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IMPACT OF ALGAE PROCESSING ON FISH GUT HOMEOSTASIS

UNIVERSIDADE DO ALGARVE
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Resumo

O aumento da produção aquícola intensiva é importante para garantir a necessidade de peixe para consumo humano. É assim reconhecida por este motivo como um recurso essencial para o desenvolvimento de um uso mais conservado e sustentável dos oceanos. O desenvolvimento da aquacultura de uma produção extensiva para uma produção mais intensiva, destacou a importância do bem-estar animal face ao stress a que o peixe está sujeito, como um fator importante na obtenção de uma produção mais sustentável e para garantir um melhor produto para o consumidor. De forma a garantir o bem-estar animal dos peixes de aquacultura, têm vindo a ser desenvolvidos métodos com uma abordagem mais natural e amiga do ambiente. Uma opção, são os alimentos funcionais, ou seja, alimentos que melhoram uma ou mais atividades alvo no organismo, além dos efeitos da nutrição fundamental.

A introdução de microalgas como ingrediente funcional na alimentação de peixes tem criado altas expectativas devido à sua riqueza em compostos biologicamente ativos, com muitos benefícios com potencial funcional, tais como a estimulação imunológica e antioxidante. A utilização de microalgas não é já aplicada na produção aquícola em alta escala, pois a estrutura da membrana celular pode ser um dos principais fatores que reduz o acesso aos compostos intracelulares das microalgas. A rutura celular apresenta-se desta forma, como um processo opcional para aumentar a biodisponibilidade dos compostos dentro das células das microalgas. A homogeneização de alta pressão é o método eficaz de rutura física da estrutura da parede celular, sem adicionar novas substâncias à suspensão de microalga, para qualquer espécie de microalga.

Neste estudo o intestino é o órgão com relevância, pois é um dos órgãos digestivos adaptados à estratégia de alimentação dos peixes. Caracteriza-se por ser sensível a perturbações ambientais e homeostáticas, que relacionam a saúde do peixe com a manutenção intestinal através de respostas, como a antioxidante e imunitária. A capacidade de resposta do sistema imunológico do peixe pode ser diretamente afetada pelo stress a que este fica suscetível devido à imunossupressão pelo mesmo, portanto, quanto mais exposto o peixe ao stress, maior a suscetibilidade a possíveis doenças. De forma a mitigar o efeito do stress, muitos estudos mostraram uma melhoria na trilha do intestino de peixes quando alimentados com algas, nomeadamente a nível das respostas antioxidante e imune. No intestino a resposta antioxidante ocorre na presença de espécies reativas de oxigênio (ROS). Estas por sua vez podem ser radicais livres ou não radicais derivados do oxigênio, que podem ativar uma resposta antioxidante por componentes enzimáticos e não enzimáticos. Da mesma forma, a resposta imune no intestino

é essencial para a manutenção imunológica e para o equilíbrio dinâmico dos peixes. Se o trato gastrointestinal for danificado ocorre uma resposta inflamatória. Com este propósito, o objetivo deste estudo foi comparar as respostas antioxidantes e imunológicas no intestino da Dourada (*Sparus aurata*), quando exposto a biomassa de algas processadas e não processadas.

O percurso metodológico desenvolveu-se por várias etapas: 1 - As pastas sólidas congeladas de biomassa de microalga industrial foram fornecidas pela Allmicroalgae - Natural Products, S.A. e levadas para o Laboratório Nacional de Energia e Geologia em Lisboa, Portugal, para serem processadas. *Nannocholopsis oceanica* e *Phaeodactylum tricornutum* foram processadas por homogeneização a alta pressão seguindo um modelo de superfície resposta, com os parâmetros pressão, número de passagens e concentração da suspensão num PandaPLUS 2000 homogeneizador. 2 - Cada amostra de microalga processada foi analisada quanto à integridade da membrana celular por um CytoFLEX Flow Cytometer equipado com um laser de argônio de 488 nm. 3 - Após a obtenção da ótima suspensão processada de cada alga, através de um modelo da mucosa intestinal in vitro, 18 peixes foram dissecados e os tecidos do intestino colocados numa placa de cultura de células com as algas em três diferentes concentrações (8 mg/mL, 40 mg/mL e 200 mg/mL). 4 - Dos 18 peixes apenas 9 foram para analisar os marcadores moleculares da resposta imune (ciclooxigenase 2 – *Cox2* e imunoglobulina M – *IgM*) e os marcadores da resposta antioxidante (catalase – *CAT*, glutationa peroxidase – *GPx* e fator 2 relacionado ao fator nuclear eritróide 2 – *Nrf2*).

Este estudo demonstrou que a microalga processada por um homogeneizador de alta pressão funciona como um alimento funcional, mantendo a capacidade imunológica para os genes selecionados no intestino da Dourada. Os resultados obtidos na análise da resposta imune demonstraram, ainda, que a disrupção aumenta o potencial da microalga como imunoestimulante para os genes selecionados para a mucosa intestinal da dourada, com maior relevância a *Nannochloropsis oceanica* processada, na concentração de 8 mg/mL e 40 mg/mL, em relação à *N. oceanica* não processada. Em contrapartida, no caso da *Phaeodactylum tricornutum* o intestino da dourada apresentou uma tendência para aumentar a estimulação da resposta imune, na concentração de 40 mg/mL, mas uma diminuição na concentração de 200 mg/mL, apresentando a concentração como um possível fator limitante. Este estudo não indicou diferenças significativas na resposta antioxidante do intestino da dourada entre alga não processada e alga processada, mas outros estudos mostram que diferenças podem ser esperadas. Desta forma os resultados do estudo sugerem uma resposta mais regulada na mucosa intestinal da dourada sempre que estimulada por algas processadas.

Pesquisas futuras são necessárias para confirmar a capacidade de melhorar a estimulação imunológica e antioxidante das algas por um homogeneizador de alta pressão. Pode

ser pelo acréscimo dos dados dos 9 peixes por analisar, utilização de diferentes marcadores ou alteração dos parâmetros de estudo.

Termos chave: alga processada, antioxidante, imune, *Nannocholopsis oceanica*, *Phaeodactylum tricornutum*

Abstract

Aquaculture fish welfare management has been developing by approaching a more naturally friendly method. The introduction of algae as a functional ingredient created the highest expectations for algae richness in biologically active compounds, with many benefits like immune and antioxidant stimulation. Unfortunately, these compounds may have a limited restriction from the cell wall membrane. Removing the cell wall limiting factor could increase algae bioavailability of compounds as well as the functional potential. For this purpose, this study compared the antioxidant and immune responses in the intestine of gilthead seabream (*Sparus aurata*), when exposed to processed lysed algae biomass and unprocessed ones. The comparison followed two factors, the treatment (disrupted or whole algae) and concentration. *Nannocholopsis oceanica* and *Phaeodactylum tricornutum* were disrupted through high-pressure homogenization and applied in different concentrations in the fish intestine tissue. The study demonstrated a potential immune capacity for the genes selected in the seabream intestine. *Nannochloropsis oceanica* disrupted at a concentration of 8 mg/mL and 40 mg/mL had better results than *N. oceanica* not disrupted. Further research is needed to complement and understand these results.

Keywords: disrupted alga, antioxidant, immune, *Nannocholopsis oceanica*, *Phaeodactylum tricornutum*

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1. Introduction

1.1. Present state of world aquaculture

In 2018, global fish production reached 179 million tons (FAO, 2020). About 156 million tons were for human consumption, of which 82 million tons of fish come from aquaculture production (FAO, 2020). With a contribution of 52% of human fish consumption (FAO, 2020). Furthermore, from 1961 to 2017, the world population tended to increase by 1.6% annually, while fish for human consumption tended to increase by 3.1% annually, which makes aquaculture production a potential source to meet this demand (FAO, 2020).

Aquaculture has been recognized as an essential resource for developing a more conserved and sustainable use of the oceans (FAO, 2018). Tending from extensive and semi-intensive to more intensive aquaculture with more value for the higher yield/unit area, maximizing the use of water and machinery (Shaalan et al., 2017).

Despite the potential of aquaculture, during production, the fish is susceptible to stress from different biotic and abiotic factors (Martínez-Álvarez et al., 2005). This stress can increase the fish's consumption of energy (Moniruzzaman et al., 2022). Leading to an unbalance of the fish's biological and physiological metabolism, negatively impacting the fish responses like the inflammatory and oxidative stress responses (Morvaridzadeh et al., 2022). Decreasing farmed fish product quality and growth, possibly promoting disease outbreaks and lowering the fish survival rate (Ezhilmathi et al., 2022). Demanding more costs associated with antibiotics to mitigate the stress results in the fish (Monteiro et al., 2021).

1.2. Importance of Fish Welfare

The welfare of farmed fish has become an essential factor in European Union legislation for the government, the consumer, and scientific people, to ensure fish welfare overall life duration during farming (Algers et al., 2009). Worldwide the Code of Conduct for Responsible Fisheries, adopted by FAO Members in 1995, has been a foundational document that marked the principles and standards for the use of fisheries and aquaculture resources, ensuring the sustainable use in balance with the environment (FAO, 1995).

As fish health could be related to different pathogenic agents, varied methods may be applied (Mondal et al., 2021). Antibiotics are a well-known treatment administrated through

oral, bath, pond sprinkle, or injection to prevent and treat aquaculture diseases (Sun et al., 2020). They can also lead to the development of bacteria antibiotic resistant and the accumulation of residues in fish tissues, leading to a need for a higher dose and becoming less reliable, and for those reasons, it is under discussion whether to avoid or eliminate it (Öztürk & Altinok, 2014; Stratev et al., 2018). For these reasons, the research for more prevention methods to decrease the need for antibiotics results in using other substances like nanoparticles, medicinal plant extracts, probiotics, and microalgae treatments in aquaculture (Mondal et al., 2021). As the number of treatments to maintain good aquaculture fish welfare increased, the development of naturally friend and healthier alternative treatments has gained more relevance in aquaculture (Mondal et al., 2021). Microalga application treatments can become the best treatments to invest. Microalgae represent the first trophic level in the food chain, with more than 30,000 species discovered, responsible for nearly 50% of the atmospheric oxygen production, improving the environmental sustainability of aquaculture production, and decreasing the fish meat in the feed (Gomez-Zavaglia et al., 2019). Be used as a functional nutrition strategy for its beneficial properties like growth stimulation, immune response enhancement, various antiviral properties, and easily practical implementation in dry or liquid form (Mondal et al., 2021). Microalgae is an option for more genuine and sustainable products, helping to maintain healthy fish aquaculture production (Fleurence et al., 2012).

1.3. Functional nutrition for fish health improvement

Functional foods are mainly defined as food containing one or more functional ingredients, improving one or more target activities in the body beyond the fundamental nutritional effects (Lordan et al., 2011).Beneficial for the health and well-being of the organism's general conditions, decreasing the risk of illness and disease (Lordan et al., 2011).

Functional feeds are generally implemented during anticipated stressful farming events and critical life stages to help the animal to maintain a lower level of stress against pathogens and to ensure good health (Kiron, 2012). Many functional ingredients can be used, depending on the required function. Examples of that are Prebiotics such as β-glucans, like mannan oligosaccharides (MOS), which are immune modulators that prevent the colonization of pathogenic bacteria, improving intestinal morphology and the epithelial brush border (Gültepe et al., 2012; J. Wang et al., 2019). Within the concept of functional feeds, microalga is of great interest with fast growth rates and the capacity to be cultured on a large scale and in different environments (e.g. wastewater, brackish, and sea waters) (Borowitzka, 2018; Wells et al., 2016).

Many bioactive compounds such as high oil contents, eicosapentaenoic acid (EPA), and pigments, give them a functional food potential, acting as an antioxidant, anti-inflammatory, prebiotic and immune stimulant (Borowitzka, 2018; Wells et al., 2016).

1.4. Algae as functional ingredients

Microalgae are rich in biologically active compounds such as polyunsaturated fatty acids (omega 3 and 6 fatty acids), polysaccharides, phospholipids, triacylglycerols, vitamins, minerals, and phenolic compounds (fucosterol and β-sitosterol) (Perera et al., 2020; Wells et al., 2016). These compounds have some acute beneficial effects on functional fish feeds. To improve fish growth and fillet quality by lipid deposition and enhances total n-3 fatty acids, EPA, and docosahexaenoic acid (DHA) contents (He et al., 2018; Peixoto et al., 2020). Or for their high interest in immune system boosting, antioxidant, anticancer, and other capacities in fucoxanthin, a carotenoid characteristic of brown algae, found in large quantities in *Phaeodactylum tricornutum* (Leong et al., 2022; Lourenço-Lopes et al., 2021; Qiu et al., 2021; Wells et al., 2016).

Some recent examples of algae applied as a functional ingredient were, the use of *Nannochloropsis sp.* as a good substitute to fish oil as the lipid source in kuruma shrimp diets, presenting similar growth performance (Oswald et al., 2019). The incorporation of an inclusion level of 15% *Chlorella sp.* into the fish diet, showing a maximum gain of 55% weight at one of the algal-based feeds, costing less to produce than the microalgae-free conventional feed, potentializing the incorporation of algae into the feeds (Yadav et al., 2020). The beneficial capacity of 5% inclusion of *Ulva ohnoi* during short periods of Senegalese sole feeding time showed an increase in the protection of the intestinal epithelium (Vizcaíno et al., 2019). The implementation of diets containing 0.5 % or 1 % inclusion of two microalgae-based products for Gilthead seabream showed an increase in feed efficiency by decreasing the feed intake by stimulating the lipid oxidative capacity and reducing the cortisol levels in plasma (Perera et al., 2020). Another blend, but with micro- and macroalgae (1.5% *Nannochloropsis sp.* and 1.5% of Gracilaria gracilis), showed increased weaned post-larvae of Senegalese sole (*Solea senegalensis*) growth performance, immune and antioxidant response (Peixoto et al., 2020).

Even with some good results, the utilization of algae can be restricted by fish's digestive capacity, limited by the tight algae cellular membrane (Lee et al., 2012; Zheng et al., 2020). To disrupt the microalgae cell wall membrane some pre-processing may be needed (Teuling et al., 2017). This pre-processing may improve intestinal nutrient uptake, decrease the loss of fish

nutrition, and enrich the feed yield before their inclusion in carnivorous fish diets (Teuling et al., 2017).

1.5. Algae cell optimization

The cell wall structure may be the principal factor that reduces the access of all intracellular compounds from microalgae by animals. As observed in a previous study by Teuling et al., (2017), the differences in fish intestine digestibility were not related to the nutrient characteristics of the algae but to the accessibility of those nutrients. To overcome this limitation, cell disruption becomes an optional process to access the compounds inside the algae cells, where lipids, proteins, and other molecules reside (Agboola et al., 2019).

Knowing the interest in improving the algae efficiency in the intestine, the most practical and effective method, may be disrupting the cell wall structure, while keeping the inner cell intact (Günerken et al., 2015; Lee et al., 2012). Extracting the homogenate (Becker et al., 1983) without changing the quality or contaminating the product to be further applied. For this case, mechanical methods are usually chosen. Although the effectiveness and safety of avoiding further chemical contamination and less dependence on the algae species, these mechanical methods have high energy and initial capital investment cost (for the equipment) (Becker et al., 1983; Harrison, 1991; Wang et al., 2015).

A mechanical method for efficient cell disruption is High-Pressure Homogenization (HPH). This method consists of processing a sample in a liquid state by high pressure (normally up to 150 MPa, but in some specialized models can go up to 400 MPa), as seen in Figure 1.1. The discharge pressure depends on the distance between the valve and the valve seat, which is regulated by a spring-loaded valve rod. There are several valve set designs to prevent cavitation, maximizing the cell disruption and, at the same time, minimizing the damage to the valve seat (Balduyck et al., 2018; Halim et al., 2013; Harrison, 1991; Lee et al., 2012; Middelberg, 1995).

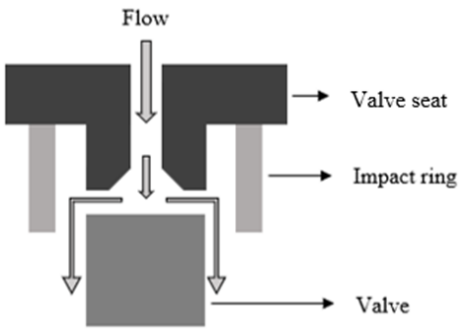

Figure 1.1-Illustration of a typical HPH scheme of the input passage for the algae suspension following the flow direction and passing through the valves, being compressed against the impact ring.

In this method, cell disruption can happen as a consequence of the collisions of the cells with the hard surfaces of the valve seat and impact ring valve, turbulence, viscous and high-pressure shear (Halim et al., 2013; Lee et al., 2012). The sudden decline in pressure, the release of gas bubbles that burst inside the cells, and the collapse of cavitation bubbles due to decreased flow velocities (Halim et al., 2013; Lee et al., 2012).Cell disruption can variate with microalgae specie due to cell wall composition and size differences (Halim et al., 2013; Lee et al., 2012).Altering the pressure and passages for each strain and each growth condition (Halim et al., 2013; Lee et al., 2012).

In the HPH method, the controllable parameters are pressure applied on the medium throughout the process, the temperature of the system, the number of passages, medium flow rate, valve, orifice design, and initial concentration of the sample (Follows et al., 1971; Lee et al., 2012). Increasing the pressure will also increase the release of cell contents per passage through the machine (Follows et al., 1971; Lee et al., 2012).

Other researchers had previously made algae bioavailability improvements for different algae species with different methods, enzymatic, physical, or both. The enzymatic treatment proved to increase the protein accessibility by enzyme species-specific relations if had a polysaccharide main cell wall with the *carrageenase* for *Chondrus crispus*, the *agarase* for *Gracilaria verrucose* and the *xylanase* for *Palmaria palmata* (Fleurence et al., 1995). These enzymatic species-specific relations were again proven in brown algae, to enrich the algae bioactivity compounds available, leading to an improvement in the antioxidant by in vitro assays, and antimicrobial response by disk diffusion method method (Sabeena et al., 2020). One physical treatment example was the increase of protein and fat digestibility of *Nannochloropsis gaditana* in the Nile tilapia intestine through bead milling of the algae before feeding the fish (Teuling et al., 2019). The implementation of both methods, through the combination of different technological processes, showed to improve the intestine nutrient accessibility and digestibility, where the best results occur for *Nannochloropsis oceanica* disrupted by enzymatic lysis, as for *Chlorella vulgaris* and *Tetraselmis sp.* the best results occurred by the vibratory grinding mill in *Dicentrarchus labrax* (European seabass) juveniles intestine (Batista et al., 2020b). Followed by the cell wall disruption of *Chlorella vulgaris* by different mechanical and chemical processes, recognizing the mechanical pre-treatments best for partial disruption and chemical processes (base-catalyzed) to achieve the highest disruption efficiency (Weber et al., 2022).

The focus on the intestine as the organ chosen for alga improvement analysis is related to the fact that the intestine is one of the digestive organs adapted to the fish feeding strategy

(Shalaby, 2020). By understanding these habits is possible to relate the feed to the functional mechanisms of fish digestive physiology (Alabssawy et al., 2019).

The mucosal epithelium is one of the functional layers of the gastrointestinal tract (Randall et al., 2011). This barrier protects against harmful intraluminal entities from the external environment and controls the homeostasis of the intestinal lumen into the circulation through a single layer of epithelial cells (Groschwitz & Hogan, 2009). The mucosal immunity response can be an innate response, such as phagocytosis, natural antibodies, and cytokine secretion, or an adaptive response through immunoglobulins (Ig) produced by the B-lymphocytes (Parra et al., 2015). Despite these responses, the immune system can be directly affected by stress due to immunosuppression, so the more exposed the fish, the higher the susceptibility to disease (Parra et al., 2015).

Many studies have shown an improvement in the intestine track of fish when fed with algae. *Lates calcarifer*, when provided with *Sargassum ilicifolium*, showed an improvement in growth performance by changing gut villus length and thickness and enhancing goblet cell counts (Zeynali et al., 2020). Higher digestion and absorption of nutrients, an increase in pancreatic digestive enzyme activities, and enhancement of the innate immune humoral and mucosal responses improved fish growth (Zeynali et al., 2020). Furthermore, *Dicentrarchus labrax*, when fed with *Gracilaria gracilis* presented an improvement in immune response, with an increase in plasmatic lysozyme and intestinal acid goblet cells (Passos et al., 2021).

As the intestine is an important organ sensitive to environmental and homeostatic disruptions, fish's health will be related to the intestinal maintenance of the antioxidant and immune response (Fu et al., 2022).

1.6. Gastrointestinal tract antioxidant response

The antioxidant response occurs in the presence of a certain level of reactive oxygen species (ROS), which can be free radicals or non-radicals oxygen-derived. While free radicals are unstable molecular species with unpaired electrons, such as hydroxyl (OH^-), superoxide (O_2^-), peroxyl (RO_2^-), and hydroperoxyl (HO_2^-) radicals (Dong et al., 2022; Phaniendra et al., 2015). Non-radicals are the conversion of radicals into hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($1O_2$) (Dong et al., 2022; Phaniendra et al., 2015). During oxygen metabolism, some ROS is formed, and the balance between the capacity to produce and to remove the ROS species (the "redox homeostasis") is important for the performance of many

functions in the cell, otherwise, it can generate oxidative stress, which may result in accumulative oxidative damage to the macromolecules of cells, such as DNA, RNA, proteins, carbohydrates, and lipids, destabilizing the cellular integrity and function necessary to keep the health status of the organism (Magnoni et al., 2017; Song et al., 2022). The antioxidant response can be constituted by enzymatic and nonenzymatic components (Ortiz et al., 2017). Some of the enzymatic is Catalase (*CAT*) with the highest turnover rates known that degrade H2O2 to water and oxygen (2H2O2 → 2H2O + O2) (Alothman et al., 2021). Glutathione peroxidase (*GPx*), is a phylogenetical enzyme, that uses glutathione as a reductant to catalyze the reduction of H2O2 or organic hydroperoxides into water or the corresponding alcohols (Brigelius-Flohé & Maiorino, 2013). Some nonenzymatic are Glutathione (a tripeptide) and Vitamine E (not synthesized, obtained through feeding), both "scavengers" of free radicals (Ortiz et al., 2017). Nuclear factor erythroid 2-related factor 2 (*Nrf2*) is a protein with a retinoid X receptor α (*RXRα*) binding to control the expression of antioxidant and detoxification enzymes (Tonelli et al., 2018). Antioxidant response markers have been previously used in studies where a functional ingredient with antioxidant proprieties was used. Like *GPx* gene expression in the gilthead seabream gut, shows a lower expression when fed with plant feedstuff-based diets than those fed with fishmeal-based diets (Basto-Silva et al., 2022). Also, Gilthead seabream postlarvae showed a higher *Nrf2* gene expression when fed a diet supplemented with a higher dose of curcumin than the fish fed a lower dose or no-supplemented diet (Xavier et al., 2022). As well as the upregulation of *Nrf2*, *CAT*, and *GPx* confirming the antioxidant property of emodin and curcumin in the intestine of Pengze crucian carp (*Carassius auratus var. Pengze*) (Yang et al., 2020). When microalgae were applied as a functional ingredient an increase in antioxidant response was observed with the highest *GPx* expression level detected in *Litopenaeus vannamei* fed with a diet supplemented with 2.5 g/kg dried *Tetraselmis suecica* (Zaki Sharawy et al., 2020). Gilthead Seabream Juveniles fed with 0.1% inclusion of *Chlorella vulgaris* peptide-enriched extract showed to increase gut lipid peroxidation and *CAT* gene expression in the gut (Reis et al., 2022).

1.7. Gastrointestinal tract immune response

The gastrointestinal tract is essential for the fish's immune maturation and dynamic balance. If an abnormal change in the dynamic balance occurs is possibly related to inflammatory stress (Ni et al., 2022). Has the gastrointestinal tract has been damaged an inflammatory response will occur. The cyclooxygenase 2 (Cox2), is inducibly expressed, in small

amounts, for epithelial repair and inflammation response (Fukata et al., 2006; Takeuchi & Satoh, 2015). The Immunoglobulin M (*IgM*), first produced as a surface-bound molecule and finally expressed in early B cell differentiation, acts as a highly effective antibody against pathogens, and in controlling B cell development, selection, and induction of central tolerance to prevent autoimmunity diseases (Keyt et al., 2020). Immune response markers have been previously analyzed in studies with a functional ingredient with immune proprieties. Some studies revealed a significant increase in *Cox2* expression in gilthead seabream intestinal when administrated with Bacillus subtilis diets (Cerezuela et al., 2013). *IgM* was proven to be a decent humoral immune parameter with a significant increase of expression for gilthead seabream fed with fenugreek supplemented diets (Awad et al., 2015). As well as the probiotic *DhBCS004* compared to no supplementation (Reyes-Becerril et al., 2021).

1.8. Objectives

Macroalgal and microalgal have many bioactive compounds with the capacity to enhance the fish intestinal response, but these compounds may have a limitation from the cell wall membrane. This study hypothesizes a higher permeability by the cell wall membrane of processed lysed algae biomass will promote higher antioxidant and immune responses in gilthead seabream (*Sparus aurata*) intestinal mucosa compared with unprocessed ones.

1.8.1. Main Objective

To evaluate the intestinal mucosal antioxidant and immune responses in the intestine of gilthead seabream (*Sparus aurata*) when exposed to processed or not processed algae. The test of the intestinal response will be with two microalgae, the *Phaeodactylum tricornutum*, and the *Nannochloropsis oceanica*.

1.8.2. Specific Objectives

Objective 1. To develop a method to optimize algae processing by breaking the cell walls homogenously.

Objective 2. To establish molecular markers of the immune response of the *in vitro* intestinal mucosa model under different conditions.

Objective 3. To establish molecular markers of the antioxidant response of the *in vitro* intestinal mucosa model under different conditions.

2. Material and Methods

Objective 1. To develop a method to optimize algae processing by breaking the cell walls homogenously.

2.1. Cell disruption by High-Pressure Homogenizer (HPH)

Industrial microalgal biomass solid frozen pastes, with 26% (w/w) dry weight for *Nannochloropsis spp*. and 19% (w/w) dry weight for *Phaeodactylum tricornutum*, were supplied by Allmicroalgae - Natural Products, S.A (Pataias, Portugal) and transported to the National Laboratory of Energy and Geology (LNEG, Lisbon, Portugal) for further analyzes.

2.1.1. HPH design test for cell integrity predictions

The cell disruption of *Nannochloropsis ocanica* and *Phaeodactylum tricornutum* was carried out in a PandaPLUS 2000 homogenizer (GEA, Germany), submitted to three high-pressure patients' homogenization (HPH) conditions to evaluate the influence of the combination of these factors on the cell integrity. For the cell disruption, the microalga culture was at different concentrations of 10 g/L, 65 g/L, and 120 g/L (ash-free dry weight) under various pressures of 100, 650, and 1200 bar and 1, 2, and 3 passages.

Alga paste was diluted with a saline media (F/2 adapted, Table II.I) to obtain the suspensions for the 15 tests. Each alga originated three different dilutions regarding the three concentrations, getting other total volumes, with 250 mL for the 10 g/L, 400 mL for the 65 g/L, and 250 mL for the 120 g/L. Between different passages in the HPH, 100 mL of biomass were used for one and then three passages and 60 mL for only two passages tests to save biomass.

To set up the HPH, tap water was introduced into the inlet feeding hopper to clean and select the pressure desired. When the suspension was diluted and homogeneous at room temperature, it was passed through the HPH (right after the water). For the one-passage treatment, the culture was added and collected at the device's outlet, while the pressure was checked for deviations in the compressing cylinder. The repetition of the one passage process on the same sample test was defined as the two and three passages tests treatments. Every test followed the same procedures, only variating the suspension cell concentration, the pressure, and the total number of passages. This procedure was repeated for the two algae equally.

Table II.I – Components to produce the marine saline media for one liter of solution.

F/2 ADAPTED RECIPE	
Chemicals	**Concentration (g/L)**
NACL	35.79
$MGSO_4.7H_2O$	7.54
KNO_3	1.02
K_2SO_4	0.87
$CACL_2.2H_2O$	0.81

2.2. Evaluation of Cell Disruption by Flow cytometry

Microalgae samples were analyzed using a CytoFLEX Flow Cytometer (Beckman Coulter Life Sciences, USA), equipped with a 488 nm argon laser to detect submicrometric particles. Fluorescence data was collected with the ratio of Side Scatter Detector (SSC) versus Forward Scatter Detector (FSC), allowing the determination of particle size and intra-cell compounds (refractive index), as well as Fluorescein isothiocyanate detector (FITC) at the green region. The cell membrane integrity was analyzed by the sensors representing the cell auto-fluorescence, chlorophyll fluorescence (670 nm filter), and Sytox Green stain (4.8 uM, 25 min in the dark, to prevent Sytox photodegradation) fluorescence detection. Sytox Green stain fluorescent probe could not enter through intact plasma membranes, leaving live cells unstained (negative cells), and penetrated dead cells with permeable plasma membranes exhibit a bright green fluorescence (positive cells). With absorption and emission maxima at 502 and 523 nm, respectively (Machado & Soares, 2015; Roth et al., 1997). This method distinguished three different cell suit populations, cells with membrane intact (a), cells with the compromised cell membrane (b), and cell debris (c).

For better observation of the measurements, optimizing the detectors and the Sytox Green stain was required. The flow cytometry parameters were optimized, comparing 1 mL of an alga non-disrupted and a death suspension (previously boiled in a water bath at 100 °C for 60 min at a concentration of 2 g/L) with Phosphate-buffered saline (PBS), to read the microalga at approximately 450 events per sec (with a total 10,000 events).

Sytox Green stain was optimized with the positive control (heat-treated cells) for the stain concentration and incubation time that produced the highest stain FITC fluorescence, leading to a 4.8 µM for 25 min in the dark.

Percentages of the population's gates plotted in the flow cytometer readings were calculated according to the following equations:

(a) $Intact\ cells\ (\%) = \frac{Non\ stained\ treated\ with\ Sytox\ cells}{Total\ untreated\ cells}$

(b) $Permeabilized\ cells\ (\%) = \frac{Stained\ treated\ with\ Sytox\ cells}{Total\ untreated\ cells}$

(c) $Disrupted\ cells\ (\%) = \frac{Total\ untreated\ cells - Total\ treated\ with\ Cytox\ cells}{Total\ untreated\ cells}$

2.3. Cell disruption model

A Second-Order Response Surface Methodology model design was carried out, with fifteen different tests and three variables (Tables II.II and II.III).

Table II.II – Experimental treatment variables.

Factor	Units	Levels		
	Coded units	-1	0	1
X1	HPH pressure (bar)	100	650	1200
X2	HPH cycles (continuous)	1	2	3
X3	Cell concentration (g/L)	10	65	120

Table II.III - Experimental Design layout.

Tests Order	Coded units			Real units		
	Pressure (bar)	Passages (n)	Alga Cell concentration (g/L)	Pressure (bar)	Passages (n)	Alga Cell concentration (g/L)
1	0	0	0	650	2	65
2	1	0	1	1200	2	120
3	0	1	1	650	3	120
4	-1	0	-1	100	2	10
5	0	0	0	650	2	65
6	0	1	-1	650	3	10
7	1	0	-1	1200	2	10
8	-1	-1	0	100	1	65
9	1	1	0	1200	3	65
10	0	-1	1	650	1	120
11	0	-1	-1	650	1	10
12	0	0	0	650	2	65
13	-1	0	1	100	2	120

14	1	-1	0	1200	1	65
15	-1	1	0	100	3	65

2.3.1. Response Surface Optimization

After the first model analyses, new data were introduced to the model. New values of pressure were predicted to obtain two levels of disruption for 120 g/L of alga cell concentration: the non-disrupted, and 90% disruption with three passages. The values of cell concentration were fixed to 120 g/L, and only variating the number of passages, defined the pressure as the factor to obtain the 90% of cell disruption (Table II.IV).

After the first model was analyzed, the same steps were done for further predictions.

When all samples were optimized, they were frozen and stored for 12 hours at -18 ºC for freeze-drying in glass Petri dishes with parafilm. The samples were freeze-dried with the help of a Freeze Dryer Heto PowerDry LL3000 (Thermo Scientific) for 72 hours at -500 ºC and stored for further use at -80 ºC in plastic cups.

Table II.IV - Optimization of selected options.

Microalgae specie	Predicted Level of Disruption (%)	Predicted Pressure (bar)	Number of passages (n)	Alga cell concentration (g/L)
*P. tricornutum*_Disrupt_α	90	Minimum	3	120
*P. tricornutum*_Disrupt_β	90	Maximum	1	120
*P. tricornutum*_Whole	0	0	0	120
*Nanno*_Disrupt_α	90	Minimum	3	120
*Nanno*_Disrupt_β	90	Maximum	1	120
*Nanno*_Whole	0	0	0	120

Objective 2. To establish molecular markers of the immune response of the in vitro intestinal mucosa model under different conditions.

and

Objective 3. To establish molecular markers of the antioxidant response of the in vitro intestinal mucosa model under different conditions.

2.4. Fish dissection and intestinal explant preparation

All procedures followed the ethical guidelines of CCMAR concerning the welfare of animals used, respecting the Portuguese (Decree-Law no. 113/2013 of the 7th of August) and European regulations (Directive 2010/63/EU) that rule the use of animals in research.

For this approach, the explant model was based on the method proposed by Peñaranda et al., (2020), standardized at GreenCoLab for gilthead seabream.

Subjects for this experiment were all delivered by the Aquaculture Research Group (CCMAR) from the Ramalhete field station (CCMAR/UAlg). All 18 fish had proximally the same size (average weight ± 200 g). All subjects were under the same environmental conditions (from the same tank) and were not fed for 24 h previously to the sampling.

The specimens were anesthetized with 2-phenoxyethanol (in a lethal dosage) and a cervical severing with a scalpel to ensure ethical euthanasia to secure no suffering for the animal. The peritoneal cavity was aseptically dissected (Figure 2.2), and the entire intestinal tract was extracted for identification and sectioned into three small anterior regions (5x2 mm) using sterile dissection material. The tissue was placed in a 48-well cell culture plate with a natural biological innocuous glue to secure the tissue in place, with 1.2 mL of IMDM, and 0.1 mL of the respective algae. Times were registered for each sample. The plate was incubated at the same temperature as the fish tanks when the plate was complete. Based on preliminary tests (standardized before the project), after five h of incubation, the sample tissues were collected. With the help of an absorbent paper, the excess medium in the tissue was taken out. Then each sample was saved for gene expression analysis and stored at -80 ºC until analysis.

Each fish had the anterior intestine assigned to three test groups, with the Control (Ct) – only Iscove's Modified Dulbecco's Medium (IMDM) from Cytiva Hyclone (no exposure), for all subjects, differing the two algae groups' tests (algae not disrupted and algae disrupted). In the protocol, every test was done in triplicate. Each fish had one alga (*Nannocholropsis sp*. or *Phaeodactylum tricornutum*) at one algae concentration (8 mg/mL, 40 mg/mL, or 120 mg/mL), with a total of 54 tests done.

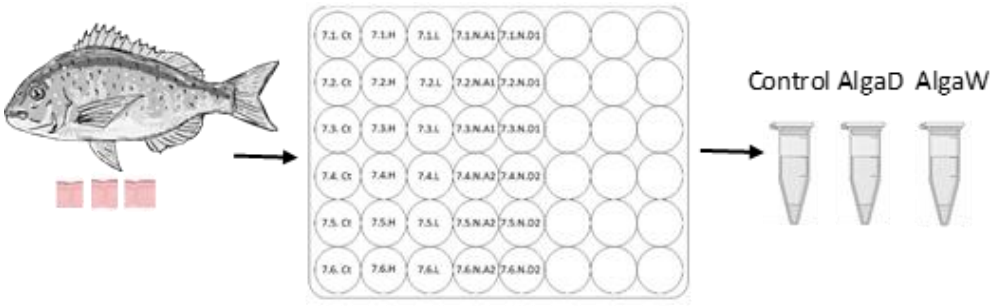

Figure 2.1- Schematization of sequence of each fish intestine sample, from fish to plate test to vials after 5 hours incubation. Three samples from fish, Control, AlgaD – disrupted, and AlgaW- Not disrupted, whole algae.

2.4. RNA purification

The protocol followed the method described in the NZYol (MB18501, Nzytech, Portugal) manual to retrieve total RNA from cell tissues with some modifications by adding the bead meals homogenization process, varying the centrifuge velocities, and an additional ethanol wash.

Only 9 fish samples were used from the 18 fish. A small part from each sample was used (average weight ±100 mg), with a cleaning process of the surgical instruments between samples, saving the resting tissue back to -80 ºC and using only the test tissue separated. Each tissue was placed in a 2 mL Eppendorf with a ceramic pearl and one mL of NZYol. Samples were homogenized in a tissue grinder two times for 5 min at 30 1/s frequencies, each with 60 sec pauses on ice. Afterward, the vials stayed for 5 min at room temperature (RT), centrifuged at 16,000 x g for 5 min at 4 ºC to separate the fat and debris from the compounds of interest, and collected 900 mL of the liquid part to a new two mL vial.

To separate de RNA from the DNA and the proteins, 200 µL of chloroform 100% was added and mixed through the vortex. The sample was left for 2-3 min in RT and it was centrifuged again at 14,000 x g for 15 min at 4 ºC. This process allowed the formation of three phases, a lower dark green organic phase (protein), a whitish interphase (DNA), and an upper aqueous phase (RNA). The upper phase (500 µL) was transferred to another 1.5 mL tube, and 500 µL of ice-cold isopropyl alcohol was added. Mixed gently and incubated for 10 min at RT, and centrifuged at 12,000 x g at 4 °C for 10 min. At this point, a whitish pellet was formed at the bottom of the vial. The supernatant was discarded, resuspended with one mL of ice-cold ethanol 75%, and centrifuged at 7,500 x g at 4 °C for 5 min. The RNA washing was done two times discarding the supernatant and repeating the process with ice-cold ethanol of 75%.

The supernatant was discarded, and with a micropipette, the remains were removed. Centrifugation of 8,000 x g at 4 °C for 30 sec and a second removal with a micropipette followed by 5 min drying under cold conditions were done to verify no remains of ethanol in the RNA

sample. Each sample was resuspended with 50 µL of DEPC-treated water by pipetting the solution up and down. Bubbles and too much mechanical exposure were avoided, during the final process. The RNA samples were stored at -80 ºC until analysis.

Like in Bunu et al., 2020 a ratio of absorbance at 260 nm and 280 nm was used to count RNA as this absorbs at 260 nm, indicating any possible contamination of protein, phenol, or other contaminants that absorb strongly at/ or near 280 nm. A ratio of approximately 2.0 ng/µL was accepted as pure for RNA. The A230/260 ratio was used as a secondary measure of nucleic acid purity, in the range of 2.0–2.2 ng/µL.

To quantify and verify the purity of the extraction, each RNA sample was examined through a NanoDrop-1000 spectrophotometer (Thermo Scientific, USA). This equipment measured 1 µL of each sample, with the ultra-purified water as blank. If the concentration was higher than 3500 ng/µL, the sample was diluted (1:1) and the dilution was measured again. All absorbance ratios were registered, and the RNA samples were stored at -80 ºC until analysis.

Visual observations of the integrities of the total RNA samples were done with a MOPS (3-(N-morpholino) propane sulfonic acid) denaturing 1.2% agarose gel electrophoresis (0.6 g agarose, 43.5 mL DEPC water, 5 mL 10X MOPS and 1.5 mL formaldehyde) for the detection of 28S and 18S ribosomal RNA bands. RNA sample concentrations were diluted to 400-500 ng/µL with DEPC water. For the running gel, the samples and the RiboRuler High Range RNA Ladder (Thermo Fisher Scientific, Massachusetts, US) were prepared, with a ratio of 2 µL of sample for 4 µL of denaturation sample and 1.5 µL of RiboRuler for 4 µL of denaturation sample (denaturation sample – 500 µL deionized formamide, 100 µL 10X MOPS, 150 µL formaldehyde, 50 µL Gelred 2%). To occur denaturation all samples and RiboRuler were incubated at 60 ºC for 10 min, followed by 5 min on ice. The gel was run at 1X MOPS Buffer and performed for 30 min at 90 Volts. The resulting gel was observed in a Nugenius Gel Documentation & Image Analysis System (Fisher Scientific - Syngene, USA). All the remains from each sample were stored at −80 °C for downstream application later.

2.5. cDNA synthesis

The cDNA was synthesized according to the manufacturer's protocol of the Thermo Revertaid H Minus first strand cDNA Synthesis kit (code: K1632). A random hexamer primer was used with 400 ng/ µL of RNA samples previously prepared.

The Reverse transcription polymerase chain reactions were performed with a total of 20 µL for each sample. It followed the component's order of preparation: the first reaction was 12 µL water nuclease-free, 1 µL random hexamer primer (5 µM, 0.01 µg/µL final concentration),

and 1 µL of specific RNA (20 µL/mL final concentration, with a 10 min at 65 ºC incubation and 5 min on ice; the second reaction added 4 µL of 5X Reaction Buffer (50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 10 mM DTT final concentration), 1 µL of RiboLock RNase Inhibitor (1 U/µL final concentration), 2 µL of 10 mM dNTP Mix (1 mM final concentration), and 1 µL ReverAid H Minus M-MulV Reverse Transcriptase (10 U/µL final concentration), with a thermocycler run. The thermocycler conditions were established as follows: 5 min at 25 ºC, 42 min at 60 ºC, and 5 min at 70 ºC respectively.

2.5.2. Confirmatory PCR

A PCR was set with the cDNA samples to secure the success of the cDNA production through visualization on an agarose 1.5%TAE Buffer Gel (Tris-Acetate-EDTA Buffer) with Green Scan and Gel Red. The procedures followed the manual protocol of the GoTaq® Flexi DNA Polymerase kit (Promega, USA), with adaptations. The cDNA obtained previously was used in a new dilution with a 20 µL total volume for each sample. It followed the components order of preparation: 9.95 µL of water nuclease-free; 4 µL of Go Taq Flexi: 5X Buffer (Ref. M890A) (1X final concentration); 1.6 µL of 25 mM MgCl2 (Promega, Ref. A351H) (2 mM final concentration); 1.25 µL of 8 mM dNTPs Mix (0.5 mM final concentration); 1 µL of Forward Elf1α Primer (0.5 µM final concentration); 1 µL of Reverse Elf1α Primer (0.5 µM final concentration); 1 µL of the cDNA; and 0.2 µL of Go Taq G2 Flexi DNA Polymerase (Ref. M7808) (0.5 U/µL final concentration) with a thermocycler run. The thermocycler conditions were established as follows: first stage with 1 cycle of 5 min at 95 ºC (initial denaturation), second stage with 35 cycles of 30 sec at 95 ºC (denaturing), 30 sec at 59 ºC (annealing), and 60 sec at 72 ºC (extension), third stage with 1 cycle of 10 min at 72 ºC and 5 min at 4 ºC respectively.

The agarose 1.5% TAE Gel was prepared with 0.75 mg of agarose for 50 mL of 1X TAE Buffer. To run the TAE Gel each PCR sample was mixed with a preparation of Green Scan with GelRed (1:500) at a ratio of 1:2. It followed the component's order of preparation: each sample had 5 µL for 4 µL of Green Scan with GelRed, and the 100 par bases ladder had 2.5 µL for 4 µL of Green Scan with GelRed. The Gel was run in 1X TAE Buffer for 30 min at 110 Volts. The resulting gel was observed in a Nugenius Gel Documentation & Image Analysis System (Fisher Scientific - Syngene, USA). All 96 PCR well plates were stored at -20 ºC until analysis.

2.6. Analysis of seabream intestinal gene expression with qPCR

From published transcriptome datasets on the database of The National Center for Biotechnology Information, two genes related to the immune response (*Cox2*, *IgM*) and three related to oxidant response (*CAT*, *GPx*, *Nrf2*) were selected (Table II.V) for analysis of relative gene expressions in seabream anterior intestines through qPCR (real-time quantitative PCR), at an initial time (t = 0 h) and at an incubation time (t = 5 h). At least two primer pairs for each gene were designed using the program GeniousPrime (Biomatters Limited, New Zealand).

All primers were tested by a PCR at annealing temperatures of 55 ºC, 57 ºC, and 59 ºC to select the optimal. A 1X TAE Buffer gel was run with the same protocol previously done to select the best primer temperature and pair.

Table II.V - Primer sequences of candidate genes (reference and target genes) in the qPCR assay.

Gene	GeneBank ID	Primer Forward (5'->3')	Primer Reverse (5'->3')	Length (bp)
	Reference Gene			
EF-1α	AF184170	GGAGATGCACCACGAGTCTC	GCGTTGAAGTTGTCAGCTCC	150
	Target Genes			
Cox2	AM296029	GACATCATCAACACTGCCTCC	GATATCACTGCCGCCTGAGT	150
IgM	JQ811851	GACAACCTCAGCGTCCTTCA	CTTTTGAGTCTGCAGCGTCG	150
CAT	JQ308823	CGACATGGTGTGGGACTTCT	CGCTCACCATTGGCATTGAC	150
GPx	KC201352	TTTACGCCCTGACAGCCAAT	AGTAACGACTGTGGAGCTCG	150
Nrf2	XM_030427725	TGAAGGAGGAGAAGGAGCGT	AGTACTCGGACGGCGAGTAT	150

2.6.1. Primer Dynamic Range

A dynamic range was done in triplicate to include 5 dilutions 1:3. This allowed us to highlight the efficiency of the primers.

All primers had efficiency within an acceptable range (between 110% and 130%). The cDNA was diluted in a ratio of 1:3 for the first dilution (D1) and a ratio of 1:9 for the second dilution (D2).

2.6.2. Pfaff Method Relative Gene expression

The gene of interest was analyzed using the SybrGreen Power Up Master Mix Kit protocol (Thermo Fisher Scientific, USA), on a BioRad CFX96 Real-Time system (San Francisco, USA). A total volume of 10 µl/well was used for each qPCR reaction, 5 µl of PowerUpTM SYBRTM Green Master Mix (2X), 1.75 µl of Nuclease-Free Water, 0.625 µl of Forward primer, and 0.625 µl of Reverse primer, with 2 µl of the diluted cDNA template. All 96-well PCR plates had a nuclease-free water control (NTC). A duplicate of each sample was done to verify the gene expression result. The thermal cycling conditions only variate the annealing temperature between genes, having the same conditions for the rest of the process: 10 min at 95 °C, followed by 39 cycles of 95 °C for 15 sec, annealing temperature for 30 sec, and 72 °C for 30 sec.

A melt curve reaction was completed after the qPCR for each gene to guarantee the efficiency of the process (only one peak was observed).

2.7. Statistical Analysis

For all statistical tests, the significance level was set for a P-value of 0.05.

The first objective cell disruption model was as follows:

The further optimization of cell disruption (response of interest) was modeled and analyzed through a Response Surface Methodology design (RSM design). ANOVA analyses were performed to quantify the statistical difference between the parameters (pressure, number of passages, and alga cell concentration), the level of significance (P-value < 0.05 %), and the Error and lack of fit of the model. For the optimization, the same procedure was followed. All with the help of the program Minitab 19 software (Minitab LLC, USA).

In second and third objectives for the gene expression were analyzed using the XLSTAT software (version 2018, Addinsoft, USA). Data were analyzed for normality by a Shapiro-Wilk test and homogeneity of variance through Levene's test. Data were analyzed by a two-way

ANOVA (factors were: algae process and concentration levels) and when significant differences were obtained, Tukey's post hoc test was performed for multiple comparisons. Significant differences were accepted at P-value<0.05.

3. Results

Objective 1. To develop a method to optimize algae processing by breaking the cell walls homogenously.

3.1. Evaluation of Cell Disruption by Flow cytometry

Nannochloropsis oceanica and *Phaeodactylum tricornutum* negative and positive control gates were created to identify and calculate the percentages of the sub-populations plotted in the flow cytometer readings. The sub-populations originated four gates visually exemplified in figure 3.1 for *N. oceanica*. The intact untreated cells (Gate 1), the intact heat-treated cells (Gate 2), the permeabilized heat-treated cells (Gate 3), and the unpermeabilised untreated cells (Gate 4).

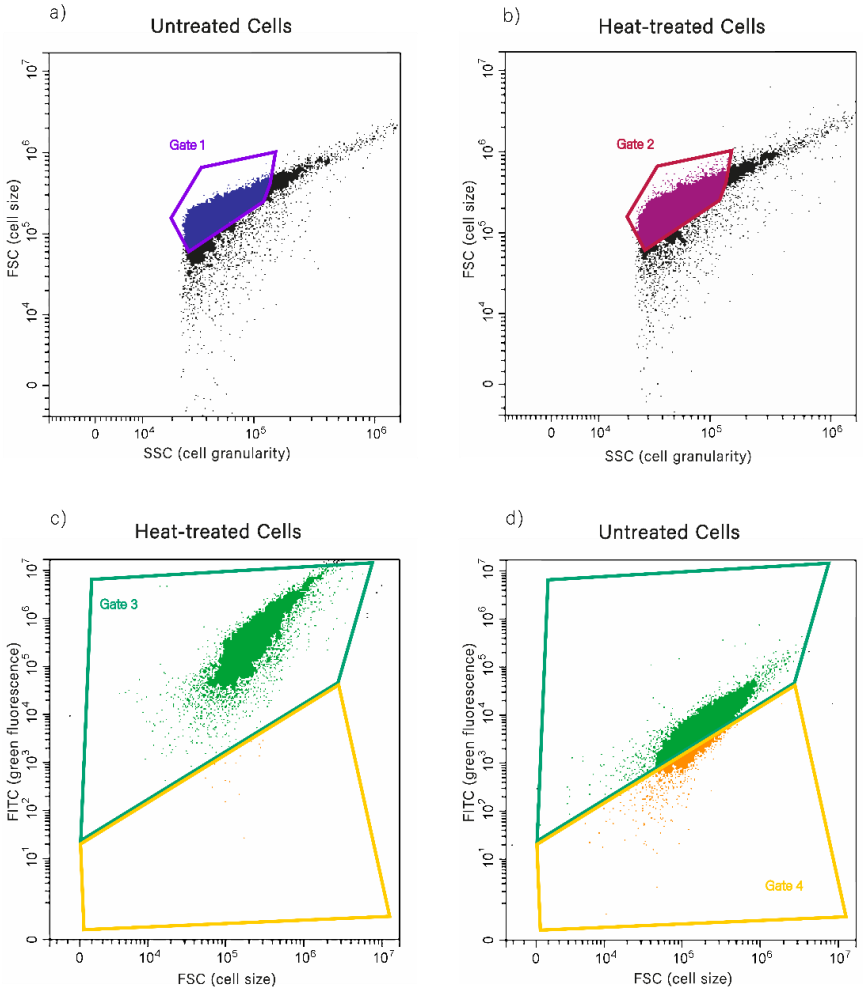

Figure 3.1- Flow cytometry gates for Nannochloropsis oceanica using the detectors FSC/SSC and FITC/FSC dot plots. Untreated cells were used as a negative control (a, d) and heat-treated cells were used as a positive control (b, c). Gate 1 represented 81.1±3.7%, Gate 2 represented 72.9±3.1%, Gate 3 represented 92.5±2.0% and Gate 4 represented 27.4±14.4% of the observations respectively mean ± standard deviation (n=4).

N. oceanica gates were used for further comparison with data obtained from the HPH disruption treatment of *N. oceanica*, and *P. tricornutum* gates were used for further comparison with data obtained from the HPH disruption treatment of *P. tricornutum*.

Through the observation of the *N. oceanica* gates, Gate 4 showed almost all untreated cells were already permeabilized, with only 27.4±14.4% (mean ± standard deviation) of observations representing the unpermeabilised cells. *P. tricornutum* gates were with 61.8 ±2.0% for Gate 1, 78.9±1.7% for Gate 2, 98.5±0.4 % for Gate 3, and 95.7±1.1% for Gate 4 of the represented observations respectively.

3.2. Recovered Suspensions

Cells disruption results of *N. oceanica* and *P. tricornutum* were obtained (Table III.I) from different pressure, the number of passages, and cell concentration suspensions of high-pressure homogenization. *Nannochloropsis oceanica* minimum disruption was 71.8% for 100 bar, 1 passage, and a cell concentration suspension of 65 g/L, and maximum disruption was 92.7% for 650 bar, 1 passage, and a cell concentration suspension of 120 g/L.

Table III.I - Box Becken Design Model algae cell disruption results. Phaeodactylum tricornutum was obtained from Chlorophyll/FSC based with and Nannochloropsis oceanica was obtained from FSC/SSC based.

	Alga Cell Disrutpion %	
Test Order	***Phaeodactylum tricornutum***	***Nannochloropsis oceanica***
1	98.5	81.8
2	99.2	85.7
3	99.1	89.2
4	91.4	76.9
5	98.0	78.9
6	99.5	90.5
7	99.7	87.6
8	74.6	71.8
9	99.5	85.9
10	88.0	92.7
11	88.8	88.0
12	95.1	85.6
13	78.1	78.3
14	96.2	81.8
15	86.4	76.8

Phaeodactylum tricornutum minimum disruption was 74.6% for 100 bar, 1 passage, and a cell concentration suspension of 65 g/L, and maximum disruption was 99.7% for 1200 bar, 2 passages, and a cell concentration suspension of 10 g/L.

The percentage contribution of each independent variable and their interactions with the dependent variable is shown in Table III.II and Table III.III. The significance of the model was validated by the P-Values. *N. oceanica* (Table III.II) R^2, adjusted R^2, and predicted R^2 values were 0.9241, 0.7876, and 0.4229 respectively. In addition, the Model F-value of 6.77 indicates a statistically significant difference within the model. The “Lack of Fit P-value” of 0.757 secures the capacity of this model to fit the data. The Linear regression of Pressure (P-value of 0.005) and the Square Pressure*Pressure (P-value of 0.012) and Cell concentration*Cell concentration (P-value of 0.012) was statistically significant for the disruption.

Table III.II - ANOVA analysis of the response surface quadratic polynomial model assessed using Box-Behnken Design for Nannochloropsis oceanica.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	461.061	51.229	6.77	0.024
Linear	3	182.727	60.909	8.05	0.023
Pressure	1	173.367	173.367	22.90	0.005
Number of passages	1	8.326	8.326	1.10	0.342
Cell concentration	1	1.034	1.034	0.14	0.727
Square	3	266.021	88.674	11.71	0.011
Pressure*Pressure	1	111.578	111.578	14.74	0.012
Number of passages * Number of passages	1	22.743	22.743	3.00	0.144
Cell concentration*Cell concentration	1	112.790	112.790	14.90	0.012
2-Way Interaction	3	12.314	4.105	0.54	0.674
Pressure* Number of passages	1	0.163	0.163	0.02	0.889
Pressure*Cell concentration	1	2.921	2.921	0.39	0.562
Number of passages *Cell concentration	1	9.230	9.230	1.22	0.320
Error	5	37.850	7.570		
Lack-of-Fit	3	14.745	4.915	0.43	0.757
Pure Error	2	23.106	11.553		

P. tricornutum (Table III.III) R^2, adjusted R^2, and predicted R^2 values were 0.9612, 0.8914, and 0.4823 respectively. In addition, the Model F-value of 13.77 indicates a statistically significant difference within the model. The "Lack of Fit P-value" of 0.257 secures the capacity of this model to fit the data. The Linear regression of Pressure (P-value of 0.000) and Number of passages (P-value of 0.005), and the Square Pressure*Pressure (P-value of 0.018) and Number of passages * Number of passages (P-value of 0.74) were statistically significant for the disruption.

Table III.III - ANOVA analysis of the response surface quadratic polynomial model assessed using Box-Behnken Design for Phaeodactylum tricornutum.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	889.692	98.855	13.77	0.005
Linear	3	713.713	237.904	33.13	0.001
Pressure	1	514.695	514.695	71.69	0.000
Number of passages	1	170.745	170.745	23.78	0.005
Cell concentration	1	28.274	28.274	3.94	0.104
Square	3	116.181	38.727	5.39	0.050
Pressure*Pressure	1	87.372	87.372	12.17	0.018
Number of passages*Number of passages	1	36.321	36.321	5.06	0.074
Cell concentration*Cell concentration	1	0.173	0.173	0.02	0.883
2-Way Interaction	3	59.797	19.932	2.78	0.150
Pressure*Number of passages	1	18.165	18.165	2.53	0.173
Pressure*Cell concentration	1	41.584	41.584	5.79	0.061
Number of passages *Cell concentration	1	0.048	0.048	0.01	0.938
Error	5	35.900	7.180		
Lack-of-Fit	3	28.976	9.659	2.79	0.275
Pure Error	2	6.923	3.462		

The final regression equation for the experimental parameters and actual factors (x1, x2 and x3 on Table II.II), was determined as:

$$N.oceanica \text{ Cell Disruption } (\%) = 78{,}83 + 0{,}03466\,x1 - 6{,}87x2 - 0{,}1574\,x3 - 0{,}000018\,x1^2 + 2{,}48\,x2^2 + 0{,}001827\,x3^2 - 0{,}00037x1 \times x2 - 0{,}000028\,x1 \times x3 - 0{,}0276\,x2 \times x3$$

$$P.tricornutum \text{ Cell Disruption } (\%) = 60{,}77 + 0{,}03631\,x1 + 19{,}55\,x2 - 0{,}0982\,x3 - 0{,}000016x1^2 - 3{,}14\,x2^2 - 0{,}000072\,x3^2 - 0{,}00387x1 \times x2 + 0{,}000107\,x1 \times x3 + 0{,}0020\,x2 \times x3$$

As the Cell Disruption (%) response surface model Equation was constructed, an optimization was performed, establishing the pressure as the predicted factor for a certain disruption level (Disrupt_α and Disrupt_β) for *N. oceanica* and *P. tricornutum* (Table III.IV). *N. oceanica* obtained a Cell disruption of 78.52±0.98 %, for 3 passages with a pressure of 234 bar while for 1 passage the pressure was 559 bar. *P. tricornutum* obtained a Cell disruption of 87.55±2.13 %, for 3 passages with a pressure of 262 bar while for 1 passage the pressure was 613.7 bar.

Table III.IV – RSM optimization to analyze the pressure needed for different number of passages (1 and 3) while obtaining the same cell disruption.

	RSM final optimizations				
ID Description	Pressure (bar)	Alga cell concentration (g/L)	Number of passages (n)	Cell disruption (%)	Cell disruption mean ± SD (%)
*P.tricornutum*_ Disrupt_α	234.0	120.0	3.0	77.8	78.52 ± 0.98
P.tricornutum _ Disrupt_β	559.0	120.0	1.0	79.2	
P.tricornutum _Whole	0.0	120.0	0.0	0.0	Not Defined
*N. oceanica*_ Disrupt_α	262.0	120.0	3.0	89.1	87.55 ± 2.13
N. oceanica _ Disrupt_β	613.7	120.0	1.0	86.0	
N. oceanica _Whole	0.0	120.0	0.0	0.0	Not Defind

Objective 2. To establish molecular markers of the immune response of the in vitro intestinal mucosa model under different conditions.

and

Objective 3. To establish molecular markers of the antioxidant response of the in vitro intestinal mucosa model under different conditions.

3.3. Analysis of tissue explant

3.3.1. RNA and cDNA extraction

All the intestinal samples' RNA extractions were implemented with the parameters of stated purity and integrity (Figure 3.2). As well, all the synthesized cDNA was validated by a PCR with a standard gene EF1α (Figure 3.3).

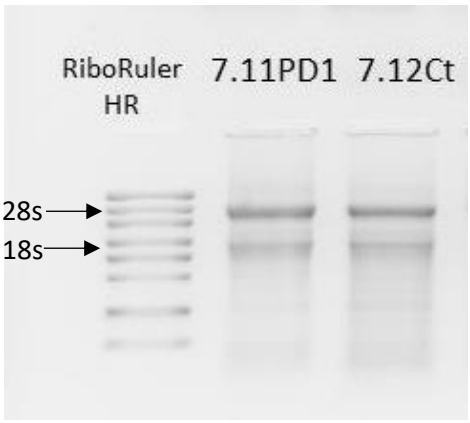

Figure 3.2-Visualization of the integrity of RNA from the intestine of gilthead seabream (Sparus aurata) in 0.6% agarose gel. Sample 7.11PD1 with 1424.941 ng/uL of Nucleic Acid, 2.046 at A260/230 and 2.264 at A260/280. Sample 7.12Ct with 1145.263 ng/uL of Nucleic Acid Ladder, 2.037 at A260/230 and 2.163 at A260/280.

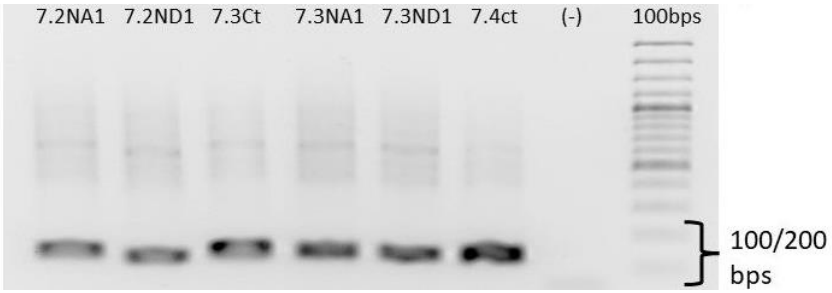

Figure 3.3-Visualization of gut cDNA verification from the intestine of gilthead seabream (Sparus aurata) in 1.5% agarose gel. The amplification of the cDNA PCR product with a 100 bps ladder showing the band in the region between the 100 and 200 bps.

3.3.2. Standardization of New primer

A new primer was standardized for the nuclear factor E2-related factor 2 gene, shown in Figure 3.4. Between the two pairs of primers, the Nrf2_2 with the temperature standardization of annealing of 55 ºC was selected as the best amplification, according to the larger expected size of the amplicon (between 100 and 200 bps), without any other amplification. The Nrf2_1 was not selected for presenting more than one amplification at a temperature of 57 ºC and 59 ºC and possible dimer at the negative control at 55 ºC, 57 ºC, and 59 ºC.

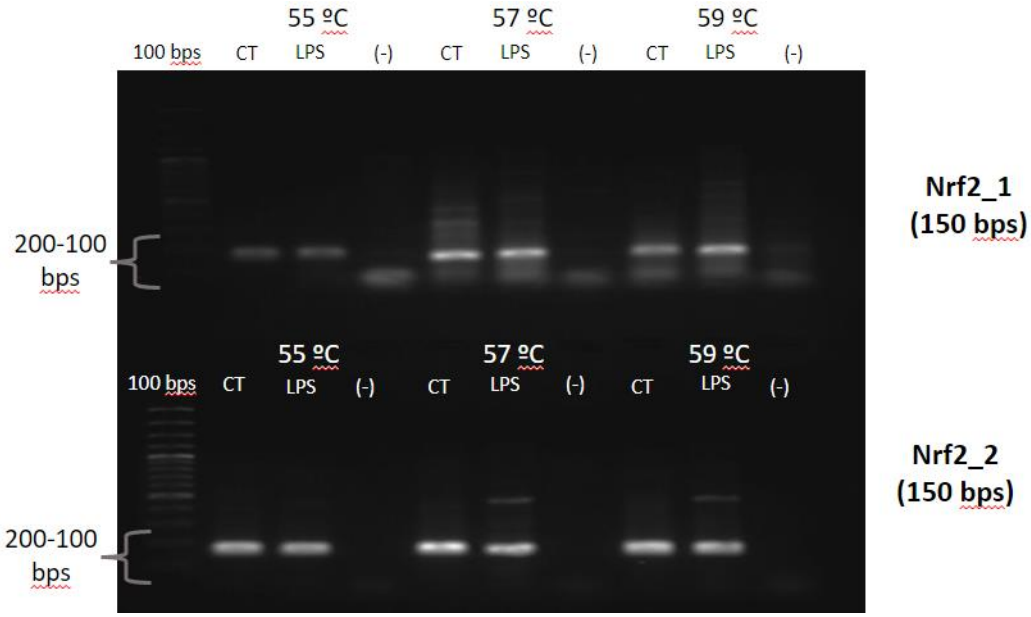

Figure 3.4-Visualization of PCR product based on S. aurata intestine cDNA in 1.5% agarose gel. The amplification of 2 pairs of primers designed for the Nrf2 gene (Nrf2_1 and Nrf2_2) was observed. They were evaluated at 3 temperatures: 55°C, 57°C, 59°C.

A dynamic range analysis for the corresponding primers of the Nrf2 gene (Figure 3.5) was implemented, with a ct within 18 and 22 (Figure 3.5A). The Melting curve proved to be no contamination or nonspecific product, with a specific amplification at 82,50 ºC (Figure 3.5B). The standard curve obtained (Figure 3.5C) an efficiency near 100% (of 126.1%) with a coefficient R^2= 0.763, securing the parameters to evaluate the relative expression.

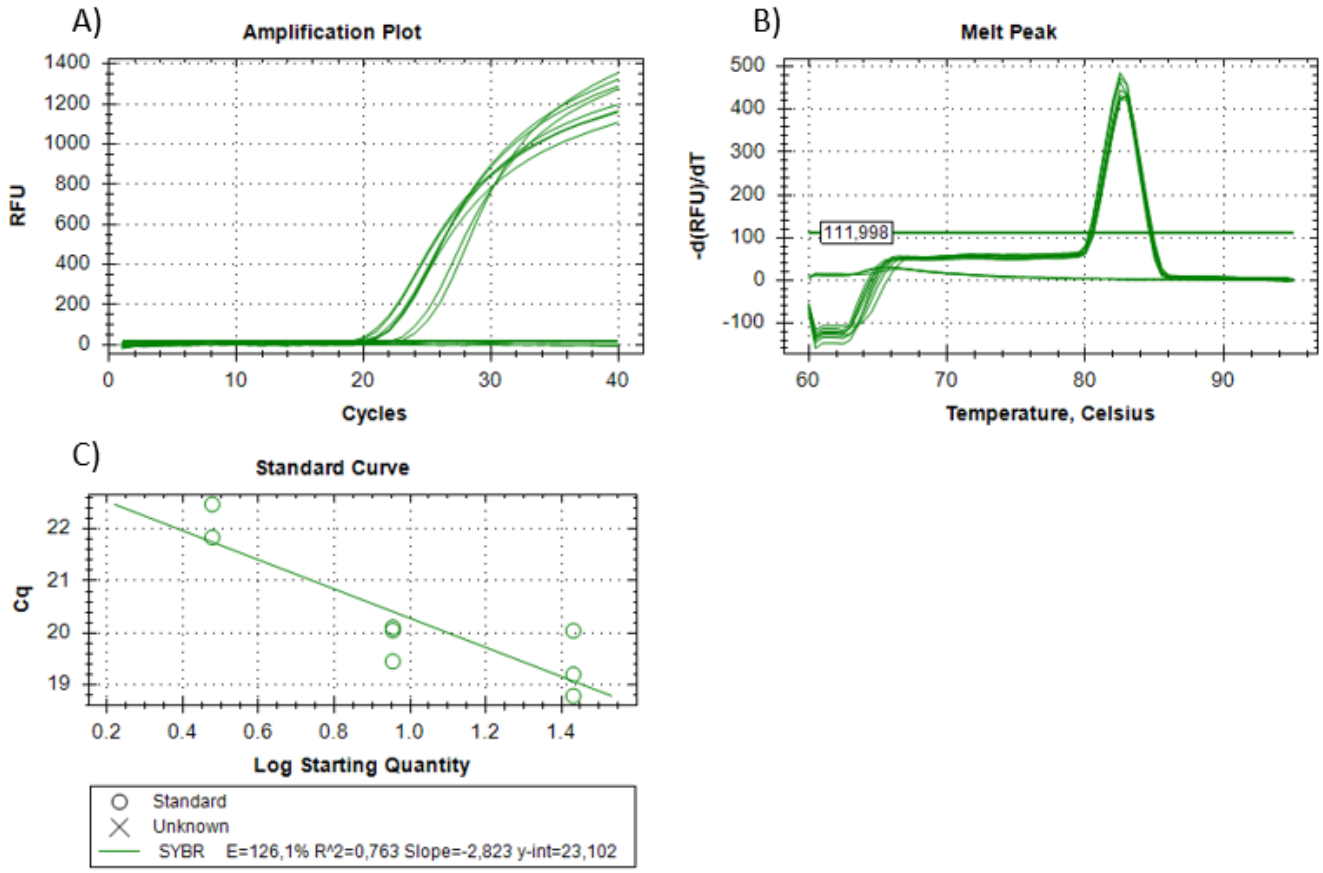

Figure 3.5- Dynamic range for the standardization of the Nfr2 gene primers by qPCR. Samples of different dilutions of 1/3, 1/9, 1/27, and a negative control were used to A) Amplification curve (RFU - relative fluorescence units; Cycles), B) Melting curve, and C) Standard curve. An efficiency of 126.1% was obtained.

3.3.3. Genetic Expression

Immune response molecular markers

The expression of seabream *Cox2* and IgM was marked in tissues exposed to different cell concentration suspensions of whole algae or disrupted and a control group without algae (Figures 3.6 to 3.9). *Cox2 (*Figure 3.6) showed a significant difference between treatments, showing the Disrupted *N. oceanica* group at 8 mg/mL with significance over the Control group (P-value of 0.011), and a significant difference between treatment and the concentration interaction, showing Disrupted *N. oceanica* at 8 mg/mL with significant difference to Whole *N. oceanica* at 8 mg/mL (P-value of 0.017).

IgM (Figure 3.8) showed no significant difference between the Whole *N. oceanica* and Disrupted *N. oceanica*, but a significant difference between whole *N. oceanica* concentrations (P-value of 0.015), with 200 mg/mL Whole *N. oceanica* with significant improvement over the 8

mg/mL Whole *N. oceanica* (P-value of 0.49). *Phaeodactylum tricornutum* expressed no significant difference between the groups in all markers (Figures 3.7 and 3.9).

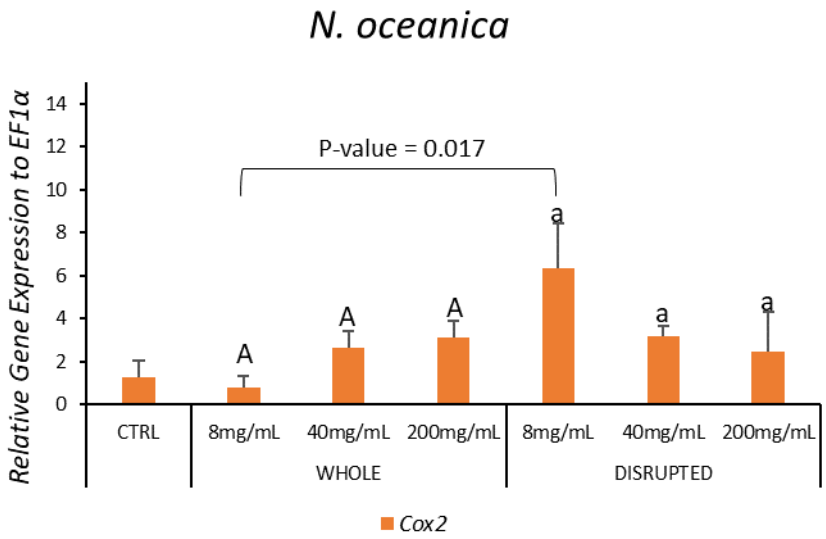

Figure 3.6 - Relative mRNA expression of immune related gene Cox2 in intestinal explant of Sparus aurata incubated with Nannochloropsis oceanica whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

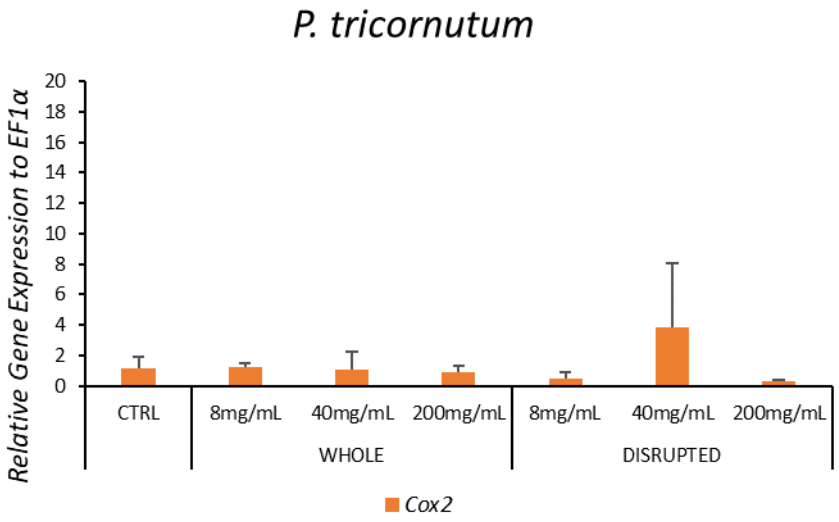

Figure 3.7- Relative mRNA expression of immune related gene Cox2 in intestinal explant of Sparus aurata incubated with Phaeodactylum tricornutum whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

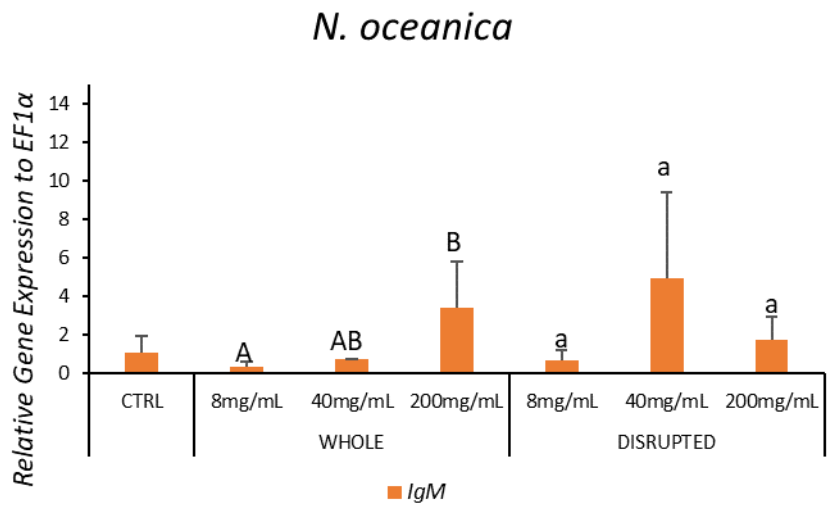

Figure 3.8- Relative mRNA expression of immune related gene IgM in intestinal explant of Sparus aurata incubated with Nannochloropsis oceanica whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

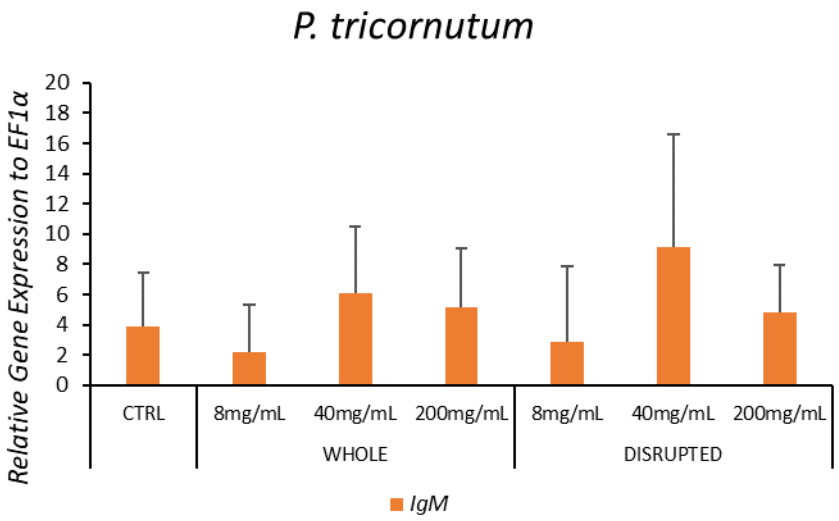

Figure 3.7 - Relative mRNA expression of immune related gene IgM in intestinal explant of Sparus aurata incubated with Phaeodactylum tricornutum whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

Antioxidant response molecular markers

The expression of seabream *GPx*, *CAT*, and *Nrf2* were marked in tissues exposed to different cell concentration suspensions of Whole algae or Disrupted algae and a Control group (Figures 7.1 to 7.6). *N. oceanica* indicates no significant difference between the groups (Figure 7.1 to 7.3), although it may appear to express at 8 mg/mL Disrupted *N. oceanica* in *GPx* (Figure 7.1) and *CAT* (Figure 7.2). *P. tricornutum* expressed no significant difference between the groups in all markers (Figures 7.4 to 7.6).

4. Discussion

Through a high pressure homogenizer the microalgae was disrupted and applied in the invitro intestinal mucosal model to compare the stimulation response from the antioxidant and immune molecular markers. The study results suggested a higher regulated response in gilthead seabream intestinal mucosa stimulated by processed lysed algae compared with unprocessed algae.

Seabream immune response for the selected gene for the intestinal mucosa had a stronger and significant upregulation for the *Cox2* gene at the 8 mg/mL concentration of *N. oceanica* disrupted, when compared to the Whole algae. Even when no significant difference was obtained between treatments (the whole and disrupted algae), between concentrations a significant upregulation of the *IgM* relative gene in the intestine was observed with the increase of algae concentration from the Whole *N. oceanica* of 8 mg/mL to 200 mg/mL, showing a correlation between the concentration of algae and the stimulation capacity (Amaro et al., 2019). This response was previously observed, when a feed composed of 25% macroalga (*U. rigida*) and 75% of microalgae mixture had the highest antioxidant response compared to a feed composed of 50% macroalga and 50% of microalgae mixture against the two biological radicals O_2 and NO^- on Pacific oysters (Amaro et al., 2019). Similar result with zebrafish increasing significantly in *Cox2* relative gene expression in the gut when fed with dietary supplementation with the LC-PUFA-rich microalga *Lobosphaera incisa* compared to no supplementation (Nayak et al., 2020). *N. oceanica,* known to be a microalgae rich in polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and arachidonic acid, with important activity in the immune and stress response, when disrupted, it may increase the bioavailability of intracellular compounds (Ferreira et al., 2021; Xiao et al., 2015; Zhang et al., 2021), and upregulate the expression of the response of the immune genes on fish intestine tissue (in this case the *Cox2* gene). In addition, as all components were possibly more available to the intestine (from disruption or higher

concentration), an increment effect from the cell compounds may be upregulating the *Cox2* gene response at 8 mg/mL for disrupted *N. oceanica* and *IgM gene* response *at 200 mg/L* for whole *N. oceanica* of the fish intestine, and this way increasing the immune response (Cornish & Garbary, 2010).

Despite *P. tricornutum* having no significant improvement between treatments and concentrations, the seabream intestinal tissue showed a higher tendency to express more *Cox2* when exposed to 40 mg of disrupted algae, supporting a positive effect of this algae as a functional ingredient until a certain concentration (Akbary & Aminikhoei, 2018). Similar result occurs when water-soluble polysaccharide extract from the green alga *Ulva rigida* (WPU) was fed to grey mullet (*Mugil cephalus*), presenting an increase in the immune response with a supplemented feed with 10 mg/kg of the WPU but a decrease with 15 mg/kg of the WPU (Akbary & Aminikhoei, 2018). The tendency may be correlated to the presence of many compounds like fucoxanthin, which was shown to exhibit anti-inflammatory, and anti-oxidative effects and proteins (Neumann et al., 2018). The implementation of microalgae in feed for upregulation of the immune response has been successfully implemented for *Sparus aurata* through oral fed supplemented with *Nannochloropsis guadinata* and *Phaeodactylum tricornutum* compounds, significantly increasing some of the immune response genes like immunoglobulin M, β-defensin, T cell receptor β and others more (Cerezuela et al., 2012).

The *P. tricornutum* results could be related to the loss of nutrient content during the mechanical process temperature and time of exposure as referred for a double extrusion by Sørensen et al., (2021), or by the shear stress and oxidation that can occur during the HPH disruption (Günerken et al., 2015). The different responses from both algae could be related to the composition of each alga (Batista et al., 2020a), both were proven to contain great amounts of EPA, but *P. tricornutum* was the only one with DHA (Conde et al., 2021). The different intracellular composition and the external composition (different cell walls) between *N. oceanica* and *P. tricornutum* (Zhao et al., 2022; Tesson et al., 2009), may lead to different responses regarding the external parameters from the disruption process. This different capacity to resist differently to the same abiotic stress from both microalgae may be supported by the different responses by other external factors besides pressure, like the high light intensity that showed only to mainly impact the capacity of *P. tricornutum* to grow, or the ammonium that only decreased the *N. oceanica* capacity to produce lipids (Huete-Ortega et al., 2018). This factor was shown in Table III.I, where *P. tricornutum* was more fragile than *N. oceanica* (needed less pressure to be disrupted).

Seabream antioxidant response selected genes for the intestinal mucosa showed no significant difference when in contact with whole algae or disrupted algae for both algae,

possibly to the denaturation of the compounds after the treatment (Yong et al., 2021). However, it has been shown to be possible to use the HPH disruption method for *P. tricornutum* and *N. oceanica* and retain the antioxidant compounds (Al-Zuhair et al., 2017; Gilbert-López et al., 2017). Two of the selected relative genes have been proven to upregulate (CAT and GPx relative genes) for zebrafish (Danio rerio) gut when fed with *Lobosphaera incisa* (Nayak et al., 2020). *P. tricornutum* has proven to be capable of improving the antioxidative and immune responses actions, as it contains β-glucans, that when available showed to be a potent source with antioxidant and immunomodulatory capacity in *Senegalese sole* (Carballo et al., 2018). Other alga showed the results expected for the antioxidant response, with Ulva rigida, *Gracilaria gracilis,* and *Fucus vesiculosus* total percentage of 5% fed, increasing the enzymatic (superoxide dismutase, catalase, glutathione-S-transferase, glutathione reductase, and glutathione peroxidase) and the non-enzymatic (total glutathione) genes stimulation in gilthead seabream as genoprotective properties (Pereira et al., 2019). Hoseinifar et al., (2018) *showed that Danio rerio* fed with 0.25, 0.5 and 1% of *Gracilaria gracilis* powder had a significant increase in catalase and superoxidase dismutase gene expressions (antioxidant stimulated gene). Since the increase of disruption of the alga cellular wall has been associated with the increase of the digestibility, and a correlation between digestibility and antioxidant response may occur in the digestive tract of rats (Martínez et al., 2022), the same assumption may be possible with disrupted alga in the fish digestive tract. Following this concept, a similar study with extruded feeds increased the digestibility of *N. oceanica* for *Salmo salar* (Gong et al., 2018), or through a vibratory grinding mill for European seabass, without changing the nutritional composition of the feed (Batista et al., 2020b), presuming to increase the antioxidant response. Both in the antioxidant response and in the immune response, when the microalgae presented more available compounds, it may express a up regulation effect, as shown by Amaro et al., (2019). Future analysis must be done, through more input from the 9 remaining fish from the study, using different genes like superoxide dismutase for oxidative stress and β-defensin for immune response (Espinosa et al., 2020; Reis et al., 2021), or by changing cell concentrations applied in the fish intestine.

5. Conclusion

This study demonstrates that disrupted alga by high-pressure homogenizer can be used as a functional feed with a potent immune capacity for the genes selected in seabream intestine. More specifically, by obtaining a suspension at 120 g/L of *Nannochloropsis oceanica* 89.1% disrupted from high pressure homogenizer and using the suspension at a concentration of 8 mg/mL and 40 mg/mL, it will obtain higher responses over *N. oceanica* not disrupted. *Phaeodactylum tricornutum* did not stimulate the tissue differently regarding the treatment or the concentration, probably from the denaturation of compounds from the high-pressure homogenizer. The use of a high-pressure homogenizer with an experimental design based on response surface methodology needs furthermore confirmation as a useful tool to optimize disruption conditions.

Future research needs to be implemented to confirm the capacity to improve algae antioxidant and immune stimulation. This could be done by analysing the 9 remaining fish from the study, using different genes like superoxide dismutase for oxidative stress and β-defensin for immune response (Espinosa et al., 2020; Reis et al., 2021), or by changing cell concentrations applied in the fish intestine.

6. Reference

Agboola, J. O., Teuling, E., Wierenga, P. A., Gruppen, H., & Schrama, J. W. (2019). Cell wall disruption: an effective strategy to improve the nutritive quality of microalgae in african catfish (*Clarias gariepinus*). Aquaculture nutrition, *25*(4), 783–797. https://doi.org/10.1111/ANU.12896

Alabssawy, A. N., Khalaf-Allah, H. M. M., & Gafar, A. A. (2019). Anatomical and histological adaptations of digestive tract in relation to food and feeding habits of lizardfish*, Synodus variegatus* (Lacepède, 1803). Egyptian journal of aquatic research, *45*(2), 159–165. https://doi.org/10.1016/J.EJAR.2019.05.006

Algers, B., Blokhuis, H. J., Bøtner, A., Broom, D. M., Costa, P., Domingo, M., Greiner, M., Hartung, J., Koenen, F., Müller-Graf, C., Morton, D. B., Osterhaus, A., Pfeiffer, D. U., Raj, M., Roberts, R., Sanaa, M., Salman, M., Sharp, J. M., Vannier, P., & Wierup, M. (2009). General approach to fish welfare and to the concept of sentience in fish. EFSA Journal, *7*(2), 954. https://doi.org/10.2903/J.EFSA.2009.954

Alothman, M., Ispas-Szabo, P., & Mateescu, M. A. (2021). Design of catalase monolithic tablets for intestinal targeted delivery. Pharmaceutics 2021, Vol. 13, Page 69, 13(1), 69. https://doi.org/10.3390/PHARMACEUTICS13010069

Al-Zuhair, S., Ashraf, S., Hisaindee, S., Darmaki, N. al, Battah, S., Svistunenko, D., Reeder, B., Stanway, G., & Chaudhary, A. (2017). Enzymatic pre-treatment of microalgae cells for enhanced extraction of proteins. Engineering in life sciences, 17(2), 175–185. https://doi.org/10.1002/ELSC.201600127

Amaro, H. M., Rato, A., Matias, D., Joaquim, S., Machado, J., Gonçalves, J. F. M., Vaz-Pires, P., Ozorio, R. O. A., Pereira, L. F., Azevedo, I. C., Sousa-Pinto, I., & Catarina Guedes, A. (2019). Alga diet formulation – an attempt to reduce oxidative stress during broodstock conditioning of pacific oysters. Aquaculture, 500, 540–549. https://doi.org/10.1016/j.aquaculture.2018.10.060

Awad, E., Cerezuela, R., & Esteban, M. Á. (2015). Effects of fenugreek (*Trigonella foenum graecum*) on gilthead seabream (*Sparus aurata L.*) immune status and growth performance. Fish & shellfish immunology, 45(2), 454–464. https://doi.org/10.1016/J.FSI.2015.04.035

Balduyck, L., Bruneel, C., Goiris, K., Dejonghe, C., & Foubert, I. (2018). Influence of high pressure homogenization on free fatty acid formation in *Nannochloropsis sp.* European journal of lipid science and technology, 120(4), 1700436. https://doi.org/10.1002/EJLT.201700436

Basto-Silva, C., García-Meilán, I., Couto, A., Serra, C. R., Enes, P., Oliva-Teles, A., Capilla, E., & Guerreiro, I. (2022). Effect of dietary plant feedstuffs and protein/carbohydrate ratio on gilthead seabream (*Sparus aurata*) gut health and functionality. Fishes, *7*(2). https://doi.org/10.3390/FISHES7020059

Batista, S., Pereira, R., Oliveira, B., Baião, L. F., Jessen, F., Tulli, F., Messina, M., Silva, J. L., Abreu, H., & Valente, L. M. P. (2020). Exploring the potential of seaweed *Gracilaria gracilis* and microalga *Nannochloropsis oceanica*, single or blended, as natural dietary ingredients for

european seabass *Dicentrarchus labrax*. Journal of applied phycology, 32(3), 2041–2059. https://doi.org/10.1007/S10811-020-02118-Z

Batista, S., Pintado, M., Marques, A., Abreu, H., Silva, J. L., Jessen, F., Tulli, F., & Valente, L. M. P. (2020). Use of technological processing of seaweed and microalgae as strategy to improve their apparent digestibility coefficients in european seabass (*Dicentrarchus labrax*) juveniles. Journal of applied phycology, 32(5), 3429–3446. https://doi.org/10.1007/S10811-020-02185-2

Becker, T., Ogez, J. R., & Builder, S. E. (1983). Downstream processing of proteins. Biotechnology advances, 1(2), 247–261. https://doi.org/https://doi.org/10.1016/0734-9750(83)90591-8

Borowitzka, M. A. (2018). Biology of Microalgae. Microalgae in health and disease prevention, 23–72. https://doi.org/10.1016/B978-0-12-811405-6.00003-7

Brigelius-Flohé, R., & Maiorino, M. (2013). Glutathione peroxidases. Biochimica et biophysica acta (BBA) - General subjects, 1830(5), 3289–3303. https://doi.org/10.1016/J.BBAGEN.2012.11.020

Carballo, C., Chronopoulou, E. G., Letsiou, S., Maya, C., Labrou, N. E., Infante, C., Power, D. M., & Manchado, M. (2018). Antioxidant capacity and immunomodulatory effects of a chrysolaminarin-enriched extract in *Senegalese sole*. Fish & shellfish immunology, 82, 1–8. https://doi.org/10.1016/J.FSI.2018.07.052

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

Cerezuela, R., Meseguer, J., & Esteban, M. Á. (2013). Effects of dietary inulin, *Bacillus subtilis* and microalgae on intestinal gene expression in gilthead seabream (*Sparus aurata L.*). Fish & shellfish immunology, 34(3), 843–848. https://doi.org/10.1016/J.FSI.2012.12.026

Conde, T. A., Neves, B. F., Couto, D., Melo, T., Neves, B., Costa, M., Silva, J., Domingues, P., & Domingues, M. R. (2021). Microalgae as sustainable bio-factories of healthy lipids: evaluating fatty acid content and antioxidant activity. Marine drugs, 19(7), 357. https://doi.org/10.3390/MD19070357

Cornish, M. L., & Garbary, D. J. (2010). Antioxidants from macroalgae: potential applications in human health and nutrition. Algae, 25(4), 155–171. https://doi.org/10.4490/ALGAE.2010.25.4.155

Dong, C., Fang, W., Yi, Q., & Zhang, J. (2022). A comprehensive review on reactive oxygen species (ROS) in advanced oxidation processes (AOPs). Chemosphere, 308, 136205. https://doi.org/10.1016/J.CHEMOSPHERE.2022.136205

Espinosa, C., García Beltrán, J. M., Messina, C. M., & Esteban, M. Á. (2020). Effect of *Jasonia glutinosa* on immune and oxidative status of gilthead seabream (*Sparus aurata L.*). Fish & shellfish immunology, 100, 58–69. https://doi.org/10.1016/J.FSI.2020.02.068

Ezhilmathi, S., Ahilan, B., Uma, A., Felix, N., Cheryl, A., & Lingam, R. S. S. (2022). Effect of stocking density on growth performance, digestive enzyme activity, body composition and gene

expression of asian seabass reared in recirculating aquaculture system. Aquaculture research. https://doi.org/10.1111/ARE.15725

FAO. (1995). Code of conduct for responsible fisheries. FAO. https://www.fao.org/publications/card/en/c/e6cf549d-589a-5281-ac13-766603db9c03/

FAO. (2018). The state of world fisheries and aquaculture 2018 - Meeting the sustainable development goals. www.fao.org/publications

FAO. (2020). The state of world fisheries and aquaculture 2020. Sustainability in action. Rome. https://doi.org/10.4060/ca9229en

Ferreira, M., Teixeira, C., Abreu, H., Silva, J., Costas, B., Kiron, V., & Valente, L. M. P. (2021). Nutritional value, antimicrobial and antioxidant activities of micro- and macroalgae, single or blended, unravel their potential use for aquafeeds. Journal of applied phycology, 33(6), 3507–3518. https://doi.org/10.1007/S10811-021-02549-2

Fleurence, J., Massiani, L., Guyader, O., & Mabeau, S. (1995). Use of enzymatic cell wall degradation for improvement of protein extraction from *Chondrus crispus*, *Gracilaria verrucosa* and *Palmaria palmata*. Journal of applied phycology 1995 7:4, 7(4), 393–397. https://doi.org/10.1007/BF00003796

Fleurence, J., Morançais, M., Dumay, J., Decottignies, P., Turpin, V., Munier, M., Garcia-Bueno, N., & Jaouen, P. (2012). What are the prospects for using seaweed in human nutrition and for marine animals raised through aquaculture? Trends in food science & technology, 27(1), 57–61. https://doi.org/10.1016/J.TIFS.2012.03.004

Follows, M., Hetherington, P. J., Dunnill, P., & Lilly, M. D. (1971). Release of enzymes from bakers' yeast by disruption in an industrial homogenizer. Biotechnology and bioengineering, 13(4), 549–560. https://doi.org/10.1002/BIT.260130408

Fu, Q., Li, Y., Zhao, S., Wang, H., Zhao, C., Zhang, P., Cao, M., Yang, N., & Li, C. (2022). Comprehensive identification and expression profiling of immune-related lncRNAs and their target genes in the intestine of turbot (*Scophthalmus maximus L*.) in response to *Vibrio anguillarum* infection. Fish & shellfish immunology, 130, 233–243. https://doi.org/10.1016/J.FSI.2022.09.004

Fukata, M., Chen, A., Klepper, A., Krishnareddy, S., Vamadevan, A. S., Thomas, L. S., Xu, R., Inoue, H., Arditi, M., Dannenberg, A. J., & Abreu, M. T. (2006). *Cox-2* is regulated by Toll-Like Receptor-4 (*TLR4*) signaling: role in proliferation and apoptosis in the intestine. Gastroenterology, 131(3), 862–877. https://doi.org/10.1053/J.GASTRO.2006.06.017

Gilbert-López, B., Barranco, A., Herrero, M., Cifuentes, A., & Ibáñez, E. (2017). Development of new green processes for the recovery of bioactives from *Phaeodactylum tricornutum*. Food research international, 99, 1056–1065. https://doi.org/10.1016/J.FOODRES.2016.04.022

Gomez-Zavaglia, A., Prieto Lage, M. A., Jimenez-Lopez, C., Mejuto, J. C., & Simal-Gandara, J. (2019). The potential of seaweeds as a source of functional ingredients of prebiotic and antioxidant value. Antioxidants, 8(9). https://doi.org/10.3390/ANTIOX8090406

Gong, Y., Guterres, H. A. D. S., Huntley, M., Sørensen, M., & Kiron, V. (2018). Digestibility of the defatted microalgae *Nannochloropsis sp*. and *Desmodesmus sp*. when fed to Atlantic

salmon, *Salmo salar*. Aquaculture nutrition, 24(1), 56–64. https://doi.org/10.1111/ANU.12533

Groschwitz, K. R., & Hogan, S. P. (2009). Intestinal barrier function: molecular regulation and disease pathogenesis. Journal of allergy and clinical immunology, 124(1), 3–20. https://doi.org/10.1016/J.JACI.2009.05.038

Gültepe, N., Hisar, O., Salnur, S., Hoşsu, B., Tansel Tanrikul, T., & Aydin, S. (2012). Preliminary assessment of dietary mannanoligosaccharides on growth performance and health status of gilthead seabream *Sparus auratus*. Journal of aquatic animal health, 24(1), 37–42. https://doi.org/10.1080/08997659.2012.668508

Günerken, E., D'Hondt, E., Eppink, M. H. M., Garcia-Gonzalez, L., Elst, K., & Wijffels, R. H. (2015). Cell disruption for microalgae biorefineries. Biotechnology advances, 33(2), 243–260. https://doi.org/10.1016/J.BIOTECHADV.2015.01.008

Halim, R., Rupasinghe, T. W. T., Tull, D. L., & Webley, P. A. (2013). Mechanical cell disruption for lipid extraction from microalgal biomass. Bioresource technology, 140, 53–63. https://doi.org/10.1016/J.BIORTECH.2013.04.067

Harrison, S. T. L. (1991). Bacterial cell disruption: A key unit operation in the recovery of intracellular products. Biotechnology advances, 9(2), 217–240. https://doi.org/https://doi.org/10.1016/0734-9750

He, Y., Lin, G., Rao, X., Chen, L., Jian, H., Wang, M., Guo, Z., & Chen, B. (2018). Microalga *Isochrysis galbana* in feed for *Trachinotus ovatus*: effect on growth performance and fatty acid composition of fish fillet and liver. Aquaculture international, 26(5), 1261–1280. https://doi.org/10.1007/S10499-018-0282-Y

Hoseinifar, S. H., Yousefi, S., Capillo, G., Paknejad, H., Khalili, M., Tabarraei, A., van Doan, H., Spanò, N., & Faggio, C. (2018). Mucosal immune parameters, immune and antioxidant defence related genes expression and growth performance of zebrafish (*Danio rerio*) fed on *Gracilaria gracilis* powder. Fish and shellfish immunology, 83, 232–237. https://doi.org/10.1016/J.FSI.2018.09.046

Huete-Ortega, M., Okurowska, K., Kapoore, R. V., Johnson, M. P., Gilmour, D. J., & Vaidyanathan, S. (2018). Effect of ammonium and high light intensity on the accumulation of lipids in *Nannochloropsis oceanica* (CCAP 849/10) and *Phaeodactylum tricornutum* (CCAP 1055/1). Biotechnology for biofuels, 11(1), 1–15. https://doi.org/10.1186/S13068-018-1061-8

Keyt, B. A., Baliga, R., Sinclair, A. M., Carroll, S. F., & Peterson, M. S. (2020). Structure, function, and therapeutic use of *IgM* antibodies. Antibodies, 9(4), 53. https://doi.org/10.3390/ANTIB9040053

Kiron, V. (2012). Fish immune system and its nutritional modulation for preventive health care. Animal feed science and technology, 173(1–2), 111–133. https://doi.org/10.1016/J.ANIFEEDSCI.2011.12.015

Lee, A. K., Lewis, D. M., & Ashman, P. J. (2012). Disruption of microalgal cells for the extraction of lipids for biofuels: processes and specific energy requirements. Biomass and bioenergy, 46, 89–101. https://doi.org/10.1016/J.BIOMBIOE.2012.06.034

Leong, Y. K., Chen, C. Y., Varjani, S., & Chang, J. S. (2022). Producing fucoxanthin from algae – recent advances in cultivation strategies and downstream processing. Bioresource technology, 344, 126170. https://doi.org/10.1016/J.BIORTECH.2021.126170

Lordan, S., Ross, R. P., & Stanton, C. (2011). Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. Marine drugs 2011, Vol. 9, Pages 1056-1100, 9(6), 1056–1100. https://doi.org/10.3390/MD9061056

Lourenço-Lopes, C., Fraga-Corral, M., Jimenez-Lopez, C., Carpena, M., Pereira, A. G., Garcia-Oliveira, P., Prieto, M. A., & Simal-Gandara, J. (2021). Biological action mechanisms of fucoxanthin extracted from algae for application in food and cosmetic industries. Trends in food science & technology, 117, 163–181. https://doi.org/10.1016/J.TIFS.2021.03.012

Magnoni, L. J., Martos-Sitcha, J. A., Queiroz, A., Calduch-Giner, J. A., Gonçalves, J. F. M., Rocha, C. M. R., Abreu, H. T., Schrama, J. W., Ozorio, R. O. A., & 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

Martínez, R., García-Beltrán, A., Kapravelou, G., Mesas, C., Cabeza, L., Perazzoli, G., Guarnizo, P., Rodríguez-López, A., Vallejo, R. A., Galisteo, M., Aranda, P., Prados, J., López-Jurado, M., Melguizo, C., & Porres, J. M. (2022). In vivo nutritional assessment of the microalga *Nannochloropsis gaditana* and evaluation of the antioxidant and antiproliferative capacity of its functional extracts. Marine drugs 2022, Vol. 20, Page 318, 20(5), 318. https://doi.org/10.3390/MD20050318

Martínez-Álvarez, R. M., Morales, A. E., & Sanz, A. (2005). Antioxidant defenses in fish: biotic and abiotic factors. Reviews in fish biology and fisheries, 15(1–2), 75–88. https://doi.org/10.1007/S11160-005-7846-4

Middelberg, A. P. J. (1995). Process-scale disruption of microorganisms. Biotechnology advances, 13(3), 491–551. https://doi.org/10.1016/0734-9750(95)02007-P

Mondal, H., Chandrasekaran, N., Mukherjee, A., & Thomas, J. (2021). Viral infections in cultured fish and shrimps: current status and treatment methods. Aquaculture international 2021, 1–36. https://doi.org/10.1007/S10499-021-00795-2

Moniruzzaman, M., Mukherjee, M., Kumar, S., & Chakraborty, S. B. (2022). Effects of salinity stress on antioxidant status and inflammatory responses in females of a “near threatened” economically important fish species *Notopterus chitala*: a mechanistic approach. Environmental science and pollution research, 1, 1–12. https://doi.org/10.1007/S11356-022-21142-9

Monteiro, M., Sousa, C., Coutinho, F., Castro, C., Fontinha, F., Guerreiro, I., Pousão, P., Matos, E., Díaz-Rosales, P., Oliva-Teles, A., Enes, P., & Couto, A. (2021). functional feeds to tackle meagre (A*rgyrosomus regius*) stress: physiological responses under acute stressful handling conditions. Marine drugs 2021, Vol. 19, Page 598, 19(11), 598. https://doi.org/10.3390/MD19110598

Morvaridzadeh, M., Estêvão, M. D., Qorbani, M., Heydari, H., Hosseini, A. sadat, Fazelian, S., Belančić, A., Persad, E., Rezamand, G., & Heshmati, J. (2022). The effect of chromium intake on oxidative stress parameters: A systematic review and meta-analysis. Journal of trace

elements in medicine and biology, 69, 126879. https://doi.org/10.1016/J.JTEMB.2021.126879

Neumann, U., Derwenskus, F., Gille, A., Louis, S., Schmid-Staiger, U., Briviba, K., & Bischoff, S. C. (2018). *Bioavailability and safety of nutrients from the microalgae Chlorella vulgaris, Nannochloropsis oceanica and Phaeodactylum tricornutum in C57bl/6 mice*. https://doi.org/10.3390/nu10080965

Ni, Q., Zhang, P., Li, Q., & Han, Z. (2022). Oxidative stress and gut microbiome in inflammatory skin diseases. Frontiers in cell and developmental biology, 10, 497. https://doi.org/10.3389/FCELL.2022.849985

Ortiz, G. G., Pacheco Moisés, F. P., Mireles-Ramírez, M., Flores-Alvarado, L. J., González-Usigli, H., Sánchez-González, V. J., Sánchez-López, A. L., Sánchez-Romero, L., Díaz-Barba, E. I., Santoscoy-Gutiérrez, J. F., & Rivero-Moragrega, P. (2017). Oxidative stress: love and hate history in central nervous system. Advances in protein chemistry and structural biology, 108, 1–31. https://doi.org/10.1016/BS.APCSB.2017.01.003

Oswald, A. T. O., Ishikawa, M., Koshio, S., Yokoyama, S., Moss, A. S., & Serge, D. (2019). Nutritional evaluation of *Nannochloropsis* powder and lipid as alternative to fish oil for kuruma shrimp, *Marsupenaeus japonicus*. Aquaculture, 504, 427–436. https://doi.org/10.1016/J.AQUACULTURE.2019.02.028

Öztürk, R. Ç., & Altinok, I. (2014). Türkiye'de görülen bakteriyel ve viral bali{dotless}k hastali{dotless}klari{dotless}. Turkish journal of fisheries and aquatic sciences, 14(1), 275–297. https://doi.org/10.4194/1303-2712-V14_1_30

Parra, D., Reyes-Lopez, F. E., & Tort, L. (2015). Mucosal immunity and B Cells in teleosts: effect of vaccination and stress. Frontiers in immunology, 6(JUN). https://doi.org/10.3389/FIMMU.2015.00354

Passos, R., Correia, A. P., Pires, D., Pires, P., Ferreira, I., Simões, M., do Carmo, B., Santos, P., Pombo, A., Afonso, C., & Baptista, T. (2021). Potential use of macroalgae *Gracilaria gracilis* in diets for european seabass (*Dicentrarchus labrax*): health benefits from a sustainable source. Fish & shellfish immunology, 119, 105–113. https://doi.org/10.1016/J.FSI.2021.09.033

Peixoto, D., Silva, J., & Morais M. (2020). Algae as functional ingredients in *Senegalese sole* microdiets. Hatchery feed & managemen*t*. https://doi.org/10.13140/RG.2.2.29582.38720

Peñaranda, D. S., Bäuerl, C., Tomás-Vidal, A., Jover-Cerdá, M., Estruch, G., Pérez Martínez, G., & Martínez Llorens, S. (2020). Intestinal explant cultures from gilthead seabream (S*parus aurata, L*.) allowed the determination of mucosal sensitivity to bacterial pathogens and the impact of a plant protein diet. International journal of molecular sciences 2020, Vol. 21*,* Page 7584, 21(20), 7584. https://doi.org/10.3390/IJMS21207584

Pereira, V., Marques, A., Gaivão, I., Rego, A., Abreu, H., Pereira, R., Santos, M. A., Guilherme, S., & 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 & pharmacology, 219, 12–24. https://doi.org/10.1016/J.CBPC.2019.01.006

Perera, E., Sánchez-Ruiz, D., Sáez, M. I., Galafat, A., Barany, A., Fernández-Castro, M., Vizcaíno, A. J., Fuentes, J., Martínez, T. F., Mancera, J. M., Alarcón, F. J., & Martos-Sitcha, J. A. (2020). Low dietary inclusion of nutraceuticals from microalgae improves feed efficiency and modifies intermediary metabolisms in gilthead sea bream (*Sparus aurata*). Scientific reports, 10(1). https://doi.org/10.1038/s41598-020-75693-3

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: properties, sources, targets, and their implication in various diseases. *Indian journal of clinical biochemistry, 30(1*), 11–26. https://doi.org/10.1007/S12291-014-0446-0

Qiu, S., Shen, Y., Wu, Z., Zhang, X., & Ge, S. (2021). Effects of algae subtype and extraction condition on extracted fucoxanthin antioxidant property: A 20-year meta-analysis. Algal research, 53, 102161. https://doi.org/10.1016/J.ALGAL.2020.102161

Randall, K. J., Turton, J., & Foster, J. R. (2011). Explant culture of gastrointestinal tissue: A review of methods and applications. In cell biology and toxicology, 27(4), 267–284. https://doi.org/10.1007/s10565-011-9187-5

Reis, B., Gonçalves, A. T., Santos, P., Sardinha, M., Conceição, L. E. C., Serradeiro, R., Pérez-sánchez, J., Calduch-giner, J., Schmid-staiger, U., Frick, K., Dias, J., & Costas, B. (2021). Immune status and hepatic antioxidant capacity of gilthead seabream S*parus aurata* juveniles fed yeast and microalga derived β-glucans. Marine drugs, 19(12), 653. https://doi.org/10.3390/MD19120653

Reis, B., Ramos-Pinto, L., Cunha, S. A., Pintado, M., da Silva, J. L., Dias, J., Conceição, L., Matos, E., & Costas, B. (2022). *Chlorella vulgaris* extracts as modulators of the health status and the inflammatory response of gilthead seabream juveniles (*Sparus aurata*). *Marined*, *20*(7). https://doi.org/10.3390/md20070407

Reyes-Becerril, M., Angulo, C., Angulo, M., & Esteban, M. Á. (2021). Probiotic properties of *Debaryomyces hansenii BCS004* and their immunostimulatory effect in supplemented diets for gilthead seabream (*Sparus aurata*). *Aquaculture research*, *52*(6), 2715–2726. https://doi.org/10.1111/ARE.15123

Sabeena, S. F., Alagarsamy, S., Sattari, Z., Al-Haddad, S., Fakhraldeen, S., Al-Ghunaim, A., & Al-Yamani, F. (2020). Enzyme-assisted extraction of bioactive compounds from brown seaweeds and characterization. Journal of applied phycology, 32(1), 615–629. https://doi.org/10.1007/S10811-019-01906-6

Scientific concepts of functional foods in europe consensus document. (1999). British journal of nutrition, 81(4), 1–27. https://doi.org/doi:10.1017/S0007114599000471

Shaalan, M., El-Mahdy, M., Saleh, M., & El-Matbouli, M. (2017). Aquaculture in egypt: insights on the current trends and future perspectives for sustainable development, 26(1), 99–110. https://doi.org/10.1080/23308249.2017.1358696

Shalaby, W. (2020). Comparative morphological and histological studies on the adaptation of esophagus and stomach to the feeding habits in some coral reef fishes at Hurghada, red sea, Egypt. Egyptian journal of aquatic biology and fisheries, 24(5), 289–306. https://doi.org/10.21608/EJABF.2020.105059

Song, L., Li, C., Wu, F., & Zhang, S. (2022). Dietary intake of diosgenin delays aging of male fish *Nothobranchius guentheri* through modulation of multiple pathways that play prominent

roles in ROS production. Biogerontology, 23(2), 201–213. https://doi.org/10.1007/S10522-022-09955-0

Sørensen, S. L., Ghirmay, A., Gong, Y., Dahle, D., Vasanth, G., Sørensen, M., & Kiron, V. (2021). Growth, chemical composition, histology and antioxidant genes of atlantic salmon (*Salmo salar*) fed whole or pre-processed *Nannochloropsis oceanica* and T*etraselmis s*p. Fishes, 6(3), 23. https://doi.org/10.3390/FISHES6030023

Stratev, D., Zhelyazkov, G., Noundou, X. S., & Krause, R. W. M. (2018). Beneficial effects of medicinal plants in fish diseases. Aquaculture international, 26(1), 289–308. https://doi.org/10.1007/S10499-017-0219-X

Sun, R., Chen, J., Pan, C., Sun, Y., Mai, B., & Li, Q. X. (2020). Antibiotics and food safety in aquaculture. Journal of agricultural and food chemistry, 68(43), 11908–11919. https://doi.org/10.1021/ACS.JAFC.0C03996

Takeuchi, K., & Satoh, H. (2015). NSAID-induced small intestinal damage - roles of various pathogenic factors. Digestion, 91(3), 218–232. https://doi.org/10.1159/000374106

Teuling, E., Schrama, J. W., Gruppen, H., & Wierenga, P. A. (2017). Effect of cell wall characteristics on algae nutrient digestibility in Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarus gariepinus*). Aquaculture, 479, 490–500. https://doi.org/10.1016/J.AQUACULTURE.2017.06.025

Teuling, E., Wierenga, P. A., Agboola, J. O., Gruppen, H., & Schrama, J. W. (2019). Cell wall disruption increases bioavailability of *Nannochloropsis gaditana* nutrients for juvenile Nile tilapia (*Oreochromis niloticus*). Aquaculture, 499, 269–282. https://doi.org/10.1016/J.AQUACULTURE.2018.09.047

Tonelli, C., Chio, I. I. C., & Tuveson, D. A. (2018). Transcriptional regulation by *Nrf2.* Antioxidants and redox signaling, 29(17), 1727–1745. https://doi.org/10.1089/ARS.2017.7342

Vizcaíno, A. J., Fumanal, M., Sáez, M. I., Martínez, T. F., Moriñigo, M. A., Fernández-Díaz, C., Anguis, V., Balebona, M. C., & Alarcón, F. J. (2019). Evaluation of *Ulva ohnoi* as functional dietary ingredient in juvenile Senegalese sole (*Solea senegalensis*): Effects on the structure and functionality of the intestinal mucosa. Algal research, 42. https://doi.org/10.1016/j.algal.2019.101608

Wang, D., Li, Y., Hu, X., Su, W., & Zhong, M. (2015). Combined enzymatic and mechanical cell disruption and lipid extraction of green alga *Neochloris oleoabundans*. *International* Journal of molecular sciences, 16(4), 7707–7722. https://doi.org/10.3390/ijms16047707

Wang, J., Lei, P., Gamil, A. A. A., Lagos, L., Yue, Y., Schirmer, K., Mydland, L. T., Øverland, M., Krogdahl, Å., & Kortner, T. M. (2019). Rainbow trout (*Oncorhynchus mykiss*) intestinal epithelial cells as a model for studying gut immune function and effects of functional feed ingredients. Frontiers in immunology, 10(FEB), 152. https://doi.org/10.3389/FIMMU.2019.00152

Weber, S., Grande, P. M., Blank, L. M., & Klose, H. (2022). Insights into cell wall disintegration of *Chlorella vulgaris*. PLoS ONE, 17(1). https://doi.org/10.1371/JOURNAL.PONE.0262500

Wells, M. L., Potin, P., Craigie, J. S., Raven, J. A., Merchant, S. S., Helliwell, K. E., Smith, A. G., Camire, M. E., & Brawley, S. H. (2016). Algae as nutritional and functional food sources:

revisiting our understanding. Journal of applied phycology 2016, 29(2), 949–982. https://doi.org/10.1007/S10811-016-0974-5

Xavier, M. J., Navarro-Guillén, C., Lopes, A., Colen, R., Teodosio, R., Mendes, R., Oliveira, B., Valente, L. M. P., Conceição, L. E. C., & Engrola, S. (2022). Effects of dietary curcumin in growth performance, oxidative status and gut morphometry and function of gilthead seabream postlarvae. Aquaculture reports, 24, 101-128. https://doi.org/10.1016/J.AQREP.2022.101128

Xiao, Y., Zhang, J., Cui, J., Yao, X., Sun, Z., Feng, Y., & Cui, Q. (2015). Simultaneous accumulation of neutral lipids and biomass in *Nannochloropsis oceanica* IMET1 under high light intensity and nitrogen replete conditions. Algal research, 11, 55–62. https://doi.org/10.1016/J.ALGAL.2015.05.019

Yadav, G., Meena, D. K., Sahoo, A. K., Das, B. K., & Sen, R. (2020). Effective valorization of microalgal biomass for the production of nutritional fish-feed supplements. Journal of cleaner production, 243, 118697. https://doi.org/10.1016/J.JCLEPRO.2019.118697

Yang, G., Yu, R., Qiu, H., Wu, H., Yan, Q., Chen, W., Ding, L., Kumar, V., Wen, C., & Peng, M. (2020). Beneficial effects of emodin and curcumin supplementation on antioxidant defense response, inflammatory response and intestinal barrier of Pengze crucian carp (Carassius auratus var. Pengze). Aquaculture nutrition, 26(6), 1958–1969. https://doi.org/10.1111/ANU.13137

Yong, S. X. M., Song, C. P., & Choo, W. S. (2021). Impact of high-pressure homogenization on the extractability and stability of phytochemicals. Frontiers in sustainable food systems, 4. https://doi.org/10.3389/FSUFS.2020.593259

Zaki Sharawy, Z., Ashour, M., Abbas, E., Ashry, O., Helal, M., Nazmi, H., Kelany, M., Kamel, A., Hassaan, M., Rossi Jr, W., El-Haroun, E., & Goda, A. (2020). Effects of dietary marine microalgae*, Tetraselmis suecica,* on production, gene expression, protein markers and bacterial count of Pacific white shrimp *Litopenaeus vannamei*. https://doi.org/10.1111/are.14566

Zeynali, M., Nafisi Bahabadi, M., Morshedi, V., Ghasemi, A., & Torfi Mozanzadeh, M. (2020). Replacement of dietary fishmeal with *Sargassum ilicifolium* meal on growth, innate immunity and immune gene mRNA transcript abundance in *Lates calcarifer* juveniles. Aquaculture nutrition, 26(5), 1657–1668. https://doi.org/10.1111/ANU.13111

Zhang, L., Ye, S. C., Chen, W. B., Han, J. C., Tian, J. J., Zhang, Y. B., Xu, J. L., Cao, J. Y., & Qin, C. (2021). Screening the rate-limiting genes in the ω6 polyunsaturated fatty acid biosynthesis pathway in *Nannochloropsis oceanica*. Algal research, 57, 102342. https://doi.org/10.1016/J.ALGAL.2021.102342

Zheng, L. X., Chen, X. Q., & Cheong, K. L. (2020). Current trends in marine algae polysaccharides: The digestive tract, microbial catabolism, and prebiotic potential. International journal of biological macromolecules, 151, 344–354. https://doi.org/10.1016/J.IJBIOMAC.2020.02.168

7. Annexes

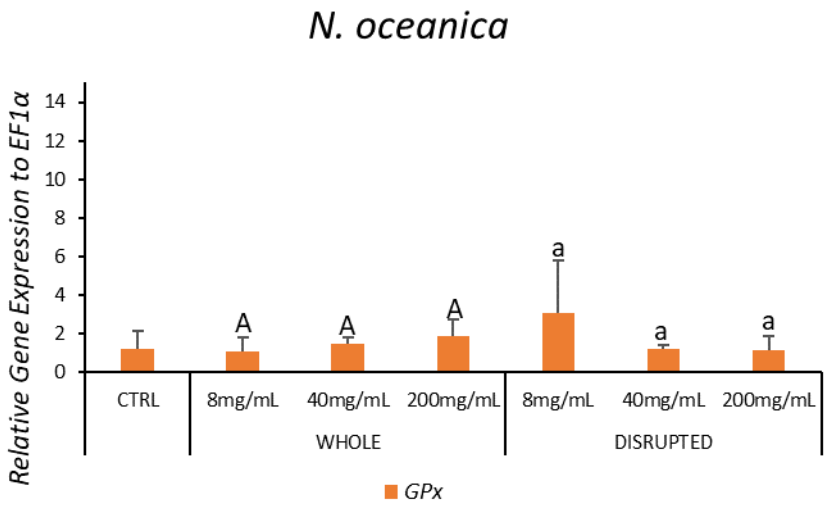

Figure 7.1- Relative mRNA expression of antioxidant related gene GPx in intestinal explant of Sparus aurata incubated with Nannochloropsis oceanica whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

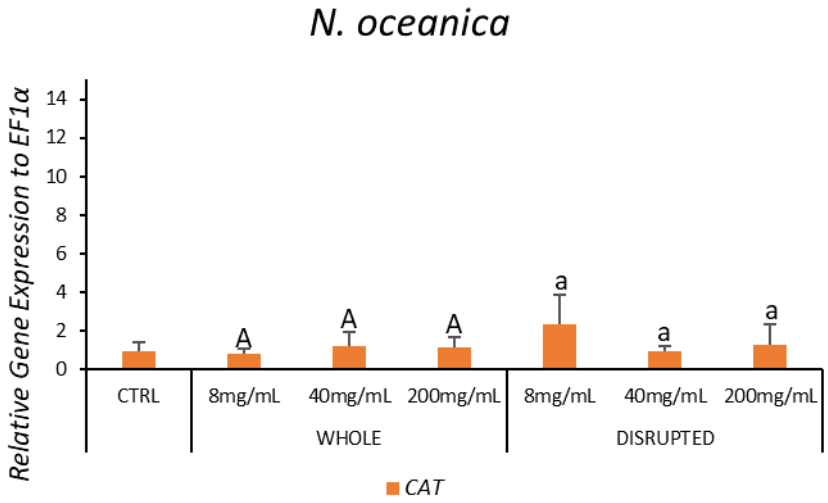

Figure 7.2- Relative mRNA expression of antioxidant related gene CAT in intestinal explant of Sparus aurata incubated with Nannochloropsis oceanica whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

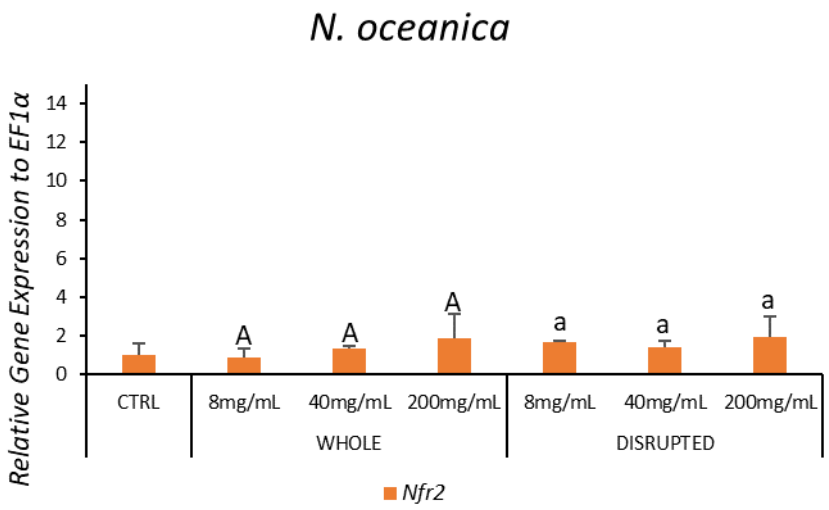

Figure 7.3- Relative mRNA expression of antioxidant related gene Nfr2 in intestinal explant of Sparus aurata incubated with Nannochloropsis oceanica whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

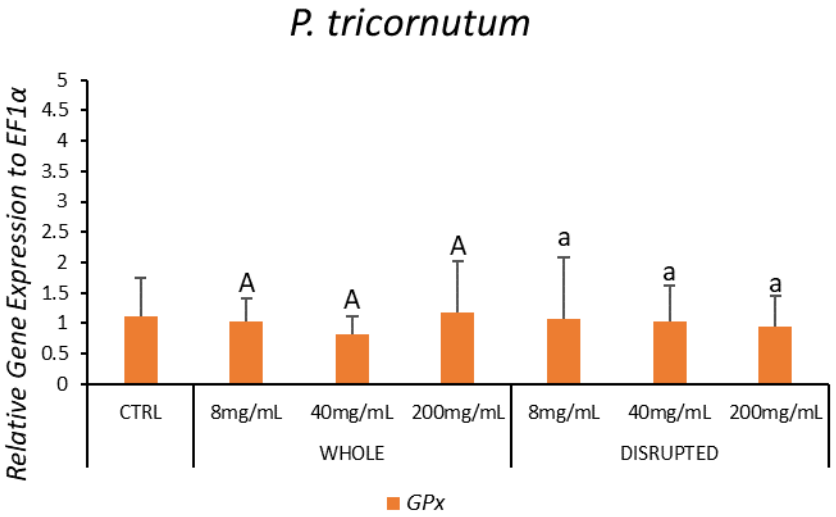

Figure 7.4- Relative mRNA expression of antioxidant related gene GPx in intestinal explant of Sparus aurata incubated with Phaeodactylum tricornutum whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

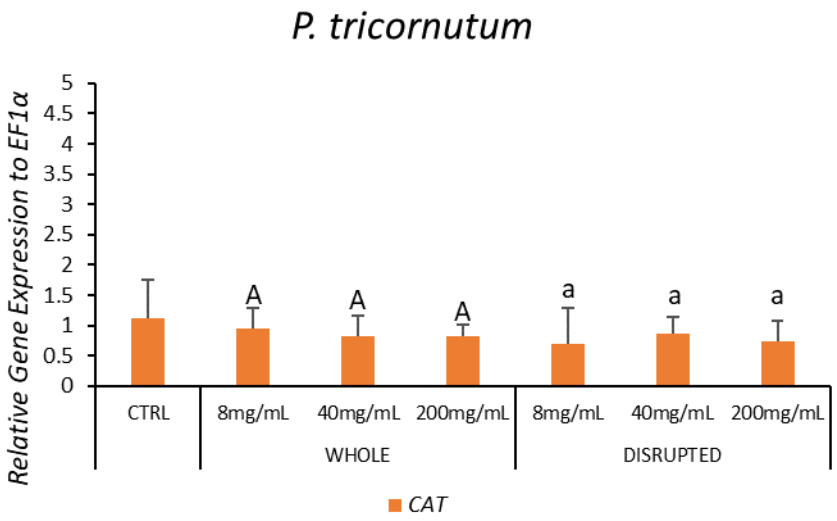

Figure 7.5- Relative mRNA expression of antioxidant related gene CAT in intestinal explant of Sparus aurata incubated with Phaeodactylum tricornutum whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

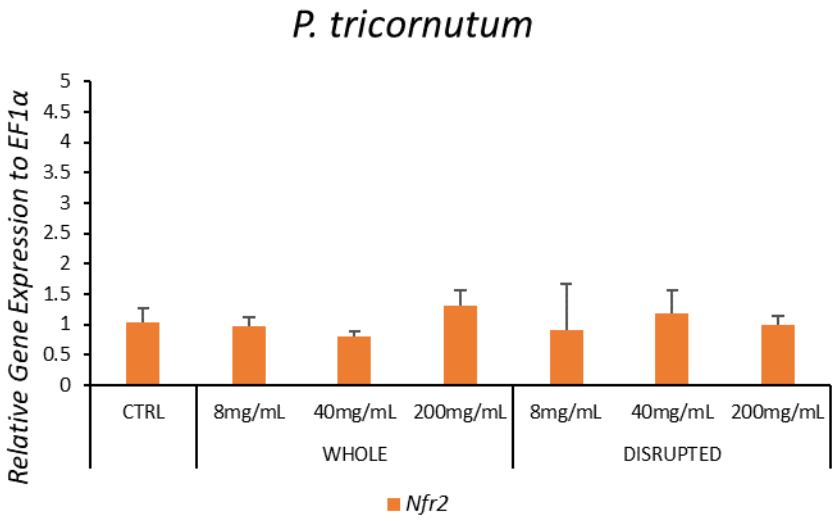

Figure 7.6- Relative mRNA expression of antioxidant related gene Nfr2 in intestinal explant of Sparus aurata incubated with Phaeodactylum tricornutum whole and disrupted biomass. Bars indicate mean +/- SD. Data were normalized with the EF1α gene. Different letters indicate significant differences (uppercase letters for whole biomass and lowercase letters for disrupted biomass). Two-way ANOVA with whole or disrupted algae and 8, 40 or 200 mg/mL algae concentration as variants (n=3 and α=0.05).

Table VI.I - N. oceanica Normal distribution significance level higher than 0.05.

	Shapiro-Wilk
Molecular Marker	P-value
Cox2	0.069
IgM	0.893
GPx	0.382
CAT	0.981
Nfr2	0.075

Table VI.II – P. tricornutum Normal distribution significance level higher than 0.05.

	Shapiro-Wilk
Molecular Marker	P-value
Cox2	0.149
IgM	0.024
GPx	0.629
CAT	0.671
Nfr2	0.158

Table VI.III – N. oceanica markers expressed a homogenous variance.

	Levene's test
Molecular Marker	P-value
Cox2	0.550
IgM	0.686
GPx	0.819
CAT	0.809
Nfr2	0.497

Table VI.IV - P. tricornutum markers expressed a homogenous variance.

	Levene's test
Molecular Marker	P-value
Cox2	0.610
IgM	0.867
GPx	0.865
CAT	0.960
Nfr2	0.534