosus* promoted protection against oxidative stress, leaving organisms less susceptible to its effects. The decrease of LPO reinforces the positive effects of this extract, suggesting it may mitigate lipid peroxidation, keeping cell wall lipids integrity. In the liver, supplementation with *N. gaditana's* extract (N1F0 and N1F1) promoted an overall decrease on antioxidant activity, leaving fish more susceptible to oxidative stress. The same happened in muscle tissue, with an overall decreased activity of antioxidant enzymes. This means that supplementation with *N. gaditana* promotes lower activity of antioxidant enzymes, which makes fish more vulnerable to oxidative stress.

Overall, the inclusion of extracts from *F. vesiculosus* and *N. gaditana* seemed to have positive effects on the mitigation of stress effects. Our results showed that these extracts have anti-inflammatory properties, and the ability to reduce physiological stress response. The use of *F. vesiculosus* also showed improvements on FE, as well as enhanced ability to fight opportunist pathogens, which offers to fish fed with this supplement, short-term protection.

4. Concluding remarks

From the two algae studied, *F. vesiculosus* seems to have a stronger positive effect in aiding the organism to fight stress. This alga revealed to detain anti-inflammatory properties and antioxidant potential, to reduce stress effect on plasma metabolites, as well as enhance immune parameters, offering short-term protection against pathogens. Moreover, its use in fish diets does not negatively affect growth performance, revealing to improve FE. As per *N. gaditana*, although data revealed that it detains anti-inflammatory properties and reduce stress effects on plasma metabolites, when under chronic stress it did not offer any kind of protection to the organism, and led to the highest mortality per day.

Overall, this study showed that the supplementation of fish diets with *F. vesiculosus* extract can be a great option to be implemented as a functional ingredient in aquaculture, having the capacity to reduce physiological effects resulted from stress, and so, to improve health and welfare. Thus, further studies to fine-tune inclusion levels and duration of the feeding should be conducted.

References

Afonso, A., Lousada, S., Silva, J., Ellis, A. E., & Silva, M. T. (1998). Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout Oncorhynchus mykiss. A light and electron microscopic cytochemical study. *Diseases of Aquatic Organisms*, *34*(1), 27–37.

Agregán, R., Munekata, P. E., Domínguez, R., Carballo, J., Franco, D., & Lorenzo, J. M. (2017). Proximate composition, phenolic content and in vitro antioxidant activity of aqueous extracts of the seaweeds Ascophyllum nodosum, Bifurcaria bifurcata and Fucus vesiculosus. Effect of addition of the extracts on the oxidative stability of canola oil unde. *Food Research International*, *99*, 986–994.

Amin, M., Jusadi, D., Sukenda, & Harris, E. (2015). Growth performance, non-specific immun and antioxidant response of juvenile tilapia oreocromis sp. feeding on brewer's yeast saccharomyces cerevisiae supplemented diet. *Pakistan Journal of Biotechnology*, *12*(2), 139–148.

Andersen, S. M., Waagbø, R., & Espe, M. (2016). Functional amino acids in fish health and welfare. *Frontiers in Bioscience - Elite*. https://doi.org/10.2741/e757

Asencio-Alcudia, G., Andree, K. B., Giraldez, I., Tovar-Ramirez, D., Alvarez-González, A., Herrera, M., & Gisbert, E. (2019). Stressors Due to Handling Impair Gut Immunity in Meagre (Argyrosomus regius): The compensatory role of dietary L-tryptophan. *Frontiers in Physiology*, *10*(MAY). https://doi.org/10.3389/fphys.2019.00547

Ashley, P. J. (2007). Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science*, *104*(3–4), 199–235. https://doi.org/10.1016/j.applanim.2006.09.001

Bagni, M. (2005). Cultured Aquatic Species Information Programme. Dicentrarchus labrax. Retrieved October 27, 2019, from http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/en#tcNA00EA

Balm, P. H. M. (1997). Immune-endocrine interactions. *Fish Stress and Health in Aquaculture*, *62*, 195–221.

Barkia, I., Saari, N., & Manning, S. R. (2019). Microalgae for high-value products towards human health and nutrition. *Marine Drugs*, *17*(5), 1–29.

https://doi.org/10.3390/md17050304

Barton, B. A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. In *Integrative and Comparative Biology*. https://doi.org/10.1093/icb/42.3.517

Batista, G., Charles, M. B., Mancini Filho, J., & Vidal, N. (2009). Seaweeds as sources of antioxidant phytomedicines. *Revista Cubana de Plantas Medicinales*, *14*(2).

Beveridge, M. C. M. (2008). *Cage aquaculture*. John Wiley and Sons.

Beveridge, M. C. M., & Little, D. C. (2002). The History of Aquaculture in Traditional Societies. In *Ecological Aquaculture. The evolution of the Blue Revolution*. https://doi.org/10.1002/9780470995051.ch1

Bondioli, P., Della Bella, L., Rivolta, G., Chini Zittelli, G., Bassi, N., Rodolfi, L., … Tredici, M. R. (2012). Oil production by the marine microalgae Nannochloropsis sp. F&M-M24 and Tetraselmis suecica F&M-M33. *Bioresource Technology*, *114*, 567–572. https://doi.org/10.1016/j.biortech.2012.02.123

Bonga, S. E. W. (1997). The stress response in fish. *Physiological Reviews*, *77*(3), 591–625. https://doi.org/10.1152/physrev.1997.77.3.591

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bricknell, I., & Dalmo, R. A. (2005). The use of immunostimulants in fish larval aquaculture. *Fish and Shellfish Immunology*. https://doi.org/10.1016/j.fsi.2005.03.008

Bulut, O., Akın, D., Sönmez, Ç., Öktem, A., Yücel, M., & Öktem, H. A. (2019). Phenolic compounds, carotenoids, and antioxidant capacities of a thermo-tolerant Scenedesmus sp. (Chlorophyta) extracted with different solvents. *Journal of Applied Phycology*. https://doi.org/10.1007/s10811-018-1726-5

Camacho-Rodríguez, J., Cerón-García, M. C., González-López, C. V., Fernández-Sevilla, J. M., Contreras-Gómez, A., & Molina-Grima, E. (2013). A low-cost culture medium for the production of Nannochloropsis gaditana biomass optimized for aquaculture. *Bioresource Technology*, *144*, 57–66. https://doi.org/10.1016/j.biortech.2013.06.083

Carballo, C., Mateus, A. P., Maya, C., Mantecón, L., Power, D. M., & Manchado, M.

(2020). Microalgal extracts induce larval programming and modify growth and the immune response to bioactive treatments and LCDV in Senegalese sole post-larvae. *Fish & Shellfish Immunology*, *106*, 263–272. https://doi.org/10.1016/j.fsi.2020.07.020

Cardia, F., & Lovatelli, A. (2007). A review of cage aquaculture : Mediterranean Sea. *FAO Fisheries Technical Paper*.

Carveth, C. J., Widmer, A. M., Bonar, S. A., & Simms, J. R. (2007). An examination of the effects of chronic static and fluctuating temperature on the growth and survival of spikedace, Meda fulgida, with implications for management. *Journal of Thermal Biology*, *32*(2), 102–108. https://doi.org/10.1016/j.jtherbio.2006.11.002

Castillo, J., Teles, M., Mackenzie, S., & Tort, L. (2009). Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (Sparus aurata). *Fish & Shellfish Immunology*, *27*(3), 493–499. https://doi.org/https://doi.org/10.1016/j.fsi.2009.06.021

Catarino, M. D., Silva, A. M. S., & Cardoso, S. M. (2017). Fucaceae: A Source of Bioactive Phlorotannins. *International Journal of Molecular Sciences*, *18*(6), 1327. https://doi.org/10.3390/ijms18061327

Catarino, M., Silva, A., & Cardoso, S. (2018). Phycochemical Constituents and Biological Activities of Fucus spp. *Marine Drugs*, *16*(8), 249. https://doi.org/10.3390/md16080249

Catarino, M., Silva, A., Mateus, N., & Cardoso, S. (2019). Optimization of Phlorotannins Extraction from Fucus vesiculosus and Evaluation of Their Potential to Prevent Metabolic Disorders. *Marine Drugs*, *17*(3), 162. https://doi.org/10.3390/md17030162

Cerezuela, R., Guardiola, F. A., Meseguer, J., & Esteban, M. Á. (2012). Enrichment of gilthead seabream (Sparus aurata L.) diet with microalgae: effects on the immune system. *Fish Physiology and Biochemistry*, *38*(6), 1729–1739. https://doi.org/10.1007/s10695-012-9670-9

Chen, H., Zhou, D., Luo, G., Zhang, S., & Chen, J. (2015). Macroalgae for biofuels production: Progress and perspectives. *Renewable and Sustainable Energy Reviews*, *47*, 427–437. https://doi.org/10.1016/j.rser.2015.03.086

Circuncisão, A., Catarino, M., Cardoso, S., & Silva, A. (2018). Minerals from Macroalgae Origin: Health Benefits and Risks for Consumers. *Marine Drugs*,

16(11), 400. https://doi.org/10.3390/md16110400

Connor, T. J., Brewer, C., Kelly, J. P., & Harkin, A. (2005). Acute stress suppresses pro-inflammatory cytokines TNF-α and IL-1β independent of a catecholamine-driven increase in IL-10 production. *Journal of Neuroimmunology*, *159*(1–2), 119–128. https://doi.org/10.1016/j.jneuroim.2004.10.016

Conte, F. S. (2004). Stress and the welfare of cultured fish. In *Applied Animal Behaviour Science* (Vol. 86, pp. 205–223). https://doi.org/10.1016/j.applanim.2004.02.003

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*. https://doi.org/10.3390/molecules15107313

Diana, J. S. (2009). Aquaculture Production and Biodiversity Conservation. *BioScience*, *59*(1), 27–38. https://doi.org/10.1525/bio.2009.59.1.7

Dias, M. K. R., Oba Yoshioka, E. T., Ruiz Rodriguez, A. F., Ribeiro, R. A., Escócio Drummond Viana Faria, F. S., Almeida Ozório, R. O., & Tavares-Dias, M. (2019). Growth, physiological and immune responses of Arapaima gigas (Arapaimidae) to Aeromonas hydrophila challenge and handling stress following feeding with immunostimulant supplemented diets. *Fish and Shellfish Immunology*, *84*, 843–847. https://doi.org/10.1016/j.fsi.2018.10.045

Duncan, N. J., Estévez, A., Fernández-Palacios, H., Gairin, I., Hernández-Cruz, C. M., Roo, J., … Vallés, R. (2013). *Aquaculture production of meagre (Argyrosomus regius): Hatchery techniques, ongrowing and market*. *Advances in Aquaculture Hatchery Technology*. Woodhead Publishing Limited. https://doi.org/10.1533/9780857097460.3.519

Emre, Y., Ergün, S., Kurtoglu, A., Güroy, B., & Güroy, D. (2013). Effects of Ulva meal on growth performance og Gilthead Seabream (Sparus aurata) at different levels of dietary lipid. *Turkish Journal of Fisheries and Aquatic Sciences*. https://doi.org/10.4194/1303-2712-v13

Encarnação, P. (2016). Functional feed additives in aquaculture feeds. *Aquafeed Formulation*, 217–237. https://doi.org/10.1016/B978-0-12-800873-7.00005-1

Esteban, M. Á., Rodriguez, A., Ayala, A. G., & Meseguer, J. (2004). Effects of high doses of cortisol on innate cellular immune response of seabream (Sparus aurata L.). *General and Comparative Endocrinology*, *137*(1), 89–98.

https://doi.org/10.1016/j.ygcen.2004.02.006

FAO. (2018). *The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals*. Rome.

FAO. (2020). *The State of World Fisheires and Aquaculture 2020*. *FAO*. Rome, Italy.

Ferreira, M., Coutinho, P., Seixas, P., Fábregas, J., & Otero, A. (2009). Enriching Rotifers with “Premium” Microalgae. Nannochloropsis gaditana. *Marine Biotechnology*, *11*(5), 585–595. https://doi.org/10.1007/s10126-008-9174-x

Ferreira, R. M., Ribeiro, A. R., Patinha, C., Silva, A. M. S., Cardoso, S. M., & Costa, R. (2019). Water Extraction Kinetics of Bioactive Compounds of Fucus vesiculosus. *Molecules*, *24*(18), 1–15. https://doi.org/10.3390/molecules24183408

FishStat, F. (n.d.). Global Aquaculture Production 1950-2018.

Ganesan, P., Kumar, C. S., & Bhaskar, N. (2008). Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2007.07.005

Gbadamosi, O. K., & Lupatsch, I. (2018). Effects of dietary Nannochloropsis salina on the nutritional performance and fatty acid profile of Nile tilapia, Oreochromis niloticus. *Algal Research*, *33*(November 2017), 48–54. https://doi.org/10.1016/j.algal.2018.04.030

Giogios, I., Grigorakis, K., & Kalogeropoulos, N. (2013). Organoleptic and chemical quality of farmed meagre (Argyrosomus regius) as affected by size. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2013.05.154

Gong, Y., Sørensen, S. L., Dahle, D., Nadanasabesan, N., Dias, J., Valente, L. M. P., … Kiron, V. (2020). Approaches to improve utilization of Nannochloropsis oceanica in plant-based feeds for Atlantic salmon. *Aquaculture*, *522*(February), 735122. https://doi.org/10.1016/j.aquaculture.2020.735122

Gonzalez-Silvera, D., Herrera, M., Giráldez, I., & Esteban, M. (2018). Effects of the Dietary Tryptophan and Aspartate on the Immune Response of Meagre (Argyrosomus regius) after Stress. *Fishes*, *3*(1), 6. https://doi.org/10.3390/fishes3010006

Griffith, O. W. (1980). Determination of glutathione and glutathine disulfide using glutathione reductase and 2-vinylpyridine. *Analytical Biochemistry*, (106.1), 207–212.

Grigorakis, K., Fountoulaki, E., Vasilaki, A., Mittakos, I., & Nathanailides, C. (2011). Lipid quality and filleting yield of reared meagre (Argyrosomus regius). *International Journal of Food Science and Technology*, *46*(4), 711–716. https://doi.org/10.1111/j.1365-2621.2010.02537.x

Grutter, A. S., & Pankhurst, N. W. (2000). The effects of capture, handling, confinement and ectoparasite load on plasma levels of cortisol, glucose and lactate in the coral reef fish Hemigymnus melapterus. *Journal of Fish Biology*, *57*(2), 391–401. https://doi.org/10.1006/jfbi.2000.1312

Guerreiro, I., Pérez-Jiménez, A., Costas, B., & Oliva-Teles, A. (2014). Effect of temperature and short chain frutooligosaccharides supplementation on the hepatic oxidative status and immune response of turbot (Scophthalmus maximus). *Fish & Shellfish Immunology*, (40.2), 570–576.

Gupta, S., Cox, S., Rajauria, G., Jaiswal, A. K., & Abu-Ghannam, N. (2012). Growth inhibition of common food spoilage and pathogenic microorganisms in the presence of brown seaweed extracts. *Food and Bioprocess Technology*, *5*(5), 1907–1916.

Hang, B. T. B., Phuong, N. T., & Kestemont, P. (2014). Can immunostimulants efficiently replace antibiotic in striped catfish (Pangasianodon hypophthalmus) against bacterial infection by Edwardsiella ictaluri? *Fish and Shellfish Immunology*, *40*(2), 556–562. https://doi.org/10.1016/j.fsi.2014.08.007

Haoujar, I., Cacciola, F., Abrini, J., Mangraviti, D., Giu, D., Oulad, Y., … Senhaji, N. S. (2019). The Contribution of Carotenoids, Phenolic Compounds, and Flavonoids to the Antioxidative Properties of Marine Microalgae Isolated from Mediterranean Morocco Imane. *Molecules*, 1–17.

Harris, J., & Bird, D. J. (2000). Modulation of the fish immune system by hormones. *Veterinary Immunology and Immunopathology*, *77*(3–4), 163–176. https://doi.org/10.1016/S0165-2427(00)00235-X

He, J., Qiang, J., Yang, H., Xu, P., Zhu, Z. X., & Yang, R. Q. (2015). Changes in the fatty acid composition and regulation of antioxidant enzymes and physiology of juvenile genetically improved farmed tilapia Oreochromis niloticus (L.), subjected to short-term low temperature stress. *Journal of Thermal Biology*, *53*, 90–97. https://doi.org/10.1016/j.jtherbio.2015.08.010

Heffernan, N., Smyth, T. J., Fitzgerald, R. J., Soler-Vila, A., & Brunton, N. (2014).

Antioxidant activity and phenolic content of pressurised liquid and solid-liquid extracts from four Irish origin macroalgae. *International Journal of Food Science and Technology*. https://doi.org/10.1111/ijfs.12512

Hegazi, M. M., Attia, Z. I., & Ashour, O. A. (2010). Oxidative stress and antioxidant enzymes in liver and white muscle of Nile tilapia juveniles in chronic ammonia exposure. *Aquatic Toxicology*, *99*(2), 118–125. https://doi.org/10.1016/j.aquatox.2010.04.007

Hepher, B., & Sandbank, S. (1984). The effect of phosphorus supplementation to common carp diets on fish growth. *Aquaculture*, *36*(4), 323–332. https://doi.org/https://doi.org/10.1016/0044-8486(84)90325-9

Höglund, E., Bakke, M. J., Øverli, Ø., Winberg, S., & Nilsson, G. E. (2005). Suppression of aggressive behaviour in juvenile Atlantic cod (Gadus morhua) by L-tryptophan supplementation. *Aquaculture*, *249*(1–4), 525–531. https://doi.org/10.1016/j.aquaculture.2005.04.028

Ibañez, E., Herrero, M., Mendiola, J. A., & Castro-Puyana, M. (2012). Extraction and characterization of bioactive compounds with health benefits from marine resources: macro and micro algae, cyanobacteria, and invertebrates. In *Marine bioactive compounds* (pp. 55–98). Springer.

Imbs, T. I., Skriptsova, A. V, & Zvyagintseva, T. N. (2015). Antioxidant activity of fucose-containing sulfated polysaccharides obtained from Fucus evanescens by different extraction methods. *Journal of Applied Phycology*, *27*(1), 545–553. https://doi.org/10.1007/s10811-014-0293-7

Jiménez-Escrig, A., Jiménez-Jiménez, I., Pulido, R., & Saura-Calixto, F. (2001). Antioxidant activity of fresh and processed edible seaweeds. *Journal of the Science of Food and Agriculture*, *81*(5), 530–534.

Jiménez, M. T., Pastor, E., Grau, A., Alconchel, J. I., Sánchez, R., & Cárdenas, S. (2005). Revisión del cultivo de esciénidos en el mundo, con especial atención a la corvina Argyrosomus regius (Asso, 1801). In *Boletin - Instituto Espanol de Oceanografia*.

Jinkerson, R. E., Radakovits, R., & Posewitz, M. C. (2013). Genomic insights from the oleaginous model alga Nannochloropsis gaditana. *Bioengineered*, *4*(1), 37–41. https://doi.org/10.4161/bioe.21880

Kantachumpoo, A., & Chirapart, A. (2010). Components and antimicrobial activity of

polysaccharides extracted from Thai brown seaweeds. *Kasetsart Journal (Natural Science)*, *44*(2), 220–233.

Kemenade, B. M. L. V., Stolte, H. H., Metz, J., & Chadzinska, M. (2009). Neuroendocrine-immune interactions in teleost fish. *Fish Neuroendocrinology*, *28*.

Khansari, A. R., Balasch, J. C., Vallejos-Vidal, E., Parra, D., Reyes-López, F. E., & Tort, L. (2018). Comparative immune- and stress-related transcript response induced by air exposure and Vibrio anguillarum bacterin in rainbow trout (Oncorhynchus mykiss) and gilthead seabream (Sparus aurata) mucosal surfaces. *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2018.00856

Khatoon, H., Rahman, N. A., Banerjee, S., Harun, N., Suleiman, S. S., Zakaria, N. H., … Endut, A. (2014). Effects of different salinities and pH on the growth and proximate composition of Nannochloropsis sp. and Tetraselmis sp. isolated from South China Sea cultured under control and natural condition. *International Biodeterioration & Biodegradation*, *95*, 11–18.

Kieffer, J. D. (2000). Limits to exhaustive exercise in fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *126*(2), 161–179.

Kim, S., & Chojnacka, K. (2015). *Marine algae extracts: processes, products, and applications*. John Wiley and Sons.

Krivoruchko, E., Kanaan, H., Samoilova, V., Ilyina, T., & Koshovyi, O. (2017). Carboxylic acids from brown algae Fucus vesiculosus and Padina pavonica. *Ceska a Slovenska Farmacie*, *66*(5), 287–289.

Kubiriza, G. K., Árnarson, J., Sigurgeirsson, Ó., Hamaguchi, P., Snorrason, S., Tómasson, T., & Thorarensen, H. (2019). Growth and hepatic antioxidant enzyme activity of juvenile Arctic charr (Salvelinus alpinus) fed on diets supplemented with ethoxyquin, rosemary (Rosmarinus officinalis), or bladder wrack (Fucus vesiculosus). *Aquaculture International*, *27*(1), 287–301. https://doi.org/10.1007/s10499-018-0324-5

Kumar, B., Manuja, A., & Aich, P. (2012). Stress and its impact on farm animals. *Frontiers in Bioscience - Elite*. https://doi.org/10.2741/e496

Laiz-Carrión, R., Martín Del Río, M. P., Miguez, J. M., Mangera, J. M., & Soengas, J. L. (2003). Influence of Cortisol on Osmoregulation and Energy Metabolism in Gilthead Seabream Sparus aurata. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*. https://doi.org/10.1002/jez.a.10256

Li, Hua Bin, Cheng, K. W., Wong, C. C., Fan, K. W., Chen, F., & Jiang, Y. (2007). Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2006.06.022

Li, Huifang, Li, J., Tang, Y., Lin, L., Xie, Z., Zhou, J., … Chen, Z. (2017). Fucoidan from Fucus vesiculosus suppresses hepatitis B virus replication by enhancing extracellular signal-regulated Kinase activation. *Virology Journal*, *14*(1), 178.

Li, P., Mai, K., Trushenski, J., & Wu, G. (2009). New developments in fish amino acid nutrition: Towards functional and environmentally oriented aquafeeds. *Amino Acids*. https://doi.org/10.1007/s00726-008-0171-1

Lobo, G., Pereira, L. F., Gonçalves, J. F. M., Peixoto, M. J., & Ozório, R. O. A. (2018). Effect of dietary seaweed supplementation on growth performance, antioxidant and immune responses in European seabass (Dicentrarchus labrax) subjected to rearing temperature and salinity oscillations. *International Aquatic Research*, *10*(4), 321–331. https://doi.org/10.1007/s40071-018-0208-3

Lubian, L. M. (1982). Nannochloropsis gaditana sp. nov., a new marine Eustigmatophyceae, Cadiz Bay. *Lazaroa*, *4*, 287–293.

Ma, Y., Wang, Z., Yu, C., Yin, Y., & Zhou, G. (2014). Evaluation of the potential of 9 Nannochloropsis strains for biodiesel production. *Bioresource Technology*, *167*, 503–509. https://doi.org/10.1016/j.biortech.2014.06.047

Macías-Sánchez, M. D., Mantell, C., Rodríguez, M., Martínez De La Ossa, E., Lubián, L. M., & Montero, O. (2005). Supercritical fluid extraction of carotenoids and chlorophyll a from Nannochloropsis gaditana. *Journal of Food Engineering*, *66*(2), 245–251. https://doi.org/10.1016/j.jfoodeng.2004.03.021

MacKenzie, S., Iliev, D., Liarte, C., Koskinen, H., Planas, J. V, Goetz, F. W., … Tort, L. (2006). Transcriptional analysis of LPS-stimulated activation of trout (Oncorhynchus mykiss) monocyte/macrophage cells in primary culture treated with cortisol. *Molecular Immunology*, *43*(9), 1340–1348. https://doi.org/https://doi.org/10.1016/j.molimm.2005.09.005

Magnoni, L. J., Martos-Sitcha, J. A., Queiroz, A., Calduch-Giner, J. A., Gonçalves, J. F. M., Rocha, C. M. R., … Perez-Sanchez, J. (2017). Dietary supplementation of heat-treated Gracilaria and Ulva seaweeds enhanced acute hypoxia tolerance in gilthead sea bream (Sparus aurata). *Biology Open*, *6*(6), 897–908.

https://doi.org/10.1242/bio.024299

Marques, A., Marçal, R., Pereira, V., Pereira, P., Mieiro, C., Guilherme, S., … Pacheco, M. (2020). Macroalgae-enriched diet protects gilthead seabream (Sparus aurata) against erythrocyte population instability and chromosomal damage induced by aqua-medicines. *Journal of Applied Phycology*, *32*(2), 1477–1493. https://doi.org/10.1007/s10811-019-01996-2

Martins, C. I. M., Schrama, J. W., & Verreth, J. A. J. (2006). The relationship between individual differences in feed efficiency and stress response in African catfish Clarias gariepinus. *Aquaculture*, *256*(1–4), 588–595. https://doi.org/10.1016/j.aquaculture.2006.02.051

Mekdade, L., Bey, M., Hamed, B., El-kebir, F. Z., Mohamed, S., Ayad, E. A., … June, M. (2016). Evaluation of Antioxidant and Antiproliferative Activities of Nannochloropsis gaditana Extracts. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *7*(904), 904–913.

Mendis, E. (2011). Present and Future Prospects of Seaweeds in Developing Functional Foods. *Advances in Food and Nutrition Research*, *64*, 1–15. https://doi.org/10.1016/B978-0-12-387669-0.00001-6

Michalak, I., & Chojnacka, K. (2014). Algal extracts: Technology and advances. *Engineering in Life Sciences*, *14*(6), 581–591. https://doi.org/10.1002/elsc.201400139

Michalak, I., & Chojnacka, K. (2015). Algae as production systems of bioactive compounds. *Engineering in Life Sciences*, *15*(2), 160–176. https://doi.org/10.1002/elsc.201400191

Michalak, I., & Chojnacka, K. (2018). Introduction: Toward Algae-Based Products. In K. Chojnacka, P. P. Wieczorek, G. Schroeder, & I. Michalak (Eds.), *Algae Biomass: Characteristics and Applications: Towards Algae-based Products* (pp. 1–5). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-74703-3_1

Millán-Cubillo, A. F., Martos-Sitcha, J. A., Ruiz-Jarabo, I., Cárdenas, S., & Mancera, J. M. (2016). Low stocking density negatively affects growth, metabolism and stress pathways in juvenile specimens of meagre (Argyrosomus regius, Asso 1801). *Aquaculture*, *451*, 87–92. https://doi.org/10.1016/j.aquaculture.2015.08.034

Millao, S., & Uquiche, E. (2016). Antioxidant activity of supercritical extracts from

Nannochloropsis gaditana: Correlation with its content of carotenoids and tocopherols. *Journal of Supercritical Fluids*, *111*, 143–150. https://doi.org/10.1016/j.supflu.2016.02.002

Milne, D. J. (2018). *Characterisation of the immune response in two new species for fish aquaculture, Argyrosomus regius and Seriola dumerili*. University of Aberdeen.

Moazami, N., Ashori, A., Ranjbar, R., Tangestani, M., Eghtesadi, R., & Nejad, A. S. (2012). Large-scale biodiesel production using microalgae biomass of Nannochloropsis. *Biomass and Bioenergy*, *39*, 449–453. https://doi.org/10.1016/j.biombioe.2012.01.046

Monfort, M. C. (2010). *Present market situation and prospects of meagre (Argyrosomus regius),as an emerging species in Mediterranean aquaculture.* Rome.

Monteiro, M., Santos, R. A., Iglesias, P., Couto, A., Serra, C. R., Gouvinhas, I., … Díaz-Rosales, P. (2019). Effect of extraction method and solvent system on the phenolic content and antioxidant activity of selected macro- and microalgae extracts. *Journal of Applied Phycology*. https://doi.org/10.1007/s10811-019-01927-1

Murakami, R., & Hashimoto, H. (2009). Unusual Nuclear Division in Nannochloropsis oculata (Eustigmatophyceae, Heterokonta) which May Ensure Faithful Transmission of Secondary Plastids. *Protist*, *160*(1), 41–49. https://doi.org/10.1016/j.protis.2008.09.002

Nardocci, G., Navarro, C., Cortés, P. P., Imarai, M., Montoya, M., Valenzuela, B., … Fernández, R. (2014). Neuroendocrine mechanisms for immune system regulation during stress in fish. *Fish and Shellfish Immunology*, *40*(2), 531–538. https://doi.org/10.1016/j.fsi.2014.08.001

Naylor, R. L., Goldburg[2], R. J., Primavera[3], J. H., Kautsky, N., Beveridge, M. C. M., Clay#, J., … Troell, M. (2000). *Effect of aquaculture on world ®sh supplies*. *NATURE* (Vol. 405). Retrieved from www.nature.com

Nelson, D. L., & Cox, M. M. (2013). *Lehninger Principles of Biochemistry, Seventh Edition*. *Regulation*. https://doi.org/10.1016/j.jse.2011.03.016

Neveux, N., Magnusson, M., Maschmeyer, T., de Nys, R., & Paul, N. A. (2015). Comparing the potential production and value of high-energy liquid fuels and

protein from marine and freshwater macroalgae. *GCB Bioenergy*, *7*(4), 673–689. https://doi.org/10.1111/gcbb.12171

Ngugi, C. C., Oyoo-Okoth, E., Mugo-Bundi, J., Orina, P. S., Chemoiwa, E. J., & Aloo, P. A. (2015). Effects of dietary administration of stinging nettle (Urtica dioica) on the growth performance, biochemical, hematological and immunological parameters in juvenile and adult Victoria Labeo (Labeo victorianus) challenged with Aeromonas hydrophila. *Fish and Shellfish Immunology*, *44*(2), 533–541. https://doi.org/10.1016/j.fsi.2015.03.025

Onofrejová, L., Vašíčková, J., Klejdus, B., Stratil, P., Mišurcová, L., Kráčmar, S., … Vacek, J. (2010). Bioactive phenols in algae: The application of pressurized-liquid and solid-phase extraction techniques. *Journal of Pharmaceutical and Biomedical Analysis*, *51*(2), 464–470. https://doi.org/10.1016/j.jpba.2009.03.027

Ottinger, M., Clauss, K., & Kuenzer, C. (2016, January 1). Aquaculture: Relevance, distribution, impacts and spatial assessments - A review. *Ocean and Coastal Management*. Elsevier Ltd. https://doi.org/10.1016/j.ocecoaman.2015.10.015

Pangestuti, R., & Kim, S.-K. (2011). Biological activities and health benefit effects of natural pigments derived from marine algae. *Journal of Functional Foods*, *3*(4), 255–266. https://doi.org/10.1016/J.JFF.2011.07.001

Peixoto, M. J., Salas-Leitón, E., Pereira, L. F., Queiroz, A., Magalhães, F., Pereira, R., … Ozório, R. O. D. A. (2016). Role of dietary seaweed supplementation on growth performance, digestive capacity and immune and stress responsiveness in European seabass (Dicentrarchus labrax). *Aquaculture Reports*, *3*, 189–197. https://doi.org/10.1016/j.aqrep.2016.03.005

Pereira, V., Marques, A., Gaivão, I., Rego, A., Abreu, H., Pereira, R., … Pacheco, M. (2019). Marine macroalgae as a dietary source of genoprotection in gilthead seabream (Sparus aurata) against endogenous and exogenous challenges. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, *219*(February), 12–24. https://doi.org/10.1016/j.cbpc.2019.01.006

Pérez-Jiménez, A., Peres, H., Rubio, V. C., & Oliva-Teles, A. (2012). The effect of hypoxia on intermediary metabolism and oxidative status in gilthead sea bream (Sparus aurata) fed on diets supplemented with methionine and white tea. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology*, (155(3)), 506–516.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*. https://doi.org/10.1093/nar/29.9.e45

Plaza, M., Herrero, M., Cifuentes, A., & Ibáñez, E. (2009). Innovative Natural Functional Ingredients from Microalgae, 7159–7170. https://doi.org/10.1021/jf901070g

Poli, B. M., Parisi, G., Zampacavallo, G., Iurzan, F., Mecatti, M., Lupi, P., & Bonelli, A. (2003). *Preliminary results on quality and quality changes in reared meagre (Argyrosomus regius): body and fillet traits and freshness changes in refrigerated commercial-size fish*.

Qiao, H., Hu, D., Ma, J., Wang, X., Wu, H., & Wang, J. (2019). Feeding effects of the microalga Nannochloropsis sp. on juvenile turbot (Scophthalmus maximus L.). *Algal Research*, *41*(May). https://doi.org/10.1016/j.algal.2019.101540

Quéméner, L., Suquet, M., Mero, D., & Gaignon, J.-L. (2002). *Selection method of new candidates for finfish aquaculture: the case of the French Atlantic, the Channel and the North Sea coasts*. Retrieved from www.elsevier.com/locate/aquliv

Quinn, J. C., Yates, T., Douglas, N., Weyer, K., Butler, J., Bradley, T. H., & Lammers, P. J. (2012). Nannochloropsis production metrics in a scalable outdoor photobioreactor for commercial applications. *Bioresource Technology*, *117*, 164–171. https://doi.org/10.1016/j.biortech.2012.04.073

Radakovits, R., Jinkerson, R. E., Fuerstenberg, S. I., Tae, H., Settlage, R. E., Boore, J. L., & Posewitz, M. C. (2012). Draft genome sequence and genetic transformation of the oleaginous alga Nannochloropis gaditana. *Nature Communications*, *3*. https://doi.org/10.1038/ncomms1688

Reyes-Becerril, M., Guardiola, F., Rojas, M., Ascencio-Valle, F., & Esteban, M. Á. (2013). Dietary administration of microalgae Navicula sp. affects immune status and gene expression of gilthead seabream (Sparus aurata). *Fish and Shellfish Immunology*, *35*(3), 883–889. https://doi.org/10.1016/j.fsi.2013.06.026

Richard, D. M., Dawes, M. A., Mathias, C. W., Acheson, A., Hill-Kapturczak, N., & Dougherty, D. M. (2009). L-tryptophan: Basic metabolic functions, behavioral research and therapeutic indications. *International Journal of Tryptophan Research*. https://doi.org/10.4137/ijtr.s2129

Robertson, R. C., Guihéneuf, F., Bahar, B., Schmid, M., Stengel, D. B., Fitzgerald, G. F., … Stanton, C. (2015). The anti-inflammatory effect of algae-derived lipid

extracts on lipopolysaccharide (LPS)-stimulated human THP-1 macrophages. *Marine Drugs*, *13*(8), 5402–5424. https://doi.org/10.3390/md13085402

Rocha, J. M. S., Garcia, J. E. C., & Henriques, M. H. F. (2003). Growth aspects of the marine microalga Nannochloropsis gaditana. *Biomolecular Engineering*, *20*(4–6), 237–242. https://doi.org/10.1016/S1389-0344(03)00061-3

Roy, S. Sen, & Pal, R. (2015). Microalgae in Aquaculture: A Review with Special References to Nutritional Value and Fish Dietetics. *Proceedings of the Zoological Society*, *68*(1), 1–8. https://doi.org/10.1007/s12595-013-0089-9

Ruane, N. M., Huisman, E. A., & Komen, J. (2002). The influence of feeding history on the acute stress response of common carp (Cyprinus carpio). *Aquaculture*, *210*(1–4), 245–257. https://doi.org/10.1016/S0044-8486(01)00819-5

Santos, G. A., Schrama, J. W., Mamauag, R. E. P., Rombout, J. H. W. M., & Verreth, J. A. J. (2010). Chronic stress impairs performance, energy metabolism and welfare indicators in European seabass (Dicentrarchus labrax): The combined effects of fish crowding and water quality deterioration. *Aquaculture*, *299*(1–4), 73–80. https://doi.org/10.1016/j.aquaculture.2009.11.018

Scholz, M. J., Weiss, T. L., Jinkerson, R. E., Jing, J., Roth, R., Goodenough, U., … Gerken, H. G. (2014). Ultrastructure and composition of the Nannochloropsis gaditana cell wall. *Eukaryotic Cell*, *13*(11), 1450–1464. https://doi.org/10.1128/EC.00183-14

Silva, S. S., & Anderson, T. A. (1994). *Fish nutrition in aquaculture*. Springer Science and Business Media.

Singh, I. P., & Sidana, J. P. (2013). In Functional Ingredients from Algae for Foods and Nutraceuticals; Domínguez, H., Ed. Woodhead Publishing: Cambridge, UK.

Singh, S., Kate, B. N., & Banecjee, U. C. (2005). Bioactive compounds from cyanobacteria and microalgae: An overview. *Critical Reviews in Biotechnology*. https://doi.org/10.1080/07388550500248498

Sony, N. M., Ishikawa, M., Hossain, M. S., Koshio, S., & Yokoyama, S. (2019). The effect of dietary fucoidan on growth, immune functions, blood characteristics and oxidative stress resistance of juvenile red sea bream, Pagrus major. *Fish Physiology and Biochemistry*, *45*(1), 439–454. https://doi.org/10.1007/s10695-018-0575-0

Sopinka, N. M., Donaldson, M. R., Connor, C. M. O., Suski, C. D., & Cooke, S. J.

(2016). *11. Stress Indicators in Fish* (Vol. 35). Elsevier Inc. https://doi.org/10.1016/B978-0-12-802728-8.00011-4

Sopinka, N. M., Donaldson, M. R., O'Connor, C. M., Suski, C. D., & Cooke, S. J. (2016). Stress Indicators in Fish. In *Fish Physiology* (Vol. 35, pp. 405–462). Elsevier Inc. https://doi.org/10.1016/B978-0-12-802728-8.00011-4

Sørensen, M., Gong, Y., Bjarnason, F., Vasanth, G. K., Dahle, D., Huntley, M., & Kiron, V. (2017). Nannochloropsis oceania-derived defatted meal as an alternative to fishmeal in Atlantic salmon feeds. *PLoS ONE*, *12*(7), 1–20. https://doi.org/10.1371/journal.pone.0179907

Soto, J. O., Paniagua-Michel, J. D. J., Lopez, L., & Ochoa, L. (2015). Functional feeds in aquaculture. In *Springer Handbook of Marine Biotechnology* (pp. 1303–1319). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-53971-8_59

Sternberg, E. M. (2006). Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nature Reviews Immunology*, *6*(4), 318–328. https://doi.org/10.1038/nri1810

Taleb, A., Pruvost, J., Legrand, J., Marec, H., Le-Gouic, B., Mirabella, B., … Takache, H. (2015). Development and validation of a screening procedure of microalgae for biodiesel production: Application to the genus of marine microalgae Nannochloropsis. *Bioresource Technology*, *177*, 224–232. https://doi.org/https://doi.org/10.1016/j.biortech.2014.11.068

Tort, L. (2011). Stress and immune modulation in fish. *Developmental and Comparative Immunology*, *35*(12), 1366–1375. https://doi.org/10.1016/j.dci.2011.07.002

Traifalgar, R. F. M., Corre, V. L., & Serrano, A. E. (2013). Efficacy of dietary immunostimulants to enhance the immunological responses and Vibriosis resistance of juvenile Penaeus monodon. *Journal of Fisheries and Aquatic Science*, *8*(2), 340–354.

Valente, L. M. P., Custódio, M., Batista, S., Fernandes, H., & Kiron, V. (2019). Defatted microalgae (Nannochloropsis sp.) from biorefinery as a potential feed protein source to replace fishmeal in European sea bass diets. *Fish Physiology and Biochemistry*, *45*(3), 1067–1081. https://doi.org/10.1007/s10695-019-00621-w

Vallejos-Vidal, E., Reyes-López, F., Teles, M., & MacKenzie, S. (2016). The response of fish to immunostimulant diets. *Fish and Shellfish Immunology*, *56*, 34–69.

https://doi.org/10.1016/j.fsi.2016.06.028

Vandeputte, C., Guizon, I., Genestir-Denis, I., Vannier, B., & Lorenzo, G. (1994). A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biology and Toxicology*, (10.5-6), 415–420.

Vazzana, M., Cammarata, M., & Cooper, E. (2002). Confinement stress in sea bass (Dicentrarchus labrax) depress peritoneal leucocyte cytotoxicity. *Aquaculture*, *210*, 231–243. https://doi.org/10.1016/S0044-8486(01)00818-3

Veena, C. K., Josephine, A., Preetha, S. P., & Varalakshmi, P. (2007). Beneficial role of sulfated polysaccharides from edible seaweed Fucus vesiculosus in experimental hyperoxaluria. *Food Chemistry*, *100*(4), 1552–1559. https://doi.org/https://doi.org/10.1016/j.foodchem.2005.12.040

Vijayan, M. M., Pereira, C., Forsyth, R. B., Kennedy, C. J., & Iwama, G. K. (1997). Handling stress does not affect the expression of hepatic heat shock protein 70 and conjugation enzymes in rainbow trout treated with β-naphthoflavone. *Life Sciences*, *61*(2), 117–127.

Vinagre, C., Madeira, D., Narciso, L., Cabral, H. N., & Diniz, M. (2012). Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, Dicentrarchus labrax. *Ecological Indicators*, *23*, 274–279. https://doi.org/10.1016/j.ecolind.2012.04.009

Walker, A. B., & Berlinsky, D. L. (2011). Effects of partial replacement of fish meal protein by microalgae on growth, feed intake, and body composition of Atlantic cod. *North American Journal of Aquaculture*, *73*(1), 76–83. https://doi.org/10.1080/15222055.2010.549030

Wang, E., Chen, X., Wang, K., Wang, J., Chen, D., Geng, Y., … Wei, X. (2016). Plant polysaccharides used as immunostimulants enhance innate immune response and disease resistance against Aeromonas hydrophila infection in fish. *Fish and Shellfish Immunology*, *59*, 196–202. https://doi.org/10.1016/j.fsi.2016.10.039

Wang, J., Zhang, Q., Zhang, Z., & Li, Z. (2008). Antioxidant activity of sulfated polysaccharide fractions extracted from Laminaria japonica. *International Journal of Biological Macromolecules*, *42*(2), 127–132. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2007.10.003

Wang, T., Jónsdóttir, R., Liu, H., Gu, L., Kristinsson, H. G., Raghavan, S., & Ólafsdóttir,

G. (2012). Antioxidant Capacities of Phlorotannins Extracted from the Brown Algae Fucus vesiculosus. *Journal of Agricultural and Food Chemistry*, *60*(23), 5874–5883. https://doi.org/10.1021/jf3003653

Weyts, F. A. A., Cohen, N., Flik, G., & Verburg-van Kemenade, B. M. L. (1999). Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. *Fish & Shellfish Immunology*, *9*(1), 1–20. https://doi.org/https://doi.org/10.1006/fsim.1998.0170

Weyts, F. A. A., Verburg-Van Kemenade, B. M. L., & Flik, G. (1998). Characterisation of glucocorticoid receptors in peripheral blood leukocytes of carp, Cyprinus carpio L. *General and Comparative Endocrinology*, *111*(1), 1–8. https://doi.org/10.1006/gcen.1998.7080

Wieten, L., Van Der Zee, R., Goedemans, R., Sijtsma, J., Serafini, M., Lubsen, N. H., … Broere, F. (2010). Hsp70 expression and induction as a readout for detection of immune modulatory components in food. *Cell Stress and Chaperones*, *15*(1), 25–37. https://doi.org/10.1007/s12192-009-0119-8

Wojtaszek, J., Dziewulska-Szwajkowska, D., Lozińska-Gabska, M., Adamowicz, A., & Dugaj, A. (2002). Hematological effects of high dose of cortisol on the carp (Cyprinus carpio L.): Cortisol effect on the carp blood. *General and Comparative Endocrinology*, *125*(2), 176–183. https://doi.org/10.1006/gcen.2001.7725

Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., & Takriff, M. S. (2014). An overview: Biomolecules from microalgae for animal feed and aquaculture. *Journal of Biological Research (Greece)*, *21*(1), 1–10. https://doi.org/10.1186/2241-5793-21-6

Yada, T., & Nakanishi, T. B. T.-I. R. of C. (2002). Interaction between endocrine and immune systems in fish (Vol. 220, pp. 35–92). Academic Press. https://doi.org/https://doi.org/10.1016/S0074-7696(02)20003-0

Yada, T., & Tort, L. (2016). *Stress and Disease Resistance: Immune System and Immunoendocrine Interactions*. *Fish Physiology* (Vol. 35). Elsevier Inc. https://doi.org/10.1016/B978-0-12-802728-8.00010-2

Yang, S., Yan, T., Wu, H., Xiao, Q., Fu, H. M., Luo, J., … Li, S. J. (2017). Acute hypoxic stress: Effect on blood parameters, antioxidant enzymes, and expression of HIF-1alpha and GLUT-1 genes in largemouth bass (Micropterus salmoides). *Fish and Shellfish Immunology*, *67*, 449–458.

https://doi.org/10.1016/j.fsi.2017.06.035

Yeganeh, S., Teimouri, M., & Amirkolaie, A. K. (2015). Dietary effects of Spirulina platensis on hematological and serum biochemical parameters of rainbow trout (Oncorhynchus mykiss). *Research in Veterinary Science*, *101*, 84–88. https://doi.org/10.1016/j.rvsc.2015.06.002

Yuan, Y., Zhang, J., Fan, J., Clark, J., Shen, P., Li, Y., & Zhang, C. (2018). Microwave assisted extraction of phenolic compounds from four economic brown macroalgae species and evaluation of their antioxidant activities and inhibitory effects on α-amylase, α-glucosidase, pancreatic lipase and tyrosinase. *Food Research International*. https://doi.org/10.1016/j.foodres.2018.07.021

Zarate, J., & Bradley, T. M. (2003). Heat shock proteins are not sensitive indicators of hatchery stress in salmon. *Aquaculture*, *223*(1–4), 175–187.