U.PORTO

DOUTORAMENTO CIÊNCIA ANIMAL

ESPECIALIDADE EM NUTRIÇÃO

Improving health and growth in gilthead seabream through fortified nutrition: new nutraceuticals from marine bio-refineries

Bruno Filipe Reis

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2022

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D.ICBAS **2022**

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Improving health and growth in gilthead seabream through fortified nutrition: new nutraceuticals from marine bio-refineries

Thesis for applying to a Doctor degree in Animal Sciences, Specialty in Nutrition, submitted to the Institute of Biomedical Sciences Abel Salazar of the University of Porto

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This Thesis includes three scientific papers published in international peer-review journals arising from part of the results obtained in the experimental work, referenced to as:

Reis, B.; Ramos-Pinto, L.; Martos-Sitcha, J.A.; Machado, M.; Azeredo, R.; Fernández Boo, S.; Engrola, S.; Unamunzaga, C.; Calduch-Giner, J.; Conceição, L.E.C.; et al. Health status in gilthead seabream (Sparus aurata) juveniles fed diets devoid of fishmeal and supplemented with Phaeodactylum tricornutum. Journal of Applied Phycology 2021, doi:10.1007/s10811-021-02377-4.

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.; et al. Immune Status and Hepatic Antioxidant Capacity of Gilthead Seabream Sparus aurata Juveniles Fed Yeast and Microalga Derived beta-glucans. Marine Drugs 2021, 19, 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. Chlorella vulgaris Extracts as Modulators of the Health Status and the Inflammatory Response of Gilthead Seabream Juveniles (Sparus aurata). Marine Drugs 2022, 20, doi:10.3390/md20070407.

Acknowledgments

This work was mainly funded by projects VALORMAR (POCI-01-0247-FEDER-024517) funded by Compete 2020, Lisboa 2020, Algarve 2020, Portugal 2020 and the European Union through the European Regional Development Fund (ERDF), and ATLANTIDA (ref. NORTE-01-0145-FEDER-000040), supported by the Norte Portugal Regional Operational Program (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement and through ERDF. It was also supported by EU FP7 through the MIRACLES project No. 613588 and the Bio Based Industries Joint Undertaking (BBI JU) under the European Union's Horizon 2020 research and innovation programme under grant agreement No. 745754 (project MAGNIFICENT).
Bruno Reis was supported by Foundation for Science and Technology (FCT), Soja de Portugal, SA, and Sparos Lda., through grant PD/BDE/129262/2017.

Although, in the end only the candidate achieves an academic degree a PhD thesis is always a collective effort within a multidisciplinary team. So, it is important to acknowledge a number of people that range from supervisors, work colleagues but also, friends and family, that contributed either with work, support or both and ultimately made this thesis possible.

To start, I would like to thank SANFEED doctoral program in the persons of Profs. Luísa Valente and Mira da Fonseca who started this doctoral program and opened new opportunities for those who seek higher education in animal science.

I want to acknowledge my industrial supervisors Drs. Luís Conceição and Jorge Dias from Sparos and Drs. Elisabete Matos and Tiago Aires from Soja de Portugal. This Thesis would not possible without both the technical support and resources provided by these companies, namely the priceless help and expertise from Dr. Ana Gonçalves.

Also, I would like to acknowledge my supervisor Dr Benjamín Costas and co-supervisors Drs Irina Amorim and Sofia Costa Lima, for their valuable expertise and guidance that were paramount to keep me on track and overcome any moments of doubt. Nonetheless, I am particularly grateful to Dr Benjamín, with whom I had the great pleasure to work these past 4 years and for some time now, consider a good friend. I also, want to mention that without your support during particularly difficult personal problems, finishing this important chapter of my journey as a researcher would not be possible. Thank you Benjamín, Irina and Sofia.

Last but not least, thank you to all the colleagues who shared the lab with me at CIIMAR and helped during samplings and sample analysis. But also, for all the relaxing moments of companionship during which I could really feel the friendship that binds us.

A special thanks to Sergio Boo, Rita Azeredo, Marina Machado, Lourenço Ramos-Pinto aka “Lou”, Diogo Peixoto, Carla Teixeira and finally, my fellow Matosinhense, Paulo Santos aka “Paulinho”.

On a personal note, I want to dedicate this PhD first to those whom I have lost during the course of this 4 years, namely and most importantly to my mother “Locas” who always supported me to study and pursue my dreams and to my grandfather “Bô Agostinho” who became my surrogate father when I lost mine 20 years ago. I know that you both are very proud wherever you may be and I love you very much.

But also, I want to dedicate it to those who were and are always there for me. To my wife Rita and daughter Sara, who are my beloved family that helped me overcome the bad moments and appreciate all the good things life has to offer.

Thank you all.

Abstract

Health related issues are the major constraint for aquaculture expansion and sustainability, being traditional infection management strategies in animal production mostly relying on antibiotics administration. Nonetheless, this approach poses a severe risk for the emergence of drug resistant pathogens as well as other environmental risks, alongside with poor consumer perception of the industry. Therefore, alternative strategies are being developed to improve fish health and welfare through sustainable approaches. Nowadays, there is a strong body of knowledge suggesting that animal's resilience to stress and disease can be achieved through the use of feed additives with immunostimulant properties by creating tailor-made diets for each species or situation. Prophylactic measures such as immunonutrition seem a promising approach to reduce fish mortality and improve welfare in aquaculture.

Marine algae´s ability to grow in different environments and conditions as well as to produce large numbers of secondary metabolites makes them suitable raw materials for different applications. These organisms are regarded as sustainable alternative sources of bioactive compounds, mostly sought out for the development of functional foods, feeds and health products. Algae biomasses can be suitable candidates not only to enhance fish immunity but also to curtail the stressful effects of routine harvesting procedures. Marine macro- and microalgae show a natural abundance in immunostimulants and immunomodulators such as n-3 polyunsaturated fatty acids (PUFA), essential amino acids (EAA), probiotics, prebiotics, complex carbohydrates and vitamins. These are generally being suggested as supplements in aquafeeds to effectively promote growth, immune response and disease control.

This thesis describes the results of a series of studies designed to evaluate the dietary effect of different algae biomasses and extracts, given their direct or indirect roles in different physiological and immune mechanisms of gilthead seabream juveniles. The combination of classical immune indicators and high-throughput molecular biology techniques, demonstrated that nutritional health related benefits are mainly developed at short-term feeding periods. These short-lived boosting effects, reinforce the pertinence of this feeding strategy as a prophylactic measure, used in specific periods of the production cycle. Marine algae supplementation did not affect growth performance but affected fish innate immunity and oxidative stress response in a way that may confer resistance to stressors or disease. Importantly, the use of bioactive compounds-enriched extracts, underlined the importance of further processing algae biomasses in order to increase nutrient availability and bioactivity.

Overall, the current thesis emphasizes the pertinence of marine algae products supplementation as a prophylactic strategy, mainly because of their health boosting properties. Morover, it identifies important challenges for the future, such as the use of adequately processed biomasses to potentiate its effects.

Keywords

Microalgae; functional feeds; immunonutrition; gilthead seabream

Resumo

Questões relacionadas com a saúde animal são o maior constrangimento ao desenvolvimento e sustentabilidade da indústria da aquacultura. Presentemente, a principal estratégia de controlo de infecção na produção animal passa pela administração de antibióticos. No entanto, esta abordagem implica um grave risco para o aparecimento de patogéneos resistentes a antibióticos para além de outros riscos ambientais não negligenciáveis. Neste sentido, o desenvolvimento de estratégias alternativas mais sustentáveis e que melhorem a saúde animal, tem sido um objectivo recente de diferentes linhas de investigação. Hoje em dia existe uma forte evidência científica do aumento da resiliência ao stress e à doença em diferentes modelos animais com a utilização de aditivos alimentares imunoestimulantes. Estratégias profilácticas tais como a imunonutrição parecem ser abordagens promissoras para reduzir perdas de produção e aumentar o bem-estar animal em aquacultura.

A capacidade das algas marinhas para crescer em diferentes condições ambientais assim como para produzir uma miríade de metabolitos secundários torna-as matérias-primas promissoras em diferentes campos de aplicação. Estes organismos são vistos como fontes sustentáveis de compostos bioactivos, procurados principalmente para o desenvolvimento de dietas funcionais e como suplementos medicinais. As biomassas de algas podem ser utilizadas não só para melhorar a imunidade dos animais em aquacultura, mas também para mitigar o stress provocado pelos procedimentos de rotina empreendidos nas operações diárias desta indústria. Estes organismos mostram uma relativa abundância em diferentes imunoestimulantes, nomeadamente ácidos gordos polinsaturados, amino ácidos essenciais, pro- e prebióticos, hidratos de carbono complexos e vitaminas. Todas estas moléculas e compostos têm sido sugeridos e alguns utilizados com sucesso como suplementos alimentares em aquacultura, promovendo o crescimento, a resposta imunitária e o controlo de doenças.

Esta tese descreve os resultados obtidos em diferentes ensaios experimentais com juvenis de dourada. Estes ensaios foram desenhados com o objectivo de avaliar o efeito de várias biomassas e extractos de algas, considerando o seu papel directo ou indirecto em diferentes mecanismos fisiológicos e imunitários.

A análise de biomarcadores clássicos de imunidade aliada à análise por diferentes técnicas de biologia molecular demonstraram que os efeitos imunoestimulantes obtidos através da nutrição são maioritariamente desenvolvidos em períodos de alimentação curtos. Estes efeitos transitórios reforçam a pertinência do uso desta estratégia

alimentar como uma medida profiláctica que pode ser usada em períodos específicos dos ciclos de produção.

A suplementação de dietas de juvenis de dourada com biomassas e extractos de algas marinhas não comprometeu o crescimento animal nos vários ensaios realizados. Teve também um efeito na imunidade inata e na resposta ao stress oxidativo no sentido de conferir uma maior resiliência à doença e ao stress. Para além disto, o uso de extractos enriquecidos em diferentes compostos bioactivos, enfatizou a importância do processamento das biomassas no sentido de aumentar a disponibilidade e bioactividade dos diferentes compostos.

No geral esta tese reforçou a importância da suplementação das dietas de dourada com algas de origem marinha, devido sobretudo às suas propriedades imunoestimulantes e utilidade como uma estratégia profiláctica. Ao mesmo tempo, identifica importantes desafios futuros, nomeadamente o processamento adequado das biomassas de forma a potenciar os seus efeitos.

Palavras-chave

Microalgas; dietas funcionais; imunonutrição; dourada

INDEX

Chapter I

General Introduction

1. General Introduction

1.1 Aquaculture future challenges/Immunonutrition

Portugal has a long tradition of fish and seafood consumption, which in 2017 amounted to 56.84 kg/year per capita according to the Food and Agriculture Organization (FAO) [1]. In the last 15 years, Portuguese aquaculture production has more than doubled (6,802 tons in 2004 compared to 14,337 in 2019) and in 2019 marine fish accounted for 46% of total production yield [2]. Globally, aquaculture has been bridging the gap between long stagnated wild fisheries captures and consumer demand. In 2020, aquaculture represented 49% of the 178 million tonnes of seafood products consumed worldwide [3]. Furthermore, in a recent report, FAO stated that 35.4 percent of wild fish stocks were estimated as fished at a biologically unsustainable level and therefore already overfished in 2019[3]. In the future, with a growing human population as well as a worldwide increase in average fish consumption per capita (9.0 kg in 1961 to 20.5 kg in 2019), sustained and unprecedented demand for fresh seafood products will expectedly be on the rise [3]. To meet such demand and increase efficiency, fish species are mostly cultured in semi-intensive and intensive production systems as they can produce greater yields. However, higher productivity often comes at the expense of fish welfare, as fish are reared at high stocking densities amounting to higher stress, nutrient pollution and poorer water quality [4]. On top of that, routine harvesting practices such as crowding, size sorting and transportation also create favourable circumstances for opportunistic bacteria to thrive and disease outbreaks to occur. Currently, health related issues are the major constraint for aquaculture expansion and sustainability, with an estimate 10% loss due to infectious agents worldwide, accounting for >10 billion USD lost in revenue annually [5; 6]. Until recently, antibiotics and other chemotherapeutics were the main disease management tools used in animal production. However, routine use of antibiotics poses a severe risk for the emergence and expansion of drug resistant pathogens, environmental pollution and accumulation of chemicals in aquatic animal tissues, ultimately leading to public health risks [7; 8; 9]. This issue has been mitigated in recent years with more restrictive legislation and regulations. Still, studies point to a 67% increase in antibiotic administration for livestock in lower to middle-income countries until 2030 [8]. Besides antibiotics, vaccination is valued as an important tool for fish disease management and prevention in aquaculture. However, lack of vaccines for specific diseases, poor performance or high administration costs still limit their application [4; 5; 8]. Complementary, prophylactic measures such as immunonutrition, which aims not only

to fulfil basic dietary requirements for growth but also generate additional health benefits, seem a promising approach to reduce fish mortality and improve welfare in aquaculture. Natural immunostimulants and/or immunomodulators such as n-3 polyunsaturated fatty acids (PUFA), essential amino acids (EAA), probiotics, prebiotics, complex carbohydrates and vitamins are generally being suggested as supplements in aquafeeds to effectively promote growth, immune response and disease control in aquatic animals [10; 11]. In this sense, marine macro- and microalgae can be regarded as promising candidates for new nutritional strategies due to their natural abundance in biologically active compounds [12; 13; 14; 15; 16].

1.2 Innate immunity/inflammatory response

1.2.1 Innate immune system in teleosts

Traditionally, the vertebrate's immune system is divided into innate and adaptive responses, however a great body of evidence shows that both are complementary. Normally, the innate immune response precedes and activates the adaptive one, while the latter sustains and amplifies the first, and both co-operate to maintain alostasis [17]. Differences reside in the ability of the adaptive response to recognize specific antigens and to differentiate memory cells, which in case of reinfection is a major advantage, making the secondary response much faster and efficient.

Vertebrates show both innate and adaptive response. Fish as lower vertebrates are at a crucial point of evolution between a powerful innate immune system and a developing adaptive one that is characterized by a limited repertoire of immunoglobulins, low affinity and limited memory cells' maturation [18]. Also, acquired immunity is energetically costly since it relies on high metabolic activity for the rapid secretion of different chemokines and the differentiation of highly specialized cells. Teleosts, being poikilothermic animals, show an environmental temperature -dependent metabolic rate, which at low temperatures can be disadvantageous. A sluggish metabolism is not compatible with a rapid adaptive immune response [17; 18]. Nonetheless, fish thrive in the aquatic environment accounting for 40% of all the vertebrate species, this adaptive success is only possible, among other traits, due to a highly competent innate immune system [19]. In fact, fish possess innate immune components more active and diverse than mammals. Antimicrobial peptides (AMPs) are major protective components in the animal kingdom which provide an early defence system against a wide range of microbial pathogens [20; 21]. Fish express all of the major classes of AMPs (defensins, cathelicidins, hepcidins, histone-derived peptides) and a fish-specific class, called piscidins, these peptides exhibit broad-spectrum antimicrobial activity [21]. They also show different

isoforms of complement proteins (C3 and factor b) and in consequence, higher spontaneous activity of the complement alternative pathway [17; 18; 19] which is a trait of immunocompetence. Furthermore, in fish the kidney is the major hematopoietic organ, with the cranial section (head kidney) being responsible for leucocyte production. It also displays an endocrine function including the release of corticosteroids by interrenal cells, in addition to several other hormones [18].

In an aquatic environment, animals are constantly in contact with a myriad of pathogenic microorganisms, therefore, fish skin and mucus as well as the mucosal epithelia of gills and gut are the first lines of defence against external stressors and pathogens [18; 19]. These are highly specialized external barriers that are equipped with different cell types producing mucus, AMPs, agglutinins, amongst others, thereby conferring physical and chemical protection [18; 19]. In case external barriers are breached, cellular and humoral components from the innate immune system are activated. The innate immune system's main feature is being non-specific, i.e. it does not rely on previous recognition of the invading antigens, but it is also both constitutive and inducible by external signals [19]. Constitutive mechanisms ensure an immediate response to a danger signal, while lacking the potential to amplify the response. On the other hand, inducible mechanisms are mediated through receptor/ligand activation and have the ability to mount very strong and efficient immune responses [22]. In inducible innate responses, non-self recognition is made by germline-encoded pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) [17; 23]. The most studied and best characterized group are the toll-like receptors (TLRs) that can recognize conserved microbial patterns, such as the peptidoglycan, lipopolysaccharide (LPS), lipoteichoic acid (LTA) and other bacterial endotoxins. Virus-host interaction is also enabled by the ability of these receptors to recognize nucleic acid variants such as double-strand RNA, commonly associated with viruses [23]. After recognition and signal transduction, a cascade of events is initiated (Fig. 1) leading to the transcription of antimicrobial and pro-inflammatory genes, which results in the production of different cytokines namely, type I interferon (IFNα and IFNβ), interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNF-α) [24; 25; 26].

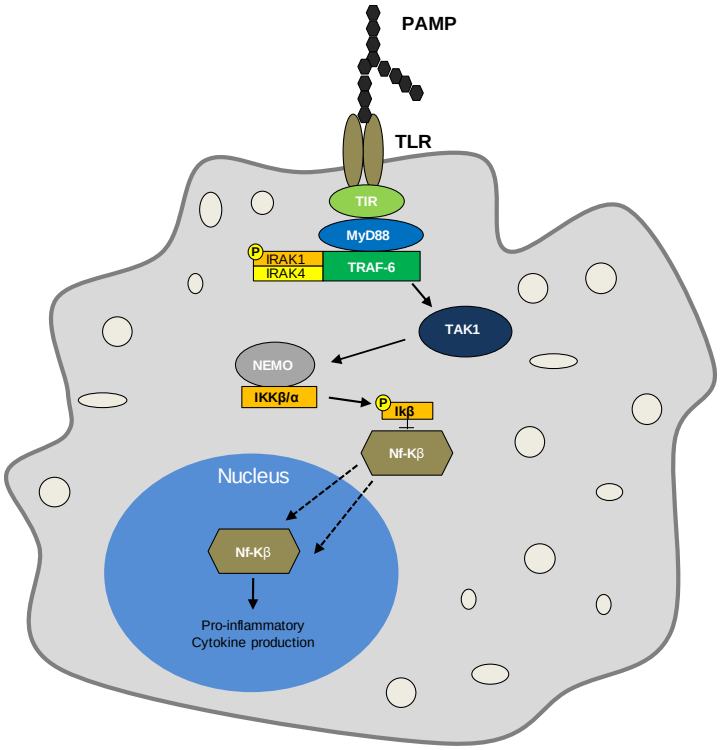

Figure 1. (Adapted from Kataria *et al.* [27]) Receptor/ligand interaction of membrane TLRs with PAMPs initiates a cascade of events in macrophage cells. Starting with the dimerization and formation of a cytoplasmic toll-interleukin domain (TIR) which becomes able to bind to the myeloid differentiation primary response gene 88 (MyD88) and IL-1 receptor-associated kinase (IRAK-1). Which is then phosphorylated and binds to the tumor necrosis factor associated receptor-6 (TRAF6). Downstream activation of the pathway leads to the phosphorylation of inhibitor of kB (IkB) and consequent dissociation from nuclear factor-kB (NF-kB) so that NF-kB can translocate to the nucleus and initiate transcription and translation of different cytokines.

That, in turn, activates antimicrobial and pro-inflammatory responses, initiating the inflammatory process. Circulating leucocytes such as myeloid cells are activated, which translates into the production and release of several mediators namely, eicosanoids (e.g. prostaglandins, leukotrienes, resolvins) and cytokines (e.g. IL-6, IL-1, IL-8) [24]. Additionally, myeloid cells are responsible for the production of several humoral components (e.g. lysozyme, complement factors, antiproteases and proteases, peroxidase, reactive oxygen species (ROS), nitric oxide (NO)) which take part in the innate response [17]. The inflammation process leads to the recruitment of phagocytes to the site of infection, first neutrophils followed by macrophages [19]. Although inflammation is initiated by pro-inflammatory mediators, control and regulation of the inflammatory response are needed to restrain or minimize self-damage. Once the pathogens are cleared, inflammation enters the resolution phase which is characterised by the production of anti-inflammatory mediators (cytokines, protectins and resolvins). These compounds promote tissue repair by stopping the recruitment of additional neutrophils and the uptake of neutrophils by macrophages. Therefore, inflammation is a process

under the tight control of opposite but complementary signals, essential for microbe clearance and self-preservation [24].

1.2.2 Inflammation and oxidative stress

Inflammation can be triggered both by microbial infection or tissue damage. As mentioned above, it is an essential adaptive response aimed at eliminating invading pathogens, inducing tissue repair and restoring homeostasis [25]. In situations of persistent cellular stress, homeostasis restoration may not be possible, resulting in a prolonged inflammatory response. It is well established that under inflammatory states, with the consequent activation and stimulation of immune cells there is an enhancement in oxidant generation [28]. Activated neutrophils and macrophages generate large amounts of ROS during the oxidative burst, an essential mechanism for the elimination of invading pathogens. However, under chronic inflammatory conditions, the activation of phagocytes can become a major source of tissue injury. Conditions that significantly induce excessive ROS production may originate biomolecular damage that exceeds the cell capacity of repair, leading to cell necrosis and death. Under these circumstances, necrotic cells release damage-associated molecular patterns (DAMPs) arising from oxidative stress, such as oxidized lipids and proteins that act as potent danger signals able to trigger inflammatory cascades through binding to different PRRs [28]. Consequently, as a way to prevent oxidative injury, animal cells developed complex antioxidant mechanisms that rely on antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPX; glutathione reductase, GR amongst others) and non-enzymatic antioxidants, especially glutathione (GSH) which along with glutathione disulphide (GSSG) forms the major redox couple in animal cells [29; 30].

In summary, there is a close relationship between inflammation and oxidative stress. Inflammatory mechanisms increase the production of ROS as a part of the inflammatory response and in turn the cellular redox imbalance may lead to tissue injury that perpetuates the inflammatory state. Hence, antioxidant endogenous mechanisms are paramount for controlling inflammation, through the detoxification of oxygen radicals and prevent cell injury.

1.3 Immunonutrition: prophylactic measure in aquaculture

1.3.1 Immunonutrition in fish: algae-supplemented feeds

As discussed above, several challenges are impending upon aquaculture industry. As pressure increases to satisfy demand, more intensive production methods may translate

into favourable conditions for disease outbreaks to occur [4]. Disease management tools traditionally available, such as large-scale antibiotics administration, pose a risk for public health and a non-negligible environmental burden [9], and are currently illegal in many countries. Vaccination, although a valuable prophylactic measure, is still only available for a limited number of pathogens and fish species [4; 5]. Based on the knowledge that nutritional factors can modulate the immune response, alternative prophylactic measures such as new nutritional strategies for fish in aquaculture, are currently being investigated. Balanced nutrition is paramount for growth and general homeostasis but also to maintain high-energy physiological processes. The immune response is an energetically demanding process dependent on readily available nutrients. During an infectious event the immune response may significantly increase the metabolic need for specific nutrients which shortage or depletion might impair the response. Immunonutrition is thus, the potential to modify inflammatory or immune responses through the provision of specific nutrients in an amount above what is normally available in the diet to satisfy requirements under homeosthasis [31]. Natural immunostimulants such as prebiotics, β-glucans, amino acids (AA), nucleotides and vitamins are examples of functional feed additives that have been added to fish feeds to enhance fish immune function in a wide range of species [11; 32]. Both micro- and macroalgae biomasses show a wide repertoire of bioactive compounds [12; 14; 15; 16]. Polysaccharides such as (1,3/1,6)-β-glucans (BGs) can activate and enhance fish immune responses [33;34; 35] by direct interaction with specific cell receptors (PRRs) [32] or acting as a prebiotic [36] enhancing the growth of commensal microbiota. Also, sulphated polysaccharides (SPs) which are found in several micro- and macroalgal species have already been proven to have several important health-related properties, such as immunomodulatory ability, antimicrobial, anti-inflammatory and antioxidant, making them promising immunonutrients [37; 38]. Furthermore, compounds such as polyphenols and carotenoids are effective free radical scavengers reported to have immunostimulating effects in fish [10; 32; 39]. In recent years, the role of algae in aquaculture has extended further with its use as a potential immunostimulant to commercially important aquaculture species (Table 1).

Table 1. Current knowledge on dietary inclusion/supplementation with marine algae in farmed fish.

Algae species	*Target species*	*Dietary inclusion/ supplementation*	*Algae effects*	*References*
Chlorella vulgaris (CV)	*Salmo salar*	20% cell-ruptured CV + 20% soybean meal (SBM) replacing 40% of fishmeal (FM) protein	Prevented SBM induced enteropathy; Reduced growth	(40)
***Nannochloropsis* sp**	*Gadhus morhua*	15% replacement of FM protein algae biomass	Improved growth performance	(41)
***Tetraselmis* sp**	*Salmo salar* *Cyprinus carpio* *Litopenaeus vannamei*	5 and 10% replacement of FM protein with algae biomass	No negative effects on growth performance	(42)
Phaeodactylum tricornutum	*Salmo salar*	6% replacement of FM protein with algae biomass		(43)
***Dunaliella* sp**	*Litopenaeus vannamei*	1–2% inclusion of β-carotenes enriched microalgal meal	Increased survival rate after infection (White spot syndrome)	(44)
Nannochloropsis gaditana ***Tetraselmis chuii*** ***Phaeodactylum tricornutum***	*Sparus aurata*	0.5 and 1% algae biomass inclusion in feed	Improved innate immune defenses	(45)
Haematococcus pluvialis	*Oncorhynchus mykiss*	0.3% algae biomass inclusion in feed	Enhanced liver antioxidant defenses	(46)
Gracilaria vermiculophylla	*Sparus aurata*	Diet supplemented with 5% heat-treated *Gracilaria* biomass	Increased survival rate after hypoxia; Improved antioxidant capacity	(47)
***Gracilaria* sp**	*Dicentrarchus labrax*	Diet supplemented with 5% *Gracilaria* aqueous extract	Increased resistance to *Phdp* pathogenic challenge; Improved antioxidant capacity	(48)
Ulva ohnoi	*Solea senegalensis*	i.p injection with *Ulva* SPs (0.5 mg/fish)	Immunomodulatory; Improved inflammatory response against *Phdp* pathogenic challenge	(49)
Ulva rigida	*Scophthalmus maximus*	Cell incubation (head-kidney phagocytes) with *Ulva* SPs at 0-100 µL/mL	Increased IL-1β expression; Increased respiratory burst activity	(50)

1.3.2 Marine macro- and microalgae potential as nutraceuticals

Marine ecosystems are a vast reservoir of biodiversity able to produce a myriad of natural products. Marine micro (bacteria; fungi) and macro organisms (sponges; tunicates; bryozoans; molluscs) are sources of high-value bioactive compounds that present new opportunities for diverse applications in food, feed, biomedical and pharmaceutical industries, amongst others [51; 52; 53; 54]. Apart from bacteria and animals, marine algae are on their own a plentiful source of good quality nutrients, coupling a balanced nutritional profile with the presence of bioactive molecules. These unique features can be harnessed to develop new functional feeds and foods so that their basic nutritional value is improved, enhancing its health benefits, but also curtailing the risk of illness for individuals [55; 56; 57]. Based on their supply of amino acids, ω-3 and ω-6 PUFA,

vitamins, minerals, pigments and complex polysaccharides [34; 54; 58; 59], algae seem promising candidates for fish feed supplementation (Table 2).

Marine macroalgae have been traditionally used in direct human consumption as food, mostly in Eastern Asia. Nowadays, marine macroalgae are significantly harvested for the extraction of gelling and stabilising agents (agar, carrageenan and algin), with important applications in the cosmetic and food industries [60; 61]. Additionally, they can be natural sources of nutrients for animal feeds, but also of bioactive molecules with antioxidant and immunostimulatory activities [62; 63]. Several studies have shown that moderate levels (2.5–10% of the diet) of macroalgae in fish feeds resulted in improved growth performance, feed utilisation efficiency, physiological activity and intestinal microbiota [43; 64; 65]. Also, improving innate immune parameters [66], disease resistance [48; 67] and stress response [47].

Marine microalgae are likewise increasingly sought for their enormous potential to act as bio-refineries. These organisms seem the most promising candidates for this holistic approach, that intends to extract value-added compounds from different fractions at low energy consumption and almost zero waste [68]. They show immense potential for different technological applications, such as biomass for biofuel production, biofilters to remove contaminants [58] or as a source of natural immunostimulants. Microalgae species can contain 30–60% protein, 10–20% lipid, and 5–15% carbohydrate [69]. Furthermore, they show balanced amino acid profiles and high levels of essential amino acids and PUFAs [70; 71]. Microalgal lipids often contain arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and these highly unsaturated fatty acids (HUFA) are usually present in high concentrations (EPA can range from 7 to 34% of total fatty acids in EPA-rich microalgal species) [69]. For these reasons, they have been used for direct or indirect nutrition of the larval stages of many aquaculture fish, shellfish, and invertebrates [72]. Unlike macroalgae, they are rich sources of HUFA which are essential for growth and survival of marine fish larvae [73]. Microalgae are also an excellent source of: 1) antioxidants, in the form of pigments such as fucoxanthin, β-carotene, astaxanthin and lutein; 2) immunomodulatory, complex carbohydrates namely β-glucans and SPs [58]. Several studies report beneficial effects on both fish immune and antioxidative responses when microalgae biomasses and bioactive compounds are incorporated in fish feeds [35; 74; 75; 76; 77], as well as increased disease resistance [44; 48; 49] and the potential to prevent soybean meal-induced enteropathy [40].

However, there are also drawbacks when using marine algal species in aquafeeds, especially when used at high inclusion levels. Several studies done with different macroalgae showed detrimental effects on feed utilisation and growth when fish were

fed diets with inclusion levels higher than 10% [64; 78; 79; 80]. Anti-nutritional factors (lectins, tannins, phytic acid, protease and amylase inhibitors) might in part explain such outcomes [79; 81; 82; 83]. Yet, most importantly is the presence of resistant and poorly digestible cell walls [84] in both macro- and microalgae, that diminishes nutrient availability. In the wild, fish in higher trophic levels ingest algae derived nutrients indirectly, through an upward passage of nutrients in the food chain [85]. When algae are orally given to fish the cell wall may restrict the access of gut enzymes to the inner-cell components and so, algal biomass utilisation depends mainly on fish ability to digest it [80]. Recent works in Atlantic salmon [86], Nile tilapia [87] and European seabass [88] indicate that the digestibility, i.e. the nutritional value of algae species, increases after disrupting the algal cell wall by appropriate processes. Cell wall rupture can be achieved employing mechanical, chemical and enzymatic methods [89]. Mechanical methods such as bead milling and high-pressure homogenization can be used to disrupt cell walls while preserving the integrity of inner-cell nutrients [86; 87; 88].

Table 2. Health applications/effects of main bioactive compounds found in marine algae.

Group of compounds	*Bioactive compounds*	*Algae source*	*Activity/Applications*	*References*
PUFA	EPA	***Phaeodactylum tricornutum;Tetraselmis* sp.*; Nannochloropsis* sp.*;***	Nutraceutical; antimicrobial; anti-inflammatory	(55; 86; 87; 88; 89)
	DHA	***Schyzochytrium* sp.*; Crypthecodinium* sp.*; Isochrysis galbana***	Nutraceutical; anti-inflammatory; poultry feed additive	(55; 86; 87 88)
	Arachidonic acid	***Parietochloris incisa; Porphiridium cruentum***	Prostaglandin precursor; nutritional supplement	(55; 66; 86; 88; 89)
PIGMENTS	β-carotene	***Dunaliella salina; Haematococcus* sp.**	Pro-vitamin A; anti-inflammatory; antioxidant	(55; 67; 87; 88)
	Astaxanthin	***Haematococcus pluvialis; Chlorella zofigiensis***	Aquaculture pigmenter; antioxidant; anti-inflammatory; anticancer	(55; 87; 90)
	Lutein	***Chlorella prothecoides; Dunaliella salina***	Antioxidant; anti-inflammatory; anticancer	(67; 87; 91; 92)
	Fucoxanthin	***Phaeodactylum tricornutum; Isochrysis* sp.**	Antioxidant	(87; 93; 94)
PROTEINS & PEPTIDES	Bulk proteins	***Arthrospira platensis; Phaeodactylum tricornutum***	Health food supplement	(55; 95)
	Phycobiliproteins	***Porphiridium cruentum;Porphyridium* sp.*; Gracilaria* sp.**	Antioxidant; anti-inflammatory; natural pigments (used in cosmetics and food); nutritional supplement	(88; 89; 96; 97; 98)
	peptides	***Chlorella pyrenoidosa; Chlorella vulgaris; Navicula incerta; Spirulina platensis***	Antioxidant; immune-modulatory	(87; 99; 100)
POLYSACCHARIDES	Sulphated extracellular polysaccharides	***Phaeodactylum tricornutum; Porphiridium cruentum***	Anti-inflammatory; Immune-modulatory	(33; 101)
	β-(1,3)-glucans	***Chlorella vulgaris; Laminaria* sp.**	Anti-inflammatory; Immune-modulatory; vaccine adjuvants	(34; 87; 102; 103)
	Sulphated polysaccharides	***Ulva rigida; Ulva* sp.*; Undaria pinnatifida***	Drug carriers; immunostimulatory; hepatoprotective	(34; 104)
PHENOLIC COMPOUNDS	Phenols	***Spirulina maxima; Nannochloropsis* sp.**	Antioxidant	(87)

Additionally, marine microalgae large-scale culture is still in its beginning, and opposite to agricultural crops, often lacks the industrial level production needed to create an economy of scale that can not only ensure cost-effective products, but also a steady supply and consistent nutrient profiles [55; 109; 110]. While some sectors, particularly renewable energy and food industries, have driven technological advances in microalgae cultivation and high-value compound extraction, there is still a long way until economic viability. Presently, algal biomasses and derived products continue to be less competitive than terrestrial crops and conventional animal-derived raw materials used in feed formulation [85]. Nonetheless, there is a large interest in these phytoplanktonic organisms not only because they produce a myriad of metabolites of interest, but also because they show simple growth requirements and the capacity to modulate their metabolism

according to environmental conditions [37; 111; 112]. Under nitrogen-depleted conditions the production and accumulation of energy-rich compounds such as starch and lipids is favoured. Metabolites secretion or accumulation is modulated through different abiotic factors (e.q. light, temperature, pH, salinity, nutrient, dissolved oxygen concentration and presence of toxic compounds) that can be manipulated to meet industrial and market needs. Furthermore, marine algal-based ingredients could be advantageous over terrestrial plant raw materials for the production of aquafeeds, since marine algae cultivation does not need to compete for valuable resources (freshwater; arable land), needed for human food crops [85].

1.4 Selected algae biomasses for the current thesis. Why?

Different marine micro and macroalgae species were chosen not only due to their ecological relevance and availability, but also and foremost, because they show significant levels of health beneficial compounds (Table 3).

Table 3. Main compounds profile and protein content of target algae species.

	Microalgae				***Macroalgae***	
Habitat	Marine			Freshwater	Marine	
Taxonomic group	Bacillariophyta	Eustigmatophyceae	Prasinophyceae	Trebouxiophyceae	Chlorophyta	Rodophyta
Algae species	*Phaeodactylum* sp.	*Nannochloropsis* sp.	*Tetraselmis striata*	*Chlorella vulgaris*	*Ulva rigida*	*Gracilaria* sp.
PUFA	Mainly EPA	EPA & DPA	EPA & DHA			Arachidonic acid
Photosyntetic pigments	Chlorophyls a, c1, c2	Chlorophyl a, c	Chlorophyl a, b	Chlorophyl a, b	Chlorophyl a, b	Chlorophyl a, Phycoerythrin
Accessory pigments	β-carotene; Fucoxanthin	β-carotene; Violaxanthin	β-carotene; lutein	β-carotene; lutein	Xanthophylls	-
Complex polysaccharides	SEP; β-glucans	β-glucans	SPs	SPs; β-glucans	Ulvan (SP)	Agar; SP
Other compounds	-	Phenolic compounds	-	Functional peptides	-	Phycobiliproteins
Protein (% DW)	24-49	35-55	30-50	30-50	-	-
References	(66; 67)	(66; 67)	(66; 67)	(66; 67)	(109; 110)	(56)

Tetraselmis sp., *Phaeodactylum* sp. and *Nannochloropsis* sp. are among the major microalgae species cultivated for feeds in fish (mainly at larval stages), shellfish and shrimp aquaculture [115]. *Tetraselmis* sp. are green microalgae mainly exploited as a food source for molluscs and enrichment of larvae feeds [116]. They show a rich content of n-3 PUFAs and starch-like polysaccharides making them an interesting energy source. The main pigments are chlorophyll and lutein, the latter pigment being found at such levels that strains from the *Tetraselmis* genus have been considered as a possible alternative commercial source [117]. Water soluble polysaccharides extracted from *Tetraselmis* sp. showed promising antioxidant and antifungal activities *in vitro* [118]. These microalgae have been previously used to supplement gilthead seabream diets, with 5

and 10% *Tetraselmis suecica* biomass causing a positive effect on seabream fry intestinal mucosa due to an increase of total enterocyte absorption surface [119].

Phaeodactylum sp. are marine diatoms particularly rich in EPA, but also carotenoids, mainly fucoxanthin and polysaccharides such as β-glucans [97; 120; 121]. Furthermore, polysaccharide extracts from *P. tricornutum* showed anti-inflammatory activity in a mammalian inflammation model and immunostimulatory activity in phagocytic assays [105]. Previous experiments with gilthead seabream fed diets supplemented with 5 and 10% *P. tricornutum* whole biomass for 4 weeks showed a general increase in innate immune parameters activity. Immunostimulant effects were attributed to the presence of β-1,3-glucans [45].

Nannochloropsis species are unicellular, planktonic organisms with subspherical or cylindrical cells [122]. They contain high contents of EPA and pigments such as chlorophyll a and xantophylls, thus are widely used in fish hatcheries live feed feeding and “green water" technique [123; 124]. Dietary supplementation with *Nannochloropsis* sp*.* biomasses in levels up to 5% has been associated with decreased lipid peroxidation and enhanced antioxidant capacity in turbot juveniles [125].

Among selected marine macroalgae, *Gracilaria* sp*.* (Rhodophyta) is one of the most common genus within the red algae group. They constitute a great source of ARA and lipids (e.g. prostaglandins and steroids) which are key players in inflammation and phagocytosis [126]. Additionally, these macroalgae are rich in complex polysaccharides with health-related bioactive properties [127] and pigments such as phycobiliproteins that have been proven to have antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective and neuroprotective effects [128]. *Gracilaria gracilis*-supplemented diets (1%) increased the expression of intestinal catalase and had a positive effect on zebrafish (*Danio rerio*) immune system without compromising growth performance [129]. Additionally, a 5% *Gracilaria* sp. inclusion in rainbow trout diets led to fish innate immune response enhancement [130].

Finally, *Ulva rigida* is a marine macroalgae (Chlorophyta), belonging to the ubiquous *Ulva* genus commonly found in Portuguese coastal waters [131]. *Ulva* sp. show abundance in cell wall SPs which are described to have antioxidant as well as macrophage-stimulating activities [13; 113].

Besides marine-derived algae species, *Chlorella vulgaris* is one of the most relevant freshwater species in the world and one of the first microalgae to be mass cultivated since the 1960s. In the following decades, the demand increased with its usage as feed for rotifers and fingerlings in aquaculture which meant that production steadily rose [132]. More recently, *Chlorella* sp. have been reported to improve growth and feed utilisation in different fish species but also to have beneficial effects in fish immunity [80]. For instance,

koi carp (*Cyprinus carpio*) haematological parameters were significantly enhanced, and an increase in the levels of IgM and C4 complement factor was observed when fish were fed a diet supplemented with 5% *C. vulgaris* [133]. Zahran and Risha [134] reported that feed supplementation with *C. vulgaris* at 10% protects Nile tilapia (*Oreochromis niloticus*) against arsenic- induced immunosuppression and oxidative stress. This microalga is among the most relevant commercial species in the world, with a real economy of scale making it cost-competitive to produce and to obtain at high quantities. Hence, it was deemed relevant to explore under the scope of this thesis.

In summary, algae used in this work cover a wide range of algal phyla, orders and genera, representing several important commercial and local species. Due to its diversity they show different structural features and metabolite production. However, they all share a common trait, which is the ability to produce and express several compounds of interest with a broad field of application, namely in nutraceuticals development.

1.5 Gilthead seabream relevance as target species

Gilthead seabream (*Sparus aurata*) is a marine carnivorous species of high commercial value which has been extensively farmed along the Mediterranean coast with production steadily rising over the last years [135; 136]. In 2018, gilthead seabream production reached 168,9 thousand tonnes of live weight valued at around EUR 304 million [137]. In Portugal, gilthead seabream farming has varied in the last 15 years with production peaking at 1,95 thousand tonnes in 2019 and accounting for approximately 30% of all the marine fish farmed in the country [2]. As it is perceivable, gilthead seabream is an important high-value food item for human consumption and research efforts have been focusing on improving feed formulations to diminish fish oil and fishmeal dependency without affecting growth performance and fillet quality [138]. This has been the driving force in the last decades for research in aquaculture. However, as a result of intensive production the aquaculture sector is becoming more vulnerable to endemic and emerging diseases [3]. Diseases are one of the major reasons constraining the expansion of the aquaculture industry worldwide. Gilthead seabream production faces several challenges when it comes to the management of disease outbreaks, mostly relying in therapeutic measures such as antibiotics and chemical disinfectants [135; 139], whose use is increasingly constrained in Europe. In face of limited disease management strategies, the development of alternatives that strengthen fish stress resistance and immunological status seems a promising approach. Due to the emerging need for more in-depth studies in this topic, together with the relevance of gilthead seabream for Mediterranean aquaculture, seabream was selected as the model species in the present thesis.

1.6 Thesis main objectives

Nowadays, there is a strong body of knowledge that resilience to stress and disease can be achieved through the use of functional ingredients in animal production. Given their richness in bioactive metabolites with antioxidant and immunomodulatory potential, algae seem suitable candidates not only to enhance fish immunity but also to curtail the stressful effects of routine harvesting procedures.

This thesis main goal was to evaluate the potential of algae biomasses and extracts as nutraceuticals for incorporation in gilthead seabream feeds. Particularly, the effects of dietary supplementation on target species growth performance, oxidative and health status and inflammatory response. Finally, this work intends to modestly contribute, to the ultimate goal of providing sustainable and ethically sound alternatives to the use of chemotherapeutics and antibiotics in aquaculture, thus contributing to the societal challenges released by the European Framework Programmes for Research and Innovation Horizon 2020 and Horizon Europe, and by the (Portuguese) National Ocean Strategy 2021-2030.

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Chapter II

Effects of short-term feeding algae supplemented diets in innate immune status and inflammatory response of *Sparus aurata* juveniles

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Effects of short-term feeding algae supplemented diets in innate immune status and inflammatory response of *Sparus aurata* juveniles

Abstract

This study aimed to evaluate the effects of short-term feeding with marine macro and microalgae biomasses on gilthead seabream (*Sparus aurata*) immune status and inflammatory response. Six isonitrogenous (45% protein) and isolipidic (17% fat) diets were formulated. A control diet (CTR) and five other consisting on CTR with a 2% inclusion of different algae biomass: Tetra (*Tetrasselmis striata*); Phaeo (*Phaeodactylum* sp.); Nanno (*Nannochloropsis* sp.); Grac (*Gracilaria gracilis*); Ulva (*Ulva rigida*). Diets were randomly assigned to triplicate groups of 110 fish/tank (IBW:11.7±1.0 g), fed to satiation three times a day. Fish were maintained in a recirculation seawater system (temperature 22.4 °C; salinity 35.2‰). After 1 and 2 weeks of feeding 12 fish/treatment were sampled for tissues. At the same time, 30 fish/treatment were subjected to an inflammatory insult by intraperitoneal injection of inactivated gram-negative bacteria and transferred to a similar recirculation system in duplicates, 6 fish/treatment were sampled as described above after 4 and 24 h post-injection. Blood was collected for haematological procedures, whereas plasma, liver and total gut were sampled for immune and oxidative stress parameters. Head-kidney was also collected for gene expression measurements. The use of lyophilized algal biomasses at 2% supplementation showed mild immunological and antioxidant effects irrespective of the dietary treatment. Nonetheless, the incorporation of 2% *Ulva rigida* algal biomass promoted neutrophil proliferation during the inflammatory insult at 1 week, which might be advantageous during an infectious event.

Keywords

Algae biomass; short-term feeding; innate immunity; inflammation

2.1 Introduction

Appropriate nutrition provides the requirements needed for growth and general homeostasis but also to maintain other energetically costly physiological processes, namely reproduction and immune response. The latter, is a high energetic process dependent on the availability of nutrients that must be provided through the diet. During an infectious event the immune response may significantly increase the metabolic need for specific nutrients, and lack or depletion might impair the response [1; 2]. Thus, designing fish diets that not only fulfill the basic requirements for growth but also generate additional health benefits seems a promising approach to reduce fish mortality and improve welfare in aquaculture. This strategy known as immunonutrition, intends to supplement diets with functional ingredients that have proven beneficial effects on specific biological processes and increase fish general health status and disease resistance [2]. Probiotics, β-glucans, amino acids (AA), nucleotides and vitamins are examples of functional feed additives that have been added to fish feeds to enhance fish immune function [3; 4]. However, obtaining purified compounds is often a costly and laborious process needed for extracting these compounds from complex matrices. Instead, the use of raw materials is a cost-effective and environmentally friendly approach since, there is no need for energy demanding physical processes or the use of organic solvents.

Algal species are natural sources of functional ingredients, and both micro- and macroalgae biomasses have a wide repertoire of bioactive compounds [5; 6; 7; 8]. Algae are also advantageous, due to their natural biodiversity and availability as well as their ability to grow in different environments and to accumulate or secrete metabolites [9; 10]. Furthermore, several studies report beneficial effects on fish immune and oxidative response when algal biomasses are incorporated in fish feeds [11; 12; 13; 14].

In this study, micro- and macroalgae of economically relevant species were used for feed supplementation and were chosen due to their availability in middle to large scale production systems. The proposed microalgae species were *Tetraselmis striata* which shows a rich content of PUFA and thus, are widely used in aquaculture for feeding molluscs and enrichment of larvae feeds [15; 16]. Furthermore, *Tetraselmis* sp. are a great source of vitamins, carotenoids, chlorophylls and phenolic compounds, being proposed as a supplement in animal and human nutrition [17; 18]. *Phaeodactylum* sp. are particularly rich in eicosapentaenoic acid (EPA), but also carotenoids [19; 20; 21], have worldwide dispersion and have been widely used in aquaculture feeds. Finally, *Nannochloropsis* sp. which have a great content of EPA, vitamin E, pigments like chlorophyll a and carotenoids [16; 22]. In general, microalgae are also excellent sources of complex

carbohydrates which due to their biological properties have been increasingly studied for biomedical applications. In complex organisms, endogenously produced complex carbohydrates mediate a wide variety of events in cell–cell and cell–matrix interactions that are crucial to homeostasis and immune response [23; 24]. Microalgae species referred above show different types of complex polysaccharides such as β-glucans [17; 20; 25] and at least *Phaeodactylum* sp. and *Nannochloropsis* sp. present sulphated polysaccharides (SPs) [25; 26]. Selected macroalgae species were *Gracilaria* sp. and *Ulva rigida*, the first constitutes an abundant source of arachidonic acid and lipids (e.g. prostaglandins, steroids and cholesterol) which are key players in inflammation and phagocytosis [27] but also SPs such as carrageenan [28]. Finally, *Ulva rigida* presents cell wall abundancy in ulvan (SP) which is described to have antioxidant as well as macrophage-stimulating activities [29; 30].

Therefore, the present study aimed to evaluate the effects of short-term dietary supplementation with marine macro and microalgae biomasses in gilthead seabream health status and inflammatory response.

2.2 Material and Methods

2.2.1 Diet composition

The trial comprised six isonitrogenous (46% crude protein) and isolipidic (17% crude fat) dietary treatments (Table 1). A fishmeal-based (FM) practical diet was used as control (CTR), whereas 5 experimental diets based on CTR were further supplemented with a 2% inclusion of different algae crude biomasses: Diet Tetra (*Tetrasselmis striata*); Diet Phaeo (*Phaeodactylum* sp.); Diet Nanno (*Nannochloropsis* sp.); Diet Grac (*Gracilaria* sp.); Diet Ulva (*Ulva rigida*). Diets were manufactured by SPAROS. All powder ingredients were mixed according to the target formulation in a double-helix mixer (model RM90, MAINCA, Spain) and ground (below 200 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Subsequently, the oils were added to the mixtures, which were humidified with 20–25% water and agglomerated by a low-shear and low-temperature extrusion process (ITALPLAST, Italy). Extruded pellets (1.5 mm) were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). Diets were packed in sealed plastic buckets and shipped to the research site (CIIMAR, Matosinhos, Portugal) where they were stored at room temperature in a cool and aerated emplacement. Samples of each diet were taken for analytical characterization.

Table 1. Ingredients and proximate composition of experimental diets.

Ingredients %	**CTR**	**Tetra**	**Phaeo**	**Nanno**	**Grac**	**Ulva**
Fishmeal 60[1]	10.00	10.00	10.00	10.00	10.00	10.00
Feather meal[2]	5.00	5.00	5.00	5.00	5.00	5.00
Porcine blood meal[3]	2.00	2.00	2.00	2.00	2.00	2.00
Poultry meal 65[4]	25.00	25.00	25.00	25.00	25.00	25.00
Porcine gelatin[5]	2.00	2.00	2.00	2.00	2.00	2.00
Wheat gluten[6]	2.50	2.50	2.5	2.50	2.50	2.50
Corn gluten meal[7]	5.00	5.00	5.00	5.00	5.00	5.00
Soybean meal 48[8]	12.00	12.00	12.00	12.00	12.00	12.00
Sunflower meal[9]	5.00	5.00	5.00	5.00	5.00	5.00
Wheat meal[10]	12.90	10.90	10.90	10.90	10.90	10.90
Potato starch (gelatinized)[11]	5.00	5.00	5.00	5.00	5.00	5.00
Fish oil[12]	6.90	6.90	6.90	6.90	6.90	6.90
Rapeseed oil[13]	2.30	2.30	2.30	2.30	2.30	2.30
Palm oil[14]	2.00	2.00	2.00	2.00	2.00	2.00
Premix 1%[15]	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin E[16]	0.03	0.03	0.03	0.03	0.03	0.03
Binder[17]	0.50	0.50	0.50	0.50	0.50	0.50
Antioxidant powder[18]	0.20	0.20	0.20	0.20	0.20	0.20
Sodium propionate[19]	0.10	0.10	0.10	0.10	0.10	0.10
L-Lysine[20]	0.30	0.30	0.30	0.30	0.30	0.30
DL-Methionine[21]	0.17	0.17	0.17	0.17	0.17	0.17
L-Taurine[22]	0.10	0.10	0.10	0.10	0.10	0.10
Tetrasselmis striata[23]		2.00				
Phaeodactylum sp.[24]			2.00			
Nannochloropsis sp.[25]				2.00		
Gracilaria sp.[26]					2.00	
Ulva rigida[27]						2.000
Proximate composition						
Dry matter (DM) %	93.15	93.58	94.54	95.58	96.49	94.92
Ash, % DM	8.88	9.65	9.33	9.18	9.35	9.53
Crude Protein, % DM	49.03	48.96	50.58	49.79	49.35	49.17
Crude Fat, % DM	16.89	16.97	17.45	17.09	17.16	17.23
Gross Energy (KJ g^{-1} DM)	22.65	22.48	22.86	23.00	23.29	22.61

[1]59.2% CP, 9.9% CF, Conresa, Spain; [2]82.9% CP, 11.2% CF, SONAC BV, Netherlands; [3]89.1% CP, 0.4% CF, SONAC BV, Netherlands; [4]62.4% CP, 14.5% CF, Savinor UTS, Portugal; [5]Lapi Gelatins, Italy; [6]Vital: 80.4% CP, 5.8% CF, Roquette, France; [7]61.2% CP, 5.2% CF, COPAM, Portugal; [8]Dehulled solvent extracted: 47.4% CP, 2.6% CF, Cargill, Spain; [9]Solvent extracted: 29.1% CP, 1.8% CF, Ribeiro e Sousa Lda, Portugal; [10]11.7% CP, 1.6% CF, Molisur, Spain; [11]Pregeflo P100, 90% starch, Roquette, France; [12]98.1% CF (16% EPA; 12% DHA), Sopropêche, France; [13]98.2%, JC Coimbra, Portugal; [14]Henry Lamotte Oils GmbH, Germany; [15]Vitamin and mineral premix (Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat gluten), ADM Portugal S.A, Portugal; [16]ROVIMIX E50, DSM Nutritional Products, Switzerland; [17]CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal; [18]VERDILOX, Kemin Europe NV, Belgium; [19]Disproquímica, Portugal; [20]99% Lys, Ajinomoto EUROLYSINE S.A.S, France; [21]99% Met (Rhodimet NP99), ADISSEO, France; [22]98% Tau, ORFFA, Netherlands; [23]*Tetrasselmis striata* lyophilized biomass, Allmicroalgae, Portugal; [24]*Phaeodactylum* sp. lyophilized biomass, Necton, Portugal; [24]*Nannochloropsis* sp. lyophilized biomass, Necton, Portugal; [24]*Gracilaria* sp. lyophilized biomass, Algaplus, Portugal; [24]*Ulva rigida* lyophilized biomass, Algaplus, Portugal.

2.2.2 Bacterial growth and inoculum preparation

Photobacterium damselae subsp. *piscicida* (Phdp), strain 6.1, was used for the inflammatory insult. Bacteria were routinely cultured at 22 °C in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from Difco Laboratories, NJ, USA) supplemented with NaCl to a final concentration of 1% (w/v) (TSB-1 and TSA-1, respectively) and stored at -70 °C in TSB-1 supplemented with 15% (v/v) glycerol. To prepare the inoculum for injection into the fish peritoneal cavities, stocked bacteria were cultured for 48 h at 22 °C on TSA-1. Afterwards, exponentially growing bacteria were collected and resuspended in sterile HBSS and adjusted against its growth curve to $1x10^7$ colony forming units (cfu) ml^{-1}. Plating serial dilutions of the suspensions onto TSA-1 plates and counting the number of cfu following incubation at 22 °C confirmed bacterial concentration of the inocula. Bacteria were then killed by heat at 70 °C for 10 min. Loss of bacterial viability following heat exposure was confirmed by plating resulting cultures on TSA-1 plates and failing to see any bacterial growth.

2.2.3 Fish rearing conditions

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals at CIIMAR aquaculture and animal experimentation facilities in Matosinhos, Portugal. Seawater flow was kept at 4 L min^{-1} (mean temperature 22.4 ± 1 °C; mean salinity 35.2 ± 0.7 ‰) in a recirculation system with aeration (mean dissolved oxygen above 6 mg L^{-1}). Water quality parameters were monitored daily and adjusted when necessary. Mortality was monitored daily. Diets were randomly assigned to triplicate groups of 110 fish/tank (IBW:11.7±1.0 g) that were fed to satiation three times a day for 2 weeks starting at 1.5% biomass.

2.2.4 Experimental procedures

After 1 and 2 weeks of feeding 12 fish/treatment were weighed and sampled for tissues (blood, head-kidney, liver and gut), after sacrifice with a 2-phenoxyethanol lethal dose (0.5 mL L^{-1}) [31]. Blood was collected from the caudal vein using heparinized syringes and centrifuged at 10,000 *g* for 10 min at 4 ºC to obtain plasma samples. Plasma and tissue samples were immediately frozen in liquid nitrogen and stored at -80 ºC until further analysis. At 1 and 2 weeks, 30 fish per treatment were subjected to an

inflammatory insult by intraperitoneal (i.p.) injection of heat inactivated *Phdp* (see section 2.2) and transferred to a similar recirculation system in duplicates. 6 fish/treatment were sampled as described above after 4 and 24 h post-injection.

2.2.5 Haematological procedures

The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts. For determination of WBC and RBC concentration, whole blood was diluted 1/20 and 1/200 respectively, in HBSS with heparin (30 U mL^{-1}) and cell counts were done in a Neubauer chamber. Blood smears were prepared from peripheral blood, air dried and stained with Wright's stain (Haemacolor; Merck, Darmstadt, Germany) after fixation for 1 minute with formol–ethanol (10 % formaldehyde in ethanol). Neutrophils were labelled through the detection of peroxidase activity revealed by the Antonow's technique described in Afonso *et al.* [32]. The slides were examined under oil immersion (1000x), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The relative percentage and absolute value ($\times 10^4$ mL^{-1}) of each cell type was calculated.

2.2.6 Innate humoral parameters

2.2.6.1 Bactericidal activity

Plasma bactericidal activity was determined following the method described by Machado *et al.* [33] with some modifications. *Edwardsiella tarda* (*E. tarda*) strain ACC 53.1, gently provided by Prof. Alicia Toranzo (University of Santiago, Spain) was used in the protocol. Briefly, 20 µL of plasma were mixed with 20 µL of bacteria suspension (10^8 CFU mL^{-1}) in duplicate in a flat-bottom 96-well plate, that was incubated for 2.5 h at 25 ºC (positive control: 20 µL of TSB instead of plasma). Afterwards, 25 µL of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg mL^{-1}; Sigma, St. Louis, MO, USA) were added to each well and incubated for 10 min at 25 ºC to allow the formation of formazan precipitates. Plates were then centrifuged at 2,000 *g* for 10 min the supernatant was discarded and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (Sigma, St. Louis, MO, USA). The absorbance was then measured at 560 nm in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Bactericidal activity is expressed as percentage, calculated from the difference between surviving bacteria compared to the number of bacteria from positive controls (100 %).

2.2.6.2 Antiprotease activity

The antiprotease activity was determined as described by Ellis *et al.* [34] with some modifications. Briefly, 10 µL of plasma were incubated with the same volume of trypsin solution (5 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) for 10 min at 22 °C. After incubation, 100 µL of phosphate buffer (NaH_2PO_4, 13.9 mg mL^{-1}, pH 7.0) and 125 µL of azocasein solution (20 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) were added and incubated for 1 h at 22 °C. Finally, 250 µL of trichloroacetic acid were added to the reaction mixture and incubated for 30 min at 22 °C. The mixture was centrifuged at 10,000 *g* for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred to a 96 well-plate and mixed with 100 µL of NaOH (40 mg mL^{-1}). The OD was read at 450 nm in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Phosphate buffer instead of plasma and trypsin served as blank, whereas the reference sample was phosphate buffer instead of plasma. Sample inhibition percentage of trypsin activity was calculated as follows: 100 – ((sample absorbance/Reference absorbance) x 100). All analyses were conducted in duplicates.

2.2.6.3 Peroxidase activity

Total peroxidase activity in plasma and intestine was measured following the procedure described by Quade and Roth [35]. Briefly, 10 µL of plasma and 5 µL of intestine homogenate were diluted with 140 and 145 µL, respectively, of HBSS without Ca^{2+} and Mg^{2+} in 96-well plates. Then, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma, St. Louis, MO, USA) and 50 µL of 5 mM H_2O_2 were added to the wells. The reaction was stopped after 2 min by adding 50 µL of H_2SO_4 (2M) and the optical density (OD) was read at 450 nm in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Wells without plasma or mucus were used as blanks. The peroxidase activity (units mL^{-1} tissue) was determined defining that one unit of peroxidase produces an absorbance change of 1 OD.

2.2.6.4 Nitric oxide (NO) production

NO production was measured in plasma (1:10 sample dilution) and intestine (1:5 sample dilution) samples. Total nitrite and nitrate concentrations in the sample were assessed using the Nitrite/Nitrate colorimetric method kit (Roche, Basel, Switzerland) adapted to microplates. Nitrite concentration was calculated by comparison with a sodium nitrite standard curve. Since nitrite and nitrate are endogenously produced as

oxidative metabolites of the NO molecule, these compounds are considered as indicative of NO production.

2.2.7 Analysis of oxidative stress biomarkers

Liver and intestine samples were homogenized (1:10) in phosphate buffer 0.1 M (pH 7.4) using Precellys evolution tissue lyser homogenizer.

2.2.7.1 Lipid peroxidation (LPO)

One aliquot of tissue homogenate was used to determine the extent of endogenous LPO by measuring thiobarbituric acid-reactive species (TBARS) as suggested by Bird and Draper [(36)]. To prevent artifactual lipid peroxidation, butylhydroxytoluene (BHT 0.2 mM) was added to the aliquot. Briefly, 1 ml of 100% trichloroacetic acid and 1 ml of 0.73% thiobarbituric acid solution (in Tris–HCl 60 mM pH 7.4 with DTPA 0.1 mM) were added to 0.2 ml of liver/intestine homogenate. After incubation at 100 °C during for 60 min, the solution was centrifuged at 12,000 *g* for 5 min and LPO levels were determined at 535 nm.

2.2.7.2 Total protein quantification

The remaining tissue homogenate was centrifuged for 20 min at 12,000 *g* (4 °C) to obtain the post-mitochondrial supernatant fraction (PMS). Total proteins in homogenates were measured by using Pierce™ BCA Protein Assay Kit, as described by the manufacturer.

2.2.7.3 Catalase (CAT)

CAT activity was determined in PMS by measuring substrate (H_2O_2) consumption at 240 nm according to Claiborne [(37)] adapted to microplate. Briefly, in a microplate well, 0.140 ml of phosphate buffer (0.05 M pH 7.0) and 0.150 ml H_2O_2 solution (30 mM in phosphate buffer 0.05 M pH 7.0) were added to 0.01 ml of liver/intestine PMS (0.7 mg ml^{-1} total protein). Enzymatic activity was determined in a microplate reader (BioTek Synergy HT, Winooski, VT, USA) reading the optical density at 240 nm for 2 min every 15 sec interval.

2.2.7.4 Superoxide dismutase (SOD)

SOD activity was measured according to Flohé and Otting [38] adapted to microplate by Lima *et al.* [39]. Briefly, in a microplate well, 0.2 ml of the reaction solution [1 part xantine solution 0.7 mM (in NaOH 1 mM) and 10 parts cytochrome c solution 0.03 mM (in phosphate buffer 50 mM pH 7.8 with 1 mM Na-EDTA)] was added to 0.05 ml of liver/intestine PMS (0.25 mg ml^{-1} total protein). Optical density was measured at 550 nm in a microplate reader (BioTek Synergy HT, Winooski, VT, USA) every 20 s interval for 3 min at 25° C.

2.2.7.5 Total glutathione (tGSH)

Total glutathione content in liver homogenate samples was measured based on the oxidation of glutathione by 5,5′- dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma) as described by Baker *et al.* [40] and Rodrigues *et al.* [41]. Samples were diluted in K-phosphate buffer (0.1 M pH 7.4) in order to obtain 0.7 mg mL^{-1} protein. Later, 50 µL of each sample was added to the microplate wells, followed by addition of 250 µL of a reaction solution, composed of DTNB, K-phosphate buffer (0.1 M, pH 7.4), NADPH (ß-nicotanimide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; Alfa Aesar), and glutathione reductase (Sigma, St. Louis, MO, USA). Absorbance was read at 412 nm for 3 min (1 read every 20-sec interval) in a Synergy HT microplate reader (Biotek, Winooski, VT, USA) and results were expressed as nm mg^{-1} protein.

2.2.8 Gene expression

RNA isolation from target tissue (Head-kidney) and cDNA synthesis were conducted with NZY Total RNA Isolation kit and NZY first strand cDNA synthesis kit (NZYTech, Lisbon, Portugal) following manufacturer's specifications. Real-time quantitative PCR was carried out on a CFX384 Touch Real-Time PCR system (Bio-Rad, CA, USA). Genes comprised in the assay were selected for their involvement in head-kidney health and immunity (Table 2). Specific primer pair sequences are listed in Table S1a (Appendix I). Controls of general PCR performance were included on each array. Briefly, RT reactions were diluted to obtain the equivalent concentration of 20 ng of total input RNA which were used in a 10 µL volume for each PCR reaction. PCR wells contained a 2x SYBR Green Master Mix (Bio-Rad, CA, USA) and specific primers were used to obtain amplicons 50–250 bp in length. The program used for PCR amplification included an

initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95°C denaturation for 15 s, primer annealing and extension temperature (Appendix I, Table S1a) for 1 min. The efficiency of PCR reactions was always higher than 90%, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C). Fluorescence data acquired during the PCR extension phase were normalized using the Pfaffl [(42)] method. The geometric mean of two carefully selected housekeeping genes (elongation factor 1-α (*ef1α*) and ribosomal protein S18 (*rps18*)) was used as the normalization factor to normalize the expression of target genes. For comparing the mRNA expression level of each gene in a given dietary treatment, all data values were in reference to the expression level of 0h CTRL fish.

Table 2. PCR-array layout for gene expression profiling of head-kidney in sea bream.

Function	**Gene**	**Symbol**	**Accession number**
Cytokines	Transforming growth factor-β1	*tgf-β1*	AF424703.1
	Interleukin 1 beta	*il1b*	AJ277166.2
	Interleukin 10	*il10*	XM_030418889.1
Cell markers	Macrophage colony-stimulating factor 1 receptor	*csf1r*	AM050293
	Non cytotoxic cell receptor protein	*nccrp1*	AY651258.1
Apoptosis	Caspase 1	*casp1*	AM490060
Pattern recognition receptor	Toll like receptor 1	*tlr1*	KF857322
Antimicrobial defence/ Iron recycling	β-defensin	*β-defensin*	FM158209
	Hepcidin	*hepc*	EF625901
Stress resistance	Heat-shock protein 70	*hsp70*	DQ524995.1
Reference genes	Elongation factor 1α	*ef1α*	AF184170
	Ribosomal protein S18	*rps18*	AM490061

2.2.9 Data analysis

All results are expressed as mean ± standard error (mean ± SE). Residuals were tested for normality (Shapiro-Wilk´s test) and homogeneity of variance (Levene's test). When residuals did not meet the assumptions, data was transformed by a Log_{10} or square root transformation. For gene expression data, a log_2 transformation was applied to all expression values. Two-way ANOVAs were performed, with "dietary treatment and time" as the fixed effects, followed by Tukey post-hoc tests. All statistical analyses were performed using the computer package SPSS 26 for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests.

2.3 Results

To examine the influence that algal biomasses supplementation may have on the initial inflammatory response following an inflammatory stimulus (inactivated *Phdp* i.p. injection), samples of blood, gut, liver and head kidney were collected at 4 h and 24 h post-injection. Sampling points at 1 and 2 weeks were used as time 0 h during time-course data analysis, as they represent the unstimulated animals prior to inflammatory stimulus. From this point forward, stimulus after 1 week of feeding will be time-course 1 and after 2 weeks, time-course 2.

2.3.1 Peripheral leucocyte responses

Time-course 1 total RBC concentration was higher at 24 h regardless of the dietary treatment (Table 3). Furthermore, at time-course 2, RBC numbers were significantly higher after 4 h remaining high at 24 h with no observed effect of algal supplementation, since no differences were observed between the experimental groups (Table 4). Circulating WBC numbers decreased at time-course 1 after 24 h of the inflammatory stimulus (Table 3), again with no observed effect of algal supplementation, while at time-course 2 there were no significant differences (Table 4). However, when evaluating different leucocyte types, differences were observed. Resulting in a significant neutrophil concentration increase at 4 h post-injection at both time-course 1 and 2 with a concomitant lymphocyte decrease (Fig. 1). Furthermore, at 4 h in time-course 1, fish fed Ulva diet show a significant neutrophil concentration increase when compared with all algal supplemented groups and CTR fed fish (Fig. 1A). However, at the same sampling point but in time-course 2 all algal diets are equal to CTR in neutrophil concentration, nonetheless, all algae supplemented diets show higher lymphocyte concentration than CTR (Fig. 1B). After 24 h there is a slight recovery of circulating lymphocyte numbers as well as a sharp neutrophil decrease at both time-course 1 and 2 with no observed effect of algal supplementation (Fig. 1). Thrombocyte and monocyte concentration were not affected by the different dietary treatments. Still, circulating thrombocyte concentration decreases at 4 h (time-course 1) and monocyte concentration increased from 0 to 4 h remaining high after 24 h at both time-course 1 and 2 (Tables 3 and 4).

Table 3. Absolute values of total red and white blood cells, and peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) in gilthead seabream juveniles after time-course 1. Data are the mean ± SEM (n = 6).

		Haematology					
1 week	**Diets**	*RBC (10^6 µL^{-1})*	*WBC (10^4 µL^{-1})*	*Thrombocytes (10^4 µL^{-1})*	*Lymphocytes (10^4 µL^{-1})*	*Monocytes (10^4 µL^{-1})*	*Neutrophils (10^4 µL^{-1})*
0h	**CTR**	1.55 ± 0.06	3.50 ± 0.29	1.77 ± 0.25	1.55 ± 0.17	0.05 ± 0.02	0.10* ± 0.03
	Tetra	1.36 ± 0.10	3.48 ± 0.18	1.89 ± 0.19	1.49 ± 0.18	0.05 ± 0.01	0.05* ± 0.01
	Phaeo	1.65 ± 0.10	3.25 ± 0.24	1.98 ± 0.20	1.10 ± 0.07	0.06 ± 0.02	0.07* ± 0.02
	Nanno	1.47 ± 0.12	3.53 ± 0.20	1.84 ± 0.22	1.60 ± 0.08	0.04 ± 0.02	0.05* ± 0.01
	Grac	1.71 ± 0.08	3.52 ± 0.18	1.61 ± 0.19	1.80 ± 0.18	0.07 ± 0.01	0.03* ± 0.01
	Ulva	1.60 ± 0.12	3.98 ± 0.29	1.75 ± 0.25	1.71 ± 0.08	0.05 ± 0.01	0.14* ± 0.03
4h	**CTR**	1.70 ± 0.16	3.32 ± 0.32	1.19 ± 0.18	0.20 ± 0.05	0.09 ± 0.02	1.46$^{b\#}$ ± 0.16
	Tetra	1.67 ± 0.14	4.17 ± 0.44	1.06 ± 0.06	0.10 ± 0.03	0.12 ± 0.03	1.66$^{b\#}$ ± 0.24
	Phaeo	1.74 ± 0.11	3.87 ± 0.45	0.92 ± 0.10	0.12 ± 0.03	0.10 ± 0.02	2.10$^{b\#}$ ± 0.18
	Nanno	1.62 ± 0.09	3.67 ± 0.29	1.44 ± 0.24	0.14 ± 0.02	0.15 ± 0.03	2.24$^{b\#}$ ± 0.20
	Grac	1.63 ± 0.12	4.18 ± 0.51	1.56 ± 0.30	0.17 ± 0.02	0.12 ± 0.03	2.40$^{b\#}$ ± 0.26
	Ulva	1.54 ± 0.16	3.35 ± 0.33	1.03 ± 0.21	0.17 ± 0.02	0.13 ± 0.02	3.76$^{a\#}$ ± 0.13
24h	**CTR**	1.87 ± 0.10	2.53 ± 0.23	1.50 ± 0.33	0.27 ± 0.04	0.11 ± 0.02	0.80$^{\#}$ ± 0.11
	Tetra	2.13 ± 0.13	2.48 ± 0.41	1.53 ± 0.16	0.30 ± 0.02	0.09 ± 0.02	0.72* ± 0.15
	Phaeo	1.92 ± 0.25	3.03 ± 0.34	1.54 ± 0.21	0.16 ± 0.02	0.08 ± 0.04	0.41* ± 0.14
	Nanno	1.98 ± 0.11	2.62 ± 0.46	1.61 ± 0.19	0.20 ± 0.04	0.10 ± 0.01	0.50* ± 0.10
	Grac	1.80 ± 0.10	2.57 ± 0.28	1.69 ± 0.12	0.19 ± 0.04	0.15 ± 0.03	0.44* ± 0.10
	Ulva	1.80 ± 0.08	2.48 ± 0.31	1.98 ± 0.29	0.26 ± 0.03	0.14 ± 0.02	0.46* ± 0.08
Two way-ANOVA		*RBC (10^6 µL^{-1})*	*WBC (10^4 µL^{-1})*	*Thrombocytes (10^4 µL^{-1})*	*Lymphocytes (10^4 µL^{-1})*	*Monocytes (10^4 µL^{-1})*	*Neutrophils (10^4 µL^{-1})*
Sig.	***Time***	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Diet	ns	ns	ns	0.008	ns	0.02
	Time*Diet	ns	ns	ns	ns	ns	<0.001
Diet	**CTR**	-	-	-	A	-	-
	Tetra	-	-	-	AB	-	-
	Phaeo	-	-	-	B	-	-
	Nanno	-	-	-	AB	-	-
	Grac	-	-	-	AB	-	-
	Ulva	-	-	-	A	-	-
Time	***0h***	B	A	A	A	B	-
	4h	B	A	B	C	A	-
	24h	A	B	A	B	A	-

Different superscript letters represent significant differences between diets within the same time ($p < 0.05$). Different superscript symbols represent significant differences in time within the same diet ($p < 0.05$). Different capital letters represent significant differences between diets regardless of time ($p < 0.05$).

Table 4. Absolute values of total red and white blood cells, and peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) in gilthead seabream juveniles after time-course 2. Data are the mean ± SEM (n = 6).

		Haematology					
2 weeks	**Diets**	*RBC ($10^6 \mu L^{-1}$)*	*WBC ($10^4 \mu L^{-1}$)*	*Thrombocytes ($10^4 \mu L^{-1}$)*	*Lymphocytes ($10^4 \mu L^{-1}$)*	*Monocytes ($10^4 \mu L^{-1}$)*	*Neutrophils ($10^4 \mu L^{-1}$)*
0h	**CTR**	1.57±0.10	3.52±0.18	2.16$^{\#*}$±0.17	1.25$^{\#}$±0.14	0.04±0.01	0.06* ± 0.01
	Tetra	1.41±0.07	3.20±0.18	2.06±0.12	0.98$^{\#}$±0.08	0.05±0.01	0.06* ± 0.01
	Phaeo	1.42±0.09	3.67±0.26	1.97±0.34	1.33$^{\#}$±0.21	0.05±0.01	0.07* ± 0.01
	Nanno	1.50±0.15	3.40±0.32	2.01±0.15	1.21$^{\#}$±0.16	0.08±0.03	0.09* ± 0.02
	Grac	1.38±0.07	3.55±0.24	2.10±0.19	1.30$^{\#}$±0.14	0.06±0.01	0.08* ± 0.02
	Ulva	1.27±0.10	3.82±0.21	2.40±0.37	1.20$^{\#}$±0.16	0.08±0.02	0.08* ± 0.01
4h	**CTR**	1.72±0.22	3.85±0.36	1.65*±0.20	0.10^{b*}±0.02	0.10±0.03	1.23$^{ab\#}$± 0.22
	Tetra	1.65±0.08	3.98±0.24	1.79±0.20	0.22^{ab*}±0.03	0.08±0.02	1.28$^{ab\#}$± 0.19
	Phaeo	1.49±0.07	4.05±0.24	2.59±0.15	0.26^{a*}±0.04	0.11±0.04	0.78$^{b\#}$± 0.07
	Nanno	1.60±0.13	3.72±0.41	2.35±0.34	0.30^{a*}±0.04	0.09±0.03	1.69$^{a\#}$± 0.26
	Grac	1.59±0.13	3.97±0.30	2.54±0.22	0.35^{a*}±0.04	0.16±0.03	1.11$^{ab\#}$± 0.08
	Ulva	1.51±0.09	3.92±0.14	2.33±0.15	0.51^{a*}±0.09	0.19±0.04	1.45$^{ab\#}$± 0.16
24h	**CTR**	1.71±0.10	3.72±0.30	2.96$^{\#}$±0.34	0.55$^{\#}$±0.12	0.14±0.05	0.37$^{\$}$±0.10
	Tetra	1.51±0.09	3.83±0.24	2.96±0.18	0.50*±0.11	0.14±0.03	0.49$^{\$}$± 0.08
	Phaeo	1.55±0.08	4.02±0.25	2.19±0.11	0.43*±0.11	0.15±0.03	0.33$^{\#*}$± 0.07
	Nanno	1.66±0.12	3.92±0.47	2.14±0.11	0.42*±0.07	0.13±0.02	0.38*± 0.07
	Grac	1.64±0.08	4.07±0.17	2.46±0.26	0.40*±0.06	0.22±0.02	0.57$^{\#}$± 0.10
	Ulva	1.68±0.12	3.10±0.21	2.92±0.17	0.54*±0.05	0.09±0.02	0.29*± 0.04
Two way-ANOVA		*RBC ($10^6 \mu L^{-1}$)*	*WBC ($10^4 \mu L^{-1}$)*	*Thrombocytes ($10^4 \mu L^{-1}$)*	*Lymphocytes ($10^4 \mu L^{-1}$)*	*Monocytes ($10^4 \mu L^{-1}$)*	*Neutrophils ($10^4 \mu L^{-1}$)*
Sig.	***Time***	0.002	ns	0.002	<0.001	<0.001	<0.001
	Diet	ns	ns	ns	0.008	ns	ns
	Time*Diet	ns	ns	0.020	<0.001	ns	0.049
Diet	**CTR**	-	-	-	-	-	-
	Tetra	-	-	-	-	-	-
	Phaeo	-	-	-	-	-	-
	Nanno	-	-	-	-	-	-
	Grac	-	-	-	-	-	-
	Ulva	-	-	-	-	-	-
Time	***0h***	B	-	-	-	B	-
	4h	A	-	-	-	A	-
	24h	A	-	-	-	A	-

Different superscript letters represent significant differences between diets within the same time ($p < 0.05$). Different superscript symbols represent significant differences in time within the same diet ($p < 0.05$). Different capital letters represent significant differences between diets regardless of time ($p < 0.05$).

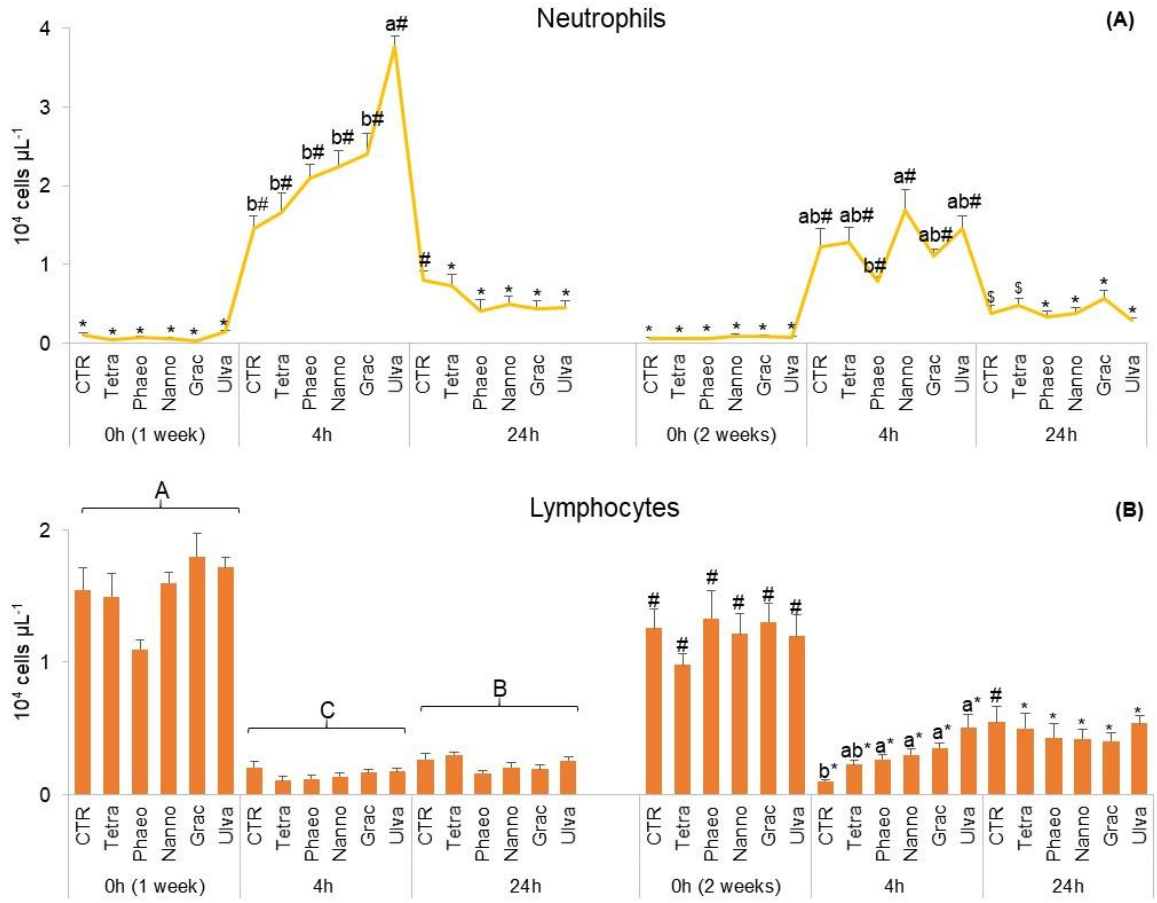

Figure 1. Absolute values of circulating lymphocytes and neutrophils in gilthead seabream after time-course 1 and 2. Data are the mean ± SEM (n = 6). Different lowercase letters represent significant differences between diets within the same time ($p < 0.05$). Different symbols represent significant differences in time within the same diet ($p < 0.05$). Different capital letters represent significant differences between diets regardless of time ($p < 0.05$).

2.3.2 Plasma humoral parameters

Plasma humoral immune parameters varied significantly throughout both time-courses (Fig. 2). Antiprotease activity decreased significantly at 4 and 24 h after stimulus at both time-course 1 and 2. Furthermore, in time-course 1, Ulva fed fish show overall lower antiprotease activity when compared to Tetra, Phaeo and Grac fed fish. Bactericidal activity increased in time-course 2 at 4 h, decreasing at 24 h but, still remaining higher than prior to stimulus. Peroxidase activity decreased at 24 h in time-course 1, while in time-course 2 this same biomarker followed an opposite trend increasing at 24 h. Finally, in time-course 1 at 0 h, Phaeo fed fish show NO production than CTR, while in time-course 2, NO production increases at 24 h regardless of the dietary treatment.

2.3.3 Gut innate immune and oxidative stress biomarkers

Intestinal peroxidase activity decreased at 24 h in time-course 1, while in time-course 2 this same biomarker followed an opposite trend increasing at 24 h. NO production followed a similar trend in both time-courses, decreasing at 4 h and 24 h regardless of the dietary treatment (Fig. 3).

Oxidative stress biomarkers show, significant differences in time regardless of the dietary treatment (Fig. 4). LPO increases at 4 h and 24 h after stimulus in both time-courses. SOD activity decreases after 4 h, followed by an increase at 24 h in both time-course 1 and 2. While, CAT activity decreases after 4 h in time-course 1 and after 24 h in time-course 2.

2.3.4 Liver oxidative stress biomarkers

Hepatic oxidative stress biomarkers showed significant differences between different sampling points regardless of the dietary treatments (Fig. 5), namely, LPO which decreases after 4 h post-stimulus returning to pre-stimulus values after 24 h in both time-courses. Total GSH (tGSH) decreased significantly at 24 h at both time-course 1 and 2. Regarding enzymatic activities, SOD decreased in time-course 1 after 24 h, however, in time-course 2 there is a sharp activity increase after 4 h followed by a decrease at 24 h. Additionally, in time-course 1, 24 h after stimulus Nanno fed fish show higher CAT activity than Tetra (Fig. 5C). While, in time-course 2, CAT activity decreases at 24 h regardless of the dietary treatment.

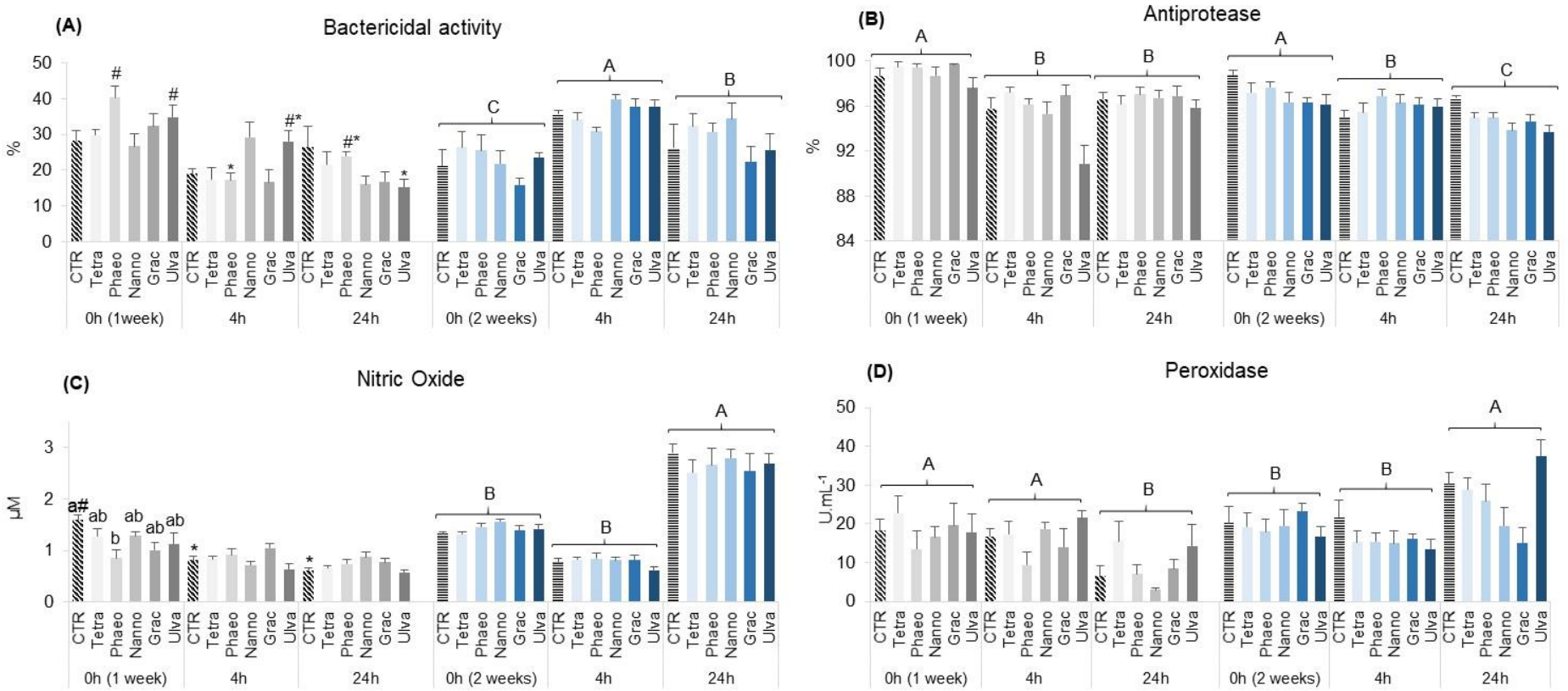

Figure 2. Plasma immune parameters of gilthead seabream juveniles after time-courses 1 and 2; (A) Bactericidal activity; (B) Antiprotease activity; (C) Nitric oxide; (D) Peroxidase activity. Data are the mean ± SEM (n = 9). Different lowercase letters represent significant differences between diets within the same time ($p < 0.05$). Different symbols represent significant differences in time within the same diet ($p < 0.05$). Different capital letters represent significant differences in time regardless of diet ($p < 0.05$).

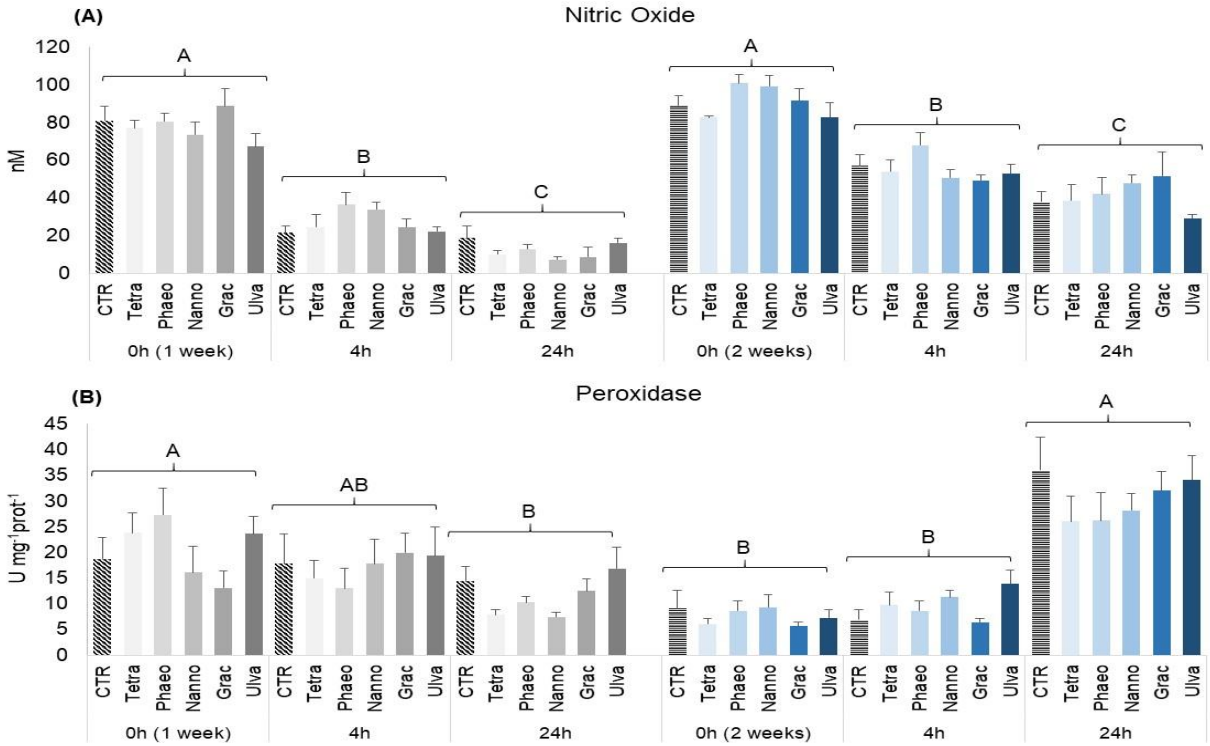

Figure 3. Gut immune parameters of gilthead seabream juveniles after time-courses 1 and 2; (A) Nitric oxide; (B) Peroxidase activity. Data are the mean ± SEM (n = 9). Different capital letters represent significant differences in time regardless of diet ($p < 0.05$).

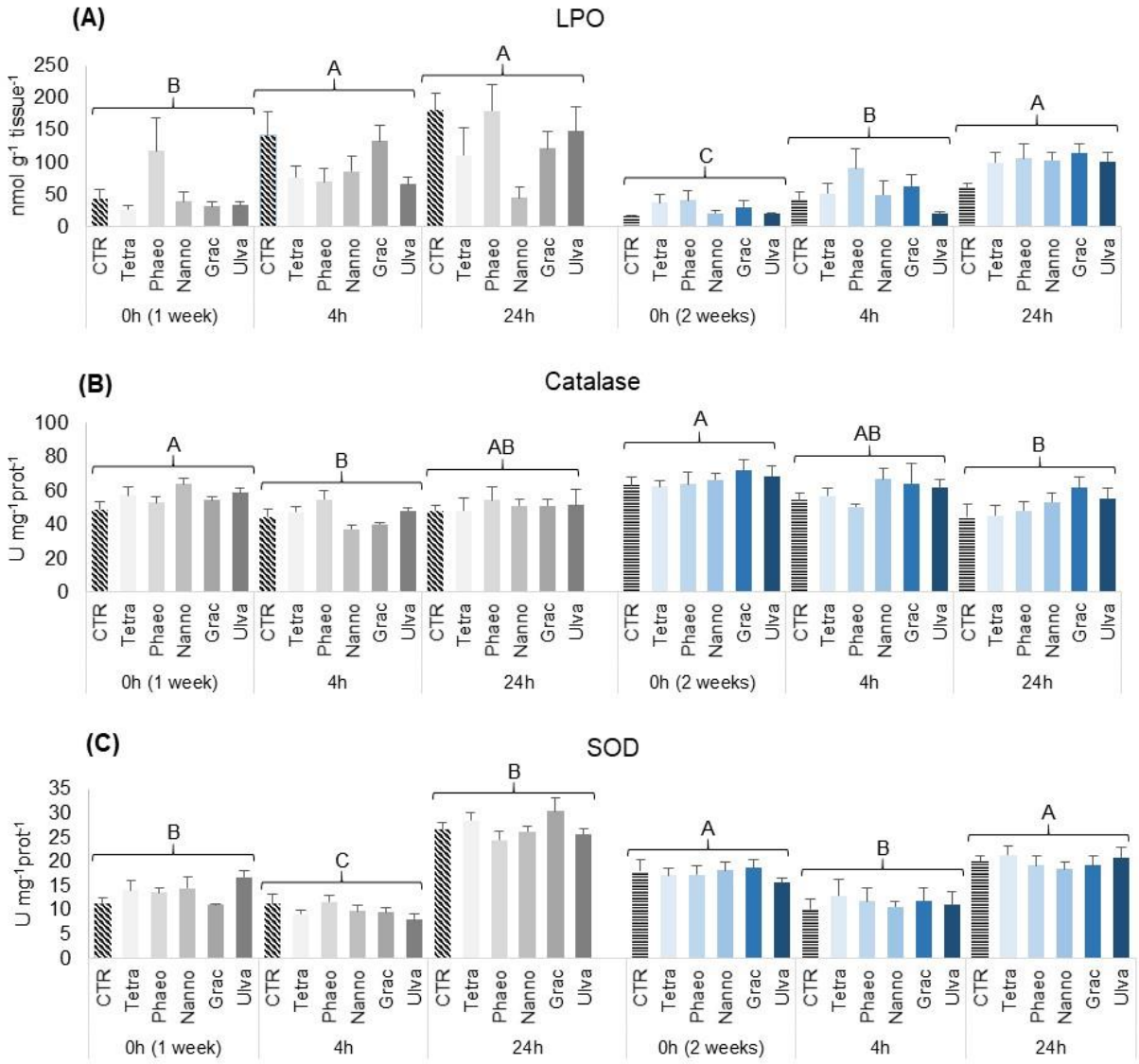

Figure 4. Gut oxidative stress parameters of gilthead seabream juveniles after time-courses 1 and 2; (A) Lipid peroxidation (LPO); (B) Catalase activity; (C) Superoxide dismutase activity (SOD). Data are the mean ± SEM (n = 9). Different capital letters represent significant differences in time regardless of diet ($p < 0.05$).

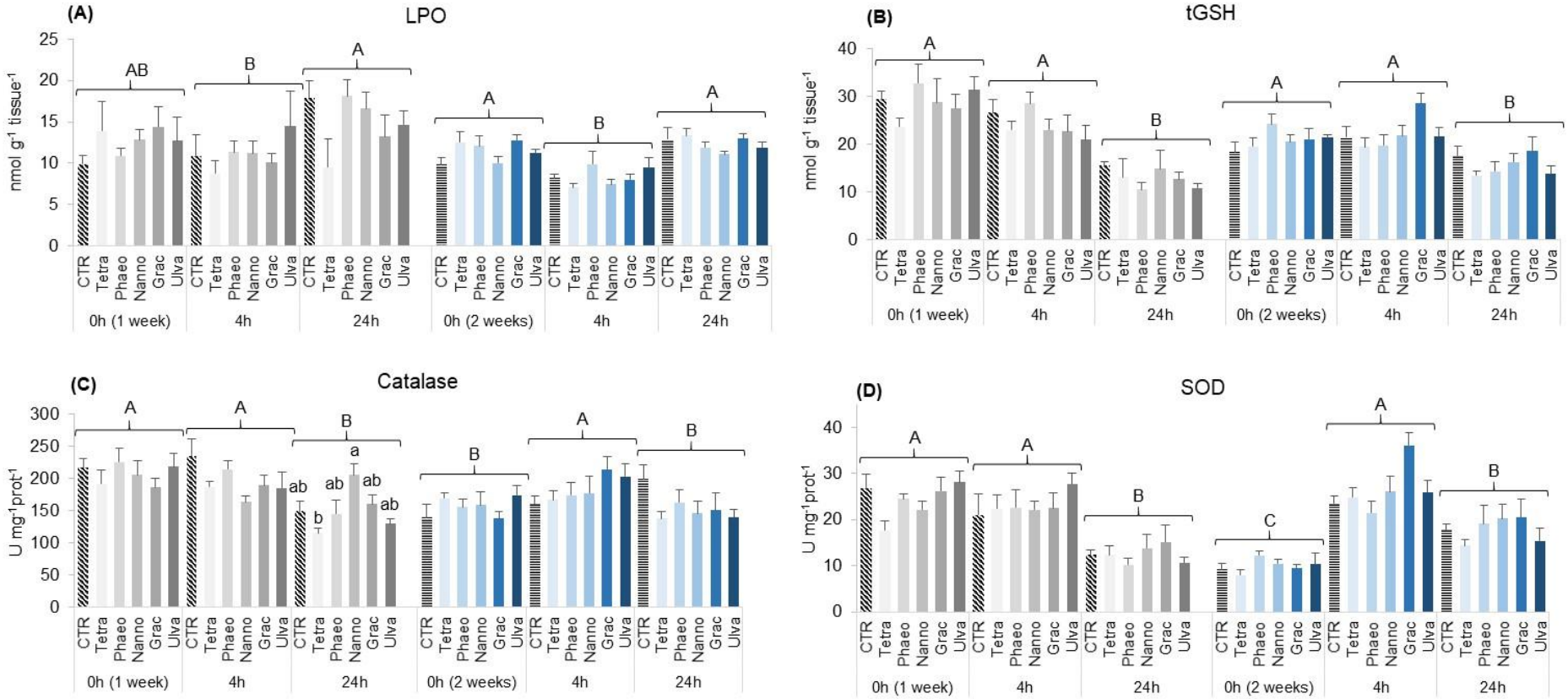

Figure 5. Liver oxidative stress parameters of gilthead seabream juveniles after time-courses 1 and 2; (A) Lipid peroxidation (LPO); (B) Total glutathione (tGSH); (C) Catalase activity; (D) Superoxide dismutase activity (SOD). Data are the mean ± SEM (n = 9). Different lowercase letters represent significant differences between diets within the same time ($p < 0.05$). Different capital letters represent significant differences in time regardless of diet ($p < 0.05$).

2.3.5 Head-kidney gene expression analysis

To evaluate the expression of immune, stress and microbial recognition related genes in the inflammatory response (Tables 5 and 6), cDNA was isolated from fish head kidney. In response to the inflammatory insult, il1β expression levels increased from 0 h to 4 h in time-course 1. Gene up-regulation was also observed at 4 h for *casp1*, *β-defensin*, *il10* and *tlr1*, while, *hepcidin*, *csf1r* and *nccrp1* were down-regulated. At 24 h with the exception of *nccrp1*, genes are down-regulated (Table 5). In time-course 2, *hsp70*, *tlr1*, *il10*, *caspase1* and *β-defensin* are up-regulated at 4 h, and *nccrp1* and *csf1r* at 24h. *il1β* was down-regulated at 24 h (Table 6). *tgf-β1* gene is up-regulated at 24h except for Grac fed fish.

Table 5. Relative gene expression profiling of anterior intestine in gilthead seabream juveniles fed experimental diets in time-course 1. Data are the mean ± SEM (n=6). All data values for each gene were in reference to the expression level of 0h CTRL fish. Different symbols represent significant differences in time within the same diet ($p < 0.05$). Different capital letters represent significant differences between diets regardless of time ($p < 0.05$).

1 week	Diets	*il1-β*	*il-10*	*tgf-β1*	*caspase 1*	*nccrp1*	*csfr1*	*β-defensin*	*hepcidin*	*hsp70*	*tlr1*
0h	**CTR**	1.29 ± 0.48	1.25 ± 0.34	1.24 ± 0.43	1.42 ± 0.43	1.21 ± 0.31	2.03 ± 1.34	1.64 ± 0.88	1.10 ± 0.19	1.14 ± 0.28	1.11 ± 0.21
	Tetra	0.86 ± 0.16	0.82 ± 0.15	0.72 ± 0.16	1.51 ± 0.33	1.20 ± 0.39	0.66 ± 0.09	1.96 ± 1.05	2.75 ± 1.13	0.88 ± 0.10	0.56 ± 0.17
	Phaeo	0.80 ± 0.37	1.44 ± 0.35	1.03 ± 0.25	1.74 ± 0.56	1.50 ± 0.36	0.96 ± 0.18	1.28 ± 0.73	1.78 ± 0.66	1.49 ± 0.32	1.24 ± 0.25
	Nanno	1.41 ± 0.49	0.64 ± 0.11	0.63 ± 0.11	1.89 ± 0.27	0.75 ± 0.18	1.11 ± 0.33	2.67 ± 1.56	1.18 ± 0.35	1.10 ± 0.13	0.71 ± 0.13
	Grac	0.90 ± 0.35	0.91 ± 0.23	0.92 ± 0.19	2.20 ± 0.47	0.95 ± 0.22	0.51 ± 0.10	1.37 ± 0.30	0.92 ± 0.29	1.10 ± 0.10	0.68 ± 0.10
	Ulva	1.44 ± 0.30	0.66 ± 0.18	0.82 ± 0.11	2.45 ± 0.81	0.74 ± 0.10	0.87 ± 0.19	0.91 ± 0.26	1.04 ± 0.23	0.99 ± 0.10	0.65 ± 0.21
4h	**CTR**	15.64 ± 6.19	3.98 ± 0.88	0.52 ± 0.16	2.61 ± 0.54	0.55 ± 0.12	0.46 ± 0.10	2.17 ± 0.63	0.78 ± 0.17	0.82 ± 0.14	2.34 ± 0.60
	Tetra	14.82 ± 5.11	6.46 ± 1.92	0.84 ± 0.10	3.14 ± 0.42	0.57 ± 0.15	0.80 ± 0.24	6.39 ± 4.55	0.49 ± 0.13	1.09 ± 0.24	2.55 ± 0.29
	Phaeo	14.57 ± 10.38	2.87 ± 1.03	0.48 ± 0.15	1.83 ± 0.29	0.32 ± 0.03	0.24 ± 0.07	3.50 ± 1.28	1.11 ± 0.39	0.67 ± 0.12	1.06 ± 0.19
	Nanno	14.39 ± 7.38	5.40 ± 1.27	0.79 ± 0.25	3.42 ± 0.73	0.55 ± 0.10	0.58 ± 0.14	10.83 ± 7.78	0.38 ± 0.07	1.17 ± 0.35	2.36 ± 0.55
	Grac	11.86 ± 2.53	3.47 ± 0.74	0.71 ± 0.32	2.42 ± 0.79	0.38 ± 0.08	0.52 ± 0.25	1.34 ± 0.40	0.53 ± 0.24	0.64 ± 0.06	2.06 ± 0.71
	Ulva	10.39 ± 4.66	6.74 ± 2.33	1.00 ± 0.48	3.49 ± 1.11	0.38 ± 0.10	0.45 ± 0.11	23.15 ± 12.69	0.41 ± 0.03	1.28 ± 0.25	2.91 ± 0.84
24h	**CTR**	6.69 ± 2.95	1.27 ± 0.29	0.41 ± 0.18	2.56 ± 0.36	1.43 ± 0.35	0.60 ± 0.20	2.16 ± 0.80	0.31 ± 0.08	1.53 ± 0.42	1.02 ± 0.24
	Tetra	6.64 ± 3.63	0.61 ± 0.07	0.36 ± 0.11	1.89 ± 0.34	1.69 ± 0.54	0.40 ± 0.11	4.35 ± 1.74	0.87 ± 0.35	0.95 ± 0.18	0.61 ± 0.13
	Phaeo	7.75 ± 5.27	0.45 ± 0.06	0.59 ± 0.08	1.65 ± 0.40	1.21 ± 0.11	0.45 ± 0.12	1.77 ± 0.70	0.60 ± 0.14	0.83 ± 0.18	0.77 ± 0.26
	Nanno	31.69 ± 18.85	0.86 ± 0.44	0.66 ± 0.40	1.41 ± 0.08	2.80 ± 0.73	0.34 ± 0.07	14.25 ± 12.22	1.11 ± 0.39	0.81 ± 0.29	1.72 ± 0.71
	Grac	16.38 ± 13.34	0.51 ± 0.14	0.35 ± 0.11	2.52 ± 0.85	2.38 ± 1.26	0.65 ± 0.16	5.39 ± 1.79	1.78 ± 1.24	1.17 ± 0.49	0.92 ± 0.30
	Ulva	17.33 ± 10.77	0.77 ± 0.21	0.51 ± 0.15	2.17 ± 0.54	1.88 ± 0.61	0.52 ± 0.15	7.97 ± 4.20	1.34 ± 0.44	0.98 ± 0.25	0.53 ± 0.11
Two way-ANOVA		*il1-β*	*il-10*	*tgf-β1*	*caspase 1*	*nccrp1*	*csfr1*	*β-defensin*	*hepcidin*	*hsp-70*	*tlr1*
Sig.	***Time***	<0.001	<0.001	<0.001	0.013	<0.001	0.001	0.001	<0.001	ns	<0.001
	Diet	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Time*Diet	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Diet	**CTR**	-	-	-	-	-	-	-	-	-	-
	Tetra	-	-	-	-	-	-	-	-	-	-
	Phaeo	-	-	-	-	-	-	-	-	-	-
	Nanno	-	-	-	-	-	-	-	-	-	-
	Grac	-	-	-	-	-	-	-	-	-	-
	Ulva	-	-	-	-	-	-	-	-	-	-
Time	***0h***	C	B	A	B	B	A	B	A	-	B
	4h	A	A	AB	A	C	B	A	B	-	A
	24h	B	B	B	AB	A	B	A	B	-	B

Table 6. Relative gene expression profiling of anterior intestine in gilthead seabream juveniles fed experimental diets in time-course 2. Data are the mean ± SEM (n=6). All data values for each gene were in reference to the expression level of 0h CTRL fish. Different symbols represent significant differences in time within the same diet ($p < 0.05$). Different capital letters represent significant differences between diets regardless of time ($p < 0.05$).

2 weeks	Diets	*il1-β*	*il-10*	*tgf-β1*	*caspase 1*	*nccrp1*	*csfr1*	*β-defensin*	*hepcidin*	*hsp70*	*tlr1*
0h	**CTR**	2.51 ± 1.48	1.12 ± 0.22	1.05* ± 0.15	1.01 ± 0.06	1.59 ± 0.77	1.09 ± 0.19	3.77 ± 2.67	2.27 ± 1.63	1.03 ± 0.11	1.02 ± 0.08
	Tetra	2.04 ± 1.05	1.72 ± 0.35	1.88* ± 0.50	1.52 ± 0.14	0.73 ± 0.17	1.34 ± 0.25	3.76 ± 1.38	1.40 ± 0.63	1.42 ± 0.29	1.75 ± 0.37
	Phaeo	5.44 ± 4.42	1.85 ± 0.65	3.37* ± 1.64	1.96 ± 0.43	1.80 ± 0.45	1.51 ± 0.46	1.28 ± 0.28	3.39 ± 1.25	1.99 ± 0.82	2.19 ± 1.15
	Nanno	2.02 ± 0.43	2.36 ± 0.86	2.17* ± 0.66	1.19 ± 0.24	1.34 ± 0.46	1.43 ± 0.29	1.22 ± 0.48	6.39 ± 3.47	2.09 ± 1.00	1.21 ± 0.40
	Grac	1.26 ± 0.71	1.46 ± 0.51	3.25 ± 0.75	1.77 ± 0.29	1.19 ± 0.46	1.55 ± 0.20	1.04 ± 0.39	1.06 ± 0.27	1.82 ± 0.42	1.59 ± 0.40
	Ulva	14.93 ± 10.50	0.96 ± 0.23	1.75* ± 0.56	1.41 ± 0.48	1.74 ± 0.49	1.28 ± 0.27	2.93 ± 1.00	3.23 ± 1.07	1.54 ± 0.30	1.53 ± 0.50
4h	**CTR**	10.42 ± 7.64	8.19 ± 1.39	6.56$^{\#}$ ± 1.55	4.65 ± 0.90	0.81 ± 0.16	2.86 ± 0.87	2.19 ± 0.96	0.92 ± 0.27	4.16 ± 1.06	9.33 ± 2.26
	Tetra	19.16 ± 17.96	14.01 ± 5.71	18.06$^{\#}$ ± 7.89	4.19 ± 1.48	1.38 ± 0.38	2.41 ± 0.56	110.31 ± 56.57	1.99 ± 0.82	5.43 ± 2.28	8.92 ± 4.21
	Phaeo	11.16 ± 8.89	9.06 ± 2.85	6.87$^{\#*}$ ± 1.27	2.83 ± 0.69	1.26 ± 0.32	1.93 ± 0.30	67.25 ± 64.66	3.14 ± 1.96	6.01 ± 2.28	5.35 ± 0.94
	Nanno	8.03 ± 2.97	15.91 ± 5.02	8.16$^{\#*}$ ± 1.13	3.97 ± 0.67	1.42 ± 0.45	2.16 ± 0.60	37.64 ± 26.42	1.17 ± 0.07	4.37 ± 0.96	6.74 ± 2.07
	Grac	0.56 ± 0.24	5.68 ± 1.01	3.83 ± 1.43	4.40 ± 2.32	0.89 ± 0.17	2.20 ± 0.82	52.80 ± 49.68	1.47 ± 0.84	5.03 ± 0.95	4.47 ± 0.92
	Ulva	7.07 ± 5.42	11.98 ± 3.98	4.63$^{\#*}$ ± 1.80	3.22 ± 0.35	0.86 ± 0.28	1.43 ± 0.19	135.5 ± 69.14	1.25 ± 0.33	3.15 ± 0.32	7.43 ± 1.35
24h	**CTR**	0.60 ± 0.29	6.73 ± 2.72	14.31$^{\#}$ ± 3.06	2.71 ± 0.75	4.33 ± 1.07	4.06 ± 0.59	2.04 ± 1.32	2.54 ± 1.04	4.14 ± 1.20	8.74 ± 2.08
	Tetra	0.73 ± 0.43	5.69 ± 1.49	12.38$^{\#}$ ± 3.82	2.31 ± 0.45	5.86 ± 2.74	5.86 ± 1.27	31.87 ± 28.83	3.66 ± 1.27	4.23 ± 1.24	3.06 ± 0.46
	Phaeo	1.06 ± 0.86	4.56 ± 0.75	15.82$^{\#}$ ± 4.90	2.66 ± 0.52	3.18 ± 1.18	3.67 ± 0.70	0.41 ± 0.12	2.85 ± 0.87	3.20 ± 0.81	4.42 ± 0.84
	Nanno	1.12 ± 0.52	7.87 ± 2.88	21.23$^{\#}$ ± 12.46	2.43 ± 0.82	4.21 ± 1.46	3.98 ± 0.87	1.00 ± 0.52	2.56 ± 1.51	4.23 ± 1.68	7.69 ± 2.42
	Grac	0.42 ± 0.13	5.93 ± 1.43	12.99 ± 3.29	3.42 ± 0.71	2.11 ± 0.48	5.62 ± 1.16	1.72 ± 0.50	4.13 ± 2.15	2.58 ± 0.50	4.13 ± 0.58
	Ulva	1.18 ± 0.99	5.02 ± 0.93	23.29$^{\#}$ ± 3.74	4.37 ± 1.36	4.47 ± 0.67	4.77 ± 1.15	33.50 ± 32.96	2.29 ± 0.75	4.60 ± 1.33	5.05 ± 0.79
Two way-ANOVA		*il1-β*	*il-10*	*tgf-β1*	*caspase 1*	*nccrp1*	*csfr1*	*β-defensin*	*hepcidin*	*hsp-70*	*tlr1*
Sig.	***Time***	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	ns	<0.001	<0.001
	Diet	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Time*Diet	ns	ns	0.018	ns	ns	ns	ns	ns	ns	ns
Diet	**CTR**	-	-	-	-	-	-	-	-	-	-
	Tetra	-	-	-	-	-	-	-	-	-	-
	Phaeo	-	-	-	-	-	-	-	-	-	-
	Nanno	-	-	-	-	-	-	-	-	-	-
	Grac	-	-	-	-	-	-	-	-	-	-
	Ulva	-	-	-	-	-	-	-	-	-	-
Time	***0h***	A	C	-	B	B	C	B	-	B	B
	4h	A	A	-	A	B	B	A	-	A	A
	24h	B	B	-	A	A	A	B	-	A	A

2.4 Discussion

For some years now, research has focused on the nutritional value of different macro- and microalgae species and the potential for these feedstuffs to substitute FM in aquafeeds. However, several constraints limit utilization, mainly, the production of algae biomasses in such a scale to fulfil fish nutritional needs at a competitive cost [43]. Alternatively, the recognized algae biomasses value as feed additives for immunonutrition [44] means, low percentages of incorporation of specific algae. Fish are given supplemented diets during short periods of time to improve innate immunity [45]. Therefore, in the present study, the potential beneficial health effects of adding 2% crude algal biomasses to a practical dietary formulation (low FM) during 2 weeks of feeding were explored. To better discriminate dietary modulation of the innate immune system in different experimental groups, an inflammatory insult was devised at 1 and 2 weeks of feeding. A clear immune response at both stimuli was observed through the time-dependent response pattern of peripheral leucocytes, plasma and gut immune parameters and liver oxidative stress biomarkers.

Peripheral cell dynamics were abruptly changed at 4 h post-stimulus in both time-courses, as observed by the sharp increase in circulating neutrophils and a significant decrease in lymphocytes. A dietary effect was perceivable following the aforementioned results, in time-course 1 at 4 h fish fed Ulva supplemented diet showed significantly higher neutrophil numbers. Ulvan is the major complex carbohydrate present in *U. rigida,* mainly composed by glucuronic acid and sulphated rhamnose [29]. Castro *et al.* [46] reported that ulvan extracted from *U. rigida*, induced increased respiratory burst and expression of IL-1β mRNA in turbot head kidney leucocytes. Former studies, show that sulphated polysaccharides (SPs) are recognized by different pattern recognition receptors (PRRs) found on the surface of phagocytes, leading to cell activation and cytokine production [29; 46; 47; 48]. Furthermore, in time-course 2 at 4 h post-stimulus, fish fed algae supplemented diets, with the exception of Tetra diet, show higher lymphocyte numbers than CTR. SPs enhanced lymphocyte proliferation as well as improved humoral and cellular immunity in different animal models after an immune challenge [49; 50]. It has been previously suggested, that sulphated residues presence and location in the polysaccharide molecule are very important features, possibly responsible for the biological activities of these molecules, as desulphation leads to a total loss or significant activity reduction [46; 51; 52; 53]. SPs present in different algae species differ in structure and sulphation degree which might, explain the milder effect from *Tetraselmis striata* supplemented diet.

Several studies with different fish species, reveal a tendency of dietary micro- and macroalgae supplementation to stimulate or modulate the innate immune parameters [11; 13; 14; 54; 55]. Cerezuela *et al*. [11] reported a significant increase in serum complement, phagocytic and respiratory burst activity in gilthead seabream fed diets supplemented with 5 and 10% *P. tricornutum* whole biomass for 2 weeks. Additionally, a blend of *Tetraselmis suecica* and *Tisochrysis lutea* used to replace 15% of the FM protein in European seabass diets elicited an increase in plasma antiprotease, lysozyme and peroxidase activities in seabass juveniles [14].

In our study, plasma and gut innate immune parameters were largely not affected by the supplementation with 2% algae biomasses even after stimulation. Accordingly, Peixoto *et al.* [56] reported absence of effects on plasma peroxidase and alternative complement pathway (ACH50) activities and only mild effects on lysozyme activity when seabass is fed diets supplemented with *Gracilaria* sp. and *Ulva* sp. at 2.5%. However, in the present study fish fed Phaeo diet show lower plasma NO concentration than CTRL. *P. tricornutum* is a major source of fucoxanthin, a pigment found in diatoms and brown-algae, which presents antioxidant and anti-inflammatory activities [57; 58; 59; 60]. In mice, intravenously injected fucoxanthin exerted anti-inflammatory effects *in vivo*, by suppressing the production of NO at the inflammation site. Authors, suggest this was achieved through the inhibition of inducible nitric oxide synthase (iNOS) expression [57]. In the present study, the effect was prior to the inflammatory insult, fucoxanthin mediated effects are dose-dependent [57; 60] and the levels present in Phaeo diet might not be high enough to prevent NO production once an inflammatory response has been triggered.

Cell protection from the detrimental action of reactive oxygen species (ROS), is the primary function of cellular antioxidant defences [61]. However, in case of an inflammatory insult ROS production increases beyond the levels of normal cellular metabolism, as a consequence of innate immune cell activation and activity [62]. When the balance between oxidants formation and antioxidant capacity is disrupted the build-up of ROS molecules leads to an oxidative imbalance, thereby, oxidative damage accumulates resulting in potential pathophysiological events [63]. Immediate deleterious effects are, damage in key biological structures, such as cell membranes due to lipid peroxidation [64; 65].

Enzymatic activities in fish, including CAT, are known to be modulated by nutritional and environmental conditions [66]. Magnoni *et al.* [67] reported an antioxidant protective effect in gilthead seabream fed algae supplemented diets (5% *Gracilaria vermiculophylla* or *Ulva lactuca*) against hypoxia induced acute stress. Higher survival rates after hypoxia from fish fed algae diets, were attributed to lower hepatic lipid peroxidation due to algal antioxidant activity. CAT activity decrease during the stressful event, suggests that the

surplus of antioxidants and free radical scavengers provided in the diet were in part responsible for the protective effect [67]. In the present study, it was not possible to ascertain a dietary effect in liver and gut antioxidant defences both prior and after the inflammatory insult.

Transcriptional changes of different immunological relevant genes were analysed in gilthead seabream head-kidney. There is a clear time-dependent response pattern to stimuli at both weeks. Most notably, resulting in an increase of *tlr1*, *il1-β*, *il10* and *β-defensin* gene expression at 4 h post-stimulus, indicating that an inflammatory response was underway. Toll-like receptors are transmembrane proteins that recognize different PAMPs, TLR-1 dimerizes with TLR-2 to recognize different bacterial molecular patterns such as peptidoglycans and lipoproteins [68]. In macrophages, TLR engagement leads to the production and secretion of several cytokines namely IL1-β [69; 70]. Increased IL1-β expression is a classical marker of inflammation, this is a pro-inflammatory cytokine that acts as an inducer of different components of the acute phase response among other functions [69]. However, algal biomasses did not modulate the response and there is not a clear dietary effect.

Recently, a great number of studies have been made to understand fish ability to utilize algal biomasses as a protein source or as a nutraceutical, still information about fish ability to digest complex polysaccharides is very scarce [71]. Algae as well as plants show complex cell walls that, omnivorous fish species cannot digest or when they do, digestibility is very low [71]. This happens because, they lack or show a very limited repertoire of digestive enzymes able to hydrolyse the ß-glycosidic bonds of complex polysaccharides, such as cellulases, ß-xylanases, ß-glucanases and ß-galactases [71; 72; 73].

In summary, the use of lyophilized algal biomasses with intact cell walls combined with seabream poor ability to digest these complex structures, might explain the mild immunological and antioxidant effects found in the present study. Indeed, recent works in Nile tilapia [74], Atlantic salmon [75], and European seabass [76] show that the disruption of algal cell walls improved feed nutritional value by improving nutrient digestibility and the ability of fish to utilize algae biomasses. Nonetheless, the incorporation of 2% *Ulva rigida* algal biomass promoted neutrophil proliferation in time-course 1, which might be advantageous during an infectious event. Furthermore, during the inflammatory insult at 2 weeks the incorporation of 2% algal biomass promoted a general effect of lymphocyte proliferation.

Future works should aim, to use disrupted cell wall algal biomass and compare it with whole-biomass, in order to ascertain, if broader bioactivity can be expected when these algal products are orally given to gilthead seabream as a part of their diet.

2.5 Acknowledgements

This work was supported by VALORMAR project (POCI-01-0247-FEDER-024517) funded by Compete 2020, Lisboa 2020, Algarve 2020, Portugal 2020 and the European Union through FEDER. BR was supported by Foundation for Science and Technology (FCT), Soja de Portugal, SA, and Sparos Lda., through grant PD/BDE/129262/2017. BC was supported by FCT through grant IF/00197/2015.

2.6 Ethics Statement

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. CIIMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). The protocol was approved by the CIIMAR Animal Welfare Committee.

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Chapter III

Health status in gilthead seabream (*Sparus aurata)* juveniles fed diets devoid of fishmeal and supplemented with *Phaeodactylum tricornutum*

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Health status in gilthead seabream (*Sparus aurata)* juveniles fed diets devoid of fishmeal and supplemented with *Phaeodactylum tricornutum*

Abstract

To enhance fish general health, feeds can be supplemented with health promoting additives, reducing the need to use chemotherapeutics. Incorporation of marine algae biomasses in aquafeeds has been shown to improve fish immune status by enhancing innate immune response. This study intended to evaluate the effects of microalgae *Phaeodactylum tricornutum* incorporation in feed by two different processes, either as freeze-dried biomass or broken cell wall biomass, on fish health status and performance. Triplicate groups of gilthead seabream juveniles (13.3 ± 0.3 g) were either fed a control diet (CTRL) with an extreme (i.e. 0% fishmeal), nutritionally balanced, formulation, or two experimental diets formulated as the CTRL with 1% inclusion of *P. tricornutum* at the expense of wheat meal: BC diet contains *P. tricornutum* broken cells and WC diet microalgae whole cells. After 2 and 12 weeks of feeding, blood was collected for haematological procedures whereas plasma and mucus were sampled for immune parameters. Head-kidney, liver and white skeletal muscle were also collected for gene expression measurements. No major differences were observed in haematological nor plasma humoral parameters after 12 weeks, irrespective of dietary treatment. Arrays of 29-31 genes were analyzed in the different tissues, revealing an early dietary effect (2 weeks) in a tissue-specific pattern. In liver, the major effect was found in the GH/IGF axis and in muscle there was a late down-regulation of myostatin (*mstn*) gene, mainly due to WC diet, even though all fish had similar growth performance. Regarding the head-kidney, BC diet led to alpha-2-macroglobulin (*a2m*) gene up-regulation. Also, the same treatment showed increased mucus alternative complement pathway and bactericidal activity at 2 and 12 weeks, respectively. Hence, it seems that BC diet has a potential stimulatory effect that might be relevant as a prophylactic measure before a predictable stressful event.

Keywords

Gilthead seabream*; Phaeodactylum tricornutum*; fishmeal-free diet; feed additive; innate immunity; immunonutrition

3.1 Introduction

Fish production has strongly depended on fishmeal (FM) as the major protein source in aquafeeds, mainly because of its high nutritional value and balanced amino acid (AA) profile. Simultaneously, aquaculture has greatly expanded in the last decades, increasing the demand for marine resources. In order to reduce the industry's pressure over these raw materials, FM replacement has been a key point of research, more importantly in species with high protein requirements [1] such as gilthead seabream (*Sparus aurata*). Often, this has been successfully achieved by partially replacing FM by plant protein (PP) feedstuffs [2; 3; 4; 5]. However, total FM replacement by alternative protein sources can lead to impaired growth performance and immune status [6; 7], mostly because of an unbalanced amino acid profile [8]. Also, the presence of antinutritional factors (ANFs) in PP can damage the intestinal epithelium and promote enteritis in carnivorous fish species [9; 10]. Aquaculture intensification and sustainability led to new challenges for farmed species since there is a need to adapt not only to the inclusion of new dietary ingredients, but also to cope with the challenges arising from intensive fish production. Repeated exposure to stressful conditions caused by routine farming practices (high stocking densities, crowding, size sorting and transportation) [11; 12; 13] can lead to poor growth performance and immunocompromised fish, especially if nutritional requirements are not met. To avoid these negative effects, feeds are often supplemented with essential amino acids (EAA) [3; 14; 15] and in some cases specialized processing techniques are employed to neutralize ANFs [16; 17]. Concomitantly, fish feed additives are also used as alternative strategies to reduce and prevent adverse effects of extreme diet formulation and stress on aquaculture fish [13; 18; 19].

Marine organisms are a plentiful source of new biologically active compounds such as polysaccharides, polyphenols, functional peptides, or fatty acids, amongst others [20; 21; 22]. These compounds can act as additives for the development of new functional feeds reported to have immunostimulating effects in fish [22; 23; 24]. Incorporation of marine algae extracts in aquafeeds has been shown to improve growth and survival of commensal bacteria in fish gastrointestinal tract, or even improving host immune status and enhancing innate immune responses (including increased lysozyme and alternative complement pathway activity, phagocytic and neutrophil activation in fish) [22; 25; 26]. *Phaeodactylum tricornutum* is a marine microalgae rich in polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic acid (EPA), but also β-glucans and fucoxanthin [27; 28; 29]. PUFA are paramount to promote optimal growth and general health of farmed fish [30]. Their administration, in mammals, changed the membrane fatty acid composition of phagocytic cells and enhanced their phagocytic activity [31]. β-1,3 glucans are glucose

polysaccharides produced by several organisms, namely algae which can activate and enhance fish immune response [32; 33; 34]. They show repeating patterns on their structure that are recognized in the gut by cell pattern recognition receptors (PRR), leading to the activation of the host´s innate immune cells [35]. Both *in vitro* and *in vivo* studies have shown fucoxanthin anti-inflammatory effects, by inhibiting the production of pro-inflammatory mediators including interleukin 1 β (IL-1β), tumor necrosis factor (TNF-α) and interleukin-6 (IL-6). Furthermore, fucoxanthin exhibited antioxidant effects by protecting zebrafish embryos against high glucose caused oxidative damage [36]. *P. tricornutum* has already been used either as a FM alternative protein source or an additive to promote immunostimulation [26; 37]. Nonetheless, when microalgae are orally given to fish the cell wall restricts the access of gut enzymes to the inner-cell components. Recent works in Atlantic salmon [38], Nile tilapia [39] and European seabass [40] indicate that the digestibility thus, the nutritional value of algae species is dependent on disrupting the algal cell wall by appropriate processes. In fact, this same studies where algae species were tested as FM substitutes after being processed through mechanical cell-wall rupture methods, show that nutrient digestibility significantly improved when compared to whole-cell biomasses. There are several ways to achieve cell wall rupture through mechanical, physical, chemical and enzymatic methods [41]. Mechanical methods such as bead milling and high-pressure homogenization can be used to disrupt cell walls, while preserving the integrity of inner-cell nutrients [38; 39; 40].

More in-depth studies are required to explore the potential of *P. tricornutum* as an immunostimulant and health promoter in animals as a strategy to curtail the possible detrimental effects of total FM substitution for more sustainable ingredients. This study intended to evaluate the effects of dietary supplementation with *P. tricornutum* incorporated in feed by two different processes, either as whole cells (intact cell wall) or broken cells (ruptured cell wall through high-pressure homogenization) on health status and growth performance of gilthead seabream juveniles. To our knowledge, this is the first study where there is a comparison between the use of *P. tricornutum* intact biomass and ruptured cell wall biomass to allow higher availability of inner-cell bioactive compounds.

3.2 Material & Methods

3.2.1 Phaeodactylum biomasses

Microalgae *Phaeodactylum tricornutum* (wild strain) biomass was produced by Fitoplancton Marino (Spain) in photobioreactors. One fraction of the intact biomass was

freeze-dried and named *Phaeodactylum* whole-cells biomass (WC). A fraction of the same initial biomass was subjected to a mechanical process for cell disruption, which consisted of high-pressure homogenization. The exact details of the cell disintegration process are not disclosed due to industrial confidentiality. After freeze-drying, it originated the *Phaeodactylum* broken-cells biomass (BC). On a dry basis, the composition of biomasses was 34% crude protein, 10% crude lipid and 29% ash. Although not measured in the *Phaeodactylum* biomasses used in the present study, by a differential calculation on the sum of protein, fat, ash, we estimate that the remaining fraction (approx. 27%) is composed of carbohydrates. This range of total carbohydrates is in accordance to measured values found in the literature [(42)]. Relevant also is the fact that *P. triconutum* is often associated to high levels of chrysolaminarin, a water soluble linear polymer of β(1→3) and β(1→6) linked glucose.

3.2.2 Diet composition

The study comprised three fishmeal-free diets. A control diet (CTRL) formulated with moderate levels of poultry meal (10%) and high levels of plant ingredients (soy protein concentrate, wheat gluten, corn gluten meal, soybean meal, guar meal, and rapeseed meal) as major protein sources. A blend of fish, soybean and rapeseed oils was used as major lipid source. This control formulation served as basis for the two additional diets, which comprised a 1% inclusion of *P. tricornutum* biomass, either as whole-cells (diet WC) or broken cells (diet BC). In both cases, algae biomasses were incorporated at the expenses of wheat. All diets were supplemented with selected crystalline amino acids and an inorganic phosphate source to avoid any nutritional deficiencies. Diets were isoproteic (crude protein, 50% dry matter) and presented similar levels of crude lipids (17.8-18.9% dry matter) and gross energy content (23.0-23.3 kJ g^{-1} dry matter) (Table 1). Diets were manufactured by SPAROS Lda. (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulations in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 250 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets, with a pellet size of 2.0 mm, were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (83 kg h^{-1}), screw speed (232 rpm), water addition in barrel 1 (300 mL min^{-1}), temperature barrel 1 (36-38ºC), temperature barrel 3 (107-111 °C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). The blend of oils was added post-extrusion by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature.

Table 1. Ingredients and proximate composition of experimental diets

	Dietary treatments		
	CTRL	**BC**	**WC**
Ingredients (%)			
Poultry meal[1]	10.00	10.00	10.00
Soy protein concentrate[2]	14.00	14.00	14.00
Wheat gluten[3]	10.44	10.44	10.44
Corn gluten meal[4]	11.00	11.00	11.00
Guar meal[5]	9.00	9.00	9.00
Soybean meal 48[6]	15.20	15.20	15.20
Rapeseed meal[7]	3.00	3.00	3.00
Wheat meal[8]	5.50	4.50	4.50
Fish oil[9]	9.20	9.20	9.20
Soybean oil[10]	3.00	3.00	3.00
Rapeseed oil[10]	3.00	3.00	3.00
Vitamin and mineral premix[11]	1.00	1.00	1.00
Binder[12]	0.20	0.20	0.20
Antioxidant[13]	0.20	0.20	0.20
Sodium propionate[14]	0.10	0.10	0.10
Monocalcium phosphate[15]	3.00	3.00	3.00
L-Histidine[16]	0.30	0.30	0.30
L-Lysine[16]	1.20	1.20	1.20
L-Threonine[16]	0.25	0.25	0.25
L-Tryptophan[16]	0.11	0.11	0.11
DL-Methionine[17]	0.30	0.30	0.30
Phaeodactylum (broken cells)[18]		1.00	
Phaeodactylum (whole cells)[18]			1.00
Proximate composition			
Dry matter (DM), %	93.9	95.6	95.8
Ash, % DM	7.0	8.5	8.5
Crude protein, % DM	49.9	50.4	50.1
Crude fat, % DM	18.9	17.8	18.2
Gross energy (kJ g^{-1} DM)	23.3	23.1	23.0

[1] Poultry meal: 62.4% crude protein (CP), 14.5% crude fat (CF), SAVINOR UTS, Portugal; [2] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; [3] VITEN: 82% CP, 2.1% CF, Roquette, France; [4] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal; [5] Guar Korma: 55.3% CP, 7.8% CF, KFEED Ltd, Bulgaria; [6] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, CARGILL, Spain; [7] Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal; [8] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal; [9] Sopropêche, France; [10] JC Coimbra, Portugal; [11] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alphatocopherol acetate, 100mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1 mg; nicotidin acid, 200 mg; folic acid, 15mg; ascorbic acid, 1000 mg; inositol, 500mg; biotin, 3 mg; calcium panthotenate, 100mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate. 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middling's; [12] Kieselguhr, LIGRANA GmbH, Germany; [13] Paramega PX, Kemin Europe NV, Belgium; [14] PREMIX LDA., Portugal; [15] ALIPHOS MONOCAL, 22.7% P, ALIPHOS, Belgium; [16] Ajinomoto EUROLYSINE S.A.S., France; [17] Rhodimet NP99, ADISSEO, France; [18] Test *Phaeodactylum* biomasses: 34% CP, 10% CF, Fitoplancton Marino, Spain

3.2.3 Fish rearing conditions

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Fish were assigned to 1 m^3 tanks at Centre of Marine Sciences (CCMAR) facilities (Faro, Portugal). Seawater flow was kept at 2 $L.min^{-1}$ (mean temperature 23 ± 2.6 °C; mean salinity 34 ± 0.7 ‰) in a flow-through system with aeration (mean dissolved oxygen above 5 mg L^{-1}). Water quality parameters were monitored daily and adjusted when necessary. Mortality was monitored daily. Diets were randomly assigned in triplicate (150 animals/tank). Fish (initial body weight (IBW): 13.3 ± 0.3 g; ~2.0 $kg.m^{-3}$ initial stocking density) were fed to visual satiety by hand, twice daily for 12 weeks.

3.2.4 Feeding trial and tissue sampling

The feeding trial lasted 12 weeks and feed consumption for each experimental unit was registered weekly. At 2 and 12 weeks, eighteen fish per tank were individually weighed and twelve were sampled for blood, skin mucus and tissues (head-kidney, liver and white skeletal muscle), the other six were stored at −20 °C until analysis of proximate composition and amino acids content. Prior to sampling, fish were fasted for 24 hours and then sacrificed with a tricaine methanesulfonate lethal dose (200 mg L^{-1}). Blood was collected from the caudal vein using heparinized syringes and centrifuged at 10,000 × *g* during 10 min at 4 ºC to obtain plasma samples. Skin mucus and tissue samples were immediately frozen at -80 ºC until further analysis.

3.2.5 Growth parameters and feed utilization

Calculations were done as follows: Daily growth index (DGI) (%/day) = ((Final weight $(Wf)^{1/3}$ – Initial weight $(Wi)^{1/3}$)/days) × 100; Feed conversion ratio (FCR) = apparent feed intake (g/fish)/(Wf − Wi); Protein efficiency ratio (PER) = (Wf − Wi)/crude protein intake; Daily intake (g/kg ABW/day) = dry matter or nutrient intake (g or mg)/((Wf + Wi)/2) (kg)/days; Nutrient retention: 100 × (Final body weight (FBW) × final carcass nutrient content – IBW × initial carcass nutrient content)/nutrient intake.

3.2.6 Haematological procedures

The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts, haematocrit (Ht) and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were also calculated as follows: MCV (μm^3) = (Ht/RBC) × 10; MCH ($pg.cell^{-1}$) = (Hb)/ RBC × 10; MCHC (g 100 mL^{-1}) = (Hb/Ht) × 100. For determination of WBC and RBC concentration, whole blood was diluted 1/20 and 1/200 respectively, in HBSS with heparin (30 U mL^{-1}) and cell counts were done in a Neubauer chamber. Blood smears were prepared from peripheral blood, air dried and stained with Wright's stain (Haemacolor; Merck) after fixation for 1 minute with formol–ethanol (10 % formaldehyde in ethanol). Neutrophils were labeled through the detection of peroxidase activity revealed by the Antonow's technique described in Afonso *et al.* [43]. The slides were examined under oil immersion (1000×), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The relative percentage and absolute value (×10^4 mL^{-1}) of each cell type was calculated.

3.2.7 Innate humoral parameters

3.2.7.1 Peroxidase activity

Total peroxidase activity in plasma and mucus was measured following the procedure described by Quade and Roth [44]. Briefly, 10 µL of plasma and 20 µL of mucus were diluted with 140 and 130 µL, respectively, of HBSS without Ca^{2+} and Mg^{2+} in 96-well plates. Then, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 µL of 5 mM H_2O_2 were added to the wells. The reaction was stopped after 2 min by adding 50 µL of H_2SO_4 (2M) and the optical density (OD) was read at 450 nm in a Synergy HT microplate reader (Biotek). Wells without plasma or mucus were used as blanks. The peroxidase activity (units mL^{-1} plasma or mucus) was determined defining that one unit of peroxidase produces an absorbance change of 1 OD.

3.2.7.2 Bactericidal activity

Plasma bactericidal activity was determined following the method of Machado *et al.* [45]. *Photobacterium damselae* subsp. *piscida* (Phdp), strain PP3, was used. Briefly, 20 µL of sample were mixed with 20 µL of Phdp (1 × 10^6 cfu mL^{-1}) in duplicate in a U-shaped

96-well plate and incubated for 2.5 h at 25 ºC (20 µL of TSB were added instead of plasma to 2 wells and served as positive control). Afterwards, 25 µL of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (2.4 mM, Sigma) were added to each well and incubated for 10 min at 25 ºC to allow the formation of formazan precipitates. Plates were then centrifuged at 2,000 × *g* for 10 min and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity is expressed as percentage, calculated from the difference between bacteria surviving compared to the number of bacteria from positive controls (100%).

3.2.7.3 Protease activity

The protease activity was determined in plasma and mucus as described by Ross *et al.* [46] with some modifications. Briefly, 100 µL of sodium bicarbonate buffer ($NaHCO_3$ 60mM, pH 8.3) (SBB) and 125 µL of azocasein solution (20 mg mL^{-1} in SBB) were added to 10 µL of plasma, whereas for mucus 100 µL of sample was used and mixed with 100 µL of azocasein solution, both reaction mixtures were incubated for 19 h at 30 °C. Finally, 250 µL of 10% (m/v) trichloroacetic acid solution (TCA) were added to both reactions. Mixtures were centrifuged at 6,000 × *g* for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred to a 96 well-plate and mixed with 100 µL of NaOH (1M). The OD was read at 450 nm in a Synergy HT microplate reader. SBB in place of plasma or mucus served as a blank, whereas the reference sample was a trypsin solution in place of plasma or mucus. Sample trypsin activity ratio was calculated as follows: (sample absorbance/reference absorbance) x 100. All analyses were conducted in duplicates.

3.2.7.4 Antiprotease activity

The anti-protease activity was determined as described by Ellis *et al.* [47] with some modifications. Briefly, 10 µL of plasma were incubated with the same volume of trypsin solution (5 mg mL^{-1} in SBB) for 10 min at 22 °C in a 1.5 mL microcentrifuge tube. After incubation, 100 µL of phosphate buffer (NaH_2PO_4 100mM, pH 7.0) and 125 µL of azocasein solution (20 mg mL^{-1} in SBB) were added and incubated for 1 h at 22 °C. Finally, 250 µL of 10% (m/v) TCA were added to the reaction mixture and incubated for 30 min at 22 °C. The mixture was centrifuged at 10,000 × *g* for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred to a 96 well-plate and mixed with 100 µL of NaOH (1M). The OD was read at 450 nm in a Synergy HT

microplate reader. Phosphate buffer in place of plasma and trypsin served as blank, whereas the reference sample was phosphate buffer in place of plasma. Sample inhibition percentage of trypsin activity was calculated as follows: 100 – ((sample absorbance/Reference absorbance) x100). All analyses were conducted in duplicates.

3.2.7.5 Complement pathway (ACH_{50})

Alternative complement pathway (ACP) activity was estimated as described by Sunyer and Tort [(48)]. The following buffers were used: GVB (isotonic veronal buffered saline), pH 7.3, containing 0.1 % gelatin; EDTA-GVB, which is GVB with the addition of 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM $MgCl_2$ and 10 mM EGTA. Horse red blood cells (HRBC; Probiologica Lda, Portugal) were used for ACP determination. HRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells mL^{-1}. 10 µL of HRBC suspension were then added to 40 µL of serially diluted plasma or mucus in Mg-EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with regular shaking. The reaction was stopped by adding 150 µL of cold EDTA-GVB. Samples were then centrifuged and haemolysis was estimated by measuring the OD of the supernatant at 414 nm in a Synergy HT microplate reader (Biotek). The ACH50 units were defined as the concentration of plasma giving 50% haemolysis of HRBC. All analyses were conducted in triplicates.

3.2.8 Gene expression

Total RNA from target tissues (liver, head-kidney, white skeletal muscle) was extracted using the MagMAX™-96 for microarrays total RNA isolation kit (Life Technologies, Carlsbad, CA, USA) after tissue homogenization in TRI reagent. RNA yield in all tissues was 50–100 µg determined by Nanodrop (Thermo Scientific, Wilmington, DE, USA) 260 and 280 nm UV absorbance ratios (A260/280) of 1.9–2.1. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. RT reactions were incubated for 10 min at 25 ºC and 2 h at 37 ºC. Negative control reactions were run without reverse transcriptase. Real-time quantitative PCR was carried out on a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using 96-well PCR array layouts designed for simultaneously profiling a panel of 31 genes for liver samples and 29 genes for head kidney and muscle samples (Summarized in Appendix II, Table S1b). Genes comprised in the arrays were selected for their involvement in fish growth,

antioxidant status and health performance. Specific primer pair sequences are listed in Appendix II, Table S2b. Controls of general PCR performance were included on each array, being performed all the pipetting operations by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, RT reactions were diluted to obtain the equivalent concentration of 660 pg of total input RNA which were used in a 25 µL volume for each PCR reaction. PCR-wells contained a 2× SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 µM were used to obtain amplicons of 50–150 bp in length. The program used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The efficiency of PCR reactions was always higher than 90%, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C), and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the PCR extension phase were normalized using the delta–delta Ct method [(49)]. β-Actin (*actb*) was tested for gene expression stability using GeNorm software (M score = 0.21) and it was used as housekeeping gene in the normalization procedure. Fold-change calculations were done in reference to the expression ratio between BC or WC and CTRL fish. For comparing the mRNA expression level of a panel of genes in a given dietary treatment, all data values were in reference to the expression level of a specific gene in CTRL fish. In liver, gene expression was in reference to the expression level of *cpt1*, whereas in white skeletal muscle and head kidney gene expression was in reference to *igfr2* and *il-7*, respectively, which was arbitrarily assigned a value of 1.

3.2.9 Data analysis

All results are expressed as mean ± standard error (mean ± SE). All residuals were tested for normality (Shapiro-Wilk´s test) and homogeneity of variance (Levene's test). When residuals did not meet the assumptions, data was transformed before analysis to account for this. Mixed-effect ANOVAs were performed, with "time" and "diet" (and their interaction) as fixed effects and "tank" as a random effect, followed by Tukey post-hoc tests. All statistical analyses were performed using the computer package SPSS for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests.

3.3 Results

3.3.1 Growth performance and nutrient intake and utilization

Growth performance and whole-body composition are presented in Table 2. At the end of the 12-week growth trial, FBW (68.5 – 64.2 g) and growth performance indicators (DGI, FCR and PER) remained unaffected by dietary treatments. All fish showed similar final whole-body composition regardless of dietary treatment.

Nutrient intake and utilization are presented in Table 3. Fish fed CTRL diet showed higher lipid intake. Dry matter and protein intake remained similar among groups. Fish fed WC diet showed higher lipid retention. Protein retention was not affected by the dietary treatments. In addition, time effects (2 weeks *vs* 12 weeks) were observed in all parameters studied in Tables 2 and 3.

3.3.2 Haematological profile

Haematological parameters such as total WBC and RBC were not affected by dietary treatments (Table 4). Ht increased from 2 to 12 weeks (Table 4) whereas diet WC showed the highest values regardless of time. Hb concentration was higher in fish fed diet BC at 12 weeks when compared to CTRL. Furthermore, Hb concentration increased from 2 to 12 weeks for this dietary treatment (Table 4). MCV increased over time during the feeding trial for all diets (Table 4). MCH and MCHC were not affected by the dietary treatments, while MCH increased during the feeding trial only in fish fed BC (Table 4). Peripheral cell dynamics changed from 2 to 12 weeks, increasing for neutrophils and decreasing for lymphocytes and monocytes regardless of dietary treatment (Table 5). Thrombocytes concentration decreased on BC-fed fish after 12 weeks (Table 5).

Table 2. Growth performance and whole-body composition of gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period.

	Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Growth	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
Final body weight (FBW) (g)	20.07 ± 0.56	20.00 ± 0.38	19.84 ± 0.34	68.45 ± 2.57	66.52 ± 0.75	64.16 ± 1.63	ns	ns	<0.01
Daily growth index (DGI)	2.32 ± 0.17	2.77 ± 0.14	2.33 ± 0.08	1.87 ± 0.06	1.84 ± 0.02	1.79 ± 0.04	ns	ns	<0.01
Feed conversion ratio (FCR)	1.58 ± 0.06	1.57 ± 0.06	1.53 ± 0.03	1.41 ± 0.03	1.44 ± 0.01	1.46 ± 0.02	ns	ns	<0.01
Protein efficiency ratio (PER)	1.38 ± 0.06	1.33 ± 0.05	1.33 ± 0.02	1.55 ± 0.03	1.47 ± 0.01	1.46 ± 0.02	ns	ns	<0.01
Final whole-body composition (% ww)									
Moisture	69.42 ± 0.01	69.01 ± 0.71	68.91 ± 0.40	66.15 ± 0.51	66.58 ± 0.44	65.51 ± 0.23	ns	ns	<0.01
Protein	15.23 ± 0.16	15.63 ± 0.18	15.66 ± 0.26	17.04 ± 0.28	16.81 ± 0.30	17.03 ± 0.06	ns	ns	<0.01
Fat	9.93 ± 0.21	9.74 ± 0.50	9.83 ± 0.12	13.05 ± 0.71	12.82 ± 0.46	13.85 ± 0.21	ns	ns	<0.01
Ash	3.87 ± 0.24	4.24 ± 0.19	4.24 ± 0.19	1.21 ± 0.05	1.26 ± 0.05	1.21 ± 0.05	ns	ns	<0.01

Initial body weight - 13.2 ± 0.08 g. Initial composition of fish (% ww) – Moisture: 72.69; Protein: 14.11; Fat: 7.90; Ash: 4.51.
Values represent mean ± standard error (n=3), ns – non significant

Table 3. Nutrient intake and utilization of gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period.

	Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)			Post-hoc Tukey Diet		
Intake (g kg^{-1} ABW^{1} day^{-1})	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**	**CTRL**	**BC**	**WC**
Dry matter	41.76 ± 1.31	42.66 ± 1.46	41.53 ± 2.14	20.16 ± 0.09	20.57 ± 0.07	20.45 ± 0.07	ns	ns	<0.01	-	-	-
Protein	19.57 ± 0.62	20.53 ± 0.70	19.92 ± 1.03	9.45 ± 0.04	9.90 ± 0.03	9.81 ± 0.04	ns	ns	<0.01	-	-	-
Lipids	8.64 ± 0.27	7.74 ± 0.26	7.33 ± 0.38	4.17 ± 0.02	3.73 ± 0.01	3.61 ± 0.01	ns	<0.01	<0.001	A	B	B
Retention (% of intake)												
Protein	24.06 ± 0.24	24.64 ± 1.33	25.53 ± 0.58	27.54 ± 0.84	25.68 ± 0.61	26.03 ± 0.37	ns	ns	0.02	-	-	-
Lipids	43.55 ± 3.07	46.66 ± 5.12	50.45 ± 0.10	50.23 ± 3.76	54.70 ± 2.30	61.16 ± 0.60	ns	0.04	<0.01	B	AB	A

[1]ABW – average body weight. Values represent mean ± standard error (n=3), ns – non significant. Different capital letters represent significant differences between diets regardless of time (*P*<0.05).

Table 4. Haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cells (RBC) and white blood cells (WBC) in gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period.

	Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)			Post-hoc Tukey Diet		
Haematology	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**	**CTRL**	**BC**	**WC**
Haematocrit (%)	31.0 ± 1.32	32.2 ± 1.46	35.0 ± 1.55	36.4 ± 1.27	39.0 ± 1.02	39.4 ± 1.58	ns	0.02	<0.01	B	A.B	A
Haemoglobin (g dL^{-1})	2.1 ± 0.17	2.0 ± 0.11*	2.5 ± 0.21	2.2 ± 0.16 ^{b}	3.1 ± 0.26$^{\# a}$	2.7 ± 0.22 ^{a,b}	0.04	0.05	0.01	-	-	-
MCV (µm^3)	114.2 ± 5.58	107.4 ± 5.51	114.6 ± 5.57	131.3 ± 8.24	134.9 ± 9.64	123.0 ± 5.50	ns	ns	<0.01	-	-	-
MCH (pg cell^{-1})	7.6 ± 0.57	6.8 ± 0.67*	8.4 ± 0.66	7.9 ± 0.63	10.6 ± 0.74$^{\#}$	8.2 ± 0.55	0.01	ns	0.03	-	-	-
MCHC (g 100 mL^{-1})	6.6 ± 0.31	6.4 ± 0.48	7.3 ± 0.46	6.0 ± 0.32	8.0 ± 0.61	6.7 ± 0.53	ns	ns	ns	-	-	-
WBC (x10^4 µL^{-1})	6.7 ± 0.84	8.5 ± 0.64*	6.1 ± 0.52	5.2 ± 0.42	4.8 ± 0.37$^{\#}$	5.2 ± 0.37	0.04	ns	<0.01	-	-	-
RBC (x10^6 µL^{-1})	2.8 ± 0.13	3.0 ± 0.15	3.0 ± 0.12	2.8 ± 0.17	3.0 ± 0.21	3.3 ± 0.17	ns	ns	ns	-	-	-

Values represent mean ± standard error (n=9), ns – non significant. Different superscript letters represent significant differences between diets within the same time (*P*<0.05). Different superscript symbols represent significant differences in time within the same diet (*P*<0.05). Different capital letters represent significant differences between diets regardless of time (*P*<0.05).

Table 5. Absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) in gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period.

	Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Peripheral blood leucocytes	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
Thrombocytes (x10^4 µL^{-1})	4.1 ± 0.47	5.7 ± 0.42*	3.9 ± 0.35	3.9 ± 0.32	3.4 ± 0.28$^{\#}$	4.1 ± 0.26	0.03	ns	0.02
Lymphocytes (x10^4 µL^{-1})	1.8 ± 0.31	2.0 ± 0.16	1.5 ± 0.24	0.4 ± 0.08	0.4 ± 0.04	0.4 ± 0.07	ns	ns	<0.001
Monocytes (x10^4 µL^{-1})	0.2 ± 0.05	0.2 ± 0.07	0.2 ± 0.04	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	ns	ns	<0.001
Neutrophils (x10^4 µL^{-1})	0.4 ± 0.13	0.5 ± 0.08	0.5 ± 0.08	0.8 ± 0.09	0.9 ± 0.12	0.7 ± 0.09	ns	ns	<0.001

Values represent mean ± standard error (n=9), ns – non significant. Different superscript symbols represent significant differences in time within the same diet (*P*<0.05).

3.3.3 Plasma & skin mucus immune parameters

Plasma humoral immune parameters, namely plasma bactericidal activity decreased from 2 to 12 weeks regardless of dietary treatment (Fig. 1a), whereas anti-protease activity (Fig. 1b), alternative complement pathway (ACH_{50}) (Fig. 1c) and peroxidase activity (Fig. 1e) increased over time. Plasma protease activity increased throughout the feeding trial only in WC-fed fish (Fig. 1d). Skin mucus bactericidal activity of BC-fed fish increased over time and at 12 weeks was higher than CTRL fed group (Fig. 2a). Mucus ACH_{50} of CTRL-fed fish increased over time from 2 to 12 weeks whereas BC- and WC-fed groups showed higher complement activity than CTRL at 2 weeks (Fig. 2b). Mucus protease activity remained unchanged throughout the feeding trial (Fig. 2c), while mucus peroxidase activity decreased throughout the feeding trial irrespective of dietary treatment (Fig. 2d).

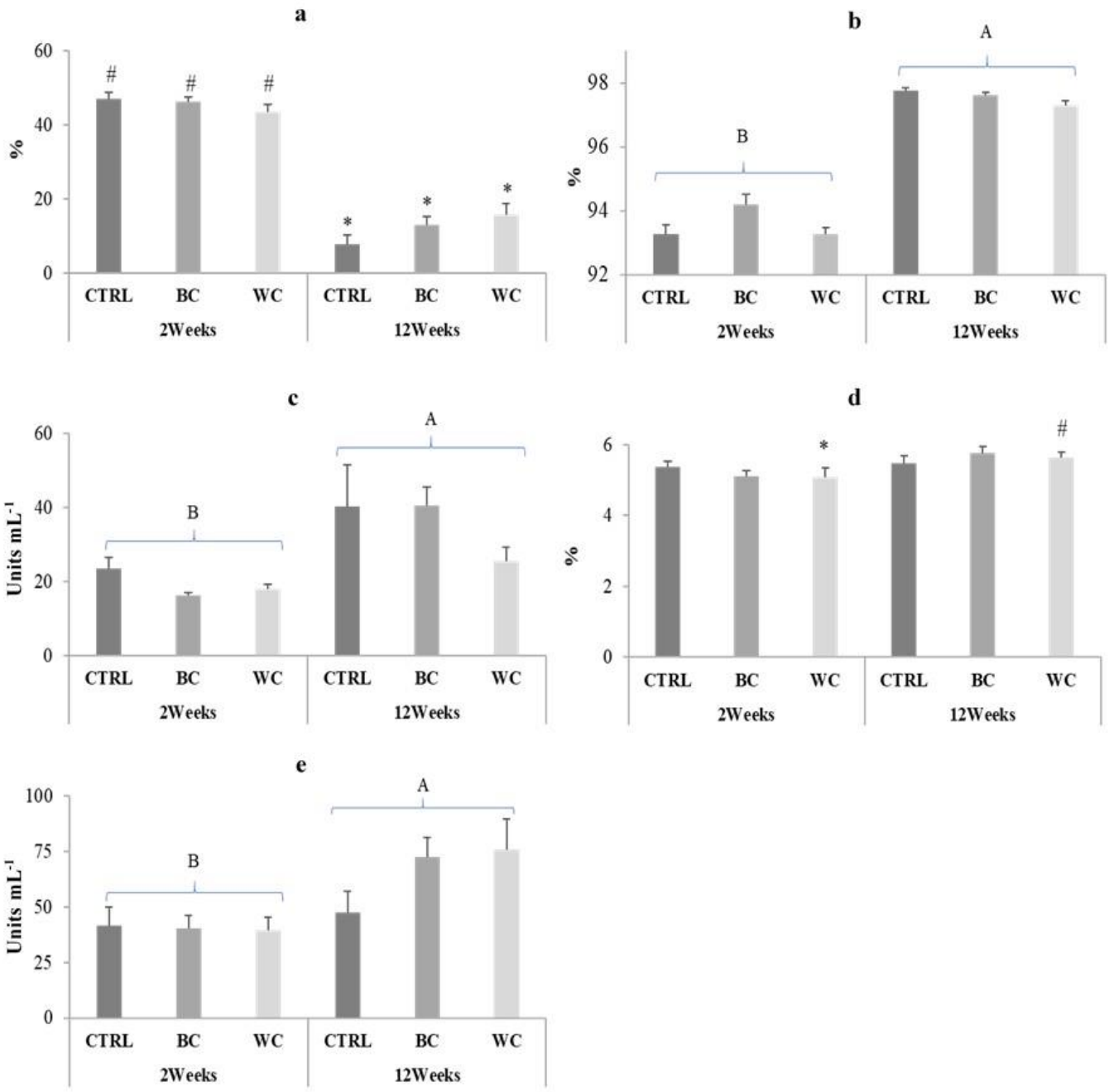

Figure 1 Plasma innate humoral parameters in gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period: Bactericidal activity (a); Anti-protease activity (b); Alternative complement pathway (c); Protease activity (d) and Peroxidase activity (e). Values represent mean ± standard error (n=12). Different superscript symbols represent significant differences in time within the same diet (P<0.05). Different capital letters represent significant differences between sampling points (P<0.05)

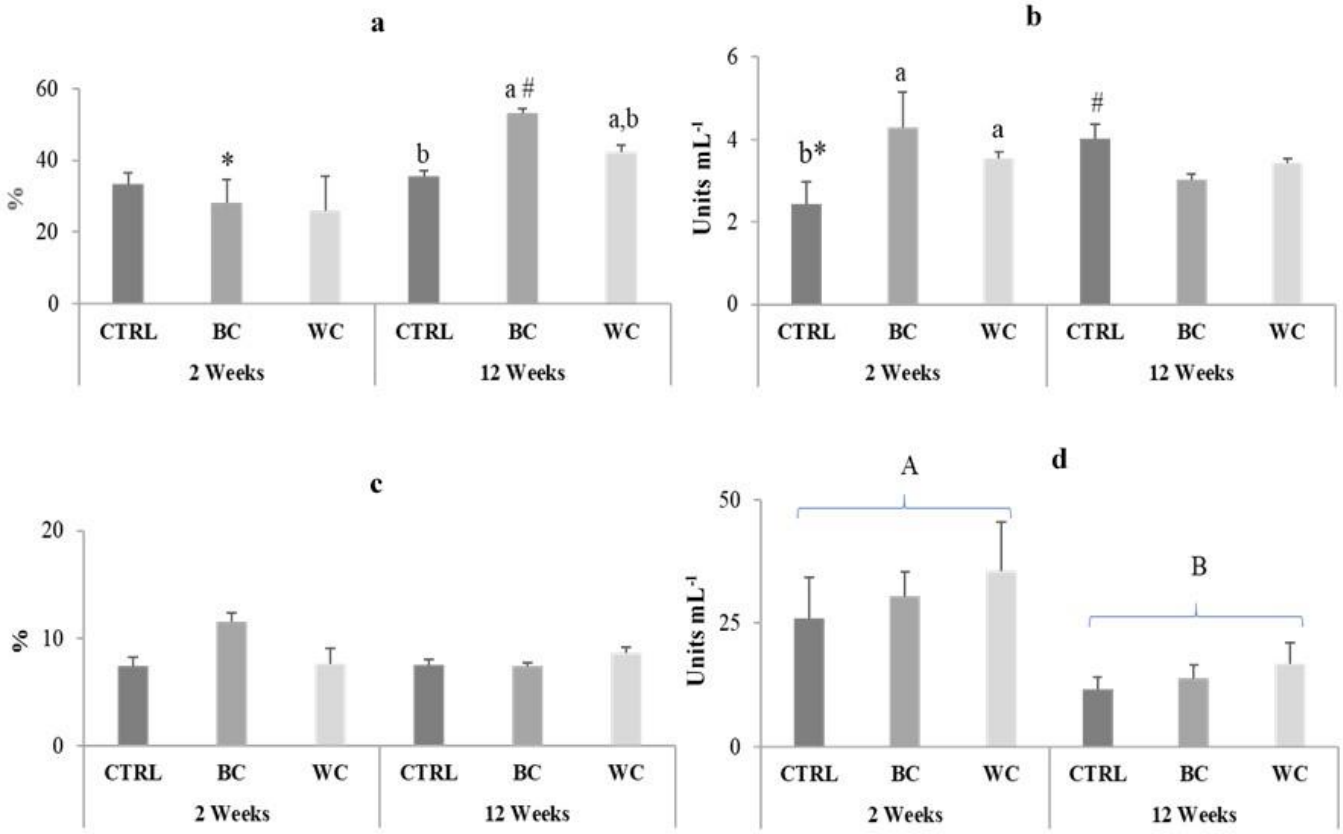

Figure 2 Mucus innate parameters in gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period: Bactericidal activity (a); Alternative complement pathway (b); Protease activity (c) and Peroxidase activity (d). Values represent mean ± standard error (n=6). Different lower-case letters represent significant differences between diets within the same time (P<0.05). Different superscript symbols represent significant differences in time within the same diet (P<0.05). Different capital letters represent significant differences between sampling points (P<0.05)

3.3.4 Gene expression

From the pathway focused array of analyzed genes it was possible to determine a dietary effect with a tissue-specific pattern. Dietary effects were found for several genes related to the growth hormone/insulin growth factor system (GH/IGF) in liver (Table 6). Insulin-like growth factor II gene (*igf-ii*) was up-regulated in WC-fed fish at 2 weeks when compared to CTRL group. BC-fed fish showed a down-regulation of insulin-like growth factor receptor I and II genes (*igfr1, igfr2*) expression at 2 weeks relative to CTRL and WC-fed fish. Hepatic insulin receptor gene (*insr*) expression was down-regulated at 2 weeks in BC-fed fish compared to CTRL. On a different pathway (cytoplasmic and lysosomal activity) calpain 1 gene (*capn1*) expression was up-regulated in WC-fed fish. Finally, catalase gene (*cat*) expression was up-regulated in WC-fed fish after 2 weeks.

Head-kidney gene expression (Table 7) also showed a dietary effect with alpha-2-macroglobulin (*a2m*) gene up-regulated at an early stage (2 weeks) in BC-fed fish. Muscle tissue (Table 8) showed only a down-regulation of myostatin gene (*mstn*) at 12 weeks in WC-fed fish. Finally, it was also possible to ascertain a clear time effect for several genes involved in different pathways, since most of the genes showed higher expression at 12 weeks especially in muscle (Table 8).

Table 6. Liver gene expression profile of gilthead seabream juveniles in response to the different dietary treatments at 2 and 12 weeks feeding time.

		Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Biological Process	**Genes**	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
GH/IGF Axis	*ghr-i*	1.17 ± 0.22	1.42 ± 0.20	1.38 ± 0.22	1.96 ± 0.18	2.39 ± 0.27	2.12 ± 0.23	ns	ns	<0.01
	ghr-ii	1.39 ± 0.11	1.63 ± 0.17	1.60 ± 0.12	0.88 ± 0.09	1.24 ± 0.18	0.99 ± 0.14	ns	ns	<0.01
	igf-i	5.75 ± 0.69	6.20 ± 0.83	6.79 ± 0.72	8.76 ± 1.24	7.64 ± 0.59	6.87 ± 0.64	ns	ns	<0.01
	igf-ii	2.01 ± 0.26*^{b}	2.62 ± 0.63*^{a,b}	4.33 ± 0.45^{a}	4.72 ± 0.53$^{\#}$	6.94 ± 1.32$^{\#}$	5.34 ± 0.74	0.02	ns	<0.01
	igfbp1a	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	ns	ns	<0.01
	igfbp2b	2.12 ± 0.15	2.28 ± 0.24	3.19 ± 0.32	1.42 ± 0.13	1.46 ± 0.10	1.45 ± 0.11	ns	ns	<0.01
	igfbp4	0.68 ± 0.06	0.61 ± 0.07	0.76 ± 0.07*	0.54 ± 0.05	0.62 ± 0.05	0.48 ± 0.04$^{\#}$	0.01	ns	<0.01
	igfr1	0.10 ± 0.01^{a}	0.04 ± 0.01^{b}	0.08 ± 0.01^{a}	0.08 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.02	0.01	ns
	igfr2	0.28 ± 0.03^{a}	0.12 ± 0.01*^{b}	0.22 ± 0.04^{a}	0.22 ± 0.02	0.21 ± 0.02$^{\#}$	0.23 ± 0.04	0.01	ns	ns
	insr	0.96 ± 0.08^{a}	0.52 ± 0.06^{b}	0.77 ± 0.11^{a,b}	0.70 ± 0.08	0.65 ± 0.05	0.72 ± 0.07	0.01	ns	ns
Cytoplasmic & lysosomal protease activity	*capn1*	0.15 ± 0.01^{b}	0.19 ± 0.02*^{a,b}	0.27 ± 0.02*^{a}	0.12 ± 0.01	0.11 ± 0.01$^{\#}$	0.10 ± 0.01$^{\#}$	<0.01	ns	<0.01
	cast	0.28 ± 0.02	0.33 ± 0.02	0.26 ± 0.02	0.53 ± 0.05	0.54 ± 0.05	0.52 ± 0.04	ns	ns	<0.01
	ctsb	1.76 ± 0.14	2.03 ± 0.21	1.97 ± 0.25	1.86 ± 0.16	2.22 ± 0.16	2.16 ± 0.18	ns	ns	ns
	ctsd	0.17 ± 0.02	0.18 ± 0.02	0.17 ± 0.02	1.03 ± 0.19	1.24 ± 0.26	0.95 ± 0.25	ns	ns	<0.01
	ctsl	6.74 ± 0.53	7.89 ± 0.88	7.88 ± 0.84	11.50 ± 0.86	12.59 ± 1.05	11.67 ± 0.68	ns	ns	<0.01
Energy sensing and oxidative metabolism	*pgc1α*	0.32 ± 0.04	0.42 ± 0.05	0.59 ± 0.11	0.17 ± 0.01	0.16 ± 0.02	0.17 ± 0.03	ns	ns	<0.01
	cpt1a	0.93 ± 0.06	0.98 ± 0.07	1.14 ± 0.08	1.09 ± 0.09	1.15 ± 0.07	1.11 ± 0.10	ns	ns	ns
	cs	0.43 ± 0.03	0.49 ± 0.03	0.50 ± 0.04	0.80 ± 0.06	0.79 ± 0.05	0.85 ± 0.07	ns	ns	<0.01
	hif-1α	0.55 ± 0.03	0.56 ± 0.02	0.63 ± 0.04	0.36 ± 0.03	0.35 ± 0.01	0.36 ± 0.02	ns	ns	<0.01
Respiration uncoupling	*ucp1*	15.18 ± 1.17	15.30 ± 1.37	19.00 ± 1.06	8.77 ± 0.88	9.80 ± 1.13	9.43 ± 0.84	ns	ns	<0.01
Antioxidant defences	*mthsp70/grp-75*	0.53 ± 0.08	0.58 ± 0.06	0.60 ± 0.07	0.70 ± 0.08	0.68 ± 0.11	0.75 ± 0.09	ns	ns	0.03
	grp-170	1.24 ± 0.15	1.29 ± 0.23	0.98 ± 0.16	1.13 ± 0.15	1.24 ± 0.19	1.05 ± 0.10	ns	ns	ns
	grp-94	3.83 ± 0.68	4.88 ± 1.00	2.41 ± 0.38	1.47 ± 0.24	2.48 ± 0.43	1.65 ± 0.22	ns	ns	<0.01
	cat	10.86 ± 0.95^{b}	11.01 ± 0.52^{b}	15.71 ± 0.94^{a}	13.15 ± 1.24	13.45 ± 1.31	12.81 ± 0.79	0.03	ns	ns
	gpx1	1.08 ± 0.06	1.29 ± 0.12	1.28 ± 0.10	0.96 ± 0.05	1.13 ± 0.06	1.09 ± 0.10	ns	ns	0.02
	gpx4	4.08 ± 0.65	3.70 ± 0.40	5.75 ± 0.46	13.82 ± 2.19	14.18 ± 1.73	14.42 ± 2.09	ns	ns	<0.01
	gr	0.24 ± 0.01	0.28 ± 0.02	0.28 ± 0.02	0.35 ± 0.02	0.35 ± 0.02	0.35 ± 0.02	ns	ns	<0.01
	prdx3	0.45 ± 0.03	0.52 ± 0.04	0.53 ± 0.04	0.68 ± 0.06	0.72 ± 0.08	0.71 ± 0.05	ns	ns	<0.01
	prdx5	0.29 ± 0.04	0.29 ± 0.04	0.27 ± 0.03	1.13 ± 0.12	1.05 ± 0.04	1.18 ± 0.14	ns	ns	<0.01
	mn-sod / sod2	0.80 ± 0.07	0.92 ± 0.09	0.91 ± 0.09	0.77 ± 0.06	0.76 ± 0.09	0.83 ± 0.05	ns	ns	ns
	h-fabp	26.47 ± 2.04	26.17 ± 1.52	31.19 ± 3.05	45.78 ± 4.31	46.60 ± 3.54	51.95 ± 4.71	ns	ns	<0.01

Values represent mean ± standard error (n=9) (Raw data), ns – non significant. Different superscript letters represent significant differences between diets within the same time (P<0.05). Different superscript symbols represent significant differences in time within the same diet (P<0.05).

Table 7. Head-kidney gene expression profile of gilthead seabream juveniles in response to the different dietary treatments at 2 and 12 weeks feeding time

		Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Biological Process	**Genes**	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
Interleukins/cytokines	*il-1β*	0.09 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.03 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	ns	ns	<0.01
	il-6	0.02 ± 0.00*$^{a.b}$	0.04 ± 0.01^{a}	0.02 ± 0.00^{b}	0.05 ± 0.01$^{\#}$	0.04 ± 0.00	0.04 ± 0.01	0.03	ns	<0.01
	il-7	1.04 ± 0.15	1.46 ± 0.18*	1.10 ± 0.15	1.00 ± 0.08	0.75 ± 0.08$^{\#}$	0.66 ± 0.07	0.03	ns	<0.01
	il-8	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	ns	ns	<0.01
	il-10	0.47 ± 0.03	0.58 ± 0.05	0.67 ± 0.05	0.67 ± 0.05	0.54 ± 0.07	0.55 ± 0.09	ns	ns	ns
	il-12	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	ns	ns	ns
	il-15	0.23 ± 0.02	0.28 ± 0.03	0.24 ± 0.03	0.33 ± 0.02	0.22 ± 0.02	0.25 ± 0.03	ns	ns	ns
	il-34	1.11 ± 0.12	1.24 ± 0.09	1.13 ± 0.10	2.18 ± 0.15	1.90 ± 0.16	1.76 ± 0.20	ns	ns	<0.01
	tnf-α	0.14 ± 0.02	0.17 ± 0.09	0.15 ± 0.02	0.17 ± 0.02	0.13 ± 0.16	0.14 ± 0.01	ns	ns	ns
Macrophages/monocytes chemokines	*csf1r1*	1.73 ± 0.19*	2.14 ± 0.12	1.89 ± 0.08	2.90 ± 0.22$^{\#}$	2.58 ± 0.19	2.33 ± 0.18	0.02	ns	<0.01
	ccr3	4.85 ± 0.59	4.55 ± 0.31	4.21 ± 0.30	5.71 ± 0.35	4.83 ± 0.33	4.58 ± 0.56	ns	ns	ns
	ck8 / ccl20	0.36 ± 0.06	0.36 ± 0.05	0.41 ± 0.09	1.30 ± 0.72	0.48 ± 0.07	0.48 ± 0.06	ns	ns	<0.01
Immunoglobulins	*igm*	76.45 ± 7.40	78.78 ± 9.83	73.14 ± 8.04	129.14 ± 16.21	107.61 ± 16.79	96.53 ± 14.03	ns	ns	0.02
	igt	0.67 ± 0.42	3.10 ± 1.20	1.64 ± 0.93	4.82 ± 1.48	2.44 ± 0.72	3.58 ± 2.35	ns	ns	ns
	igt-m	9.16 ± 0.96	10.41 ± 1.23	11.33 ± 2.37	8.35 ± 1.06	7.06 ± 1.00	10.73 ± 2.53	ns	ns	ns
	migm	12.86 ± 1.24	14.55 ± 0.92	14.17 ± 1.39	17.73 ± 1.17	14.67 ± 2.01	18.09 ± 2.704	ns	ns	ns
Anti-protease	*a2m*	0.10 ± 0.04^{b}	0.20 ± 0.02^{a}	0.13 ± 0.01^{a,b}	0.09 ± 0.02	0.11 ± 0.03	0.08 ± 0.02	0.04	ns	0.03
Antimicrobial peptide/ Iron recycling	*hepc*	67.75 ± 10.00	70.28 ± 11.93	58.88 ± 5.06	10.91 ± 1.88	15.15 ± 5.27	7.31 ± 1.92	ns	ns	<0.01
T-cell markers	*cd3e*	2.33 ± 0.37	2.71 ± 0.16	3.15 ± 0.78	3.04 ± 0.13	2.71 ± 0.37	2.83 ± 0.33	ns	ns	ns
	cd3x	2.00 ± 0.23	2.41 ± 0.52	2.06 ± 0.45	2.62 ± 0.19	2.27 ± 0.31	2.47 ± 0.29	ns	ns	ns
	cd4-full	1.51 ± 0.23	2.21 ± 0.68	1.94 ± 0.66	2.05 ± 0.13	1.51 ± 0.19	1.92 ± 0.27	ns	ns	ns
	cd8a	1.28 ± 0.24	1.88 ± 0.60	1.57 ± 0.60	1.19 ± 0.10	0.89 ± 0.14	0.98 ± 0.16	ns	ns	ns
	cd8b	0.62 ± 0.16	0.57 ± 0.11	0.43 ± 0.05	0.35 ± 0.04	0.26 ± 0.04	0.28 ± 0.04	ns	ns	<0.01
	zap70	1.55 ± 0.19	1.62 ± 0.24	1.47 ± 0.22	2.01 ± 0.18	1.61 ± 0.19	1.95 ± 0.26	ns	ns	ns
Pattern recognition receptors	*tlr1*	1.16 ± 0.05	1.46 ± 0.13*	1.30 ± 0.10	1.26 ± 0.06	1.04 ± 0.07$^{\#}$	1.13 ± 0.10	0.03	ns	0.04
	tlr2	1.44 ± 0.12	1.91 ± 0.07	1.69 ± 0.08	3.05 ± 0.15	2.91 ± 0.28	3.17 ± 0.38	ns	ns	<0.01
	tlr5	0.32 ± 0.04	0.29 ± 0.02	0.27 ± 0.02	0.48 ± 0.07	0.45 ± 0.03	0.48 ± 0.04	ns	ns	<0.01
	tlr9	0.26 ± 0.03	0.27 ± 0.02	0.30 ± 0.04	0.79 ± 0.10	0.61 ± 0.09	0.69 ± 0.09	ns	ns	<0.01
	mrc1	5.18 ± 0.66	5.63 ± 0.44	5.17 ± 0.44	5.83 ± 0.32	5.10 ± 0.30	4.16 ± 0.31	ns	ns	ns

Values represent mean ± standard error (n=9) (Raw data), ns – non significant. Different superscript letters represent significant differences between diets within the same time (*P*<0.05). Different superscript symbols represent significant differences in time within the same diet (*P*<0.05).

Table 8. Muscle gene expression profile of gilthead seabream juveniles in response to the different dietary treatments at 2 and 12 weeks feeding time.

		Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Biological Process	**Genes**	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
GH/IGF axis	*ghr-i*	3.17 ± 0.33	3.85 ± 0.55	3.28 ± 0.30	9.99 ± 1.27	10.92 ± 0.97	9.02 ± 1.21	ns	ns	<0.01
	ghr-ii	4.73 ± 0.71	6.68 ± 1.20	4.96 ± 0.71	3.97 ± 0.66	3.58 ± 0.70	3.42 ± 0.53	ns	ns	0.01
	igf-i	0.16 ± 0.01	0.15 ± 0.03	0.16 ± 0.02	0.29 ± 0.04	0.23 ± 0.04	0.20 ± 0.04	ns	ns	<0.01
	igf-ii	1.34 ± 0.12	1.31 ± 0.18	1.30 ± 0.08	2.60 ± 0.24	2.42 ± 0.22	1.94 ± 0.21	ns	ns	<0.01
	igfbp3	3.69 ± 0.30$^{\#}$	3.61 ± 0.31$^{\#}$	4.13 ± 0.11$^{\#}$	1.80 ± 0.18*	1.36 ± 0.12*	1.28 ± 0.15*	0.03	ns	<0.01
	igfbp5b	1.64 ± 0.11	1.84 ± 0.33	1.40 ± 0.05	3.57 ± 0.38	2.90 ± 0.31	2.28 ± 0.18	ns	ns	<0.01
	igfbp6b	0.28 ± 0.03	0.20 ± 0.02	0.30 ± 0.04	0.32 ± 0.04	0.22 ± 0.03	0.19 ± 0.04	0.04	ns	ns
	insr	1.65 ± 0.16	1.73 ± 0.21	2.01 ± 0.25	2.25 ± 0.19	2.24 ± 0.22	1.82 ± 0.20	ns	ns	ns
	igfr1	1.40 ± 0.09	1.48 ± 0.21	1.31 ± 0.11	2.85 ± 0.20	2.98 ± 0.22	2.69 ± 0.28	ns	ns	<0.01
	igfr2	0.98 ± 0.10	1.21 ± 0.30	0.85 ± 0.09	1.34 ± 0.09	1.73 ± 0.35	1.29 ± 0.12	ns	ns	<0.01
Muscle growth and differentiation	*myod1*	10.87 ± 0.53	11.33 ± 1.46	11.46 ± 1.07	13.90 ± 1.60	12.61 ± 0.96	11.56 ± 0.65	ns	ns	ns
	myod2	2.04 ± 0.41	2.37 ± 0.30	2.39 ± 0.26	2.28 ± 0.22	2.01 ± 0.26	1.86 ± 0.35	ns	ns	ns
	myf5	0.47 ± 0.03	0.48 ± 0.06	0.47 ± 0.04	0.47 ± 0.03	0.35 ± 0.02	0.37 ± 0.03	ns	ns	0.01
	myf6	0.45 ± 0.03	0.47 ± 0.08	0.44 ± 0.03	0.75 ± 0.06	0.73 ± 0.05	0.65 ± 0.06	ns	ns	<0.01
	mstn	2.16 ± 0.23	2.93 ± 0.72	2.36 ± 0.28	6.38 ± 1.65^{a}	6.10 ± 1.29^{a}	2.03 ± 0.36^{b}	0.02	ns	<0.01
	mef2a	15.43 ± 1.14	16.90 ± 1.67	17.57 ± 1.69	42.76 ± 3.10	44.70 ± 3.73	41.50 ± 2.95	ns	ns	<0.01
	mef2c	5.94 ± 0.22	6.38 ± 0.76	6.05 ± 0.56	12.08 ± 1.21	12.30 ± 0.88	10.92 ± 0.98	ns	ns	<0.01
	fst	0.67 ± 0.08	0.74 ± 0.08	0.77 ± 0.11	0.57 ± 0.07	0.57 ± 0.06	0.54 ± 0.06	ns	ns	<0.01
Energy sensing & oxidative metabolism	*sirt1*	0.37 ± 0.02	0.43 ± 0.06	0.36 ± 0.03	0.56 ± 0.06	0.59 ± 0.04	0.54 ± 0.03	ns	ns	<0.01
	sirt2	0.48 ± 0.02	0.47 ± 0.06	0.46 ± 0.04	0.75 ± 0.06	0.74 ± 0.07	0.61 ± 0.04	ns	ns	<0.01
	sirt5	1.03 ± 0.08	1.19 ± 0.21	1.08 ± 0.10	1.16 ± 0.10	1.16 ± 0.14	1.07 ± 0.10	ns	ns	ns
	cpt1a	10.72 ± 0.37	12.81 ± 2.67	11.09 ± 0.86	22.94 ± 1.91	23.17 ± 2.22	16.58 ± 1.56	ns	ns	<0.01
	cs	25.09 ± 1.58	30.98 ± 5.76	26.85 ± 2.41	36.41 ± 2.49	40.95 ± 3.07	33.17 ± 2.77	ns	ns	<0.01
	nd2	44.81 ± 3.28	62.25 ± 16.12	47.24 ± 6.55	88.01 ± 15.03	86.45 ± 9.55	75.30 ± 8.90	ns	ns	<0.01
	nd5	26.63 ± 1.85	35.33 ± 7.91	26.71 ± 3.14	45.55 ± 7.20	42.46 ± 4.66	38.70 ± 4.04	ns	ns	<0.01
	cox i	239.75 ± 17.83	292.41 ± 64.62	282.61 ± 26.74	320.82 ± 27.86	318.17 ± 27.74	274.24 ± 30.86	ns	ns	ns
	cox ii	123.85 ± 6.90	170.12 ± 42.93	134.07 ± 13.09	146.09 ± 23.72	132.80 ± 10.66	125.64 ± 14.72	ns	ns	ns
Respiration uncoupling	*ucp3*	14.43 ± 1.93	11.73 ± 2.44	13.47 ± 1.99	29.40 ± 4.92	37.71 ± 3.50	30.81 ± 3.09	ns	ns	<0.01
	pgc1α	0.58 ± 0.15	0.91 ± 0.60	0.42 ± 0.10	2.47 ± 0.43	2.71 ± 0.65	3.66 ± 0.49	ns	ns	<0.01

Values represent mean ± standard error (n=9) (Raw data), ns – non significant. Different superscript letters represent significant differences between diets within the same time (P<0.05). Different superscript symbols represent significant differences in time within the same diet (P<0.05).

3.4 Discussion

In the present study, the potential beneficial effects of adding *P. tricornutum* to an extreme sustainability-driven diet formulation (i.e. 0% FM) were explored, either as whole freeze-dried biomass (WC diet) or processed broken cells (BC diet). In the past, *P. tricornutum* has been successfully tested as a FM replacement ingredient and immunostimulant when incorporated as whole cell biomass in fish feeds [26; 37; 50]. Sørensen *et al.* [26] reported that *P. tricornutum* can replace up to 6% of the FM in Atlantic salmon (*Salmo salar*) feeds without adverse effects on feed utilization and growth performance over a period of 82 days of feeding. Accordingly, in the current study the incorporation of 1% *P. tricornutum* did not negatively affect growth performance over the course of the trial (12 weeks). Dietary protein in fish feeds has shifted in the last years from marine derived sources to terrestrial ones [51; 52]. This shift, although maintaining good growth performance, has the potential to negatively affect fish immune status and response to stressors [19; 53; 54].

In the current study, health status analysis was based on the haematological profile along with several humoral and cellular defense indicators after feeding dietary treatments. Haematology and peripheral cell dynamics were not strongly affected by the incorporation of 1 % *P. tricornutum*. However, BC-fed fish showed a decrease in total WBC from 2 weeks to 12 weeks which translated in a lower concentration of thrombocytes at the end of the experiment, this might indicate thrombocyte activation at an early stage (2 weeks). *P. tricornutum* biomass used in BC diet was disrupted and fractioned, possibly giving rise to cell wall fractions that can act as antigens recognized by thrombocyte cell surface receptors. *P. tricornutum* cell wall is mainly composed by sulphated polysaccharides (SPs) as glucoronomannan [55], these compounds are known to interact with different toll-like receptors (TLRs). At least in carp, peripheral thrombocytes constitutively express different TLRs and also major histocompatibility complex (MHC) II class genes [56]. Furthermore, this cell type has been reported to have phagocytic activity and the ability to ingest particulate antigens possibly acting as an antigen presenting cell [57]. RBC counts did not differ between experimental groups nonetheless, fish fed BC diet showed a significant increase in Hb concentration at 12 weeks. Iron in pennate diatoms can be stored through two previously described mechanisms: protein ferritin and vacuolar storage [58; 59]. Increased Hb concentration might be ascribed to inner-cell iron storages in *P. tricornutum* made available in BC diet. Iron is an essential element for hemoglobin synthesis and higher Hb might lead to an improved O_2 carrying capacity, increasing the animal's energy producing potential in case of a stressful situation [60].

Non-specific humoral and cellular parameters were not affected by the supplementation with 1% *P. tricornutum* either as whole (WC) or processed (BC) freeze-dried biomass. However, previous studies in fish revealed a tendency of dietary microalgae supplementation to stimulate or modulate the immune response [50; 61; 62]. A study done with gilthead seabream fed diets containing 10% *Navicula* sp. included either as whole freeze-dried biomass or as a silage preparation (SN) combined with *Lactobacillus sakei* (10^6 CFU g^{-1}), reported immunostimulant effects caused by dietary supplementation [63]. These authors found an increased leucocyte peroxidase, phagocytosis and complement activities in seabream fed the SN diet compared to those fed CTRL after 2 weeks of feeding. However, the level of supplementation was 10 times higher than in the present study, nevertheless, data appears to support the use of processed microalgae (SN), where bioactive compounds are readily available for absorption and digestion. Additionally, a probiotic effect cannot be ruled out since SN diet was combined with *L. sakei*, and lactic acid bacteria have already been described to enhance fish innate immune system [64; 65; 66]. Cerezuela *et al.* [50] reported a significant increase in serum complement, phagocytic and respiratory burst activity in gilthead seabream fed diets supplemented with 5 and 10% *P. tricornutum* whole biomass for 4 weeks. Immunostimulant effects were attributed to the presence of β-1,3-glucans. Glucans from *P. tricornutum* have a high degree of structural resemblance with laminarin, a polysaccharide extracted from brown macroalgae which stimulates macrophage phagocytic activity [28; 67].

Along with systemic defenses, the skin mucus innate immune defenses were also evaluated in the present study. Pathogens are firstly recognized in mucosal tissues, leading to a local activation of innate immunity components that will in turn activate the overall physiological response [68]. In fish skin, mucus acts as a natural barrier against a wide array of stressors and as a source of lysozyme, complement, lectins and proteolytic enzymes. Contrarily to the humoral parameters, *P. tricornutum* incorporation elicited an early response in mucus innate immune components with BC and WC diet showing increased complement activity at 2 weeks. Sulfated cell wall polysaccharides similar to those found in *P. tricornutum* are able to induce macrophage activation [69; 70]. The presence of these complex hydrocarbons in both microalgae supplemented diets might explain this early alternative complement pathway stimulation in mucus as it is described that activated macrophages can locally secrete complement proteins [71; 72; 73]. At 12 weeks BC fed fish also showed higher bactericidal activity than CTRL. Several authors have tested different plant or herbal based immunostimulants effect on skin mucosal immunity [74; 75; 76]. In common carp fed palm fruit extract, skin mucus lysozyme and protease activity were elevated after eight weeks of feeding [74]. Similarly, Guardiola *et*

al. [76], reported an increase of mucus peroxidase and protease activities, as well as an enhancement of the antioxidant status in animals fed diets with 10% fenugreek seeds. Although effects in mucosal immunity seem promising, it is important to keep in mind that in the present study fish were not stimulated by an inflammatory agent or a live bacteria challenge, in addition to the lower level of inclusion (1%) used in the present study, which might explain the lack of response obtained for most of the humoral parameters.

Transcriptional changes of metabolic, health and growth biomarkers were analyzed in different tissues (i.e. liver, head-kidney and white skeletal muscle). This integrated approach allows an understanding of growth performance and health status, at molecular level, of fish in the given sampling points. From 2 to 12 weeks, several genes were modulated due to *P. tricornutum* dietary supplementation mostly at an early stage (2 weeks). Major impact was found in the GH/IGF system in liver, where Igfs play a key role on animal's growth and development, directly stimulating cell proliferation and differentiation. Previous studies in gilthead seabream juveniles indicated that the somatotropic axis can be affected by changes in feed protein source and level of essential fatty acids [6; 19; 77; 78]. WC-fed fish showed a hepatic upregulation of *igf-ii* at 2 weeks and the downregulation of *mstn* in muscle at the end of the growth trial. Muscle growth in fish depends on myocyte proliferation. Igf-II is a powerful proliferation factor in muscular tissue while Mstn was found to be a potent inhibitor of myoblast proliferation and fiber hypertrophy [79; 80]. Thus, present results point to a positive effect at the transcriptional level in the somatotropic axis from the *P. tricornutum* WC-supplemented diet. Still, in the present study no tendency for increased FBW was perceivable at 12 weeks. Sørensen *et al*. [26] also reported the absence of negative effects after feeding Atlantic salmon for 82 days with a diet where 6 % FM was replaced by *P. tricornutum* whole-cell biomass. However, it is important to point out that the level of microalgae incorporation was higher in the latter study in comparison to the present one. Both Igfs and insulin induce complex effects on metabolism, Igf-I acts primarily as a promoter of cell differentiation and growth and insulin as a regulator to maintain metabolic homeostasis. Still, physiological effects depend on specific binding to the homologous receptor. In gilthead seabream, Igfr-I is mainly expressed in muscle tissue but also in liver, promoting muscle growth and enhanced metabolism [79; 81]. BC-fed fish showed hepatic down regulation of *insr*, *igfr1* and *igfr2* mRNA transcripts, while muscle expression levels were similar among groups. Despite lower hepatic expression levels found in BC-fed fish, it was not possible to ascertain any growth or metabolic impairment between groups. In a different experiment, Ramos-Pinto *et al.* [19] fed gilthead seabream juveniles with a FM-free diet supplemented with tryptophan, revealing the same trend for

early down-regulation of GH/IGF axis in liver without compromising growth performance and metabolism. Additionally, dietary supplementation with *P. tricornutum* whole cells induced changes in other hepatic biological processes such as upregulation of *capn1* at 2 weeks. Calpains are cytoplasmic proteases with a regulatory or signaling function in proteolysis, affecting intracellular protein turnover and muscle growth [82; 83]. Finally, *cat* was up-regulated in WC-fed fish at an early stage. *P. tricornutum* cell wall is rich in sulfated cell wall polysaccharides a common feature shared with different algae species. Previously, cell wall sulfated polysaccharides have been reported to increase serum catalase activity in fish at 0.1% supplementation [84] and also, *cat* expression in a murine macrophage cell line [85]. The head-kidney was also evaluated to determine the effect of dietary treatments on the gene expression patterns of several relevant immune-related transcripts. Diet-related effects on gene expression were promoted by the broken cells diet at 2 weeks: BC group was fed the disrupted cell wall biomass, making microalgae cell contents more available to these fish namely, β-glucans. In *P. tricornutum*, β-glucans are located inside the cell in vacuoles [28]. Once available, these polysaccharides are known to have immunostimulatory effects in fish [67]. At present, the mechanism by which β-glucans are recognized in fish is not fully elucidated, although it is thought that this recognition follows the same pattern as in higher vertebrates. These molecules are recognized by leucocyte surface receptors, mainly by C-type lectin and Toll-like receptors, which activate the transcription of the proinflammatory cytokines IL-1β, TNF-α and IL-6 [67; 86]. BC-fed fish showed higher *il-6* levels than CTR and WC-fed fish, although the increase was only significant between the two microalgae treatments. Interleukin 6 is a pleiotropic cytokine, with both pro- and anti-inflammatory functions. During inflammatory processes, this cytokine is produced by activated cells inducing an acute phase response (APR) and the production of acute phase proteins (APP) [87; 88]. Furthermore, BC fed fish show *a2m* up-regulation at 2 weeks when compared to CTR-fed fish. Alpha-2-macroglobulin is an APP that acts as a non-specific protease inhibitor involved in host defense mechanisms, inhibiting both endogenous and exogenous proteases.

The use of immunostimulants is generally beneficial for fish health status, but effects depend primarily on nutrient or nutraceutical bioavailability. BC diet showed the highest overall immunostimulatory effect and these were mainly felt at an early stage (2 weeks). Which, when compared to previously reported results suggests that they depend on dose and length of administration. In this study, the level of incorporation was low (1% or 10 g/kg feed), which can partially explain the mild immunostimulatory effect reported. Nonetheless, there were promising results in mucus innate immunity, which emphasize the pertinence of further evaluating the inclusion of physically treated *P. tricornutum*

biomasses in the context of a short-term feeding period before a predictable stressful event or disease outbreak. In future works, different levels of supplementation higher than 1% should be tested, followed by an inflammatory insult, in order to evaluate fish immune response.

3.5 Acknowledgements

This work has been funded under the EU FP7 by the MIRACLES project No. 613588: Multi-product Integrated bioRefinery of Algae: from Carbon dioxide and Light Energy to high-value Specialties and by national funds through FCT - Foundation for Science and Technology within the scope of UIDB/04423/2020 and UIDP/04423/2020. The views expressed in this work are the sole responsibility of the authors and do not necessary reflect the views of the European Commission. B. Reis, L. Ramos-Pinto, B. Costas and S. Engrola were supported by the Portuguese Foundation for Science and Technology (PD/BDE/129262/2017, PD/BDE/114436/2016, IF/00197/2015 and IF/00482/2014, respectively).

3.6 Ethics statement

CCMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). The protocol was approved by the CCMAR Animal Welfare Committee.

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Chapter IV

Immune Status and Hepatic Antioxidant Capacity of Gilthead Seabream *Sparus aurata* Juveniles Fed Yeast and Microalga Derived β-glucans

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Immune Status and Hepatic Antioxidant Capacity of Gilthead Seabream *Sparus aurata* Juveniles Fed Yeast and Microalga Derived β-glucans

Abstract

This work aimed to evaluate the effects of dietary supplementation with β-glucans extracted from yeast (*Saccharomyces cerevisiae*) and microalga (*Phaeodactylum tricornutum*) on gene expression, oxidative stress biomarkers and plasma immune parameters in gilthead seabream (*Sparus aurata*) juveniles. A practical commercial diet was used as the control (CTRL), and three others based on CTRL were further supplemented with different β-glucan extracts. One was derived from *S. cerevisiae* (diet MG) and two different extracts of 21% and 37% *P. tricornutum*-derived β-glucans (defined as Phaeo21 and Phaeo37), to give a final 0.06% β-glucan dietary concentration. Quadruplicate groups of 95 gilthead seabream (initial body weight: 4.1 ± 0.1 g) were fed to satiation three times a day for 8 weeks in a pulse-feeding regimen, with experimental diets intercalated with the CTRL dietary treatment every 2 weeks. After 8 weeks of feeding, all groups showed equal growth performance and no changes were found in plasma innate immune status. Nonetheless, fish groups fed β-glucans supplemented diets showed an improved anti-oxidant status compared to those fed CTRL at both sampling points (i.e., 2 and 8 weeks). The intestinal gene expression analysis highlighted the immunomodulatory role of Phaeo37 diet after 8 weeks, inducing an immune tolerance effect in gilthead seabream intestine, and a general down-regulation of immune-related gene expression. In conclusion, the results suggest that the dietary pulse administration of a *P. tricornutum* 37% β-glucans-enriched extract might be used as a counter-measure in a context of gut inflammation, due to its immunetolerant and anti-oxidative effects.

Keywords

Saccharomyces cerevisiae; *Phaeodactylum tricornutum*; *Sparus aurata*; β-glucans; pulse feeding; immune tolerance

4.1 Introduction

Aquaculture is the fastest growing food-sector related industry, and as practices become more intensive, the risk of disease outbreaks increases accordingly [1; 2]. In fact, animal health-related issues are nowadays the major constraint for aquaculture expansion and sustainability [3]. To date, one of the main strategies to cope with disease outbreaks in aquaculture has been the use of antibiotics. Although this issue has been mitigated in recent years with more restrictive legislation and regulations, antibiotics are still routinely used, leading to the emergence of new antibiotic-resistant bacteria [1]. In addition to vaccination, an alternative strategy to the use of antibiotics is the adoption of prophylactic measures through nutrition, such as the incorporation of immunostimulants and prebiotics in feeds to enhance fish disease resistance and general health [2; 4; 5].

Marine microalgae are a rich source of bioactive compounds [6] that are drawing increasing attention considering their use in different applications including functional feeds [7; 8]. *Phaeodactylum tricornutum* is a marine diatom, unicellular brown microalgae rich in several health beneficial compounds such as β-glucans (BGs) [9; 10; 11]. BGs can be naturally found as cell wall components in bacteria, yeast, fungi, plants, micro- and macro-algae, and due to their promising biological activities, BGs have been extensively studied in vertebrates [2; 12; 13; 14]. These polysaccharides can act as a prebiotic, enhancing the growth of commensal microbiota and by directly stimulating the innate immune system through interaction with specific cell receptors [4]. BGs bioactivity depends on their degree of branching, size and molecular structure [15]. However, those with higher biological activity show a common pattern: a repeating chain of (l-3)-linked β-D-glucopyranosyl units with randomly branched single β-D -glucopyranosyl units attached by l-6 or 1–4 linkages [2; 15]. These repeating patterns, a feature shared with bacterial lipopolysaccharides (LPS), can be recognized by the host's cell pattern recognition receptors (PRR) and are termed pathogen-associated microbial patterns (PAMPs). Upon recognition they can elicit an inflammatory response and activate the host's innate immune cells [12].

In mammals, dectin-1 is the best described BGs receptor, considered to be the most important for recognition and signal transduction. It is a C-type lectin receptor (CLR) which is predominantly expressed on cells from both the monocyte/macrophage and neutrophil lineages [12; 16]. In a former study, European common carp (*Cyprinius carpio*) macrophages were activated with curdlan, a dectin-1-specific BG ligand in mammals, showing that immune modulatory effects in carp macrophages could be triggered by a member of the CLR family, although different from dectin-1 receptor [17]. In teleosts, the specific receptors involved in the recognition of BGs and consequent downstream

signaling remains to be elucidated [14; 18; 19; 20]. In contrast, the beneficial effects of BGs in fish innate immune response are well documented. Most of the studies focusing on fish showed that oral administration of BGs not only benefits innate immune response, such as the increase of phagocytic capacity, oxidative burst, lysozyme and complement activity [21; 22; 23; 24], but also modulates immune gene expression in different organs [25; 26; 27]. However, the use of BG-rich microalgae cell extracts as feed supplements to modulate both the systemic and local immune response is still poorly explored. Therefore, the present study aimed to evaluate the effects of β-glucans extracted from microalga (*P. tricornutum*) and yeast (*Saccharomyces cerevisiae*), when applied as dietary supplements for juveniles of a valuable fish species for European aquaculture such as gilthead seabream (*Sparus aurata*).

4.2 Materials and Methods

4.2.1 P. tricornutum Extracts

Chrysolaminarin-rich biomass from *P. tricornutum* (SAG 1090-1b) grown under nitrogen-depleted conditions in flat panel airlift reactors was harvested and concentrated via centrifugation to 250–270 g L^{-1} (Clara 20, Alfa Laval). Afterwards, the biomass was frozen at -20 °C. For further processing, the biomass was thawed and diluted to 100 g L^{-1} with deionized water. The cell disruption was performed according to Derwenskus *et al.* [28] with a ball mill (PML-2, Bühler). Phaeo21 was freeze dried after cell disruption (VaCo 5, Zirbus), while Phaeo37 was centrifuged and the supernatant freeze-dried (Avanti J-26 XP, Beckman Coulter).

4.2.2 Diet Composition

The trial comprised four isonitrogenous (63% crude protein) and isolipidic (17% crude fat) diets (Table 4). A high-quality, practical diet was used as control (CTRL) and 3 experimental diets based on CTRL were supplemented with either a commercial product derived from *S. cerevisiae* (diet MG) or different extracts of *P. tricornutum* (diets Phaeo21 and Phaeo37), to obtain a final concentration of 0.6 g β-glucans per kg of feed (0.06%) in all supplemented diets. Diets were manufactured by SPAROS. All powder ingredients were mixed according to the target formulation in a double-helix mixer (model RM90, MAINCA, Spain) and ground (below 200 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Subsequently, the oils were added to the mixtures, which were humidified with 20–25% water and agglomerated by a low-shear and low-

temperature extrusion process (ITALPLAST, Italy). Extruded pellets (1.5 mm) were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). Diets were packed in sealed plastic buckets and shipped to the research site (Riasearch, Murtosa, Portugal) where they were stored at room temperature in a cool and aerated emplacement. Samples of each diet were taken for analytical characterization.

Table 4. Ingredients and proximate composition of experimental diets.

Ingredients %	**CTRL**	**MG**	**Phaeo21**	**Phaeo37**
Fishmeal [1]	20.00	20.00	20.00	20.00
Fish protein hydrolysate [2]	8.00	8.00	8.00	8.00
Squid meal [3]	21.00	21.00	21.00	21.00
Krill meal [4]	16.50	16.50	16.50	16.50
Wheat gluten [5]	11.50	11.50	11.50	11.50
Wheat meal [6]	0.29	0.19		0.13
Vitamin and mineral premix [7]	2.00	2.00	2.00	2.00
Lecithin [8]	4.30	4.30	4.30	4.30
Fish oil [9]	6.50	6.50	6.50	6.50
Binders, antioxidant and other additives [10]	9.91	9.91	9.91	9.91
Yeast beta-glucans [11]		0.10		
Algae beta-glucans Phaeo21 [12]			0.29	
Algae beta-glucans Phaeo37 [13]				0.16
Proximate composition				
Dry matter (DM) %	94.60	94.20	94.20	94.50
Ash, % DM	9.60	9.50	9.50	9.50
Crude protein, % DM	62.90	62.80	62.80	62.90
Crude fat, % DM	17.10	17.10	17.10	17.10
Gross energy (kJ g^{-1} DM)	22.90	22.90	22.90	22.90

[1] Super Prime: 66.3% CP, 11.5% CF, Pesquera Diamante, Peru; [2] CPSP 90: 82% CP 9% CF, Sopropêche, France; [3] Squid meal without guts: 83% CP, 4% CF, Sopropêche, France; [4] Krill meal: 61.1% CP, 17.4% CF, Aker Biomarine, Norway; [5] VITEN: 82% CP, 2.1% CF, Roquette, France; [6] Wheat meal: 10.2% CP; 1.2% CF, MOLISUR, Spain; [7] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 50 mg; retinyl acetate, 40000 IU; DL-cholecalciferol, 4000 IU; thiamin, 60 mg; riboflavin, 60 mg; pyridoxine, 40 mg; cyanocobalamin, 0.2 mg; nicotinic acid, 400 mg; folic acid, 30 mg; ascorbic acid, 1000 mg; inositol, 1000 mg; biotin, 6 mg; calcium panthotenate, 200 mg; choline chloride, 2000 mg, betaine, 1000 mg. Minerals (g or mg/kg diet): copper sulphate, 18 mg; ferric sulphate, 12 mg; potassium iodide, 1 mg; manganese oxide, 20 mg; sodium selenite, 0.02 mg; zinc sulphate, 27.5 mg; sodium chloride, 800 mg; excipient wheat middling's; [8] LECICO GmbH, Germany; [9] Sopropêche, France; [10] Confidential blend of constant binders and other additives; [11] Macrogard, 67.2% beta-glucans, Biorigin, Brazil; [12] Beta-glucan rich biomass of microalgae *(Phaeodactylum tricornutum* from SAG culture collection*)* with 21% beta-glucans; [13] Beta-glucan rich extract of microalgae *(Phaeodactylum tricornutum* from SAG culture collection*)* with 37% beta-glucans.

4.2.3 Fish Rearing Conditions and Feeding Trial

Fish were reared in a seawater recirculation system with aeration (mean dissolved oxygen above 6 mg L^{-1}) and water flow at 3 L min^{-1} (mean temperature 24.1 ± 0.6 °C; mean salinity 18.7 ± 0.1‰). Water quality parameters were monitored daily (mean dissolved oxygen 6.4 ± 1.0 mg L^{-1}; mean unionized ammonia levels 0.001 ± 0.002 mg L^{-1}). Diets were randomly assigned to quadruplicate groups of 95 gilthead seabream juveniles (initial body weight: 4.1 ± 0.1 g) that were fed to satiation three times a day for 8 weeks in a pulse-feeding regimen. Accordingly, in fish fed the different experimental diets, the CTRL diet was intercalated every 2 weeks, as shown in Figure 6.

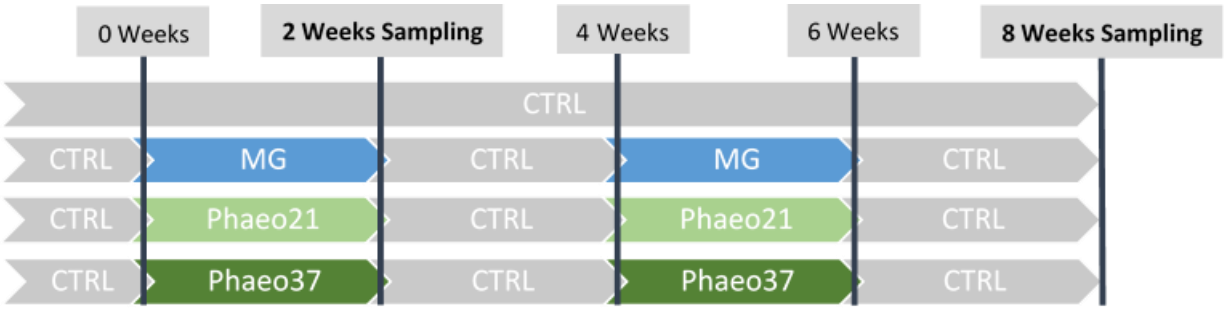


Figure 6. Schematic overview of the experimental design.

4.2.4 Sampling Procedures

Fish were individually weighed at the beginning and after 2 and 8 weeks of the feeding trial and feed consumption for each experimental replicate was registered daily. After 2 and 8 weeks, three fish per tank were euthanized with a 2-phenoxyethanol lethal dose (0.5 mL L^{-1}) [29], weighed and sampled for tissues (blood, head-kidney, liver and gut). Blood was collected from the caudal vein using heparinized syringes and centrifuged at 10,000× *g* during 10 min at 4 °C to obtain plasma samples. Plasma, head-kidney and liver samples were immediately frozen at −80 °C, and anterior intestine was preserved in RNA later until further analysis.

4.2.5 Haematological Procedures

Blood smears were prepared from peripheral blood, air dried and stained with Wright's stain (Haemacolor; Merck) after fixation for 1 min with formol–ethanol (10% formaldehyde in ethanol). Neutrophils were labelled through the detection of peroxidase activity revealed by the Antonow's technique described by Afonso *et al.* [30]. The slides

were examined under oil immersion (1000×), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The relative percentage of each cell type was calculated.

4.2.6 Innate Humoral Parameters

4.2.6.1 Bactericidal activity

Plasma bactericidal activity was determined following the method described by Machado *et al.* [31] with some modifications. *Edwardsiella tarda* (*E. tarda*) strain ACC 53.1, gently provided by Prof. Alicia Toranzo (University of Santiago, Spain) was used in the protocol. Briefly, 20 µL of plasma were mixed with 20 µL of bacteria suspension (10^8 CFU mL^{-1}) in duplicate in a flat-bottom 96-well plate that was incubated for 2.5 h at 25 °C (positive control: 20 µL of TSB instead of plasma). Afterwards, 25 µL of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg mL^{-1}; Sigma, St. Louis, MO, USA) was added to each well and incubated for 10 min at 25 °C to allow the formation of formazan precipitates. Plates were then centrifuged at 2000× *g* for 10 min, the supernatant was discarded and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (Sigma, St. Louis, MO, USA). The absorbance was then measured at 560 nm. Bactericidal activity is expressed as a percentage calculated from the difference between surviving bacteria compared to the number of bacteria from positive controls (100%).

4.2.6.2 Antiprotease activity

Antiprotease activity was determined as described by Ellis *et al.* [32] with some modifications. Briefly, 10 µL of plasma were incubated with the same volume of trypsin solution (5 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) for 10 min at 22 °C. After incubation, 100 µL of phosphate buffer (NaH_2PO_4, 13.9 mg mL^{-1}, pH 7.0) and 125 µL of azocasein solution (20 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) were added and incubated for 1 h at 22 °C. Finally, 250 µL of trichloroacetic acid were added to the reaction mixture and incubated for 30 min at 22 °C. The mixture was centrifuged at 10,000× *g* for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred to a 96-well plate and mixed with 100 µL of NaOH (40 mg mL^{-1}). The OD was read at 450 nm in a Synergy HT microplate reader. Phosphate buffer instead of plasma and trypsin served as blank, whereas the reference sample was phosphate buffer instead of plasma. The

sample inhibition percentage of trypsin activity was calculated as follows: 100 − ((sample absorbance/reference absorbance) × 100). All analyses were conducted in duplicate.

4.2.6.3 Immunoglobulin M

Plasma immunoglobulin M (IgMs) levels were measured by an ELISA assay. Briefly, plasma samples were diluted (1:100) in Na_2CO_3 (50 mM, pH = 9.6). Diluted plasma samples (100 µL in duplicate) were incubated overnight (4 °C) in a 96 well plate, using Na_2CO_3 (100 µL) as a negative control. The samples (antigen) were then removed and 300 µL of blocking buffer (5% low fat milk in 0.1% Tween 20) was added to each well and incubated for 1 h at 22 °C. This mixture was then removed, followed by three consecutive washes with 300 µL of T-TBS (0.1% Tween 20). After properly cleaning and drying the wells, 100 µL of the anti-seabream primary IgM monoclonal antibody (1:200 dilution in blocking buffer; Aquatic Diagnostics, UK) was added to each well and incubated for 1 h at 22 °C. The primary antibody was then removed by aspiration, with three consecutive washes being performed. Afterwards, the anti-mouse IgG-HRP, secondary antibody (1:1000 dilution in blocking buffer; SIGMA), was added and incubated for 1 h at 22 °C, then removed by aspiration. The wells were again washed three times and 100 µL of TMB substrate solution for ELISA (BioLegend #421101), was added to each well and incubated for 5 min. The reaction was stopped after 5 min by adding 100 µL of H_2SO_4 2 M and the optical density was read at 450 nm.

4.2.7 Analysis of Oxidative Stress Biomarkers

Liver samples were thawed and homogenized (1:10) in phosphate buffer 0.1 M (pH 7.4) using Precellys evolution tissue lyser homogenizer.

4.2.7.1 Lipid peroxidation (LPO)

One aliquot of tissue homogenate was used to determine the extent of endogenous LPO by measuring thiobarbituric acid-reactive species (TBARS) as suggested by Bird and Draper [33]. To prevent artifactual lipid peroxidation, butylhydroxytoluene (BHT 0.2 mM) was added to the aliquot. Briefly, 1 mL of 100% trichloroacetic acid and 1 mL of 0.73% thiobarbituric acid solution (in Tris–HCl 60 mM pH 7.4 with DTPA 0.1 mM) were added to 0.2 mL of liver homogenate. After incubation at 100 °C for 60 min, the solution was centrifuged at 12,000× *g* for 5 min and LPO levels were determined at 535 nm.

4.2.7.2 Total protein quantification

The remaining tissue homogenate was centrifuged for 20 min at 10,000× *g* (4 °C) to obtain the post mitochondrial supernatant fraction (PMS). Total proteins in homogenates were measured by using Pierce™ BCA Protein Assay Kit, as described by the manufacturer.

4.2.7.3 Catalase (CAT)

CAT activity was determined in PMS by measuring substrate (H_2O_2) consumption at 240 nm according to Claiborne [34] adapted to microplate. Briefly, in a microplate well, 0.140 mL of phosphate buffer (0.05 M pH 7.0) and 0.150 mL H_2O_2 solution (30 mM in phosphate buffer 0.05 M pH 7.0) were added to 0.01 mL of liver PMS (0.7 mg ml^{-1} total protein). Enzymatic activity was determined in a microplate reader (BioTek Synergy HT) reading the optical density at 240 nm for 2 min every 15 sec interval.

4.2.7.4 Superoxide dismutase (SOD)

SOD activity was measured according to Flohé and Otting [35] adapted to microplate by Lima *et al.* [36]. Briefly, in a microplate well, 0.2 mL of the reaction solution [1 part xantine solution 0.7 mM (in NaOH 1 mM) and 10 parts cytochrome c solution 0.03 mM (in phosphate buffer 50 mM pH 7.8 with 1 mM Na-EDTA)] was added to 0.05 mL of liver PMS (0.25 mg ml^{-1} total protein). Optical density was measured at 550 nm in a microplate reader (BioTek Synergy HT, Winooski, VT, USA) every 20-s interval for 3 min at 25° C.

4.2.7.5 Total glutathione (tGSH)

tGSH content was determined with PMS fraction at 412 nm using a recycling reaction of reduced glutathione (GSH) with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) excess [37; 38]. TG content is calculated as the rate of TNB^{2-} formation with an extinction coefficient of DTNB chromophore formed, ε = $14.1 \times 10^3\ M^{-1}cm^{-1}$.

4.2.8 Gene Expression

Total RNA isolation from target tissue (anterior intestine) was conducted with NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal) following the manufacturer's specifications. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. RT reactions were incubated for 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without reverse transcriptase. Real-time quantitative PCR was carried out on an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) using a 96-well PCR array layout with 44 genes designed for simultaneously profiling of anterior intestine (Table 5). Genes comprised in the array were selected for their involvement in gut integrity, health, immunity and signal transduction. Specific primer pair sequences are listed in Appendix III, Table S3c. Controls of general PCR performance were included on each array. Briefly, RT reactions were diluted to obtain the equivalent concentration of 660 pg of total input RNA which were used in a 25-µL volume for each PCR reaction. PCR wells contained a 2× SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 µM were used to obtain amplicons 50–150 bp in length. The program used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The efficiency of PCR reactions was always higher than 90%, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C), and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the PCR extension phase were normalized using the delta–delta Ct method [(39)]. Beta-actin (*actb*) was tested for gene expression stability using GeNorm software (M score = 0.13) and it was used as housekeeping gene in the normalization procedure. For comparing the mRNA expression level of a panel of genes in a given dietary treatment, all data values were in reference to the expression level of claudin 12 (*cldn12*) in CTRL fish, which was arbitrarily assigned a value of 1.

Table 5. PCR-array layout for gene expression profiling of anterior intestine in seabream.

Function	Gene	Symbol	GenBank
Epithelia integrity	proliferating cell nuclear antigen	*pcna*	KF857335
	transcription factor HES-1-B	*hes1-b*	KF857344
	krueppel-like factor 4	*klf4*	KF857346
	claudin-12	*cldn12*	KF861992
	claudin-15	*cldn15*	KF861993
	cadherin-1	*cdh1*	KF861995
	cadherin-17	*cdh17*	KF861996
	tight junction protein ZO-1	*tjp1*	KF861994
	desmoplakin	*dsp*	KF861999
	gap junction Cx32.2 protein	*cx32.2*	KF862000
	coxsackievirus and adenovirus receptor homolog	*cxadr*	KF861998
Nutrient transport	intestinal-type alkaline phosphatase	*alpi*	KF857309
	liver type fatty acid-binding protein	*fabp1*	KF857311
	intestinal fatty acid-binding protein	*fabp2*	KF857310
	ileal fatty acid-binding protein	*fabp6*	KF857312
Mucus production	mucin 2	*muc2*	JQ277710
	mucin 13	*muc13*	JQ277713
Interleukins	tumor necrosis factor-alpha	*tnf-alpha*	AJ413189
	interleukin 1 beta	*il1b*	AJ419178
	interleukin 6	*il6*	EU244588
	interleukin 7	*il7*	JX976618
	interleukin 8	*il8*	JX976619
	interleukin 10	*il10*	JX976621
	interleukin 12 subunit beta	*il12b*	JX976624
	interleukin 15	*il15*	JX976625
	interleukin 34	*il34*	JX976629
Cell markers	cluster differentiation 4	*cd4*	AM489485
	cluster differentiation 8 beta	*cd8b*	KX231275
	C-C chemokine receptor 3	*ccr3*	KF857317
	C-C chemokine receptor 9	*ccr9*	KF857318
	C-C chemokine receptor 11	*ccr11*	KF857319
	C-C chemokine ck8 / C-C motif chemokine ligand 20	*ck8/ ccl20*	GU181393
	macrophage colony-stimulating factor 1 receptor	*csf1r*	AM050293
Ig production	immunoglobulin M	*igm*	JQ811851
	immunoglobulin T membrane-bound form	*igt-m*	KX599201
Pathogen associated microbial pattern (PAMP)	galectin 1	*lgals1*	KF862003
	galectin 8	*lgals8*	KF862004
	toll like receptor 2	*tlr2*	KF857323
	toll like receptor 5	*tlr5*	KF857324
	toll like receptor 9	*tlr9*	AY751797
	CD209 antigen-like protein D	*cd209d*	KF857327
	CD302 antigen	*cd302*	KF857328
	macrophage mannose receptor 1	*mrc1*	KF857326
	fucolectin	*fcl*	KF857331

4.2.9 Data Analysis

All results are expressed as mean ± standard error (mean ± SE). Residuals were tested for normality (Shapiro–Wilk´s test) and homogeneity of variance (Levene's test). When residuals did not meet the assumptions, data were transformed by a Box-Cox transformation. One-way ANOVAs were performed for all datasets, with "dietary treatment" as the fixed effect, followed by multiple comparison Tukey post-hoc tests. The factor "time" was not considered in the analysis since it is not a goal of the study to evaluate how time affects the measured variables, and groups were treated independently for 2 and 8 weeks.

In an attempt to discriminate and classify individuals by the existing groups, a multivariate canonical discriminant analysis was performed on the physiological dataset (obtained from blood, plasma and liver tissues analyses) to evaluate the linear combinations of the original variables that will best separate the groups (discriminant functions). Each discriminant function explains part of total variance of the dataset and is loaded by variables contributing the most for that variation. Discriminatory effectiveness was assessed by Wilk's λ test, and the distance between groups' centroids was measured by squared Mahalanobis distance, and Fisher's F statistic was applied to infer significance. All statistical analyses were performed using the computer package SPSS 26 for WINDOWS.

Gene expression results were evaluated with an unsupervised multivariate analysis by principal component analysis (PCA) as an unbiased statistical method to observe intrinsic trends in the dataset, using EZ-INFO® v3.0 (Umetrics, Sweden). To achieve the maximum separation among the groups, a supervised multivariate analysis by partial least-squares discriminant analysis (PLS-DA) was sequentially applied, using EZ-INFO® v3.0 (Umetrics, Sweden). Potential differential genes were selected according to the Variable Importance in the Projection (VIP) values. Variables with VIP > 1 were considered to be influential for the separation of samples in PLS-DA analysis [40; 41; 42]. The level of significance used was $p \leq 0.05$ for all statistical tests. Heat map of transcript levels were produced with the R package gplots, using the average linkage method and Euclidean distance.

4.3 Results

4.3.1 Growth Performance

Growth performance data are presented in Table 1. At the end of the trial (8 weeks), all fish showed similar final whole-body weight, regardless of dietary treatment. All groups showed similar feed conversion ratio (FCR) and relative growth rate (RGR) values (1.2 and 3.8% day^{-1}, respectively).

Table 1. Growth performance parameters in gilthead seabream juveniles after 8 weeks of feeding regimen. Data are the mean ± SEM (n = 4).

	8 Weeks			
DIETS	**CTRL**	**MG**	**Phaeo21**	**Phaeo37**
IBW	**4.18 ±** 0.04	**4.12 ±** 0.02	**4.12 ±** 0.05	**4.15 ±** 0.05
FBW	**41.36 ±** 0.81	**42.48 ±** 0.43	**42.08 ±** 0.53	**41.93 ±** 0.97
RGR	**3.77 ±** 0.03	**3.83 ±** 0.02	**3.82 ±** 0.03	**3.8 ±** 0.03
FCR	**1.20 ±** 0.01	**1.19 ±** 0.01	**1.21 ±** 0.02	**1.19 ±** 0.04

IBW: initial body weight (g); FBW: final body weight (g); RGR: relative growth rate (% average body weight/day) and FCR: feed conversion ratio.

4.3.2 Haematological Profile and Humoral Parameters

Peripheral cell dynamics were analysed at both sampling points (Table 2). The relative percentage of circulating lymphocytes increased in fish-fed Phaeo21 compared to CTRL and Phaeo37 groups after 2 weeks of feeding. In contrast, the same cell type showed decreased percentages in fish fed MG and Phaeo21 dietary treatments compared to those fed CTRL in the second (8 weeks) sampling. Furthermore, after 8 weeks, peripheral thrombocytes were higher in MG and Phaeo37 compared to CTRL. Monocytes and neutrophils numbers remained unaltered among dietary groups at both sampling points. Plasma humoral parameters (i.e., bactericidal activity, antiprotease activity and IgM) remained unchanged among dietary treatments at both 2 and 8 weeks (Table 3).

Table 2**.** Percentage values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) in gilthead seabream juveniles after 2 and 8 weeks of feeding regimen. Data are the mean ± SEM (n = 12).

	2 Weeks				8 Weeks			
DIETS	**CTRL**	**MG**	**Phaeo21**	**Phaeo37**	**CTRL**	**MG**	**Phaeo21**	**Phaeo37**
CELLS (%)								
Thrombocytes	**65.2 ±** 2.0	**63.0 ±** 2.2	**60.3 ±** 1.8	**64.7 ±** 1.6	**71.2** ^{b} **±** 2.6	**78.5** ^{a} **±** 1.4	**76.4** ^{a,b} **±** 2.5	**81.0** ^{a} **±** 0.8
Lymphocytes	**24.1** ^{b} **±** 1.5	**28.3** ^{a,b} **±** 1.9	**30.7** ^{a} **±** 1.4	**24.8** ^{b} **±** 1.3	**18.4** ^{a} **±** 2.5	**13.0** ^{b} **±** 1.0	**13.1** ^{b} **±** 2.6	**13.4** ^{a,b} **±** 0.8
Monocytes	**5.3 ±** 0.8	**3.5 ±** 0.7	**4.4 ±** 0.7	**5.2 ±** 0.4	**3.4 ±** 0.6	**2.4 ±** 0.7	**3.1 ±** 0.6	**2.0 ±** 0.5
Neutrophils	**4.4 ±** 1.0	**4.1 ±** 0.9	**4.1 ±** 0.7	**4.8 ±** 0.7	**4.7 ±** 0.4	**5.6 ±** 0.6	**4.3 ±** 0.7	**3.6 ±** 0.6

Different superscript letters indicate significant differences between diets (p < 0.05) within the same sampling point.

Table 3. Plasma immune parameters of gilthead seabream juveniles after 2 and 8 weeks feeding (antiprotease activity, bactericidal activity and immunoglobulin M). Data are the mean ± SEM (n = 12). Different letters indicate significant differences between dietary treatments (p < 0.05).

	2 Weeks				8 Weeks			
DIETS	**CTRL**	**MG**	**Phaeo21**	**Phaeo37**	**CTRL**	**MG**	**Phaeo21**	**Phaeo37**
Antiprotease act (%)	**95.7 ±** 0.6	**96.0 ±** 0.5	**95.5 ±** 0.7	**95.4 ±** 0.6	**97.9 ±** 0.2	**98.1 ±** 0.1	**97.9 ±** 0.2	**98.2 ±** 0.2
Bactericidal act (%)	**45.0 ±** 6.3	**35.5 ±** 4.0	**40.3 ±** 6.6	**45.4 ±** 4.4	**53.3 ±** 7.2	**56.5 ±** 5.8	**57.4 ±** 6.8	**61.8 ±** 4.8
IgM (OD 450 nm)	**0.31 ±** 0.03	**0.32 ±** 0.03	**0.37 ±** 0.05	**0.29 ±** 0.03	**0.62 ±** 0.05	**0.53 ±** 0.03	**0.47 ±** 0.05	**0.55 ±** 0.03

4.3.3 Oxidative Stress Biomarkers

Hepatic oxidative stress biomarkers showed significant differences at both sampling points (Fig. 1). Lipid peroxidation decreased in seabream-fed MG and Phaeo37 diets compared to those fed CTRL at 2 weeks (early sampling), while the extent of lipid peroxidation was similar among groups after 8 weeks (final sampling). Catalase (CAT) and superoxide dismutase (SOD) activities were not affected by the dietary treatments at 2 weeks, however, CAT activity increased in Phaeo21 compared to Phaeo37 group at 8 weeks. SOD activity was enhanced in Phaeo37-fed fish compared to those fed CTRL and MG dietary treatments. Total glutathione remained unaltered among dietary groups at both sampling points.

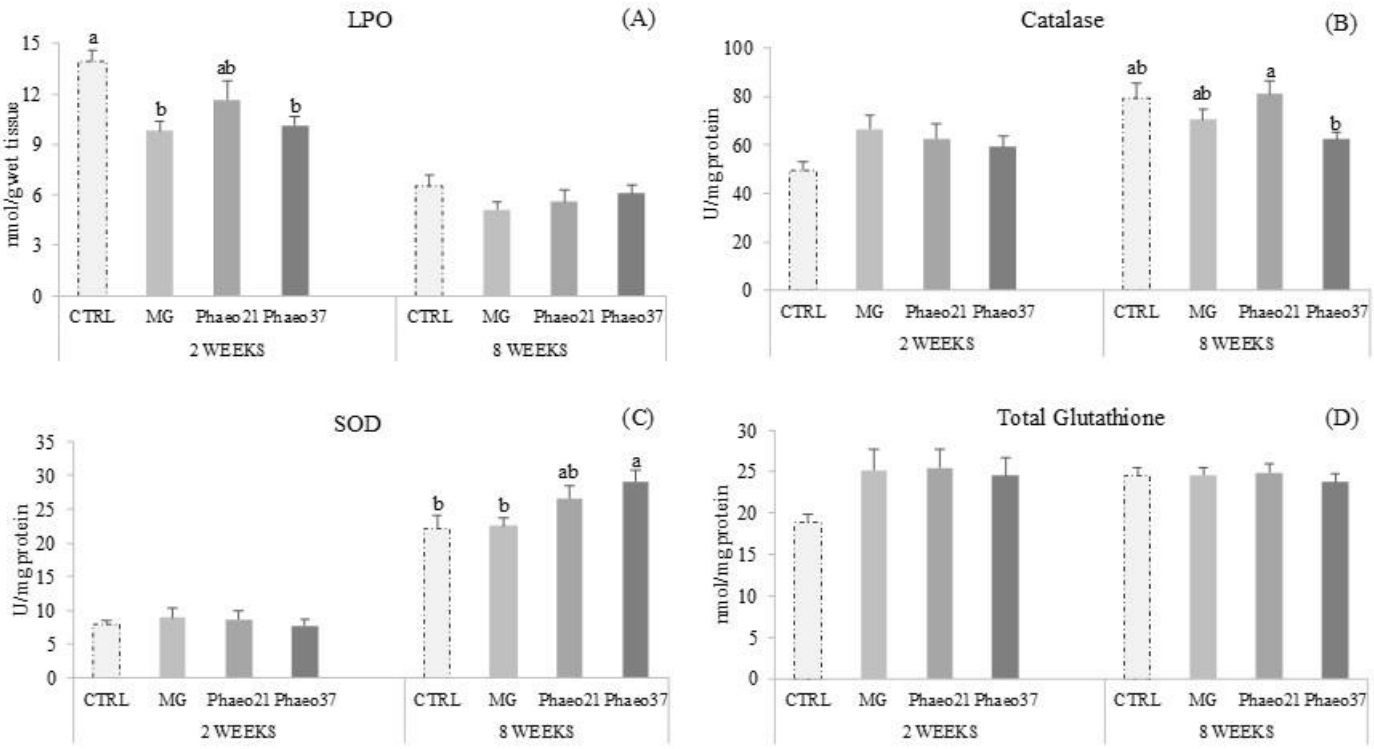


Figure 1. Liver oxidative stress biomarkers of gilthead seabream juveniles after 2 and 8 weeks feeding. Lipid peroxidation (LPO) (**A**); Catalase activity (CAT) (**B**); Superoxide dismutase activity (SOD) (**C**) and Total Glutathione (**D**). Data are the mean ± SEM (n = 12). Different lowercase letters indicate significant differences between dietary treatments ($p < 0.05$).

4.3.4 Multivariate Analysis from Physiological Parameters

An overall multivariate analysis combining raw data from haematological, humoral and hepatic oxidative stress biomarkers (using PCA-DA) was performed to discriminate the physiological effects caused by the experimental diets both at 2 and 8 weeks of feeding (Fig. 2). The first two discriminant functions accounted for 95% of dataset variability at 2 weeks. Group discrimination was significant (Wilk's lambda = 0.3, p = 0.01) highlighting the differences between CTRL and BG groups (p < 0.03). This discrimination was loaded by lower lipid peroxidation, higher CAT and tGSH and to a lesser extent higher antiproteases activity in BG groups. At 8 weeks, groups were discriminated (Wilk's lambda = 0.2; p < 0.001) and the first two discriminant factors accounted for 84% of dataset variability. CTRL and MG dietary treatments were significantly discriminated from the Phaeo groups (p < 0.04) and this separation was loaded by a higher SOD activity and thrombocyte percentage in the last groups.

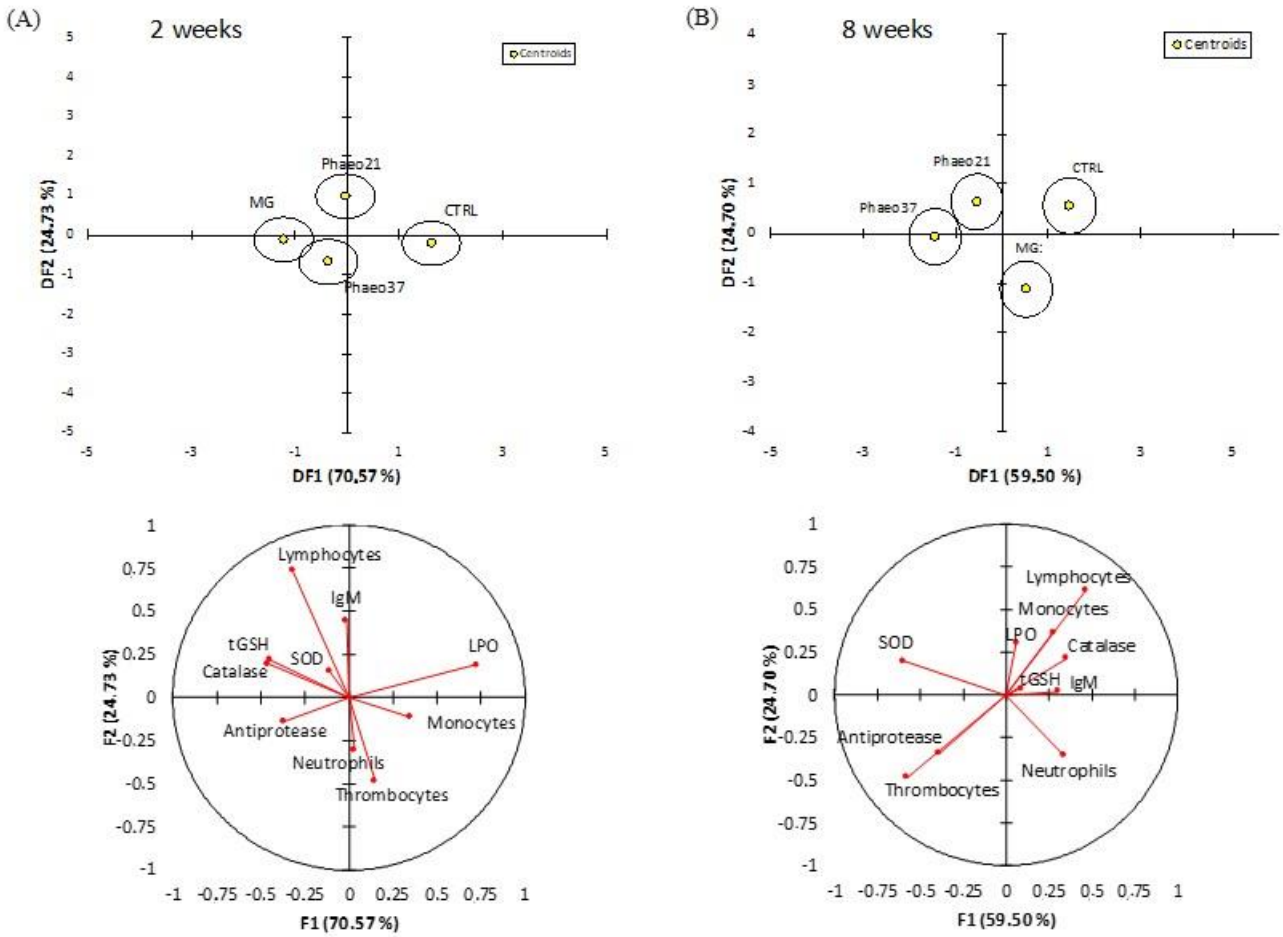


Figure 2. Discriminant analysis of experimental groups based on all physiological biomarkers analysed in the target tissues (yellow marker indicates the centroids of each group) and variables loads for DF1 and DF2 at 2 and 8 weeks. (**A**) 2 weeks; (**B**) 8 weeks.

4.3.5 Gene Expression Analysis

Different pathways represented in this gene array showed significant dietary effects in the proximal intestine. Proliferating cell nuclear antigen gene (*pcna*) was down-regulated in Phaeo37-fed fish at 2 weeks (Fig. 3A). In contrast, different genes belonging to different molecular and cellular pathways were down-regulated in seabream-fed Phaeo37 after 8 weeks of feeding, in particular, fucolectin (*fcl*) (Fig. 3D), as well as gap junction cx32.2 protein (*cx32.2*) (Fig. 3B) genes. Moreover, the latter gene was down-regulated in fish fed MG as well as interleukin 10 (*il10*) (Fig. 3C). Complete relative gene expression profile of the anterior intestine is provided as supplementary material in Appendix III (Tables S1c and S2c). According to the supplementary tables, it was found that the mRNA expression levels of the other analysed genes showed no statistically significant differences among experimental groups.

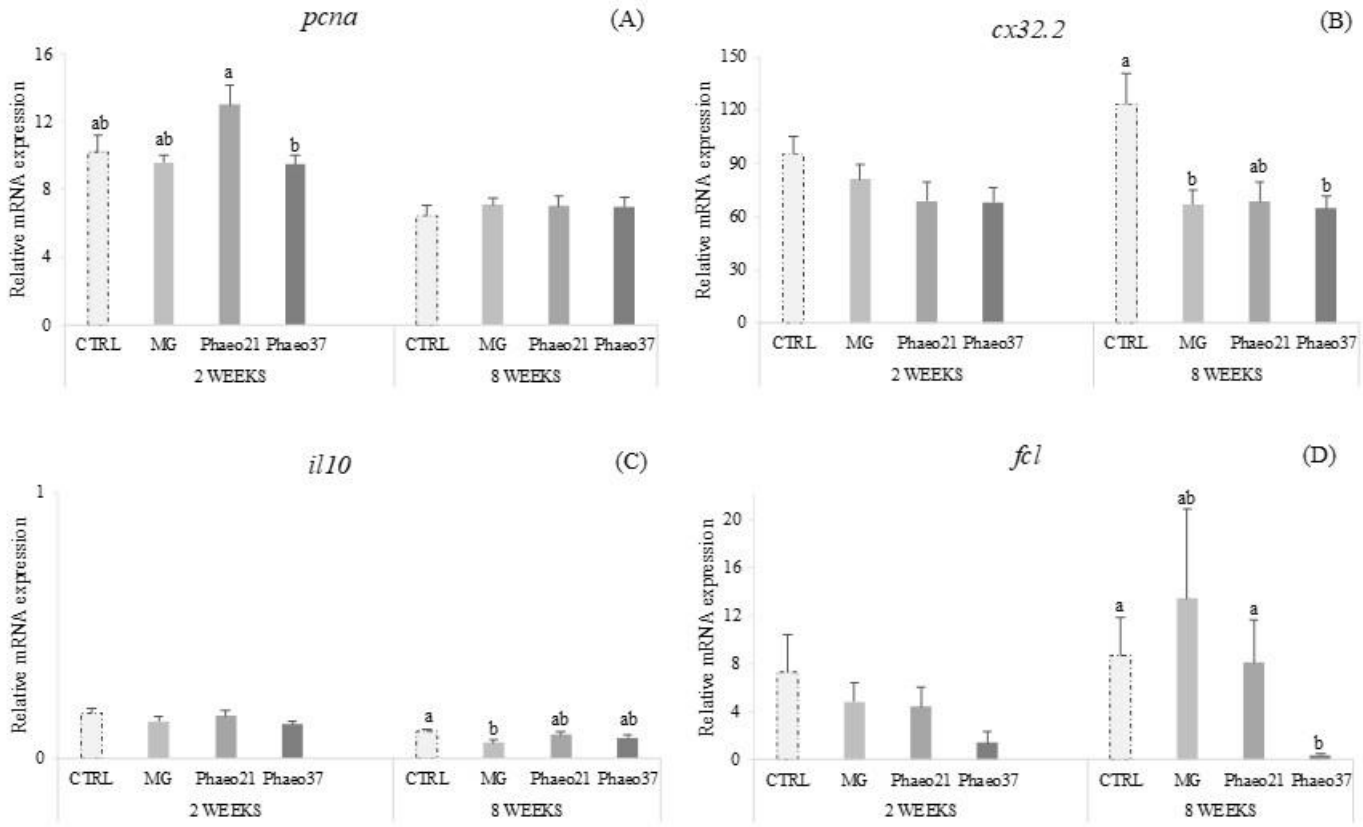


Figure 3. Relative mRNA expression of *pcna* (**A**), *cx32.2* (**B**), *il10* (**C**) and *fcl* (**D**) genes in the anterior intestinal tissue of gilthead seabream juveniles fed the experimental diets for 2 and 8 weeks. Data are the mean ± SEM (n = 9). All data values for each gene were in reference to the expression level of *cldn12* of CTRL fish with an arbitrary assigned value of 1. p values result from one-way ANOVA. Different lowercase letters indicate significant differences among dietary treatments ($p < 0.05$).

Further differences between fish fed the experimental diets in comparison to CTRL were highlighted by a clustering heatmap of gene expression after 8 weeks of feeding (Fig. 4). This approach pointed to Phaeo37 as the experimental diet more apart from CTRL in the gene expression pattern. In order to get a clearer picture of the dietary effect on intestinal gene expression, an overall multivariate analysis combining raw data from the different genes (using PLS-DA) in CTRL and Phaeo 37 groups was performed. For the 2 weeks feeding period, the model was not able to show a clear separation between experimental groups (data not shown). However, at 8 weeks, the same approach showed that expression patterns can be summarized through two main components that explain 88.11% of total variance (Fig. 5A). On the one hand, component 1 (63.59% of total variance, X-axis) appeared to be mostly related to diet effect, as it was able to clearly separate Phaeo37- and CTRL-fed fish. On the other hand, component 2 (19.52% of total variance, Y-axis) appeared to account for inner group variability. A total of 20 genes showed a VIP value > 1, highlighting their contribution to diet differences (Fig. 5B).

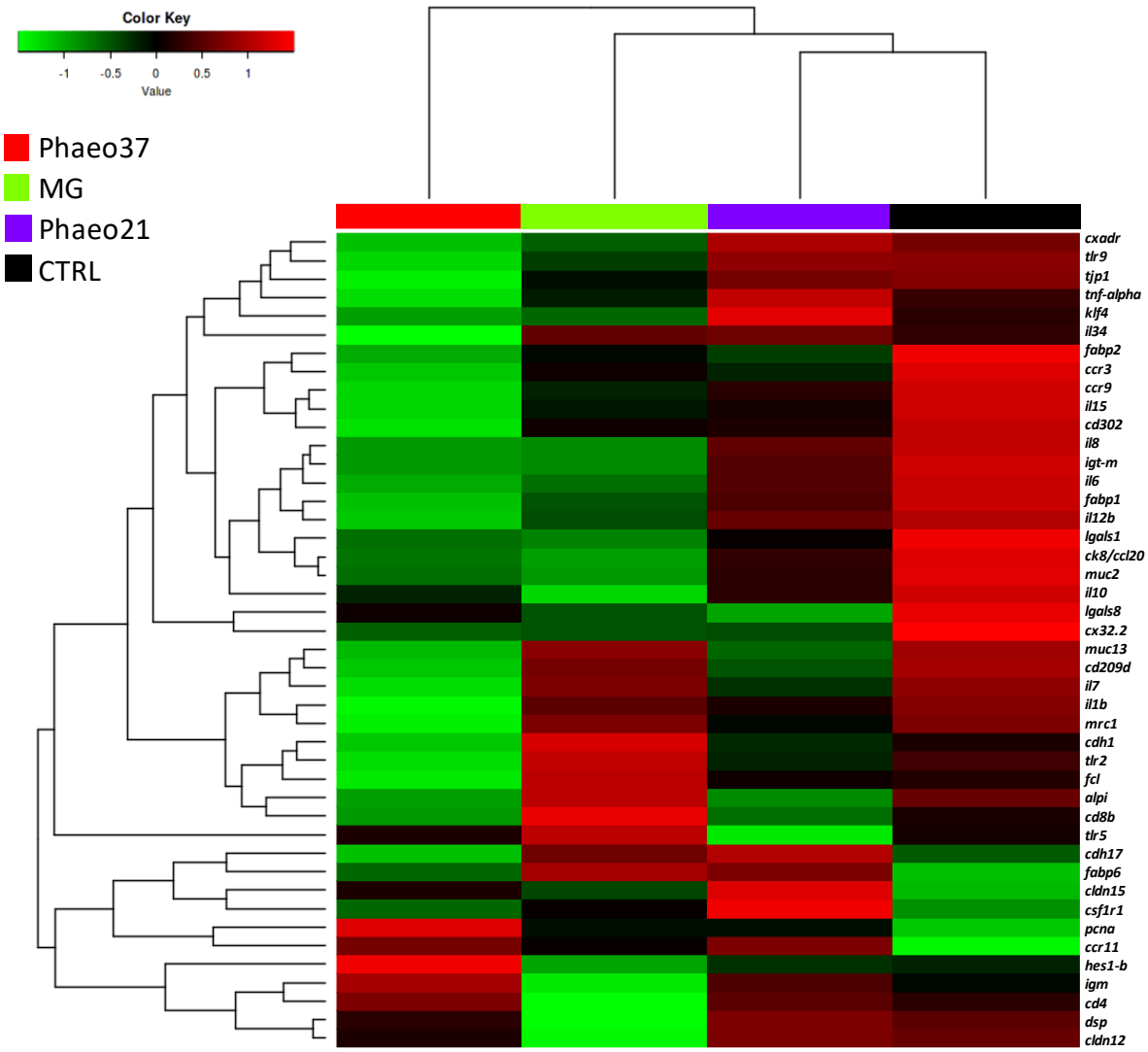
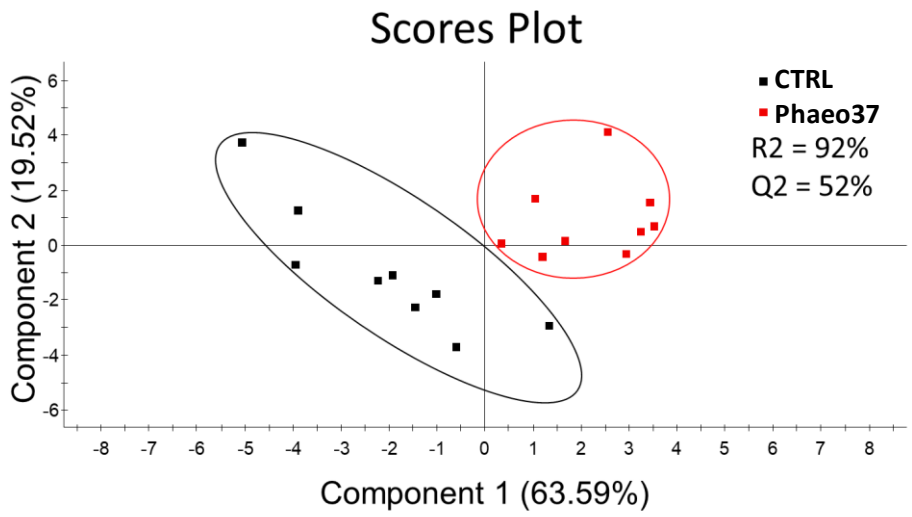


Figure 4. Heat map showing the normalized mRNA levels of selected genes in the anterior intestinal tissue of gilthead seabream juveniles after 8 weeks of feeding. Each block represents the mean mRNA level quantified by qPCR (*n* = 9).

(A)



(B)

Gene	VIP[2] / FC
fcl	1.38 / -22.72
cd209d	1.27 / -4.14
il15	1.26 / -3.06
mrc1	1.29 / -2.53
cx32.2	1.71 / -1.91
il7	1.13 / -1.90
Igt-m	1.35 / -1.77
ccr3	1.22 / -1.76
tlr9	1.49 / -1.51
il8	1.04 / -1.46
fabp2	1.65 / -1.45
il12b	1.14 / -1.40
fabp1	1.10 / -1.34
ccr9	1.18 / -1.32
il34	1.20 / -1.32
lgals1	1.02 / -1.31
ck8/ccl20	1.20 / -1.27
tjp1	1.48 / -1.24
cxadr	1.13 / -1.19
muc13	1.02 / -1.11

Figure 5. (**A**) Partial least square discriminant analysis (PLS-DA) score plots of all gene expression biomarkers analysed in the proximal intestine of gilthead seabream juveniles along the two main components at 8 weeks. (**B**) Ordered list of markers by variable importance (VIP) in projection of PLS-DA model for group differentiation, as well as the fold-change (FC) in comparison to CTRL. Markers with VIP values > 1 after the first and second main components are represented.

4.4 Discussion

Intensive fish production creates stressful conditions that negatively affect immune function [43], increasing the risk of infection caused by opportunistic bacteria. Therefore, preventive strategies that can improve aquatic animal health and reduce the risk of disease outbreaks must be adopted, such as the use of prebiotics and immunostimulants in feed formulation [44; 45; 46]. Immunostimulant and prebiotic activities after β-(1,3; 1,6)-glucans administration are well-recognized; thus, these compounds have been suggested as potential nutraceuticals or vaccine adjuvants to enhance immune responses [4; 47; 48]. For that purpose, in the present study, gilthead seabream juveniles were fed microalgae (*P. tricornutum*) derived BGs in a 2-week cycle pulse-feeding regimen. This nutritional strategy was outlined, as care must be taken not to exhaust the fish immune system due to immunostimulant overexposure after long administration periods [49; 50; 51; 52]. Moreover, intermittent administration seems a suitable approach as BGs apparently can induce long-lived effects in fish [53]. Several studies report increased innate immune parameters and pathogen resistance at least 2 weeks after BGs oral and intra-peritoneal (i.p.) administration [22; 54; 55].

Studies where BGs were orally administered to fish not only showed immunostimulatory effects but in some cases, improved growth performance [23; 24; 56; 57]. Dawood *et al.* [58] showed that supplementing red seabream diets up to 0.1% (g/kg feed) with a commercial BG product (85% purity) for 8 weeks improved final body weight and growth performance as well as lysozyme activity and higher tolerance against a low-salinity stress test when compared with a BG-free fed group. In the current study, β-glucan supplementation did not affect fish growth performance over the course of the trial (8 weeks), independently from its source. However, it did show immunomodulatory effects and improved oxidative stress status in accordance with the findings reported in other studies [23; 59; 60; 61; 62]. Dietary treatments appeared to modulate peripheral lymphocyte numbers. Results pointed to an immunostimulatory effect of diet Phaeo21 at 2 weeks feeding, with this particular BG extract apparently affecting the adaptive arm of the immune system with a rise in circulating lymphocytes. Previous works reported increased lymphocyte percentage in comparison to other leucocytes in pompano fish (*Trachinotus Ovatus*) fed 0.05% and 0.10% [60] and Persian sturgeon (*Acipenser persicus*) fed 0.2 and 0.3% yeast BGs [63] for 8 and 6 weeks, respectively. Nonetheless, in the current study, gilthead seabream fed Phaeo21 and MG dietary treatments showed a decrease in circulating lymphocytes percentage compared to those fed CTRL after 8 weeks. Kühlwein *et al.* [64] reported no apparent effect on circulating lymphocytes when carp juveniles were fed 0.1, 1 and 2% yeast BGs continuously for 8 weeks.

On the other hand, non-specific humoral parameters (antiprotease, bactericidal activity and circulating IgM) were not affected by the supplementation with 0.06% BGs from *S. cerevisiae* or *P. tricornutum* throughout the experimental period. Accordingly, a study done in gilthead seabream fed a 0.1% supplemented feed with a macroalgae derived BG (laminarin) did not show changes in serum antiprotease activity and IgM levels after 4 weeks of feeding [23]. Yamamoto et al. [65] tested different levels, ranging from 0% to 0.8%, of microalgae (*Euglena gracilis*)-derived BGs in Nile tilapia both in vitro and in vivo. While exposing naïve head-kidney phagocytes directly to BGs facilitated the activation of immune cells increasing bactericidal activity against *Streptococcus iniae* and superoxide anion production, in vivo immune effects were found to be more moderate. Authors reported increased complement system activity but no effects on serum lysozyme and blood leucocytes respiratory burst. Still, previous studies in fish revealed a tendency of BGs oral administration to stimulate or modulate innate immune parameters [21; 22; 57; 59; 66; 67].

Reactive oxygen species (ROS) are produced as a normal by-product of cellular metabolism but in excess, they can contribute to increased oxidative stress and cause cellular damage. Antioxidant enzymatic machinery is the principal cellular protective mechanism against oxidative stress in fish tissue [68]. BGs are reported to have antioxidant properties and modulate antioxidant enzymes activity as well as inhibiting lipid peroxidation in mammals [69]. In fish, BG injection increased SOD and CAT activities in the intestine [61] and blood erythrocytes [70], suggesting that BGs could improve anti-oxidative capacity. Accordingly, hepatic lipid peroxidation decreased in Phaeo37- and MG-fed animals at an early stage (2 weeks), while hepatic SOD showed a long-term (8 weeks) stimulation pattern, with the Phaeo37-fed group showing the highest activity. Zeng *et al.* [61] reported a correlation between higher mRNA transcription of nuclear factor erythroid 2-related factor 2 (Nrf2) gene and increased SOD and CAT genes transcription, which translated in higher enzyme activity in fish injected with a 0.1% BG solution. Nrf proteins, under oxidative conditions translocate to the nucleus where they bind to the antioxidant response element (ARE) [71]. ARE is found in the promoters of several chemoprotective genes, including those involved in the response to oxidative stress [72]. Integrating all physiological responses into a multivariate analysis, dietary effects became clearer and differed between sampling points. At 2 weeks of feeding, all groups received BGs clustered together and were different from CTRL. Differences at this early stage pointed to a dietary effect mainly affecting the antioxidant defenses and most prominently decreasing lipid peroxidation corroborating results from the one-way ANOVA. Previous studies reported higher antioxidant enzyme activity and lower lipid peroxidation when fish are previously treated with BGs through different administration

routes (i.e., i.p. injection; oral route) [61; 70; 73]. After a toxicological insult, pre-treated fish with barley-derived BG were able to prevent intestinal Cu-induced lipid peroxidation [61]. Although BGs from the present study differ in origin and solubility, the observed early (2 weeks) beneficial effect was elicited by all BG-supplemented feeds, most likely due to the fact that both soluble and particulate BGs can act as exogenous ROS scavengers. Carballo *et al.* [74] reported that both a *P. tricornutum* chrysolaminarin-rich extract (soluble) and a yeast BG (particulate) show ROS scavenger activities. At a longer feeding period (8 weeks), the *P. tricornutum*-derived BG-groups showed higher SOD activity and clustered together independently from CTRL and MG. Furthermore, microalgae BG-treated groups also showed higher thrombocyte numbers. Although both Phaeo21 and 37 diets were supplemented with BG enriched extracts, other compounds such as *P. tricornutum* cell wall fragments might be present in the mixture and cannot be ruled out as immunomodulators. *P. tricornutum* cell wall is mainly composed of sulphated polysaccharides (SPs) [75], which are known to interact with different toll-like receptors (TLRs). These compounds might act as antigens recognized by cell surface receptors activating different leucocyte types. In carp, peripheral thrombocytes constitutively express different TLR genes [76] and have been reported to have phagocytic activity and the ability to ingest particulate antigens possibly acting as an antigen presenting cell [77].

In the present study, at 2 weeks proliferating cell nuclear antigen (*pcna*) gene was down-regulated in the anterior gut of Phaeo37 fed fish. Former studies, report PCNA protein expression inhibition in mammalian cancer cells treated with different glucans including laminarin [78; 79]. However, intestinal transcriptional changes were more significant at 8 weeks, where differences between CTRL and Phaeo37 gene profiles can be found. Furthermore, Phaeo37 and MG fed groups showed a down-regulation of different genes when compared to CTRL, namely *il10*, *cx32.2*, *fcl*. Hence, a multivariate analysis was performed (PLS-DA) allowing for a more comprehensive understanding of fish health status, whereas, at the same time identifying the most responsive gene biomarkers in fish intestine. VIP analysis with the first two components, highlighted that the top contributing genes for dietary differences in the gut were immune related (PRR- *fcl, cd209d, mrc1, tlr9, lgals1*; Interleukins- *il7, il8, il12β, il15, il34*; Immunoglobulin production- *igt-m*; chemokines and receptors- *ccr3, ccr9, ck8/cl20*). Phaeo37 dietary treatment caused a general down-regulation of gene transcription. Therefore, the effect of Phaeo37 supplemented diet was mostly immunomodulatory inducing a local anti-inflammatory state at molecular level, which as a consequence led to decreased immune cell activation in the gut. The down-regulation of intestinal immune-related genes can be understood as an immune tolerance effect that can be beneficial in an acute inflammation scenario, counterbalancing its negative and potentially dangerous effects. Falco *et al.* [25]

also found an anti-inflammatory effect in common carp (*Cyprinius carpio L.*) intestine, with several inflammatory genes appearing down regulated when fish were fed a yeast BG supplemented diet for a 2-week period. Furthermore, even after a challenge (i.p. injection) with live bacteria (*Aeromonas salmonicida*), fish fed BGs showed decreased intestinal *il1b*, *il6* and *tnf-alpha* expression, while showing up-regulation in the head-kidney. In this particular case, it seems that BG may be preventing an acute response to infection in the gut, without compromising the systemic response. Additionally, the same down-regulation pattern (inflammatory genes) was seen in the spleen of rainbow trout after 37 days of feeding lentinan (soluble low molecular weight glucan)-supplemented diets [(80)]. These findings support the idea that BG can have localized specific effects depending on the target tissue.

Overall, some discrepancies can be observed among previous works and data gathered in the present study, which can be explained by different BG preparations. While some studies use crude BG extracts, others use purified compounds differing in molecular weight, branching and solubility. BGs solubility/insolubility seems to play a major role in ligand/receptor recognition and consequently immune cell activation [(81; 82)]. In mammals, particulate BGs directly stimulate immune cell activation through a Dectin-1 recognition pathway, while soluble BG require complement-mediated opsonisation to activate a CR3-dependent pathway [(81; 83)]. Still, in the present work, the particulate BG diet (MG) showed only mild effects mostly related with oxidative defenses after 2 weeks of feeding. In addition to solubility, molecular weight can play an important role in the biological effects of BGs. Different authors have found that in colitis-induced rat models, the dietary administration of low and high molecular weight oat BGs reduced the inflammatory response in colon and also ameliorated the local inflammation [(84; 85)]. However, these authors found that low molecular mass BGs showed a significantly stronger anti-inflammatory effect, through the down-regulation of several pro-inflammatory cytokines and that the therapeutic effect is in evident relation with the molecular mass of the polymer. When comparing the different feeds used in the present study, Phaeo21 and 37 extracts show low molecular mass BGs (chrysolaminarin) [(10)] while, MG feed is supplemented with a high molecular weight BG (Baker's yeast) [(86; 87)]. Furthermore, *P. tricornutum* extract supplemented diets although having the same BG concentration, differ in purity, since Phaeo37 extract has a higher percentage of BGs compared to Phaeo21. Thus, the combination of low molecular mass BGs and higher extract purity might explain the higher overall immunomodulatory and oxidative protective effects of Phaeo37 dietary treatment.

In summary, novel feeds with increasingly higher percentages of terrestrial animal- and plant-derived ingredients have been shown to have anti-nutritional factors that often

cause gut inflammation in fish, a condition that might lead to impaired nutrient absorption and the disruption of normal microbiota. The use of gut anti-inflammatory compounds can have special relevance nowadays in aquaculture, both as a prophylactic and therapeutic measure, as the industry decreases the use of FM, replacing it by the ingredients referred to above. In this regard, our results indicate that the dietary administration of a *P. tricornutum* 37% enriched-BG extract might be relevant in a context of extreme dietary formulation due to its anti-inflammatory and anti-oxidative effects.

4.5 Acknowledgments

This work has received funding from the Bio Based Industries Joint Undertaking (BBI JU) under the European Union's Horizon 2020 research and innovation programme under grant agreement No. 745754 (project MAGNIFICENT). This output reflects the views only of the author(s), and the European Union and BBI JU cannot be held responsible for any use which may be made of the information contained therein. This work is also a result of the project ATLANTIDA (ref. NORTE-01-0145-FEDER-000040), supported by the Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement and through the European Regional Development Fund (ERDF), and by national funds through FCT—Foundation for Science and Technology within the scope of UIDB/04423/2020 and UIDP/04423/2020. BR and BC were supported by FCT (PD/BDE/129262/2017 and IF/00197/2015, respectively).

4.6 Ethics Statement

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals at Riasearch facilities (Portugal).

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Chapter V

Chlorella Vulgaris Extracts as Modulators of the Health Status and the Inflammatory Response of Gilthead Seabream Juveniles *(Sparus aurata)*

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Chlorella Vulgaris Extracts as Modulators of the Health Status and the Inflammatory Res*p*onse of Gilthead Seabream Juveniles *(Sparus aurata)*

Abstract

This study aimed to evaluate the effects of short-term supplementation, with 2% *Chlorella vulgaris* (*C. vulgaris*) biomass and two 0.1% *C. vulgaris* extracts, on the health status (experiment one) and on the inflammatory response (experiment two) of gilthead seabream (*Sparus aurata*). The trial comprised four isoproteic (50% crude protein) and isolipidic (17% crude fat) diets. A fish-meal-based (FM), practical diet was used as a control (CTR), whereas three experimental diets based on CTR were further supplemented with a 2% inclusion of *C. vulgaris* biomass (Diet D1); 0.1% inclusion of *C. vulgaris* peptide-enriched extract (Diet D2) and finally a 0.1% inclusion of *C. vulgaris* insoluble fraction (Diet D3). Diets were randomly assigned to quadruplicate groups of 97 fish/tank (IBW: 33.4 ± 4.1 g), fed to satiation three times a day in a recirculation seawater system. In experiment one, seabream juveniles were fed for 2 weeks and sampled for tissues at 1 week and at the end of the feeding period. Afterwards, randomly selected fish from each group were subjected to an inflammatory insult (experiment two) by intraperitoneal injection of inactivated gram-negative bacteria, following 24 and 48 h fish were sampled for tissues. Blood was withdrawn for haematological procedures, whereas plasma and gut tissue were sampled for immune and oxidative stress parameters. The anterior gut was also collected for gene expression measurements. After 1 and 2 weeks of feeding, fish fed D2 showed higher circulating neutrophils than seabream fed CTR. In contrast, dietary treatments induced mild effects on the innate immune and antioxidant functions of gilthead seabream juveniles fed for 2 weeks. In the inflammatory response following the inflammatory insult, mild effects could be attributed to *C. vulgaris* supplementation either in biomass form or extract. However, the *C. vulgaris* soluble peptide-enriched extract seems to confer a protective, anti-stress effect in the gut at the molecular level, which should be further explored in future studies.

Keywords

Functional feeds; protein hydrolysate; innate immunity; fish robustness

5.1 Introduction

In intensive farming facilities fish are reared at high densities, which may increase stress and susceptibility to diseases, resulting in lower production yields. Consequently, there is an increasing pressure for disease management strategies, beyond the use of antibiotics or vaccination. In this sense, health promoting feeds designed not only to fulfil the nutrient requirements, but also to strengthen the immune system, are viewed as a way to reduce aquaculture dependency in chemotherapeutics and mitigate its negative environmental effects [1; 2]. Novel applications based on algal products are a fast emerging and developing area, expected to reach 56.5 billion US$ by 2027 with a compound annual growth rate of 6% in the period from 2019 to 2027 [3]. The ability to grow in different environments and conditions as well as to produce large numbers of secondary metabolites makes microalgae suitable raw materials for different applications. These organisms are regarded as sustainable alternative sources of bioactive compounds, mostly sought out for the development of functional -feeds, -foods and health products [4; 5; 6].

Chlorella vulgaris is a green microalga with a wide distribution in freshwater, marine and terrestrial environments capable of rapid growth under autotrophic, mixo-trophic and heterotrophic conditions [7]. These characteristics made *C. vulgaris* a successful candidate for large-scale cultivation and commercial production [8]. As with other microalgae species, *C. vulgaris* produces a different array of health-promoting biomolecules [9; 10]. Notably, natural pigments such as lutein and astaxanthin extracted from *Chlorella* sp. show immunostimulatory and antioxidant protective effects [4; 11; 12]. Furthermore, these microalgae are characterised by a very high crude protein content (> 50%) and a balanced amino acid (AA) profile, synthesising all essential AA in a considerable amount [4]. Already, *C. vulgaris* biomass has been successfully used in aquafeeds as a source of protein, improving growth performance, oxidative status and immune response in several fish species [13; 14; 15; 16; 17]. For instance, dietary supplementation of *Chlorella* sp. at 0.4 to 1.2%, stimulated the innate immunity of gibel carp (*Carassius auratus gibelio*), namely by increasing IgM, IgD, Interleukin-22 and chemokine levels [18]. Also, Zahran and Risha [16] reported that feed supplementation with powdered *C. vulgaris* protected Nile tilapia against arsenic-induced immunosuppression and oxidative stress. Nonetheless, as with other algal biomasses, at high fishmeal replacement levels studies start to report impaired growth performances [19; 20]. Microalgae generally show thick cell walls that hinder the access of fish gut enzymes to intracellular nutrients. Hence, algae nutritional value increases if access is provided to macro and micronutrients [21; 22; 23]. Hydrolysis improves digestibility through the

application of chemical or enzymatic methods to disrupt the cell wall and hydrolyse intact proteins [24]. The enzymatic method is sometimes advantageous because of milder processing conditions and peptide bond specificity, giving rise to digestible peptides believed to be more effective than the whole protein or the free AA [24; 25]. Peptide bioactivity is influenced by molecular weight and peptide chain size [26]. In fact, low molecular weight peptides (< 3kDa) are described as having immune-stimulating or anti-inflammatory properties [26; 27; 28].

Several studies have evaluated marine protein hydrolysates (MPH) as a dietary ingredient and their effects on growth performance, immune response and disease resistance in fish [26]. Results are promising, as the dietary inclusion of MPH has been shown to induce growth, antioxidant activity and fish immunity [28; 29; 30; 31; 32], as well as improve fish immune response and disease resistance to specific bacterial infections [27; 33; 34; 35]. Moreover, regarding microalgae, different *C. vulgaris* protein hydrolysates and extracts have already been studied concerning its different bioactivities, namely, anticancer and antibacterial effects [36], as well as antioxidant and immune modulatory properties [37]. Results mentioned above suggest that *C. vulgaris* has the potential to act as a dietary supplement with nutraceutical properties and to stimulate the immune system. Therefore, the present study aimed to evaluate the effects of short-term dietary supplementation, with a 2% *C. vulgaris* biomass and a 0.1% supplementation with *C. vulgaris* soluble peptide-enriched extract, on the immune and the oxidative stress defenses (health status; experiment 1) and on the inflammatory response after an inflammatory insult (experiment 2) of gilthead seabream (*Sparus aurata*).

5.2 Material and Methods

5.2.1 *C. vulgaris* hydrolysates production

C. vulgaris was supplied, as powder, by AllMicroalgae-Natural Products, SA (Pataias, Portugal). The *C. vulgaris* hydrolysates were produced by an acid pre-treatment followed by an enzymatic hydrolysis, using a previously optimised method [38]. Briefly, *C. vulgaris* (Table 1) was mixed with an acetic acid solution (2% in deionised water) in a ratio of microalgae:water of 1:3 (w/v). The mixture was incubated for 1 h at 50 °C and 125 rpm in an orbital shaker (ThermoFisher Scientific, Waltham, MA, USA, MaxQ™ 6000). Then, deionised water was added until microalgae:water ratio reached 1:10 and the pH was adjusted to 7.5. For the enzymatic hydrolysis, first, 5% cellulase was added and the mixture was incubated for 2 h at 50 °C and 125 rpm. Secondly, 3.9% subtilisin was added

and the mixture was incubated for 2 h at 40 °C at 125 rpm. During the enzymatic hydrolysis, pH was constantly verified and adjusted to 7.5, mainly in the subtilisin hydrolysis step. To stop the hydrolysis reaction, the mixture was incubated at 90 °C for 10 min to inactivate the enzymes. The resulting solution was centrifuged at 5000× g for 20 min, and both the water-soluble peptide-enriched supernatant (Table 2) and the pellet were collected and freeze-dried for further analysis.

Table 1. Microalgae *Chlorella vulgaris* biomass composition (prior to extraction).

Nutrients	**Quantity (g/100g)**
Crude Protein	52.2
Crude Fat	7.9
Carbohydrates	10.9
Fibers	15.5
Mineral matter	11.1
Moisture	2.4

Table 2. *Chlorella vulgaris* soluble extract protein concentration and *in vitro* bioactivities.

	***Chlorella vulgaris* soluble extract**
% Protein	44.71 ± 1.75
Antioxidant activity (ORAC) (µmol TE/g of extract)	462.83 ± 39.97
Anti-hypertensive activity (iACE) (IC_{50} µg protein mL^{-1})	286.00 ± 55.00
Anti-diabetic activity (% of inhibition of α-Glucosidase enzyme in a solution with 30 mg mL^{-1} of soluble extract)	31.36 ± 3.90

5.2.2 Diet composition

The trial comprised 4 isonitrogenous (50% protein in dry matter (DM)) and isolipidic (17% fat in DM) dietary treatments. A fishmeal-based (FM), practical diet was used as control (CTR) whereas 3 experimental diets based on CTR were further supplemented with a 2% inclusion of *C. vulgaris* powdered biomass (Diet D1); 0.1% inclusion of *C. vulgaris* peptide-enriched extract (Diet D2) and finally 0.1% inclusion of *C. vulgaris* insoluble residue (Diet D3) (Table 3). Diets were manufactured by SPAROS (Olhão, Portugal). All powder ingredients were initially mixed and ground (<200 micron) in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany). Subsequently, the oils were added to the powder mixtures, which were humidified with 25% water and agglomerated by a low-shear and low-temperature extrusion process (ITALPLAST, Parma, Italy). The resulting pellets of 2.0 mm were dried in a convection oven for 4 h at 55 ºC (OP 750-UF, LTE Scientifics, Oldham, UK). Diets were packed in sealed plastic buckets and shipped to the research site (CIIMAR, Matosinhos, Portugal), where they were stored in a temperature-controlled room.

Table 3. Ingredients and proximate composition of experimental diets.

Ingredients (%)	**CTR**	**D1**	**D2**	**D3**
Fishmeal Super Prime[1]	10.00	10.00	10.00	10.00
Fish gelatin[2]	2.00	2.00	2.00	2.00
Soy protein concentrate[3]	10.00	10.00	10.00	10.00
Wheat gluten[4]	7.00	7.00	7.00	7.00
Corn gluten[5]	15.00	15.00	15.00	15.00
Soybean meal[6]	20.00	20.00	20.00	20.00
Rapeseed meal[7]	5.25	5.25	5.25	5.25
Sunflower meal[8]	5.00	5.00	5.00	5.00
Wheat meal[9]	7.00	5.00	7.00	7.00
Fish oil[10]	4.90	4.90	4.90	4.90
Soybean oil[11]	9.10	9.10	9.10	9.10
Premix 1%[12]	1.00	1.00	1.00	1.00
Binder (Celatom - Diatomite)[13]	1.00	1.00	1.00	1.00
MAP (Monoammonium phosphate)[14]	1.50	1.50	1.50	1.50
L-Lysine[15]	1.00	1.00	1.00	1.00
L-Threonine[16]	0.10	0.10	0.10	0.10
DL-Methionine[17]	0.15	0.15	0.15	0.15
Chlorella whole biomass - Algafarm	0.00	2.00	0.00	0.00
Chlorella – soluble fraction	0.00	0.00	0.10	0.00
Chlorella – Insoluble residue	0.00	0.00	0.00	0.10

[1]66.3% CP, 11.5% CF, Pesquera Diamante, Peru; [2]94% WEISHARDT, Slovakia; [3]62.2% CP, 0.7% CF, Soycomil P, ADM, Netherlands; [4]80.4% CP, 5.8% CF, VITAL, Roquette, France; [5]61.2% CP, 5.2% CF, COPAM, Portugal; [6]Dehulled solvent extracted: 47.4% CP, 2.6% CF, Cargill, Spain; [7]Solvent extracted: 34.3% CP, 2.1% CF, Ribeiro e Sousa Lda, Portugal; [8]Solvent extracted: 29.1% CP, 1.8% CF, Ribeiro e Sousa Lda, Portugal; [9]11.7% CP, 1.6% CF, Molisur, Spain; [10]98.1% CF (16% EPA; 12% DHA), Sopropêche, France; [11]98.6%, JC Coimbra, Portugal; [12]Vitamins (IU or mg/kg diet): DL-alphatocopherol acetate, 100mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1 mg; nicotidin acid, 200 mg; folic acid, 15mg; ascorbic acid, 1000 mg; inositol, 500mg; biotin, 3 mg; calcium panthotenate, 100mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate. 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middling's, Premix Lda, Portugal; [13]CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal; [14]Windmill AQUAPHOS (26% P), ALIPHOS, Netherlands; [15]99% Lys, Ajinomoto EUROLYSINE S.A.S, France; [16]98.5% Thr, Ajinomoto EUROLYSINE S.A.S, France; [17]99% Met, Rodhimet NP99, ADISSEO, France; [18]*Chlorella vulgaris* lyophilized biomass, Allmicroalgae, Portugal; [19,20]*Chlorella vulgaris* aqueous and insoluble extracts, CBQF—Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Portugal.

5.2.3 Bacterial growth and inoculum preparation

Photobacterium damselae subsp. piscicida (Phdp), strain PP3, was used for the inflammatory insult. Bacteria were routinely cultured at 22 °C in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from BD Difco™, Franklin Lakes, NJ, USA) supplemented with NaCl to a final concentration of 1% (w/v) (TSB-1 and TSA-1, respectively) and stored at -80 °C in TSB-1 supplemented with 15% (v/v) glycerol. To prepare the inoculum for injection into the fish peritoneal cavities, stocked bacteria were cultured for 48 h at 22 °C on TSA-1. Afterwards, exponentially growing bacteria were collected and resuspended in sterile HBSS and adjusted against its growth curve to $1x10^7$ colony forming units (cfu) mL^{-1}. Plating serial dilutions of the suspensions onto TSA-1 plates and counting the number of cfu following incubation at 22 °C confirmed bacterial concentration of the inocula. Bacteria were then killed by heat at 70 °C for 10 min. Loss of bacterial viability following heat exposure was confirmed by plating resulting cultures on TSA-1 plates and failing to see any bacterial growth.

5.2.4 Fish rearing conditions and feeding scheme

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals at CIIMAR aquaculture and animal experimentation facilities in Matosinhos, Portugal . The protocol was approved by the CIIMAR Animal Welfare Committee in 29/04/2020 with the reference 0421/000/000/2020 from Direção Geral de Alimentação e Veterinária (DGAV). Seawater flow was kept at 4 L min^{-1} (mean temperature 22.4 ± 1 °C; mean salinity 35.2 ± 0.7 ‰) in a recirculation system with aeration (mean dissolved oxygen above 6 mg L^{-1}). Water quality parameters were monitored daily and adjusted when necessary. Mortality was monitored daily. Diets were randomly assigned to triplicate groups of 97 fish/tank (IBW: 33.4 ± 4.1 g) that were fed to satiation three times a day for 2 weeks starting at 1.5 % biomass.

5.2.5 Experimental procedures

To examine the influence that *C. vulgaris* biomass and protein-rich extract supplementation may have on the health status (experiment 1) and the inflammatory response against bacteria (inactivated Phdp i.p. injection; experiment 2), samples of

blood and gut were collected at 1 and 2 weeks (experiment 1) and after 2 weeks of feeding at 0 h, 24 h and 48 h post-injection (experiment 2).

5.2.5.1 Health status (experiment 1)

After 1 and 2 weeks, 12 fish/treatment were weighed and sampled for tissues (blood, head-kidney, liver and gut), after being sacrificed with a 2-phenoxyethanol lethal dose (0.5 mL L^{-1}) [39]. Blood was collected from the caudal vein using heparinised syringes and centrifuged at 10,000 g for 10 min at 4 °C to obtain plasma samples. Plasma and tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

5.2.5.2 Inflammatory response (experiment 2)

At 2 weeks, 24 fish/treatment were subjected to an inflammatory insult by intraperitoneal (i.p.) injection of heat-inactivated Phdp (see section 5.2.3) and immediately transferred to a similar recirculation system in triplicates. After 24 and 48h post-injection (time-course), 9 fish/treatment were sampled as described above.

5.2.6 Haematological procedures

The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts. To determine WBC and RBC concentration, whole blood was diluted 1/20 and 1/200 respectively, in HBSS with heparin (30 U mL^{-1}) and cell counts were done in a Neubauer chamber. Blood smears were prepared from peripheral blood, air-dried and stained with Wright's stain (Haemacolor; Merck, Darmstadt, Germany) after fixation for 1 minute with formol–ethanol (10 % formaldehyde in ethanol). Neutrophils were labelled by detecting peroxidase activity revealed by Antonow's technique described in Afonso, et al. [40]. The slides were examined under oil immersion (1000x), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The relative percentage and absolute value ($\times 10^4$ mL^{-1}) of each cell type was calculated.

5.2.7 Innate humoral parameters

5.2.7.1 Antiprotease activity

The antiprotease activity was determined as described by Ellis, et al. [41] with some modifications. Briefly, 10 µL of plasma were incubated with the same volume of trypsin solution (5 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) for 10 min at 22 °C. After incubation, 100 µL of phosphate buffer (NaH_2PO_4, 13.9 mg mL-1, pH 7.0) and 125 µL of azocasein solution (20 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) were added and incubated for 1h at 22 °C. Finally, 250 µL of trichloroacetic acid were added to the reaction mixture and incubated for 30 min at 22 °C. The mixture was centrifuged at 10,000 *g* for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred to a 96 well-plate and mixed with 100 µL of NaOH (40 mg mL^{-1}). The OD was read at 450 nm in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Phosphate buffer instead of plasma and trypsin served as blank, whereas the reference sample was phosphate buffer instead of plasma. Sample inhibition percentage of trypsin activity was calculated as follows: 100 – ((sample absorbance/Reference absorbance) x 100). All analyses were conducted in duplicates.

5.2.7.2 Peroxidase activity

Total peroxidase activity in plasma and intestine was measured following the procedure described by Quade and Roth [42]. Briefly, 10 µL of plasma and 5 µL of intestine homogenate were diluted with 140 and 145 µL, respectively, of HBSS without Ca2+ and Mg2+ in 96-well plates. Then, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma-Aldrich®, Merck, Darmstadt, Germany) and 50 µL of 5 mM H_2O_2 were added to the wells. The reaction was stopped after 2 min by adding 50 µL of H_2SO_4 (2M) and the optical density (OD) was read at 450 nm in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Wells without plasma or mucus were used as blanks. The peroxidase activity (U mL^{-1} tissue) was determined, defining that one unit of peroxidase produces an absorbance change of 1 OD.

5.2.7.3 Nitric oxide (NO) production

NO production was measured in plasma (1:10 sample dilution) and intestine (1:5 sample dilution) samples. Total nitrite and nitrate concentrations in the sample were assessed using the Nitrite/Nitrate colorimetric method kit (Roche, Basel, Switzerland)

adapted to microplates. Nitrite concentration was calculated by comparison with a sodium nitrite standard curve. Since nitrite and nitrate are endogenously produced as oxidative metabolites of the NO molecule, these compounds are considered as indicative of NO production.

5.2.8 Analysis of oxidative stress biomarkers

Intestine samples were homogenized (1:10) in phosphate buffer 0.1M (pH 7.4) using Precellys evolution tissue lyser homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France).

5.2.8.1 Lipid peroxidation (LPO)

One aliquot of tissue homogenate was used to determine the extent of endogenous LPO by measuring thiobarbituric acid-reactive species (TBARS) as suggested by Bird and Draper [43]. To prevent artifactual lipid peroxidation, butylhydroxytoluene (BHT 0.2 mM) was added to the aliquot. Briefly, 1 ml of 100% trichloroacetic acid and 1 ml of 0.73% thiobarbituric acid solution (in Tris–HCl 60 mM pH 7.4 with DTPA 0.1 mM) were added to 0.2 ml of intestine homogenate. After incubation at 100°C for 60 min, the solution was centrifuged at 12,000 *g* for 5 min and LPO levels were determined at 535 nm.

5.2.8.2 Total protein quantification

The remaining tissue homogenate was centrifuged for 20 min at 12,000 *g* (4°C) to obtain the post-mitochondrial supernatant fraction (PMS). Total proteins in homogenates were measured by using Pierce™ BCA Protein Assay Kit, as described by the manufacturer (ThermoFisher Scientific, Waltham, MA, USA).

5.2.8.3 Catalase (CAT)

CAT activity was determined in PMS by measuring substrate (H_2O_2) consumption at 240 nm according to Claiborne [44] adapted to microplate. Briefly, in a microplate well, 0.140 ml of phosphate buffer (0.05 M pH 7.0) and 0.150 ml H_2O_2 solution (30 mM in phosphate buffer 0.05 M pH 7.0) were added to 0.01 ml of intestine PMS (0.7 mg ml^{-1} total protein). Enzymatic activity was determined in a microplate reader (BioTek Synergy

HT, Winooski, VT, USA) reading the optical density at 240 nm for 2 min every 15 sec interval

5.2.8.4 Superoxide dismutase (SOD)

SOD activity was measured according to Flohé and Otting [45] adapted to microplate by Lima, et al. [46]. Briefly, in a microplate well, 0.2 ml of the reaction solution [1 part xanthine solution 0.7 mM (in NaOH 1 mM) and 10 parts cytochrome c solution 0.03 mM (in phosphate buffer 50 mM pH 7.8 with 1 mM Na-EDTA)] was added to 0.05 ml of intestine PMS (0.25 mg ml^{-1} total protein). Optical density was measured at 550 nm in a microplate reader (BioTek Synergy HT, Winooski, VT, USA) every 20 sec interval for 3 min at 25° C.

5.2.9 Gene expression

RNA isolation from target tissue (Anterior gut) and cDNA synthesis was conducted with NZY Total RNA Isolation kit and NZY first-strand cDNA synthesis kit (NZYTech, Lisbon, Portugal) following manufacturer's specifications. Real-time quantitative PCR was carried out on a CFX384 Touch Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Genes comprised in the assay were selected for their involvement in gut integrity, health and immunity (Table 4). Specific primer pair sequences are listed in Table S1d (Appendix IV). Controls of general PCR performance were included on each array. Briefly, RT reactions were diluted to obtain the equivalent concentration of 20 ng of total input RNA which were used in a 10 µL volume for each PCR reaction. PCR wells contained a 2x SYBR Green Master Mix (Bio-Rad, CA, USA) and specific primers were used to obtain amplicons 50–250 bp in length. The program used for PCR amplification included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C denaturation for 15 s, primer annealing and extension temperature (Appendix IV, Table S1d) for 1 min. The efficiency of PCR reactions was always higher than 90 %, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55 - 95 °C). Fluorescence data acquired during the PCR extension phase were normalised using the Pfaffl [47] method. The geometric mean of two carefully selected housekeeping genes (elongation factor 1-α (ef1α) and ribosomal protein S18 (rps18)) was used as the normalisation factor to normalise the expression of target genes. For comparing the mRNA expression level of

each gene in a given dietary treatment, all data values were in reference to the expression level of CTR fish.

Table 4. PCR-array layout for gene expression profiling of anterior gut in sea bream.

Function	Gene	Symbol	Accession number
Intestinal epithelium protection	Mucin 2	*muc2*	JQ277710
	Mucin 13	*muc13*	JQ277713
Cytokines	Interleukin 1 beta	*il1b*	AJ277166.2
	Interleukin 34	*Il34*	JX976629.1
Pattern recognition receptors	Toll like receptor 1	*tlr1*	KF857322
Cell markers	CD8 alpha	*cd8α*	AJ878605
Antibodies	Immunoglobulin M	*igm*	AM493677
Antimicrobial defence/ Iron recycling	Hepcidin	*hepc*	EF625901
Oxidative stress defences	Heat-shock protein 70	*hsp70*	DQ524995.1
	Glutathione peroxidase	*gpx*	DQ524992
	Manganese superoxide dismutase	*Sod(mn)*	JQ308833
Reference genes	Elongation factor 1α	*ef1α*	AF184170
	Ribosomal protein S18	*rps18*	AM490061

5.2.10 Data analysis

All results are expressed as mean ± standard error (mean ± SE). Residuals were tested for normality (Shapiro–Wilk's test) and homogeneity of variance (Levene's test). When residuals did not meet the assumptions, data was transformed by a Log10 or square root transformation. For gene expression data, a log2 transformation was applied to all expression values. Two-way ANOVAs were performed in data arising from both trials one and two, with "dietary treatment and time" as the fixed effects. Analysis of variance was followed by Tukey post-hoc tests. All statistical analysis were performed using the computer package SPSS 26 for WINDOWS. The level of significance used was $p \leq 0.05$ for all statistical tests.

5.3 Results

5.3.1 Haematology/Peripheral leucocyte responses

In experiment 1, total WBC and RBC as well as MCH did not change significantly among different dietary treatments at both 1 and 2 weeks of feeding (Table 5). However, fish fed D2 presented a higher haemoglobin (Hb) concentration than D1 and D3 fed fish (Table 5). Differential leucocyte counts showed different modulation patterns between dietary treatments regardless of the sampling point (Table 6). For instance, the D1 fed

group showed lower lymphocyte numbers at both 1 and 2 weeks, when compared to the other dietary treatments (Table 6). Whereas peripheral neutrophils increased in D2 fed fish compared to those fed CTR (Table 6). Circulating monocytes were not significantly modulated by dietary treatments at either 1 or 2 weeks of feeding.

After the inflammatory insult (experiment 2), Hb increased at 24 h following inoculation with the inactivated bacteria, while MCH, total WBC and RBC remained unchanged (Table 7). Peripheral lymphocyte numbers decreased at 24 h compared to 0 h, returning to resting values at 48 h (Table 8). Circulating neutrophil levels increased at 24 h and 48 h following pathogen inoculation compared to time 0 h (Table 8). Total thrombocyte and monocyte concentrations were unaffected (Table 8).

Table 5**.** Haemoglobin, mean corpuscular haemoglobin (MCH), red blood cells (RBC) and white blood cells (WBC) in gilthead seabream juveniles after 1 and 2 weeks of feeding (experiment 1). Data are the mean ± SEM (n = 12).

Haematology	1 week				2 weeks			
Diets	**CTR**	**D1**	**D2**	**D3**	**CTR**	**D1**	**D2**	**D3**
Haemoglobin (g.dL^{-1})	0.69± 0.05	0.65± 0.04	0.81± 0.04	0.61± 0.03	0.68± 0.06	0.65± 0.02	0.74± 0.05	0.67± 0.05
MCH (pg.cell^{-1})	2.24± 0.31	1.89± 0.16	2.29± 0.23	1.87± 0.16	3.31± 0.39	3.12± 0.19	3.61± 0.19	3.24± 0.18
WBC (10^4 .µL^{-1})	1.85± 0.05	1.84± 0.12	1.96± 0.06	1.86± 0.05	3.96± 0.18	4.19± 0.21	3.73± 0.23	3.89± 0.19
RBC (10^6.µL^{-1})	3.25± 0.26	3.46± 0.21	3.79± 0.39	3.64± 0.20	1.93± 0.09	2.13± 0.16	2.06± 0.12	1.91± 0.11

2-Way ANOVA							
	Time		**Diet**				**Diet*Time**
	1 week	**2 weeks**	**CTR**	**D1**	**D2**	**D3**	
Haemoglobin (g.dL^{-1})	-	-	AB	B	A	B	ns
MCH (pg.cell^{-1})	A	B	-	-	-	-	ns
WBC (10^4 .µL^{-1})	B	A	-	-	-	-	ns
RBC (10^6.µL^{-1})	A	B	-	-	-	-	ns

Different capital letters represent significant differences in time regardless of diet and between diets regardless of time ($p < 0.05$), ns (not significant).

Table 6**.** Absolute values of peripheral blood leucocytes (thrombocytes, Lymphocytes, monocytes and neutrophils) in gilthead seabream juveniles after 1 and 2 weeks of feeding (experiment 1). Data are the mean ± SEM (n = 12).

Peripheral leucocytes	1 week				2 weeks			
Diets	**CTR**	**D1**	**D2**	**D3**	**CTR**	**D1**	**D2**	**D3**
Thrombocytes (10^4 .μL^{-1})	1.17 ± 0.08	1.30± 0.07	1.29±0.12	1.26±0.05	2.78±0.18	3.24±0.17	2.58±0.17	2.66±0.16
Lymphocytes (10^4 .μL^{-1})	0.57 ± 0.07	0.37± 0.04	0.62±0.05	0.56±0.03	1.15±0.12	0.84±0.07	1.05±0.15	1.17±0.08
Monocytes (10^4 .μL^{-1})	0.01 ± 0.00	0.02± 0.00	0.02±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.00	0.02±0.01
Neutrophils (10^4 .μL^{-1})	0.01 ± 0.00	0.01 ± 0.01	0.04±0.01	0.02±0.01	0.02±0.01	0.06±0.02	0.06±0.01	0.06±0.01

2-Way ANOVA							
	Time		**Diet**				**Diet*Time**
	1 week	**2 weeks**	**CTR**	**D1**	**D2**	**D3**	
Thrombocytes (10^4 .μL^{-1})	B	A	-	-	-	-	ns
Lymphocytes (10^4 .μL^{-1})	B	A	A	B	A	A	ns
Monocytes (10^4 .μL^{-1})	-	-	-	-	-	-	ns
Neutrophils (10^4 .μL^{-1})	B	A	B	AB	A	AB	ns

Different capital letters represent significant differences in time regardless of diet and between diets regardless of time ($p < 0.05$), ns (not significant).

Table 7**.** Haemoglobin, mean corpuscular haemoglobin (MCH), red blood cells (RBC) and white blood cells (WBC) in gilthead seabream juveniles following an inflammatory insult after 2 weeks of feeding (experiment 2). Data are the mean ± SEM (n = 9).

Haematology	0h				24h				48h			
Diets	**CTR**	**D1**	**D2**	**D3**	**CTR**	**D1**	**D2**	**D3**	**CTR**	**D1**	**D2**	**D3**
Haemoglobin (g.dL^{-1})	0.68 ± 0.06	0.65 ± 0.02	0.74 ± 0.05	0.67 ± 0.05	0.87 ± 0.10	0.79 ± 0.10	0.96 ± 0.08	0.78 ± 0.09	0.57 ± 0.05	0.83 ± 0.08	0.73 ± 0.05	0.69 ± 0.03
MCH (pg.cell^{-1})	3.31 ± 0.39	3.12 ± 0.19	3.61 ± 0.19	3.24 ± 0.18	3.60 ± 0.28	3.82 ± 0.33	3.98 ± 0.33	3.79 ± 0.34	3.09 ± 0.28	3.93 ± 0.33	3.43 ± 0.16	3.54 ± 0.13
WBC (10^{4}.µL^{-1})	3.96 ± 0.18	4.19 ± 0.21	3.73 ± 0.23	3.89 ± 0.19	4.04 ± 0.28	4.23 ± 0.28	4.09 ± 0.42	4.11 ± 0.32	4.56 ± 0.29	4.69 ± 0.26	4.19 ± 0.35	4.42 ± 0.35
RBC (10^{6}.µL^{-1})	1.93 ± 0.09	2.13 ± 0.16	2.06 ± 0.12	1.91 ± 0.11	2.20 ± 0.14	2.11 ± 0.12	2.34 ± 0.13	2.05 ± 0.09	1.86 ± 0.11	2.23 ± 0.10	2.13 ± 0.10	1.97 ± 0.09

2-Way ANOVA								
	Time			**Diet**				**Diet*Time**
	0h	**24h**	**48h**	**CTR**	**D1**	**D2**	**D3**	
Haemoglobin	B	A	B	-	-	-	-	ns
MCH	-	-	-	-	-	-	-	ns
WBC	-	-	-	-	-	-	-	ns
RBC	-	-	-	-	-	-	-	ns

Different capital letters represent significant differences in time regardless of diet ($p < 0.05$), ns (not significant).

Table 8. Absolute values of peripheral blood leucocytes (thrombocytes, Lymphocytes, monocytes and neutrophils) in gilthead seabream juveniles following an inflammatory insult after 2 weeks of feeding (experiment 2). Data are the mean ± SEM (n = 9).

Peripheral leucocytes	0h				24h				48h			
Diets	**CTR**	**D1**	**D2**	**D3**	**CTR**	**D1**	**D2**	**D3**	**CTR**	**D1**	**D2**	**D3**
Thrombocytes (10^4 .μL^{-1})	2.78 ± 0.18	3.24 ± 0.17	2.58 ± 0.17	2.66 ± 0.16	3.06 ± 0.16	3.05 ± 0.24	3.33 ± 0.29	3.26 ± 0.28	3.14 ± 0.33	3.10 ± 0.16	2.88 ± 0.19	2.93 ± 0.20
Lymphocytes (10^4 .μL^{-1})	1.15 ± 0.12	0.84 ± 0.07	1.05 ± 0.15	1.17 ± 0.08	0.75 ± 0.13	0.88 ± 0.13	0.76 ± 0.14	0.60 ± 0.10	1.09 ± 0.12	1.00 ± 0.12	0.93 ± 0.15	0.87 ± 0.07
Monocytes (10^4 .μL^{-1})	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.01
Neutrophils (10^4 .μL^{-1})	0.02 ± 0.01	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.16 ± 0.05	0.18 ± 0.04	0.14 ± 0.04	0.18 ± 0.06	0.09 ± 0.02	0.21 ± 0.07	0.20 ± 0.06	0.25 ± 0.05

2-Way ANOVA

	Time			**Diet**				**Diet*Time**
	0h	**24h**	**48h**	**CTR**	**D1**	**D2**	**D3**	
Thrombocytes	-	-	-	-	-	-	-	ns
Lymphocytes	A	B	A	-	-	-	-	ns
Monocytes	-	-	-	-	-	-	-	ns
Neutrophils	B	A	A	-	-	-	-	ns

Different capital letters represent significant differences in time regardless of diet ($p < 0.05$), ns (not significant).

5.3.2 Plasma humoral parameters

In experiment 1, plasma humoral parameters (NO production, antiprotease and peroxidase activities) remained unaffected by the different dietary treatments at both sampling points (Fig. 1A, 1B and 1C). However, antiprotease activity increased from 1 to 2 weeks of feeding, while peroxidase followed an opposite trend.

Following heat-inactivated bacteria inoculation, peroxidase activity increased after 48 h (Fig. 1E), while both NO concentration and antiprotease activity decreased at 24 and 48 h (Fig. 1D and 1F).

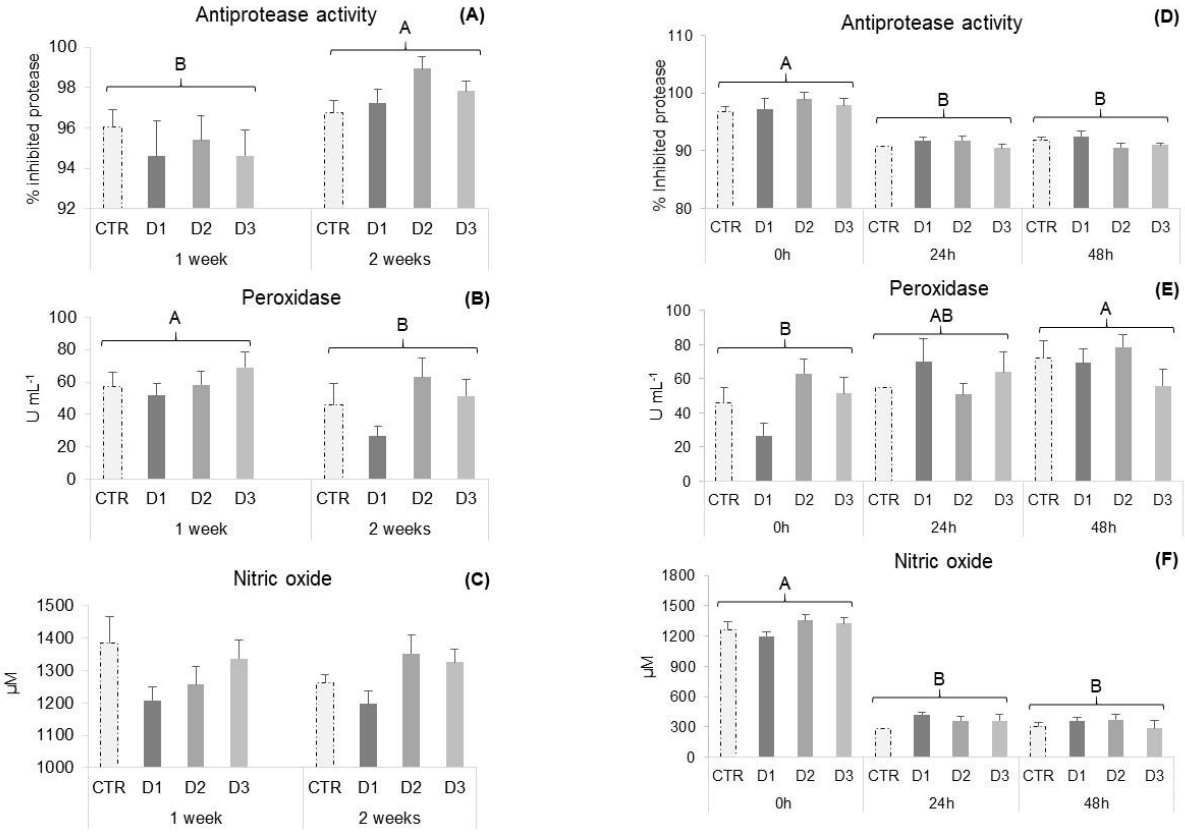

Figure 1. Plasma immune parameters of gilthead seabream juveniles. Experiment 1; (A) Antiprotease activity; (B) Peroxidase activity (C) Nitric oxide. Data are the mean ± SEM (n = 12). Experiment 2; (D) Antiprotease activity; (E) Peroxidase activity (F) Nitric oxide. Data are the mean ± SEM (n = 9) Different capital letters represent significant differences in time regardless diet ($p < 0.05$).

5.3.3 Gut innate immune and oxidative stress biomarkers

Peroxidase, NO production and SOD activity remained unchanged during the health status experiment in gut samples (Fig. 2A, 2B and 3C). Nonetheless, D2 fed fish showed increased gut lipid peroxidation compared to D3 and CTR (Fig. 3A) and catalase activity increased from 1 to 2 weeks of feeding.

In experiment 2, all measured parameters changed over time. Peroxidase activity increased from 24 to 48 h and NO production decreased after 24 and 48 h (Fig. 2C and 2D). Antioxidant defences, such as catalase activity decreased 48 h after inoculation (Fig. 3E), while lipid peroxidation increased at 24 and 48 h (Fig. 3D). Superoxide dismutase activity increases at 24 h post-stimulus and D1 fed fish have higher activity than D3 irrespective of the sampling point (Fig. 3F).

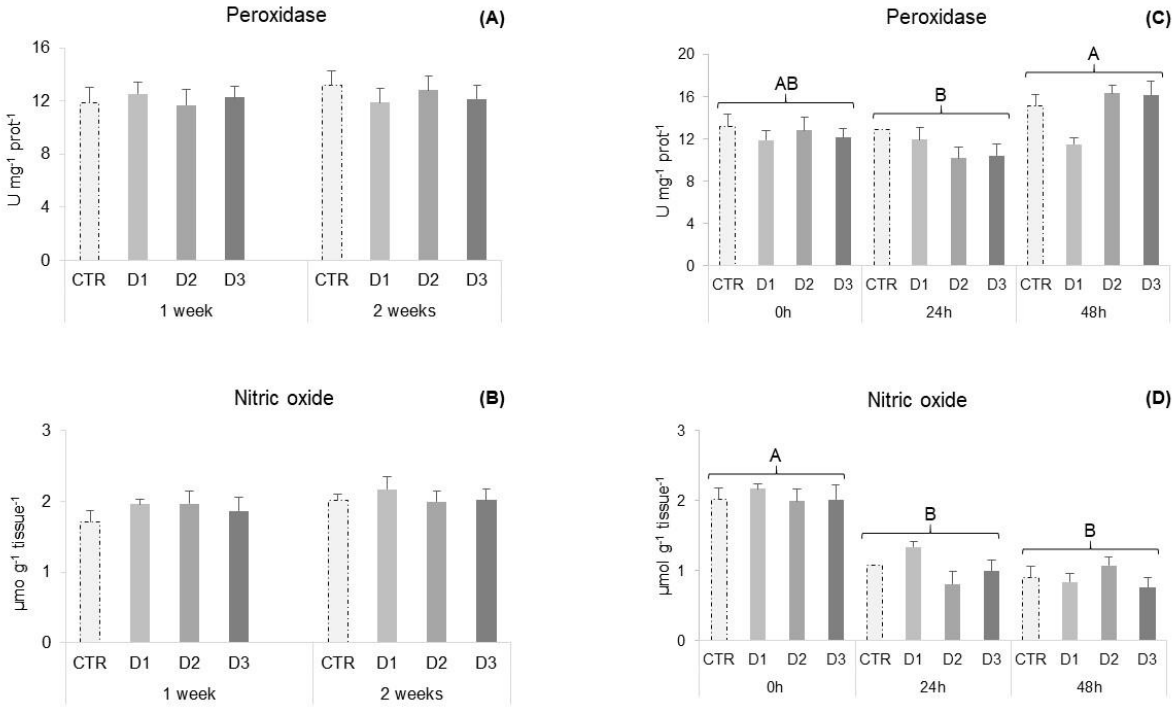

Figure 2. Gut immune parameters of gilthead seabream juveniles. Experiment 1; (A) Peroxidase activity; (B) Nitric oxide (NO). Data are the mean ± SEM (n = 12). Experiment 2; (C) Peroxidase activity; (D) Nitric oxide (NO). Data are the mean ± SEM (n = 9) Different capital letters represent significant differences in time regardless of diet ($p < 0.05$).

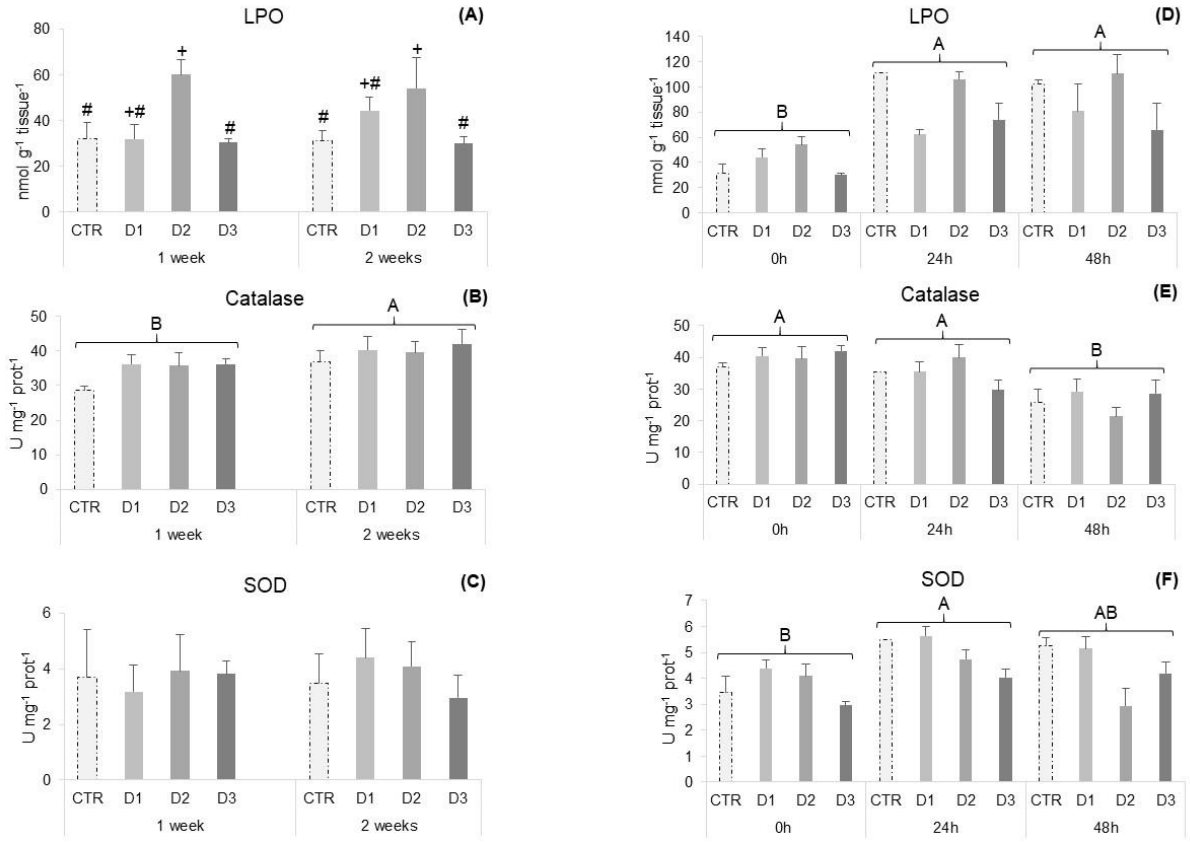

Figure 3. Gut oxidative stress parameters of gilthead seabream juveniles. Experiment 1; (A) Lipid peroxidation (LPO); (B) Catalase activity; (C) Superoxide dismutase activity (SOD). Data are the mean ± SEM (n = 12). Experiment 2; (D) Lipid peroxidation (LPO); (E) Catalase activity; (F) Superoxide dismutase activity (SOD). Data are the mean ± SEM (n = 9). Different symbols represent significant differences between diets regardless of time ($p < 0.05$). Different capital letters represent significant differences in time regardless of diet ($p < 0.05$).

5.3.4 Gut gene expression analysis

To evaluate the expression of gut health, immunity and oxidative stress related genes (Tables 9 and 10), total RNA was isolated from fish anterior intestine. In experiment one, target genes transcriptomic analysis was not able to ascertain differences attributable to the dietary treatments, which could be related to the high intraspecific variability for some target genes (Table 9). However, cd8α, hsp70 and muc2 genes expression increased from 1 to 2 weeks.

Following the inflammatory insult, changes attributed to dietary treatments were also not found in the majority of analysed genes, except for hsp70, which was down-regulated at 24 h in D2 fed fish (Table 10). Furthermore, tlr1 gene expression was up-regulated and gpx was down-regulated at 24 h in all dietary treatments.

Table 9. Relative gene expression profiling of anterior intestine in gilthead seabream juveniles after 1 and 2 weeks of feeding (experiment 1). Data are the mean ± SEM (n = 12). All data values for each gene were in reference to the expression level of CTR.

		Relative mRNA expression										
Trial 1	**Diets**	***il1-β***	***il-34***	***tlr1***	***cd8α***	***igm***	***hepcidin***	***hsp70***	***gpx***	***sod(mn)***	***muc2***	***muc13***
1 week	**CTR**	1.09 ± 0.15	1.20 ± 0.16	1.05 ± 0.10	1.52 ± 0.43	1.09 ± 0.13	1.58 ± 0.45	1.04 ± 0.09	1.11 ± 0.13	1.06 ± 0.12	1.34 ± 0.29	1.11 ± 0.17
	D1	1.04 ± 0.22	1.18 ± 0.16	1.27 ± 0.12	0.94 ± 0.27	1.17 ± 0.43	1.58 ± 0.52	1.38 ± 0.25	1.49 ± 0.29	1.28 ± 0.14	1.04 ± 0.27	1.27 ± 0.15
	D2	1.41 ± 0.18	1.37 ± 0.12	1.12 ± 0.16	1.22 ± 0.34	2.01 ± 0.63	1.13 ± 0.32	1.67 ± 0.28	1.79 ± 0.26	1.12 ± 0.14	1.35 ± 0.22	1.17 ± 0.14
	D3	1.15 ± 0.09	1.18 ± 0.12	1.28 ± 0.16	1.67 ± 0.58	1.31 ± 0.57	0.99 ± 0.19	1.77 ± 0.38	1.50 ± 0.23	1.46 ± 0.20	1.28 ± 0.18	1.34 ± 0.20
2 weeks	**CTR**	1.54 ± 0.47	1.08 ± 0.15	1.31 ± 0.34	1.23 ± 0.29	1.67 ± 0.50	3.17 ± 1.50	1.04 ± 0.16	1.67 ± 0.41	1.18 ± 0.20	1.93 ± 0.99	1.58 ± 0.58
	D1	1.61 ± 0.62	1.93 ± 0.37	1.94 ± 0.37	1.84 ± 0.42	7.39 ± 3.31	3.45 ± 1.04	1.40 ± 0.25	2.26 ± 0.33	2.25 ± 0.51	3.11 ± 0.68	1.89 ± 0.24
	D2	1.59 ± 0.47	1.43 ± 0.37	2.44 ± 0.65	2.02 ± 0.65	4.16 ± 1.53	3.43 ± 2.61	0.77 ± 0.27	1.78 ± 0.70	1.02 ± 0.29	1.86 ± 0.60	1.59 ± 0.57
	D3	1.31 ± 0.42	1.77 ± 0.54	2.09 ± 0.44	1.70 ± 0.55	3.70 ± 1.58	1.49 ± 0.52	1.56 ± 0.77	1.92 ± 0.50	1.66 ± 0.65	2.19 ± 0.63	1.92 ± 0.69

2 way-ANOVA		***il1-β***	***il-34***	***tlr1***	***cd8α***	***igm***	***hepcidin***	***hsp70***	***gpx***	***sod(mn)***	***muc2***	***muc13***
Sig.	***Time***	ns	ns	ns	0.024	ns	ns	0.009	ns	ns	0.046	ns
	Diet	ns	ns	ns	0.747	ns	ns	0.541	ns	ns	0.569	ns
	Time*Diet	ns	ns	ns	0.405	ns	ns	0.106	ns	ns	0.156	ns
Diet	**CTR**	-	-	-	-	-	-	-	-	-	-	-
	D1	-	-	-	-	-	-	-	-	-	-	-
	D2	-	-	-	-	-	-	-	-	-	-	-
	D3	-	-	-	-	-	-	-	-	-	-	-
Time	***1 week***	-	-	-	B	-	-	B	-	-	B	-
	2 weeks	-	-	-	A	-	-	A	-	-	A	-

Different capital letters represent significant differences between in time regardless of diet ($p < 0.05$).

Table 10. Relative gene expression profiling of anterior intestine in gilthead seabream juveniles following an inflammatory insult after 2 weeks of feeding (experiment 2). Data are the mean ± SEM (n = 9). All data values for each gene were in reference to the expression level of CTR.

		Relative mRNA expression										
Trial 2	**Diets**	***il1-β***	***il-34***	***tlr1***	***cd8α***	***igm***	***hepcidin***	***hsp70***	***gpx***	***sod(mn)***	***muc2***	***muc13***
0h	**CTR**	1.54 ± 0.47	1.08 ± 0.15	1.23 ± 0.34	1.31 ± 0.29	1.67 ± 0.50	3.17 ± 1.50	1.04 ± 0.16	1.67 ± 0.41	1.18 ± 0.20	1.93 ± 0.99	1.58 ± 0.58
	D1	1.61 ± 0.62	1.93 ± 0.37	1.84 ± 0.37	1.94 ± 0.42	7.39 ± 3.31	3.45 ± 1.04	1.40 ± 0.25	2.26 ± 0.33	2.25 ± 0.51	3.11 ± 0.68	1.89 ± 0.24
	D2	1.59 ± 0.47	1.43 ± 0.37	2.02 ± 0.65	2.44 ± 0.65	4.16 ± 1.53	3.43 ± 2.61	0.77 ± 0.27*#	1.78 ± 0.70	1.02 ± 0.29	1.86 ± 0.60	1.59 ± 0.57
	D3	1.31 ± 0.42	1.77 ± 0.54	1.70 ± 0.44	2.09 ± 0.55	3.70 ± 1.58	1.49 ± 0.52	1.56 ± 0.77	1.92 ± 0.50	1.66 ± 0.65	2.19 ± 0.63	1.92 ± 0.69
24h	**CTR**	0.98 ± 0.17	2.20 ± 0.36	2.19 ± 0.38	1.95 ± 0.50	6.18 ± 2.70	2.31 ± 0.83	1.41 ± 0.19[a]	1.59 ± 0.61	1.22 ± 0.30	2.98 ± 0.52	1.71 ± 0.31
	D1	0.58 ± 0.22	0.85 ± 0.36	3.47 ± 1.06	0.80 ± 0.37	1.53 ± 0.87	1.77 ± 0.57	0.68 ± 0.22[ab]	1.14 ± 0.36	1.27 ± 0.51	3.31 ± 1.19	1.13 ± 0.38
	D2	0.80 ± 0.26	1.75 ± 1.11	2.38 ± 0.59	1.31 ± 0.80	0.77 ± 0.24	1.25 ± 0.41	0.34 ± 0.14[b]*	0.89 ± 0.32	0.62 ± 0.25	3.70 ± 2.22	0.94 ± 0.25
	D3	0.96 ± 0.41	1.80 ± 0.57	2.13 ± 0.29	1.95 ± 0.53	4.90 ± 4.01	1.77 ± 0.42	1.25 ± 0.22[ab]	0.69 ± 0.20	0.98 ± 0.24	3.06 ± 0.77	2.83 ± 1.22
48h	**CTR**	0.60 ± 0.07	1.49 ± 0.26	0.90 ± 0.14	2.11 ± 0.55	5.64 ± 1.59	3.70 ± 1.84	3.28 ± 1.47	1.86 ± 0.42	2.40 ± 0.51	2.27 ± 0.52	2.56 ± 0.87
	D1	0.51 ± 0.11	1.08 ± 0.28	1.42 ± 0.33	1.37 ± 0.29	1.11 ± 0.47	1.13 ± 0.34	0.83 ± 0.16	0.84 ± 0.20	0.81 ± 0.22	1.42 ± 0.42	1.02 ± 0.24
	D2	0.84 ± 0.21	1.35 ± 0.20	0.78 ± 0.10	2.51 ± 0.27	1.54 ± 0.34	3.20 ± 0.82	1.12 ± 0.2[#]	1.07 ± 0.19	1.27 ± 0.18	2.60 ± 0.47	2.34 ± 0.48
	D3	1.94 ± 1.49	1.44 ± 0.38	1.00 ± 0.27	2.39 ± 1.14	2.59 ± 1.08	4.63 ± 2.31	1.50 ± 0.50	1.64 ± 0.20	1.46 ± 0.60	2.78 ± 0.78	1.75 ± 0.52

Two way-ANOVA		***il1-β***	***il-34***	***tlr1***	***cd8α***	***igm***	***hepcidin***	***hsp70***	***gpx***	***sod(mn)***	***muc2***	***muc13***
Sig.	***Time***	ns	ns	<0.001	ns	ns	ns	0.030	0.006	ns	ns	ns
	Diet	ns	ns	ns	ns	ns	ns	<0.001	ns	ns	ns	ns
	Time*Diet	ns	ns	ns	ns	ns	ns	0.028	ns	ns	ns	ns
Diet	**CTR**	-	-	-	-	-	-	-	-	-	-	-
	D1	-	-	-	-	-	-	-	-	-	-	-
	D2	-	-	-	-	-	-	-	-	-	-	-
	D3	-	-	-	-	-	-	-	-	-	-	-
Time	***0h***	-	-	B	-	-	-	-	A	-	-	-
	24h	-	-	A	-	-	-	-	B	-	-	-
	48h	-	-	B	-	-	-	-	AB	-	-	-

Different superscript letters represent significant differences between diets within the same time ($p < 0.05$). Different superscript symbols represent significant differences in time within the same diet ($p < 0.05$). Different capital letters represent significant differences in time regardless of diet ($p < 0.05$).

5.4 Discussion

A main feature of *C. vulgaris* is its protein content and its balanced AA profile, making it a potential source of bioactive peptides. However, the presence of rigid cell walls limits the fish's ability to access and to utilise the different nutrients inside microalgae cells. In the present study, cell wall disruption was obtained through a combination of chemical and enzymatic processes and the protein fraction was hydrolysed using a serine protease. Protein hydrolysates seem more effective than either intact protein or free AA in different applications for nutrition [25; 48]. The current study was devised using two different approaches. First, there was a 2-week feeding trial to evaluate the health status of the fish, aiming to develop future prophylactic strategies (experiment one). After 2 weeks of feeding, fish were subjected to an inflammatory insult to evaluate the inflammatory response (experiment two) and to better discriminate any immunomodulatory effect from the different dietary treatments.

The overall haematological profile from the health status experiment showed some changes, mainly exerted by *C. vulgaris* biomass and peptide-enriched extract supplemented diets (D1 and D2 diets). Fish fed diet D1 showed lower lymphocyte numbers (Table 6). Accordingly, in a previous experiment with poultry, where different preparations of *C. vulgaris* were used, animals fed a supplemented diet with 1% *Chlorella* powder showed decreased lymphocyte numbers [49]. Nonetheless, fish fed D2 diet not only had comparable lymphocyte numbers to CTR, but also showed a higher neutrophil concentration (Table 6). These higher circulating myeloid cell numbers in the D2 group might be of relevance during early responses to infection. Bøgwald et al. [50] have shown that medium-size peptides (500–3000 Da) from cod muscle protein hydrolysate, stimulated *in vivo* respiratory burst activity in Atlantic salmon (*Salmo salar*) head-kidney leucocytes. In the present study, the peptide-enriched extract protein/peptide profile (Fig. S1) is mainly composed of small to medium size particles (< 1200 Da) [38]. Size and molecular weight (MW) seem to be particularly important for peptide immunomodulatory activities, with small- to medium-sized particles showing the highest activity [26; 28; 50; 51]. However, an increased leucocyte response in fish fed the D2 diet did not translate into an improved plasma humoral parameters response (NO concentration, antiprotease and peroxidase activities) at 1 or 2 weeks (Fig. 1A; 1B and 1C), although those values tended to increase in seabream fed D2 and D3. Accordingly, former studies conducted on Coho salmon (*Oncorhynchus kisutch*) and turbot (*Scophthalmus maximus*) did not show any significant impacts on several innate immune defense mechanisms, when fish were fed MPH supplemented diets [52; 53]. Nonetheless, beneficial effects have been reported in

different fish species [26]. Khosravi et al. [33] supplemented red seabream (*Pagrus major*) and olive flounder (*Paralichthys olivaceus*) feeds with 2% krill and tilapia protein hydrolysates and supplementation improved lysozyme activity and respiratory burst in both species. Protein hydrolysates were mainly composed of small- (< 500 Da) to medium-sized peptides (500–5000 Da). Furthermore, diet D2 shows a higher Hb concentration than D1 and D3 fed fish. The extraction method employed in the *C. vulgaris* biomass to obtain the soluble extract (diet D2) might increase iron availability, since most of the intracellular iron is associated with soluble proteins and iron is an essential element for Hb production [54].

In the present study, when fish were subjected to an inflammatory insult (experiment 2), an immune response after the stimulus was observed through the time-dependent response pattern of peripheral leucocytes, plasma and gut immune parameters. Peripheral cell dynamics were significantly changed at 24 h post-stimulus, translating into a sharp increase in circulating neutrophils and a significant decrease in lymphocytes (Table 8), indicating that cells were differentiating and being recruited to the site of inflammation. Also, Hb concentration increased (Table 7) in line with a higher metabolic expenditure due to the inflammatory response, and peroxidase activity showed a clear augmentation following inflammation (Fig. 1E). Even though circulating neutrophil numbers tended to increase in D1, 2 and 3 dietary treatments at 48 h following inflammation (Table 8), it was not possible to ascertain a clear *Chlorella* whole-biomass or extracts effect, a fact that could be related to high intraspecific variability in response to the stimulus and that reinforces the need for further studies to unravel the potential of these extracts.

Hydrogen peroxide and oxygen radicals are physiologically generated within cellular compartments and their build-up leads to tissue oxidative stress and damage [55]. Free radical effects are controlled endogenously by antioxidant enzymes and non-enzymatic antioxidants and also by exogenous dietary antioxidants that prevent oxidative damage. *Chlorella* sp. contain several phytochemicals, namely carotenoids, chlorophyll, flavonoids and polyphenols, which exhibit antioxidant activities [56; 57]. Earlier studies showed a significant increase in serum SOD activity in gibel carp fed diets containing 0.8–2.0% dry *Chlorella* powder [20]. Rahimnejad et al. [14] reported increased plasma CAT activity and total antioxidant capacity (TAC) in olive flounder fed diets with 5% and 10% defatted *C. vulgaris* meal. As with other microalgae species, the antioxidant potential of *C. vulgaris* has been mainly assessed on serum and liver, though information is still scarce at the intestinal level. The intestinal epithelium, a highly selective barrier between the animal and the external environment, is constantly exposed to dietary and environmental oxidants. Consequently, it is more prone to oxidative stress and damage,

which can impact gut functionality and health [58; 59]. The dietary effects of microalgae biomass inclusion have been previously assessed on the intestine of gilthead seabream. Fish were fed diets supplemented with 0.5, 0.75 and 1.5% *Nannochloropsis gaditana* biomass and no signs of nutritional modulation were found for intestinal SOD and CAT transcription [60]. In the present study, D2 fed fish showed higher gut LPO than CTR and D3 at the end of experiment one (Fig. 3A), which could be related to the extraction method employed, since most of the pigments present in the *C. vulgaris* biomass are not present in the peptide-enriched extract, diminishing the availability of exogenous dietary antioxidants. As pigments are mostly hydrophobic, they are extracted alongside the lipid fraction present in the insoluble extract (Diet D3). Regarding the activities of key enzymes involved in intestinal redox homeostasis (CAT and SOD), these remained unchanged among experimental groups. Castro et al. [17] replaced 100% FM by *C. vulgaris* biomass in plant protein rich diets for seabass (*Dicentrarchus labrax*) and found no differences in intestinal LPO, tGSH and GSH levels between dietary treatments. However, they reported lower SOD activity and higher GSSG levels in microalgae-enriched diets, suggesting an increased risk for oxidative stress when fish are subjected to pro-oxidative conditions. Such conditions might arise during an inflammatory insult. However, in experiment 2 of the present study, lipid peroxidation increased at 24 and 48 h (Fig. 3D) post-stimulus but to the same extent for all the dietary treatments. It could be hypothesised that fish fed the D2 diet were able to cope with acute inflammation in a similar manner as the other experimental groups, despite their higher intestinal oxidative state. In other studies, *C. vulgaris* powdered biomass has been found to counteract the pro-oxidative effects of arsenic induced toxicity in both the gills and the liver of tilapia [16]. Furthermore, Grammes et al. [60] reported that substituting FM by *C. vulgaris* in aquafeeds containing 20% soybean meal (SBM) is an effective strategy to counteract soybean meal-induced enteropathy (SBMIE) in Atlantic salmon. Likely, this was by maintaining the integrity of the intestinal epithelial barrier and therefore preventing innate immune response activation and ROS generation [61; 62].

In the present study, anterior gut transcriptional changes were also evaluated to determine the effect of dietary treatments on the expression patterns of different structural (muc2 and muc13), antioxidant (hsp70; gpx and sod(mn)) and immune related genes (il1β; il34; tlr1; cd8α; igm and hepc). The transcriptomic approach employed was not able to ascertain a clear dietary modulation, at least for the great majority of genes under evaluation in both experiments 1 and 2. However, after the inflammatory insult, the hsp70 gene was down regulated in the D2 fed group after 24 h compared to those fed CTRL (Table 10). Heat shock protein 70 (HSP70) maintains cell integrity and function, and it promotes cell survival under stressful conditions [63]. Leduc et al. [28]

reported that genes involved in cellular damage response and repair were also under-expressed in seabass fed a mix of tilapia (TH) and shrimp (SH) protein hydrolysates (5% dry matter diet), mainly composed of low molecular weight peptides. In the same study, fish that were fed the SH alone showed up-regulation of intestinal immune-related genes. Although composed of small-sized peptides, TH did not show the same pattern of stimulation, following what was observed in the current work. According to the authors, the immune-stimulatory effect of the SH was due to low molecular weight peptides, but also to its origin and its degree of hydrolysis [28]. Bioactive peptides are inactive when they are part of the native protein sequence; and, after hydrolysis, bioactivity can be gained depending on specific AA sequences and the size of the newly formed peptides [25]. Nevertheless, in the present study, the observed down-regulation of hsp70 gene expression in the gut of seabream fed D2 suggests a certain degree of anti-stress and/or antioxidant properties from the *C. vulgaris* peptide-enriched extract, in line with that hypothesized above.

In summary, the *C. vulgaris* peptide-enriched extract tested in the present study seems to confer a dual modulatory effect at both peripheral (blood) and local (gut) levels. In particular, it drives the proliferation of circulating neutrophils in resting seabream, which could be of assistance to fight against opportunistic pathogens. Following an inflammatory insult, this peptide-enriched extract may protect the gut against stress, and it should be considered for further studies.

5.5 Acknowledgements

This work was supported by VALORMAR project (POCI-01-0247-FEDER-024517) funded by Compete 2020, Lisboa 2020, Algarve 2020, Portugal 2020 and the European Union through FEDER, and by national funds through Foundation for Science and Technology (FCT) within the scope of UIDB/50016/2020, UIDB/04423/2020 and UIDP/04423/2020. The views expressed in this work are the sole responsibility of the authors. B. Reis was supported by the Portuguese Foundation for Science and Technology, Soja de Portugal, SA, and Sparos Lda., through the grant PD/BDE/129262/2017. S.A. Cunha and B. Costas were supported by the Portuguese Foundation for Science and Technology, through grants SFRH/BD/144155/2019 and 2020.00290.CEECIND, respectively.

5.6 Ethics Statement

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. CIIMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). The protocol was approved by the CIIMAR Animal Welfare Committee.

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Chapter VI

General discussion, Conclusions and Future approaches

6. General discussion

Recognizing marine algae potential to modulate health-related mechanisms in fish, the current thesis proposed to evaluate the immune status and inflammatory response of gilthead seabream fed diets supplemented with different microalgae biomasses and extracts.

A holistic approach, comprising several analytical tools (i.e. biometry, innate immune and oxidative stress parameters, haematology and transcriptomics), enabled the simultaneous assessment of different physiological responses, namely growth performance, immunological status and oxidative stress response. Globally, four trials were designed to evaluate the effects of dietary algae biomasses and extracts supplementation, under different rearing scenarios and challenging conditions.

6.1 Algae biomasses

Starting with the evaluation of relevant marine algae species, diets were supplemented with 2% lyophilized macro and microalgae biomass with integral cell walls (Chapter II). The experiment was devised as a short-term period supplementation (2 weeks) with inflammatory stimulus at both 1 and 2 weeks. After 1 week, the supplementation with 2% macroalga *Ulva rigida* biomass promoted neutrophil proliferation 4 h post-inflammation, which can be advantageous during infectious events. Also, all algae supplemented diets showed a clear lymphocyte increase 4 h post-inflammation at 2 weeks. It was hypothesized that stimulatory effects are due to the presence of complex carbohydrates, particularly sulphated polysaccharides (SPs). Nonetheless, general results showed mild immunological and antioxidant effects. Algae show complex cell walls, that omnivorous fish species digest in a very limited way or are simply unable to digest. These species lack or show a very limited repertoire of digestive enzymes able to hydrolyse the ß-glycosidic bonds present in complex polysaccharides [1; 2]. Several works in fish show that cell wall disruption improves nutrient digestibility and the ability of fish to utilize algae biomasses [3; 4]. Thus, it can be inferred that cell wall disruption can be determinant for bioactive compounds availability and fish utilization.

6.2 Processed biomasses

Following the conclusion that mild effects exerted by the different algae biomasses (Chapter II) could be mainly related to the presence of complex cell walls that prevent access to valuable bioactive compounds rather than a general lack of bioactivity, a second experiment was devised (Chapter III).

P. tricornutum biomass was subjected to a mechanical treatment resulting in cell wall disruption. Diets were supplemented with 1% microalgae biomass with intact cells or disrupted cell walls and fish were fed for a 12-week period. *P. tricornutum* broken cells diet showed the highest overall immunostimulatory effect and these, were mainly observed at an early stage (2 weeks). The above mentioned diet led to head-kidney *α2m* gene upregulation and promising results in mucus innate immunity. Results showed increased mucus alternative complement pathway and bactericidal activity at 2 and 12 weeks, respectively. In *P. tricornutum*, β-glucans are located inside the cell in vacuoles and when available, these polysaccharides are known to have immunostimulatory effects in fish [5; 6; 7].

Results from the second trial reinforced the need to continue exploring this rationale, that cell wall disruption is an important feature for bioactive compounds to exert their biological effects, especially in omnivorous fish such as gilthead seabream.

In that sense, in the third experimental trial (Chapter IV) the experimental approach was refined by going a step forward and using a chrysolaminarin-enriched biomass from *P. tricornutum*, which, was mechanically treated to obtain β-glucans enriched extracts. This experiment was devised as a discontinuous feeding trial. β-glucan enriched *P. tricornutum* extracts and yeast β-glucan (benchmark food additive) supplemented diets were given to fish every two weeks for an 8-week period, while CTR fish were continuously fed with the control diet. This approach had a double purpose, firstly to avoid immunological overstimulation [8; 9] and secondly to understand if β-glucans could have long-lived effects, as some bibliography suggests [5]. Overall, results can be divided in a short-term (2 weeks) hepatoprotective effect, showed by β-glucan supplemented diets, which was independent of β-glucan origin and physical properties (i.e. molecular weight and solubility). However in the short-term, it was no immunomodulatory effect could be ascertained. Nonetheless, at 8 weeks, there was a general down-regulation of immune related genes in the intestine of fish fed Phaeo37 diet, which points to an anti-inflammatory effect. Molecular weight can play an important role in the biological effects of β-glucans. In colitis-induced rat models, the dietary administration of low molecular weight oat β-glucans reduced local inflammatory signs in colon and significantly down-regulated several pro-inflammatory cytokines [10; 11]. Furthermore, the therapeutic effect

was in evident relation to the molecular mass of the polymer. Phaeo21 and 37 extracts show low molecular mass β-glucans (chrysolaminarin). Additionally, *P. tricornutum* extracts supplemented diets although having the same β-glucan concentration, differ in purity, since Phaeo37 extract has a higher percentage of β-glucans compared to Phaeo21. Thus, the combination of low molecular mass and higher extract purity might explain the higher immunomodulatory effects of Phaeo37 dietary treatment at 8 weeks. The use of gut anti-inflammatory compounds can have special relevance in aquaculture, both as a prophylactic and therapeutic measure, as the industry decreases the use of FM, replacing it with ingredients that are not normally part of omnivorous/carnivorous fish diet (i.e. plant and terrestrial animal proteins). In this regard, results indicate that the dietary administration of a *P. tricornutum* 37% enriched- β-glucan extract might be relevant the context of extreme dietary formulations due to its anti-inflammatory and anti-oxidative effects, respectively in gut and liver.

Comprising results from chapters III and IV, it seems clear that biomass processing through appropriate processes increases algae compounds' bioactivity and enhances its value as feed additives. At least for the targeted application intended in this thesis, of boosting health-related parameters.

Additionally, a fourth experiment was carried out using *C. vulgaris* biomass and extracts (Chapter V). *C. vulgaris* biomass was physically and enzymatically treated to obtain a low molecular weight peptide-enriched extract. The experiment was conceived as a short-term period supplementation (2 weeks) with an inflammatory stimulus at 2 weeks. *C. vulgaris* peptide-enriched supplemented diet (D2 diet) showed higher neutrophil concentration following 1 week of feeding. Higher phagocytic circulating cell numbers might be of relevance during the early response to infection. After the inflammatory stimulus at 2 weeks, it was not possible to discriminate any dietary effect in the different innate immune and oxidative stress parameters measured among experimental diets. Nevertheless, the observed down-regulation of *hsp70* gene expression at 24 h in the gut of seabream fed D2 diet, suggests a certain degree of anti-stress and/or anti-oxidant properties from the *C. vulgaris* peptide-enriched extract. In summary, the *C. vulgaris* peptide-enriched extract tested in the present study seems to confer a dual modulatory effect both at peripheral (blood) and local (gut) levels.

6.3 Feeding interval: Short-term vs medium-term

Feed additives are commonly used in aquaculture. However, when addressing additives targeting innate immunity there is some debate about feeding periods. Which is the best approach to achieve immunomodulation or stimulation? Long- or short-term feeding?

In chapter II a short-term feeding approach was employed. From the results we could see that both at 1 and 2 weeks, 4 h-post the inflammatory insult there was a positive effect elicited by the algae supplemented diets in leucocyte numbers. This constitutes an encouraging outcome favouring immunonutrition, i.e. we can see a stimulatory effect after the inflammatory stimulus and not before, which means that while fish were in normal physiological conditions all energy was being channelled for growth. But when challenged, supplemented fed fish showed an improved response. Following this first approach, 2 longer-term feeding experiments were devised with sampling points at 2 weeks and a final sampling at the end of the trials. In Chapter III, gilthead juveniles were fed processed *P. tricornutum* biomasses during a 12-week period. Main immunological effects were seen at 2 weeks mainly exerted by broken cells diet. Results point to a short-term immunostimulatory effect in head-kidney and mucus. When using the same microalgae species further processed to obtain extracts specifically enriched in β-glucans (Chapter IV), there was an hepatoprotective effect at 2 weeks, translated to a lower hepatic oxidative state. As in Chapter III plasma innate immune parameters remained unaffected. However, at a longer feeding period (8 weeks) there was a localized anti-inflammatory effect in the gut. In Chapter V supplemented diets were fed to fish for a short-term period and an inflammatory stimulus similar to Chapter II was devised at 2 weeks. However, in this case only mild beneficial effects were perceived from the supplementation with *C. vulgaris* biomass and extracts. Nonetheless, the *C. vulgaris* peptide-enriched extract seemed to confer modulatory effect both at peripheral (blood) and local (gut) levels, particularly boosting proliferation of circulating neutrophils in resting seabream. While, after an inflammatory insult it seems to protect the gut against stress and should be considered for further studies.

From results obtained in Chapters II, III, IV and V, it seems clear that health-related effects of immunonutrition strategies using algal products are mainly developed at short-term feeding periods. This dynamic of short-lived boosting effects might be advantageous if this feeding strategy is outlined as a prophylactic measure, used in specific periods of the production cycle.

6.4 Sustainable prophylactic practices

Feed supplementation can be an extra cost for the fish farmer. However, if the supplemented feed exerts beneficial effects after short feeding periods, it can be used within a prevention strategy to boost fish's immune system prior to a predictable stressful event or disease outbreak, and be cost-effective. Thus, preventing infections from opportunistic pathogens, reducing the need for antibiotics administration, mortalities and disruptions in the production cycle. Furthermore, this strategy of antibiotics reduction is within the scope of EU's Farm to Fork policy, which aims, among other objectives, for food production to have a neutral or positive environmental impact. Furthermore, the use of algal-based ingredients can contribute to reduce the environmental burden of the aquaculture industry, since marine algae do not compete with human food crops for valuable resources such as freshwater and arable land.

6.5 Conclusions

This thesis supports:

1) Use of processed algal biomasses in aquafeeds for gilthead seabream;
2) Importance of further processing algae biomasses to increase bioactivity in fish;
3) Marine algae supplementation:
    - Did not compromise growth performance at the studied levels;
    - Changed fish innate immunity and oxidative stress response leading to physiological adaptations, that may confer resistance to stressors or disease;
    - Modulated the transcription of genes associated with immune and antioxidant responses

4) Feeding duration seems to be an important factor for dietary supplementation's beneficial effects. The use of functional feeds can be a promising approach for boosting fish health status, particularly during short-term feeding periods before a predictable stressful event or disease outbreak. Nonetheless, effects depend on the type of bioactive molecule(s) present in the diet. In Chapter IV β-glucans showed both short and long-term effects.

Overall, the results presented in this thesis attempt to modestly contribute to the understanding of how dietary supplementation modulates immune and stress responsiveness in gilthead seabream. Also, reinforce immunonutrition as a promising

and viable approach to the development of tailor-made feed formulations with functional properties.

6.6 Future approaches

Future research should evaluate different supplementation levels of processed marine algal biomasses and focus on specific pathways involved in fish defense mechanisms, antioxidant response and energy metabolism. Such knowledge should allow a targeted application of specific amounts of functional ingredients, for short periods before stressful events. Furthermore, a live pathogen challenge should be performed in order to study the disease resistance and survival of fish fed these supplemented diets. Thus, more in-depth studies are needed to understand the role of processed algal biomasses as immunomodulators during stress and infection, in order to meet the societal challenges towards better practices in the use of chemotherapeutic and antibiotic treatments in aquaculture.

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Appendix I

Effects of short-term feeding algae supplemented diets in innate immune status and inflammatory response of *Sparus aurata* juveniles

Table S1a. Primers for qPCR amplification in seabream.

Gene	Symbol	Annealing T °C	Primer sequence
Transforming growth factor-β1	*tgf-β1*	60	F: TCTGGGGTGGAAATGGATAC R: CTCCTGGGTTGTGATGCTTA
Interleukin-1 beta	*il1β*	60	F: TCTTCAAATTCCTGCCACCA R: CAATGCCACCTTGTGGTGAT
Interleukin-10	*il10*	57	F: AACATCCTGGGCTTCTATCTG R: GTGTCCTCCGTCTCATCTG
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	60	F: ACGTCTGGTCCTATGGCATC R: AGTCTGGTTGGGACATCTGG
Non cytotoxic cell receptor protein	*nccrp1*	60	F: ACTTCCTGCACCGACTCAAG R: TAGGAGCTGGTTTTGGTTGG
Caspase 1	*casp1*	59	F: ACGAGGTGGTGAAACACACA R: GTCCGTCTCTTCGAGTTTCG
Toll like receptor 1	*tlr1*	60	F: GGGACCTGCCAGTGTGTAAC R: GCGTGGATAGAGTTGGACTTGAG
β-defensin	*β-defensin*	60	F: CCCCAGTCTGAGTGGAGTGT R: AATGAGACACGCAGCACAAG
Hepcidin	*hepc*	60	F: GCCATCGTGCTCACCTTTAT R: CCTGCTGCCATACCCCATCTT
Heat-shock protein 70	*hsp70*	55	F: ACGGCATCTTTGAGGTGAAG R: TGGCTGATGTCCTTCTTGTG
Elongation factor 1α	*ef1α*	58	F: CTGTCAAGGAAATCCGTCGT R: TGACCTGAGCGTTGAAGTTG
Ribosomal protein S18	*rps18*	60	F: AGGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACC

Appendix II

Health status in gilthead seabream (*Sparus aurata)* juveniles fed diets devoid of fishmeal and supplemented with *Phaeodactylum tricornutum*

Table S1b. Genes included in the liver (†), head kidney (‡) and white muscle (#) pathway-focused PCR arrays.

Gene name/category	Symbol
GH/IGF system	
Growth hormone receptor I	*ghr-i* †#
Growth hormone receptor II	*ghr-ii* †#
Insulin-like growth factor-I	*igf-i* †#
Insulin-like growth factor-II	*igf-ii* †#
Insulin-like growth factor binding protein 1a	*igfbp1a* †
Insulin-like growth factor binding protein 2b	*igfbp2b* †
Insulin-like growth factor binding protein 3	*igfbp3* #
Insulin-like growth factor binding protein 4	*igfbp4* †
Insulin-like growth factor binding protein 5b	*igfbp5b* #
Insulin-like growth factor binding protein 6b	*igfbp6b* #
Insulin-like growth factor receptor I	*igfr1* †#
Insulin-like growth factor receptor II	*igfr2* †#
Insulin receptor	*insr* †#
Energy sensing and oxidative metabolism	
Sirtuin 1	*sirt1* #
Sirtuin 2	*sirt2* #
Sirtuin 5	*sirt5* #
NADH-ubiquinone oxidoreductase chain 2	*nd2* #
NADH-ubiquinone oxidoreductase chain 5	*nd5* #
Cytochrome c oxidase subunit I	*coxi* #
Cytochrome c oxidase subunit II	*coxii* #
Carnitine palmitoyltransferase 1A	*cpt1a* †#
Citrate synthase	*cs* †#
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α* †#
Hypoxia inducible factor-1 alpha	*hif-1α* †
Respiration uncoupling	
Uncoupling protein 1	*ucp1* †
Uncoupling protein 3	*ucp3* #
Interleukins and cytokines	
Interleukin-1 beta	*il-1β* ‡
Interleukin-6	*il-6* ‡
Interleukin-7	*il-7* ‡
Interleukin-8	*il-8* ‡
Interleukin-10	*il-10* ‡
Interleukin 12 subunit beta	*il12* ‡

Gene name/category	Symbol
Muscle growth and cell differentiation	
Myoblast determination protein 1	*myod1* #
Myogenic factor MYOD2	*myod2* #
Myogenic factor 5	*myf5* #
Myogenic factor 6	*myf6/mrf4/ herculin* #
Myostatin/Growth differentiation factor 8	*mstn/gdf-8* #
Myocyte-specific enhancer factor 2A	*mef2a* #
Myocyte-specific enhancer factor 2C	*mef2c* #
Follistatin	*fst* #
Antioxidant defence and molecular chaperons	
Catalase	*cat* †
Glutathione peroxidase 1	*gpx1* †
Glutathione peroxidase 4	*gpx4* †
Glutathione reductase	*gr* †
Peroxiredoxin 3	*prdx3* †
Peroxiredoxin 5	*prdx5* †
Superoxide dismutase [Mn]	*mn-sod/sod2* †
Fatty acid binding protein, heart	*h-fabp* †
Glucose-regulated protein, 170 kDa	*grp-170* †
Glucose-regulated protein, 94 kDa	*grp-94* †
70 kDa heat shock protein, mitochondrial	*mthsp70/grp-75/mortalin* †
Cytoplasmatic and lysosomal proteases	
Calpain 1	*capn1* †
Calpastatin	*cast* †
Cathepsin B	*ctsb* †
Cathepsin D	*ctsd* †
Cathepsin L	*ctsl* †
Macrophages and monocytes chemokines	
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1* ‡
C-C chemokine receptor type 3	*ccr3* ‡
C-C chemokine CK8 / C-C motif chemokine	*ck8/ccl20* ‡
Immunoglobulins	
Immunoglobulin M	*igm* ‡
Immunoglobulin M membrane-bound form	*migm* ‡
Immunoglobulin T	*igt* ‡
Immunoglobulin T membrane-bound form	*igt-m* ‡

Interleukin-15	*il-15* ‡
Interleukin-34	*il-34* ‡
Tumor necrosis factor-alpha	*tnf-α* ‡

Antiprotease	
Alpha-2-macroglobulin	*a2m* ‡

Antimicrobial peptide/Iron recycling	
Hepcidin	*hepc* ‡

T-cell markers	
Cluster of differentiation 3 epsilon chain	*cd3e* ‡
Cluster of differentiation 3 zeta chain	*cd3x* ‡
CD4-full	*cd4-full* ‡
Cluster of differentiation 8 alpha	*cd8a* ‡
Cluster of differentiation 8 beta	*cd8b* ‡
Zeta-chain-associated protein kinase 70	*zap70* ‡

Pattern recognition receptors	
Toll-like receptor 1	*tlr1* ‡
Toll-like receptor 2	*tlr2* ‡
Toll-like receptor 5	*tlr5* ‡
Toll-like receptor 9	*tlr9* ‡
Macrophage mannose receptor 1	*mrc1* ‡

Table S2b. Forward (F) and reverse (R) primers used for real-time PCR in liver, head kidney and white muscle.

Gene Name	Symbol	Acc. No.		Primer sequences (5' → 3')
70 kDa heat shock protein, mitochondrial	*mthsp70/grp-75*	DQ524993	F	TCCGGTGTGGATCTGACCAAAGAC
			R	TGTTTAGGCCCAGAAGCATCCATG
Alpha-2-macroglobulin	*a2m*	AY358020	F	TCCTGGGTGACATTCTGGGT
			R	CCGTATGGCATCCTCAGCAG
ß-actin	*actb*	X89920	F	TCCTGCGGAATCCATGAGA
			R	GACGTCGCACTTCATGATGCT
C-C chemokineCK8 / C-C motif chemokine 20	*ck8/ccl20*	GU181393	F	CCGTCCTCATCTGCTTCATACT
			R	GCTCTGCCGTTGATGGAAC
C-C chemokine receptor type 3	*ccr3*	KF857317	F	CTACATCAGCATCACCATACGCATCCT
			R	TGGCACGGCACTTCTCCTTCA
Calpain 1	*capn1*	KF444899	F	CAGAACCACAACGCCGTGAAGTTT
			R	AGGCACTGGGCTTTAAGACTCTCG
Calpastatin	*cast*	KM522786	F	CCCAAACCCGAGCCCACCAT
			R	GACAAGAAGTCCAGAGCGTCTCCAGTA
Carnitine palmitoyltransferase 1A	*cpt1a*	JQ308822	F	GTGCCTTCGTTCGTTCCATGATC
			R	TGATGCTTATCTGCTGCCTGTTTG
Catalase	*cat*	JQ308823	F	TGGTCGAGAACTTGAAGGCTGTC
			R	AGGACGCAGAAATGGCAGAGG
Cathepsin B	*ctsb*	KJ524457	F	TGGTCGAGAACTTGAAGGCTGTC
			R	GGGTCTACTGCCATTCACAT
Cathepsin D	*ctsd*	AF03619	F	CACACTGGGAGACCTGCACTATGTCAATG
			R	ATTGCCAACTTGAAGTCCGTCCATACC
Cathepsin L	*ctsl*	KM522787	F	GGGAACGGATGACCAGCCTTGT
			R	CGGTGTCATTGGCAGAGTTGTAGTTG
CD4-full	*cd4-full*	AM489485	F	TCCTCCTCCTCGTCCTCGTT
			R	GGTGTCTCATCTTCCGCTGTCT
Citrate synthase	*cs*	JX975229	F	TCCAGGAGGTGACGAGCC
			R	GTGACCAGCAGCCAGAAGAG
Cluster of differentiation 3 epsilon chain	*cd3e*	MF175240	F	GGTGTGATGTTCGTCGTCTACAAGTG
			R	TGGCAGCGTGAGTGAGTCCT
Cluster of differentiation 3 zeta chain	*cd3x*	MF175235	F	ATGGCGGTCCAGACGAGGGTTTC
			R	ACCAGCGAGGACAGGACCAGCAG
Cluster of differentiation 8 alpha	*cd8a*	EU921630	F	GCAGCAACGGTAACACGAACG
			R	CCAGTATGAGCGGAGTACAGAACA
Cluster of differentiation 8 beta	*cd8b*	KX231275	F	CCGAAATGTGGAAGACTGGAACTC
			R	CCAGTATGAGCGGAGTACAGAACA
Cytochrome c oxidase subunit I	*coxi*	KC217652	F	GTCCTACTTCTTCTGTCCCTTCCTGTTCT
			R	AGGTTTCGGTCTGTAAGGAGCATTGTAATC

Cytochrome c oxidase subunit II	*coxii*	KC217653	F	ACTGCCTACACAGGACCTTGCC
			R	GTCTGCTTCCAGGAGACGGAATTGT
Fatty acid binding protein, heart	*h-fabp*	JQ308834	F	CTGGGTGTGGGCTTCGCTAC
			R	CTCTGTGTTCTTGATGGTGCTCTG
Follistatin	*fst*	AY544167	F	GGACCAGACAAACAACGCATATTG
			R	CATAGATGATCCCGTCGTTTCCAC
Glucose-regulated protein, 170 kDa	*grp-170*	JQ308821	F	CAGAGGAGGCAGACAGCAAGAC
			R	TTCTCAGACTCAGCATTTCCAGATTTC
Glucose-regulated protein, 94 kDa	*grp-94*	JQ308820	F	AAGGCACAGGCTTACCAGACAG
			R	CTTCAGCATCATCGCCGACTTTC
Glutathione peroxidase 1	*gpx1*	DQ524992	F	GAAGGTGGATGTGAATGGAAAAGATG
			R	CTGACGGGACTCCAAATGATGG
Glutathione peroxidase 4	*gpx4*	AM977818	F	TGCGTCTGATAGGGTCCACTGTC
			R	GTCTGCCAGTCCTCTGTCGG
Glutathione reductase	*gr*	AJ937873	F	TGCGTCTGATAGGGTCCACTGTC
			R	GTCTGCCAGTCCTCTGTCGG
Growth hormone receptor I	*ghr-i*	AF438176	F	ACCTGTCAGCCACCACATGA
			R	TCGTGCAGATCTGGGTCGTA
Growth hormone receptor II	*ghr-ii*	AY573601	F	GAGTGAACCCGGCCTGACAG
			R	GCGGTGGTATCTGATTCATGGT
Hepcidin	*hepc*	AM749960	F	ACTCCTGGAAGATGCCGTATGC
			R	AACTTACACCTCCTGCGTCCAC
Hypoxia inducible factor-1 alpha	*hif-1α*	JQ308830	F	CAGATGAGCCTCTAACTTGTGGAC
			R	TTAGCAAGAATGGTGGCAAGATGAG
Immunoglobulin M	*igm*	JQ811851	F	ACCTCAGCGTCCTTCAGTGTTTATGATGCC
			R	CAGCGTCGTCGTCAACAAGCCAAGC
Immunoglobulin M membrane-bound form	*migm*	KX599199	F	GCTATGGAGGCGGAGGAAGATAACA
			R	CAGCGTCGTCGTCAACAAGCCAAGC
Immunoglobulin T	*igt*	KX599200	F	GCTGTCAAGGTGGCCCCAAAAG
			R	CAACATTCATGCGAGTTACCCTTGGC
Immunoglobulin T membrane-bound form	*igt-m*	KX599201	F	AGACGATGCCAGTGAAGAGGATGAGT
			R	CGAAGGAGGAGGCTGTGGACCA
Insulin receptor	*insr*	KM522774	F	ACGGACAGCAAGAAGGCAGAGAATC
			R	CGAAGGAGGAGGCTGTGGACCA
Insulin-like growth factor binding protein 1a	*igfbp1a*	KM522771	F	ACGGACAGCAAGAAGGCAGAGAATC
			R	CCGTTCCAAGAGTTCACACACCAG
Insulin-like growth factor binding protein 2b	*igfbp2b*	AF377998	F	AGCGATGTGTCCTGAGATAGTGAG
			R	GCACCGTGGCGTGTAGACC
Insulin-like growth factor binding protein 3	*igfbp3**	MH577191 MH577192	F	ACA GTG CCG TCC ATC CAA
			R	GCT GCC CGT ATT TGT CCA
Insulin-like growth factor binding protein 4	*igfbp4*	KM658998	F	GGCATCAAACACCCGCACAC
			R	ATCCACGCACCAGCACTTCC
Insulin-like growth factor binding protein 5b	*igfbp5b*	MH577194	F	CGACAGGGCAGTCAAAGAAGCTAACC
			R	GTCTCGAAGGCATGTGAGCAGAAGG
Insulin-like growth factor binding protein 6b	*igfbp6b*	MH577196	F	GAT TGC TCA CTG CGG ATC
			R	GGA GGG ACA GAC CTT GAA
Insulin-like growth factor receptor I	*igfr1*	KM522775	F	TCAACGACAAGTACGACTACCGCTGCT
			R	CACACTTTCTGGCACTGGTTGGAGGTC
Insulin-like growth factor receptor II	*igfr2*	KM522776	F	ACATTCGGGCAGCACTCCTAAGAT
			R	CCAGTTCACCTCGTAGCGACAGTT
Insulin-like growth factor-I	*igf-i*	AY996779	F	TGTCTAGCGCTCTTTCCTTTCA
			R	AGAGGGTGTGGCTACAGGAGATAC
Insulin-like growth factor-II	*igf-ii*	AY996778	F	TGGGATCGTAGAGGAGTGTTGT
			R	CTGTAGAGAGGTGGCCGACA
Interleukin-1 beta	*il-1β*	AJ419178	F	GCGACCTACCTGCCACCTACACC
			R	TCGTCCACCGCCTCCAGATGC
Interleukin-6	*il-6*	EU244588	F	TCTTGAAGGTGGTGCTGGAAGTG
			R	AAGGACAATCTGCTGGAAGTGAGG
Interleukin-7	*il-7*	JX976618	F	CTATCTCTGTCCCTGTCCTGTGA
			R	TGCGGATGGTTGCCTTGTAAT
Interleukin-8	*il-8*	JX976619	F	CAGCAGAGTCTTCATCGTCACTATTG
			R	AGGCTCGCTTCACTGATGG
Interleukin-10	*il-10*	JX976621	F	AACATCCTGGGCTTCTATCTG
			R	GTGTCCTCCGTCTCATCTG
Interleukin 12 subunit beta	*il12*	JX976624	F	ATTCCCTGTGTGGTGGCTGCT
			R	GCTGGCATCCTGGCACTGAAT
Interleukin-15	*il-15*	JX976625	F	GAGACCAGCGAGCGAAAGGCATCC

			R	GCCAGAACAGGTTACAGGTTGACAGGAA
Interleukin-34	*il-34*	JX976629	F	TCTGTCTGCCTGCTGGTAG
			R	ATGCTGGCTGGTGTCTGG
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293	F	TTGCGTGTGGTGAGGAAGGAAGGT
			R	AGCAGGCAGGGCAGCAGGTA
Macrophage mannose receptor 1	*mrc1*	KF857326	F	CTTCCGACCGTACCTGTACCTACTCA
			R	CGATTCCAGCCTTCCGCACACTTA
Myoblast determination protein 1	*myod1*	AF478568	F	ATGGAGCTGTCGGATATCTCTTTC
			R	GAAGCAGGGGTCATCGTAGAAATC
Myocyte-specific enhancer factor 2A	*mef2a*	KM522777	F	ATGGACGAGAGGAACAGGCAGGTTA
			R	GGCTATCTCACAGTCACATAGTACGCTCAG
Myocyte-specific enhancer factor 2C	*mef2c*	KM522778	F	TAGCAACTCCCACTCTACCAGGACAAG
			R	GGAATACTCGGCACCATAAGAAGTCG
Myogenic factor 5	*myf5*	JN034420	F	GCATGGTTGACAGCAACAGTCCAGTGT
			R	TGTCTTATCGCCCAAAGTGTCGTTCTTCAT
Myogenic factor 6	*myf6/mrf4/herculin*	JN034421	F	GCAGCAATGACAAACCAGAGAGACGGAACA
			R	TGTCTTATCGCCCAAAGTGTCGTTCTTCAT
Myogenic factor MYOD2	*myod2*	AF478569	F	CCAACTGCTCTGATGGCATGATGGATTTC
			R	GACCGTTTGCTTCTCCTGGACTCGTATG
Myostatin/Growth differentiation factor 8	*mstn/gdf-8*	AF258448	F	AAGAGCAGATCATCTACGGCAAGATCC
			R	TCAAGAGCATCCACAACGGTCTACCA
NADH-ubiquinone oxidoreductase chain 2	*nd2*	KC217558	F	TAGGTTGAATGACCATCGTA
			R	GGCTAAGGAGTTGAGGTT
NADH-ubiquinone oxidoreductase chain 5	*nd5*	KC217559	F	CCTAAACGCCTGAGCCCTGG
			R	GCTGTAAACGAGGTGGCTAGAAGG
Peroxiredoxin 3	*prdx3*	GQ252681	F	CCTAAACGCCTGAGCCCTGG
			R	ACCGTTTGGATCAATGAGGAACAGACC
Peroxiredoxin 5	*prdx5*	GQ252683	F	GAGCACGGAACAGATGGCAAGG
			R	TCCACATTGATCTTCTTCACGACTCC
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α*	JX975264	F	CGTGGGACAGGTGTAACCAGGACTC
			R	TCCACATTGATCTTCTTCACGACTCC
Sirtuin 1	*sirt1*	KF018666	F	GGTTCCTACAGTTTCATCCAGCAGCACATC
			R	CCTCAGAATGGTCCTCGGATCGGTCTC
Sirtuin 2	*sirt2*	KF018667	F	GAACAATCCGACGACAGCAGTGAAG
			R	AGGTTACGCAGGAAGTCCATCTCT
Sirtuin 5	*sirt5*	KF018670	F	CAGACATCCTAACCCGAGCAGAG
			R	CCACGAGGCAGAGGTCACA
Superoxide dismutase [Mn]	*mn-sod/sod2*	JQ308833	F	CCTGACCTGACCTACGACTATGG
			R	AGTGCCTCCTGATATTTCTCCTCTG
Toll-like receptor 1	*tlr1*	KF857322	F	GGGACCTGCCAGTGTGTAAC
			R	AGTGCCTCCTGATATTTCTCCTCTG
Toll-like receptor 2	*tlr2*	KF857323	F	CATCTGCGACTCTCCTCTCTTCCT
			R	GCGTGGATAGAGTTGGACTTGAG
Toll-like receptor 5	*tlr5*	KF857324	F	TCGCCAATCTGACGGACCTGAG
			R	CAGAACGCCGATGTGGTTGTAAGAC
Toll-like receptor 9	*tlr9*	AY751797	F	GCCTTCCTTGTCTGCTCTTTCT
			R	GCCGTAGAGGTGCTTCAGTAG
Tumor necrosis factor-alpha	*tnf-α*	AJ413189	F	CAGGCGTCGTTCAGAGTCTC
			R	CTGTGGCTGAGAGCTGTGAG
Uncoupling protein 1	*ucp1*	FJ710211	F	GCACACTACCCAACATCACAAG
			R	CGCCGAACGCAGAAACAAAG
Uncoupling protein 3	*ucp3*	EU555336	F	AGGTGCGACTGGCTGACG
			R	TTCGGCATACAACCTCTCCAAAG
Zeta-chain-associated protein kinase 70	*zap70*	MF175239	F	TGGTGAAGGAGGAGATGATGAGG
			R	GCGAACGATGTAGCGGTTGT

(*) Acc. No. MH577191: *igfbp3a*; Acc. No. MH577192:*igfbp3b*. Primers used for *igfbp3* gene expression jointly amplify both *igfbp3a*and *igfbp3b*isoforms.

Appendix III

Immune Status and Hepatic Antioxidant Capacity of Gilthead Seabream *Sparus aurata* Juveniles Fed Yeast and Microalga Derived β-glucans

Table S1c. Relative gene expression profiling of anterior intestine in gilthead seabream juveniles fed experimental diets for 2 weeks. Data are the mean ± SEM (n=9). All data values for each tissue were in reference to the expression level of *cldn12* of CTRL fish with an arbitrary assigned value of 1.

	CTRL	MG	Phaeo21	Phaeo37	*P*-value[1]
pcna	10.22 ± 0.97^{ab}	9.59 ± 0.45^{ab}	12.99 ± 1.14^{a}	9.49 ± 0.56^{b}	**0.028**
hes1-b	2.37 ± 0.30	1.86 ± 0.17	2.17 ± 0.18	1.88 ± 0.13	0.287
klf4	3.48 ± 0.23	4.24 ± 0.59	2.91 ± 0.29	3.03 ± 0.14	0.201
cldn12	1.00 ± 0.04	0.98 ± 0.10	1.01 ± 0.06	0.96 ± 0.05	0.350
cldn15	17.96 ± 1.81	15.40 ± 1.75	18.87 ± 1.28	15.33 ± 1.22	0.215
cdh1	20.23 ± 0.96	19.45 ± 1.14	19.24 ± 1.13	18.33 ± 1.03	0.647
cdh17	90.58 ± 4.54	84.13 ± 4.45	89.38 ± 3.25	77.46 ± 2.93	0.084
tjp1	0.76 ± 0.03	0.74 ± 0.04	0.74 ± 0.03	0.76 ± 0.04	0.942
dsp	7.51 ± 0.42	7.12 ± 0.64	8.10 ± 0.41	6.35 ± 0.28	0.060
cx32.2	95.30 ± 9.78	80.92 ± 8.33	68.93 ± 10.83	68.20 ± 8.00	0.160
cxadr	5.02 ± 0.34	4.25 ± 0.46	4.66 ± 0.24	4.00 ± 0.16	0.108
alpi	67.89 ± 9.78	61.19 ± 10.40	55.41 ± 9.12	65.75 ± 10.49	0.777
fabp1	119.10 ± 7.40	112.74 ± 11.75	109.79 ± 5.30	101.19 ± 6.01	0.211
fabp2	835.42 ± 131.39	772.79 ± 119.22	891.79 ± 119.74	702.29 ± 71.58	0.780
fabp6	0.13 ± 0.06	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.863
muc2	36.90 ± 2.25	34.90 ± 1.36	37.06 ± 3.94	31.92 ± 4.55	0.456
muc13	117.68 ± 7.82	101.79 ± 8.13	114.59 ± 9.31	104.98 ± 3.14	0.423
tnf-alpha	0.21 ± 0.02	0.21 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.396
il1b	0.18 ± 0.02	0.19 ± 0.02	0.15 ± 0.01	0.18 ± 0.02	0.471
il6	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.227
il7	0.82 ± 0.07	0.93 ± 0.07	0.84 ± 0.06	0.88 ± 0.05	0.634
il8	0.22 ± 0.02	0.27 ± 0.03	0.26 ± 0.03	0.24 ± 0.03	0.440
il10	0.17 ± 0.02	0.14 ± 0.02	0.16 ± 0.02	0.13 ± 0.01	0.264
il12b	0.63 ± 0.05	0.59 ± 0.05	0.68 ± 0.08	0.53 ± 0.04	0.277
il15	0.59 ± 0.03	0.60 ± 0.05	0.69 ± 0.04	0.60 ± 0.03	0.295
il34	2.66 ± 0.12	2.62 ± 0.12	2.45 ± 0.11	2.71 ± 0.13	0.491
cd4-full	0.59 ± 0.11	0.54 ± 0.08	0.56 ± 0.05	0.52 ± 0.07	0.925
cd8b	0.06 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.06 ± 0.00	0.431
ccr3	1.68 ± 0.17	1.84 ± 0.25	1.39 ± 0.08	1.72 ± 0.14	0.292
ccr9	1.21 ± 0.10	1.24 ± 0.12	1.14 ± 0.06	1.17 ± 0.10	0.962
ccr11	4.65 ± 0.72	3.31 ± 0.41	4.34 ± 0.50	3.25 ± 0.25	0.125
ck8 / ccl20	6.57 ± 0.43	6.94 ± 0.67	8.15 ± 0.96	7.65 ± 0.37	0.414
csf1r1	1.06 ± 0.07	1.15 ± 0.08	1.03 ± 0.09	0.95 ± 0.06	0.308
Igm	3.77 ± 0.66	4.05 ± 0.74	3.67 ± 0.94	3.92 ± 1.24	0.928
igt-m	0.36 ± 0.04	0.37 ± 0.06	0.39 ± 0.07	0.29 ± 0.05	0.409
lgals1	13.55 ± 1.58	12.45 ± 1.51	13.27 ± 1.59	11.60 ± 1.20	0.762
lgals8	2.84 ± 0.16	2.50 ± 0.18	2.94 ± 0.26	2.66 ± 0.18	0.420
tlr2	0.54 ± 0.04	0.47 ± 0.03	0.48 ± 0.03	0.45 ± 0.03	0.349
tlr5	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.148
tlr9	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.881
cd209d	0.16 ± 0.02	0.15 ± 0.03	0.12 ± 0.02	0.13 ± 0.02	0.724
cd302	8.40 ± 0.32	7.91 ± 0.43	8.41 ± 0.58	7.53 ± 0.43	0.437
mrc1	1.60 ± 0.09	1.94 ± 0.27	1.64 ± 0.11	1.63 ± 0.10	0.738
fcl	7.32 ± 3.06	4.82 ± 1.59	4.41 ± 1.59	1.38 ± 0.97	0.107

[1]*P* values result from one-way ANOVA. Different superscript letters in each row indicate significant differences among dietary treatments (Tukey post-hoc test *P* <0.05).

Table S2c. Relative gene expression profiling of anterior intestine in gilthead seabream juveniles fed experimental diets for 8 weeks. Data are the mean ± SEM (n=9). All data values for each tissue were in reference to the expression level of *cldn12* of CTRL fish with an arbitrary assigned value of 1.

	CTRL	MG	Phaeo21	Phaeo37	*P*-value[1]
pcna	6.48 ± 0.58	7.08 ± 0.42	7.01 ± 0.59	6.99 ± 0.59	0.788
hes1-b	0.71 ± 0.17	0.63 ± 0.04	0.70 ± 0.05	1.02 ± 0.23	0.402
klf4	2.40 ± 0.23	2.17 ± 0.21	2.70 ± 0.35	2.08 ± 0.15	0.448
cldn12	0.92 ± 0.08	0.85 ± 0.07	0.93 ± 0.08	0.91 ± 0.06	0.868
cldn15	36.90 ± 3.93	39.04 ± 3.47	44.59 ± 3.52	42.56 ± 2.86	0.397
cdh1	21.37 ± 1.03	22.56 ± 0.83	20.94 ± 1.56	19.93 ± 1.14	0.471
cdh17	67.56 ± 2.31	69.25 ± 2.03	69.82 ± 4.91	66.67 ± 3.73	0.937
tjp1	0.50 ± 0.02	0.47 ± 0.03	0.50 ± 0.05	0.41 ± 0.03	0.202
dsp	4.31 ± 0.28	3.58 ± 0.27	4.38 ± 0.44	4.20 ± 0.34	0.308
cx32.2	123.59 ± 16.89[a]	66.78 ± 8.39[b]	68.64 ± 10.75[ab]	64.56 ± 6.85[b]	**0.034**
cxadr	5.02 ± 0.41	4.46 ± 0.46	5.16 ± 0.43	4.23 ± 0.39	0.296
alpi	79.98 ± 11.58	83.98 ± 12.39	68.88 ± 10.80	67.95 ± 7.74	0.798
fabp1	220.17 ± 24.84	180.66 ± 15.00	203.05 ± 16.45	164.57 ± 14.70	0.162
fabp2	1144.00 ± 114.86	931.28 ± 93.59	879.36 ± 61.09	786.87 ± 67.85	0.094
fabp6	0.03 ± 0.01	0.05 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	**0.049**
muc2	21.84 ± 2.71	17.65 ± 2.57	19.77 ± 4.03	18.13 ± 2.64	0.737
muc13	127.18 ± 5.91	126.41 ± 7.70	117.73 ± 8.32	114.57 ± 9.25	0.516
tnf-alpha	0.19 ± 0.03	0.19 ± 0.01	0.21 ± 0.02	0.17 ± 0.02	0.534
il1b	0.13 ± 0.02	0.13 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.628
il6	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.461
il7	2.58 ± 0.72	2.50 ± 0.99	1.94 ± 0.56	1.35 ± 0.41	0.558
il8	0.24 ± 0.05	0.17 ± 0.03	0.22 ± 0.04	0.17 ± 0.02	0.584
il10	0.10 [a] ± 0.01	0.06 [b] ± 0.01	0.09 [ab] ± 0.01	0.08 [ab] ± 0.01	**0.042**
il12b	0.59 ± 0.08	0.48 ± 0.04	0.56 ± 0.04	0.42 ± 0.04	0.107
il15	4.06 ± 1.26	2.57 ± 1.19	2.84 ± 0.81	1.33 ± 0.37	0.324
il34	3.65 ± 0.39	3.78 ± 0.59	3.82 ± 0.33	2.77 ± 0.30	0.186
cd4-full	0.34 ± 0.02	0.28 ± 0.02	0.35 ± 0.04	0.35 ± 0.04	0.406
cd8b	0.09 ± 0.01	0.10 ± 0.03	0.08 ± 0.01	0.08 ± 0.01	0.906
ccr3	2.52 ± 0.51	2.00 ± 0.71	1.87 ± 0.40	1.43 ± 0.25	0.325
ccr9	3.24 ± 0.35	2.79 ± 0.16	2.93 ± 0.42	2.45 ± 0.24	0.329
ccr11	6.23 ± 1.09	7.70 ± 1.49	8.40 ± 1.48	7.40 ± 0.88	0.695
ck8 / ccl20	7.74 ± 0.62	5.90 ± 0.60	6.89 ± 0.50	6.10 ± 0.50	0.068
csf1r1	1.12 ± 0.08	1.18 ± 0.09	1.28 ± 0.08	1.14 ± 0.08	0.594
igm	5.09 ± 0.54	4.21 ± 0.67	5.40 ± 1.03	5.73 ± 0.59	0.221
igt-m	0.41 ± 0.08	0.24 ± 0.04	0.35 ± 0.04	0.23 ± 0.02	**0.039**
lgals1	11.38 ± 1.25	8.56 ± 0.52	9.60 ± 0.97	8.71 ± 0.89	0.240
lgals8	7.34 ± 1.59	5.56 ± 0.82	5.12 ± 0.72	6.11 ± 1.01	0.698
tlr2	3.23 ± 1.08	3.75 ± 1.62	2.83 ± 0.83	2.10 ± 1.03	0.737
tlr5	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.844
tlr9	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.06 ± 0.01	**0.046**
cd209d	1.54 ± 0.55	1.38 ± 0.65	0.76 ± 0.27	0.37 ± 0.15	0.361
cd302	15.69 ± 2.22	13.96 ± 1.79	14.11 ± 1.54	11.63 ± 1.50	0.401
mrc1	3.25 ± 0.81	3.22 ± 1.26	2.54 ± 0.67	1.29 ± 0.27	0.290
fcl	8.61 [a] ± 3.22	13.40 [ab] ± 7.50	8.10 [a] ± 3.53	0.38 [b] ± 0.11	**0.019**

[1]*P* values result from one-way ANOVA. Different superscript letters in each row indicate significant differences among dietary treatments (Tukey post-hoc test *P* <0.05).

Table S3c. Primers for qPCR amplification in seabream.

Gene	Symbol	GenBank	Primer sequence
Proliferating cell nuclear antigen	*pcna*	KF857335	F: CGT ATC TGC CGT GAC CTG T R: AGA ACT TGA CTC CGT CCT TGG
Transcription factor HES-1-B	*hes1-b*	KF857344	F: GCC TGC CGA TAT GAT GGA A R: GGA GTT GTG TTC ATG CTT GC
Krueppel-like factor 4	*klf4*	KF857346	F: ACA TCA CCG CAC GCA CAC R: AAC CAC AGC CCT CCC AGT C
Claudin-12	*cldn12*	KF861992	F: CTC TCA GGG CTA CAC ATC TAC CTA TGC R: ACA TTC GTG AGC GGC TGG AG
Claudin-15	*cldn15*	KF861993	F: CCG ATT GTG GAA GTA GTG GCT CTG GT R: CAG CAT CAC CCA ACC GAC GAA CC
Cadherin-1	*cdh1*	KF861995	F: TGC TCC ATA CAG CGT CAC CTT ACA R: CTC GTT CAT CCT AGC CGT CCA GTT
Cadherin-17	*cdh17*	KF861996	F: GAT GCC CGC AAC CCA GAG R: CCG TTG ATT CAC TGC CGT AGA C
Tight junction protein ZO-1	*tjp1*	KF861994	F: AAG CAG TAT TAC GGT GAC TCA R: TGC ATC CCT GGC TTG TAG
Desmoplakin	*dsp*	KF861999	F: GCA GAA GGA GCA CGA GAC CATC R: GGG TGT TCT TGT CGC AGG TGA A
Gap junction Cx32.2 protein	*cx32.2*	KF862000	F: CGA GGT GTT CTA TCT GCT CTG TA R: CTT GTG GGT GCG AGT CCT
Coxsackievirus and adenovirus receptor homolog	*cxadr*	KF861998	F: CAT CAG AGG ACT ACG AGA GG R: CAT CTT GGC AGC ATT TGG T
Intestinal-type alkaline phosphatase	*alpi*	KF857309	F: CCG CTA TGA GTT GGA CCG TGA T R: GCT TTC TCC ACC ATC TCA GTA AGG G
Liver type fatty acid-binding protein	*fabp1*	KF857311	F: GTC CTC GTC AAC ACC TTC ACC AT R: CGC CTT CAT CTT CTC GCC AGT
Intestinal fatty acid-binding protein	*fabp2*	KF857310	F: CGA GCA CAT TCC GCA CCA AAG R: CCC ACG CAC CCG AGA CTT C
Ileal fatty acid-binding protein	*fabp6*	KF857312	F: ACC CAG GAC GGC AAT ACC

			R: CGA CGG TGA AGT TGT TGG T
Mucin 2	*muc2*	JQ277710	F: ACG CTT CAG CAA TCG CAC CAT R: CCA CAA CCA CAC TCC TCC ACA T
Mucin 13	*muc13*	JQ277713	F: TTC AAA CCC GTG TGG TCC AG R: GCA CAA GCA GAC ATA GTT CGG ATA T
Tumor necrosis factor-alpha	*tnf-alpha*	AJ413189	F: CAG GCG TCG TTC AGA GTC TC R: CTG TGG CTG AGA GCT GTG AG
Interleukin-1 beta	*Il1b*	AJ419178	F: GCG ACC TAC CTG CCA CCT ACA CC R: TCG TCC ACC GCC TCC AGA TGC
Interleukin-6	*il6*	EU244588	F: TCT TGA AGG TGG TGC TGG AAG TG R: AAG GAC AAT CTG CTG GAA GTG AGG
Interleukin 7	*il7*	JX976618	F: CTA TCT CTG TCC CTG TCC TGT GA R: TGC GGA TGG TTG CCT TGT AAT
Interleukin-8	*il8*	JX976619	F: CAG CAG AGT CTT CAT CGT CAC TAT TG R: AGG CTC GCT TCA CTG ATG G
Interleukin-10	*il10*	JX976621	F: AAC ATC CTG GGC TTC TAT CTG R: GTG TCC TCC GTC TCA TCT G
Interleukin 12 subunit beta	*il12b*	JX976624	F: ATT CCC TGT GTG GTG GCT GCT R: GCT GGC ATC CTG GCA CTG AAT
Interleukin 15	*il15*	JX976625	F: GAG ACC AGC GAG CGA AAG GCA TCC R: GCC AGA ACA GGT TAC AGG TTG ACA GGA A
Interleukin 34	*il34*	JX976629	F: TCT GTC TGC CTG CTG GTA G R: ATG CTG GCT GGT GTC TGG
CD4	*cd4*	AM489485	F: TCCTCCTCCTCGTCCTCGTT R: GGTGTCTCATCTTCCGCTGTCT
CD8 beta	*cd8b*	KX231275	F: CCGAAATGTGGAAGACTGGAACTC R: CTTTGGAGGTAAGGTTGGAGGGAT
C-C chemokine receptor type 3	*ccr3*	KF857317	F: CTA CAT CAG CAT CAC CAT ACG CAT CCT R: TGG CAC GGC ACT TCT CCTTCA
C-C chemokine receptor type 9	*ccr9*	KF857318	F: TCC CTG AGT TAA TCT TCG CCC AAG TG R: TGT TGT ATT CGT TGT TCC AGT AGA CCA GAG

C-C chemokine receptor type 11	*ccr11*	KF857319	F: GCT ACG ATT ACA GTT ATG AA R: TAG ATG ATT GGG AGG AAG
C-C chemokine CK8 / C-C motif chemokine 20	*ck8/ cl20*	GU181393	F: CCG TCC TCA TCT GCT TCA TAC T R: GCT CTG CCG TTG ATG GAA C
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293	F: TTG CGT GTG GTG AGG AAG GAA GGT R: AGC AGG CAG GGC AGC AGG TA
Immunoglobulin M	*igm*	JQ811851	F: ACC TCA GCG TCC TTC AGT GTT TAT GAT GCC R: CAG CGT CGT CGT CAA CAA GCC AAG C
Immunoglobulin T membrane-bound form	*igt-m*	KX599201	F: AGA CGA TGC CAG TGA AGA GGA TGA GT R: CGA AGG AGG AGG CTG TGG ACC A
Galectin-1	*lgals1*	KF862003	F: GTG TGA GGA GGT CCG TGA TG R: ACT GTA GAG CCG TCC GAT AGG
Galectin-8	*lgals8*	KF862004	F: GGC GGT GAA CGG CGG TCA R: GCT CCA GCT CCA GTC TGT GTT GAT AC
Toll like receptor 2	*tlr2*	KF857323	F: CAT CTG CGA CTC TCC TCT CTT CCT R: ATT CAA CAA TGG AGC GGT GGA CTT
Toll like receptor 5	*tlr5*	KF857324	F: TCG CCA ATC TGA CGG ACC TGA G R: CAG AAC GCC GAT GTG GTT GTA AGA C
Toll like receptor 9	*tlr9*	AY751797	F: GCC TTC CTT GTC TGC TCT TTC T R: GCC GTA GAG GTG CTT CAG TAG
CD209 antigen-like protein D	*cd209d*	KF857327	F: CGC CAC GAG CAT GAG GAC AA R: TCT TGC CAG AAT CCA TCA CCA TCC A
CD302 antigen	*cd302*	KF857328	F: GGA CCA GAG GAA GAG CAC ATC R: GAC CAG GGC GGA CAT CAG
Macrophage mannose receptor 1	*mrc1*	KF857326	F: CTT CCG ACC GTA CCT GTA CCT ACT CA R: CGA TTC CAG CCT TCC GCA CAC TTA
Fucolectin	*fcl*	KF857331	F: CCA TAC TGC TGA ACA GAC CAA CC R: TGA TGG AGG TGA CGA TGT AGG A

Appendix IV

Chlorella vulgaris extracts as modulators of gilthead seabream juveniles (*Sparus aurata*) health status and inflammatory response

Table S1d. Primers for qPCR amplification in seabream gut.

Gene	Symbol	Annealing T °C	Primer sequence
Mucin 2	*muc2*	60	F: ACGCTTCAGCAATCGCACCAT R: CCACAACCACACTCCTCCACAT
Mucin 13	*muc13*	60	F: TTCAAACCCGTGTGGTCCAG R: GCACAAGCAGACATAGTTCGGATAT
Interleukin-1 beta	*il1β*	60	F: TCTTCAAATTCCTGCCACCA R: CAATGCCACCTTGTGGTGAT
Interleukin-34	*Il34*	60	F: CATCAGGGTTCATCACAACG R: GACTCCCTCTGCATCCTTGA
Toll like receptor 1	*tlr1*	60	F: GGGACCTGCCAGTGTGTAAC R: GCGTGGATAGAGTTGGACTTGAG
CD8 alpha	*cd8α*	60	F: CTCGACTGGTCGGAGTTAA R: TCCATCAGCGGCTGCTCGT
Immunoglobulin M	*igm*	59	F: CAGCCTCGAGAAGTGGAAAC R: GAGGTTGACCAGGTTGGTGT
Hepcidin	*hepc*	60	F: GCCATCGTGCTCACCTTTAT R: CCTGCTGCCATACCCCATCTT
Heat-shock protein 70	*hsp70*	55	F: ACGGCATCTTTGAGGTGAAG R: TGGCTGATGTCCTTCTTGTG
Glutathione peroxidase	*gpx*	60	F: GAAGGTGGATGTGAATGGAAAAGATG R: CTGACGGGACTCCAAATGATGG
Manganese superoxide dismutase	*Sod(mn)*	60	F: CCTGACCTGACCTACGACTATGG R: AGTGCCTCCTGATATTTCTCCTCTG
Elongation factor 1α	*ef1α*	58	F: CTGTCAAGGAAATCCGTCGT R: TGACCTGAGCGTTGAAGTTG
Ribosomal protein S18	*rps18*	60	F: AGGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACC

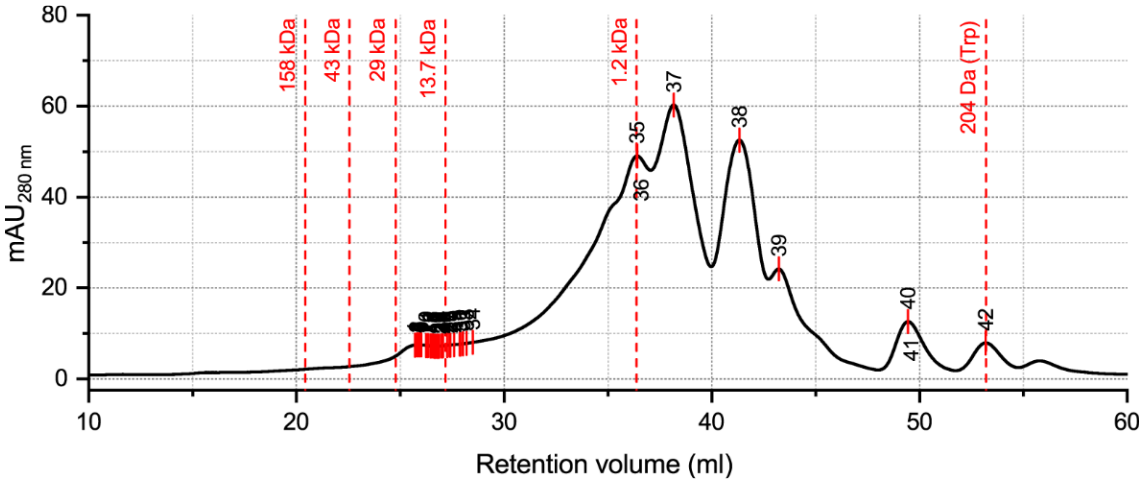

Figure S1. Protein/peptide profile of *C. vulgaris* peptide-enriched extract. Main molecular weight ranges, area of the main peak and the localization of all identified peaks as previously reported in Cunha, et al. [37].