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Functional strategies in gilthead seabream (*Sparus aurata*) juvenile nutrition: dietary microalgae supplementation for higher resilience

UAlg
UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2024

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Master's degree in Aquaculture and Fisheries

(Specialization in Aquaculture)

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Declaration of authorship of work

I declare I am the author of this work, which is original and unpublished. The sources consulted have been duly cited in the text and included in the list of references.

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Acknowledgements

This work was financially supported by “Pacto da Bioeconomia azul” (Project No. C644915664-00000026) within the WP5 Algae Vertical, funded by Next Generation EU European Fund and the Portuguese Recovery and Resilience Plan (PRR), under the scope of the incentive line “Agendas for Business Innovation” through the funding scheme C5 - Capitalization and Business Innovation.

I would like to express my gratitude to everyone who helped me accomplish my objectives and fulfilling another chapter of my academic path.

To my supervisors Sofia Engrola and Rita Teodósio for having presented me with the opportunity of collaborating in the current study, as well as the for the orientation, support provided and shared knowledge throughout the course of the Dissertation.

To Rita Colen, Ana Rebotim and Nuno Magalhães for all the assistance and knowledge provided in the experimental trials and analytical procedures. Thank you for the patience and for making me feel welcome in the group. I could not have done this otherwise. To Carolina Soares, Candela Pichel, Carlos Aleman, Alexandros Tsakogiannis, Raquel Ceriz, Ana Cavaco and Rodrigo Mendes: thank you for the entertaining moments when we needed a break from the screen to decompress and share thoughts.

To my friends Nídia Viegas, Dulce Estêvão, Sónia Monteiro and Francisco Videira for all the support during the past six years. To my high school friends David Carmona, Maxi Strobl, Dieter Vanacker and Malte Leip: thank you for your unwavering support and understanding since our days in secondary school. Despite the distance, you've been there for me throughout my academic journey.

Especially to my parents, to whom I owe everything, for their constant support, patience, and understanding over time. Thank you for giving me the necessary strength to carry on, no matter how difficult the path ahead seemed to be, and for conveying the timely messages of encouragement when I needed them most.

Thank you!

Abstract

Gilthead seabream (*Sparus aurata*), a species of high commercial value, often faces stressors inherent to aquaculture practices, which can negatively affect health, leading to diseases and economic losses. Given the pivotal role of nutrition in addressing such challenges, this study aimed to evaluate the effects of *Tetraselmis chui* extracts as functional dietary supplements to enhance fish resilience during acute stress events. A feeding trial was performed in juvenile gilthead seabream where three dietary treatments were tested in triplicate: CTRL – commercial-like diet; LOW – commercial-like diet supplemented with 0.06 % of *T. chui* extract; and HIGH – commercial-like diet supplemented with 0.12 % of *T. chui* extract. Fish were randomly distributed among 9 tanks (n = 20 fish per tank). After three weeks of feeding, growth performance indicators and oxidative status biomarkers were assessed. Fish were then exposed to various stressors and tissue samples were collected 6 h post-challenge to evaluate antioxidant responses. Oxidative status was assessed by measuring lipid peroxidation, total antioxidant status, catalase activity and total glutathione content in liver before and after the challenge. Results showed that fish fed the LOW diet significantly improved total antioxidant status after stress. Additionally, lipid peroxidation levels between pre- and post-stress events were significantly lower in the LOW and HIGH treatments compared to the control. While no significant differences were found in the remaining biomarkers, trends indicated beneficial effects in LOW and HIGH fish. This indicates that the beneficial effects are mostly observed in the LOW treatment. LOW fish exhibit less variation in LPO before and after the challenge, and significantly higher total antioxidant capacity 6 h after the stress events. The HIGH fish showed either no beneficial effects or only minimal effects. In conclusion, the 0.06 % microalgal supplementation increased stress resilience in gilthead seabream.

Keywords: *Sparus aurata*, functional feeds, redox-status, microalgae, aquaculture.

Sumário

A aquacultura tem revelado um desenvolvimento enorme para corresponder à crescente procura de produtos aquícolas saudáveis e nutritivos. Apesar dos avanços na sua produção, o bem-estar animal continua a ser um desafio, especialmente no que diz respeito à diminuição do stress induzido pelo cativeiro. Para superar este constrangimento, as dietas suplementadas com aditivos funcionais, como as microalgas, surgiram como uma estratégia para aumentar a resiliência dos peixes a fatores de stress e eventualmente melhorar o seu desempenho de crescimento.

O conceito de dietas funcionais surgiu do conceito de alimentos funcionais na nutrição humana, cujos componentes dietéticos trazem benefícios adicionais além da nutrição básica. Assim, esta abordagem promissora foca-se em incluir compostos bioativos nas dietas de peixes e crustáceos, que os ajude a enfrentar os desafios recorrentes na produção animal, como a mitigação do stress, em vez de se focar unicamente no desempenho do crescimento. É amplamente reconhecido que a nutrição adequada é fundamental para melhorar o desempenho dos peixes e a sua resistência às doenças.

Estudos recentes têm-se focado em métodos sustentáveis para melhorar as respostas antioxidantes nos peixes. As microalgas desempenham um papel fundamental nesta estratégia, dada a sua produtividade, composição rica em nutrientes (lípidos, proteínas e carboidratos) e em compostos bioativos (carotenóides, ácidos gordos poli-insaturados e vitaminas) com propriedades anti-inflamatórias, imunomoduladoras, antioxidantes e antibacterianas. Coletivamente, estes atributos tornam as microalgas candidatas ideais para corresponder às necessidades nutricionais dos animais.

A *Tetraselmis chui* é uma microalga marinha unicelular, com benefícios nutricionais consideráveis resultantes dos seus altos níveis de proteínas, lípidos e carboidratos, além de compostos bioativos como α-tocoferol (vitamina E), carotenóides (fucoxantina e β-caroteno), que apresentam propriedades antioxidantes. Dado o papel crucial da nutrição na resolução destes desafios, o presente estudo visa explorar o efeito de um extrato de *Tetraselmis chui* como suplemento funcional. Para isso, três tratamentos dietéticos foram testados para determinar se os juvenis de dourada alimentados com dietas suplementadas com microalgas seriam mais resilientes ao stress. O tecido escolhido para a análise do estado antioxidante dos peixes foi o fígado, conhecido pelo seu papel crucial na defesa antioxidante, servindo como o principal órgão desintoxicante.

O estudo consistiu num ensaio de alimentação seguido de um ensaio de desafio. O ensaio de alimentação foi realizado num sistema de aquacultura de recirculação (RAS) e cada tratamento (CTRL – dieta comercial; LOW – dieta controlo suplementada com 0.06 % de extrato de *Tetraselmis chui* e HIGH – dieta controlo suplementada com 0.12 % de extrato) foi testado em triplicado, durante um período de três semanas. Os juvenis de dourada (18,54 ± 2,48 g) foram distribuídos aleatoriamente em nove tanques, a uma densidade de 3,7 kg m^{-3} (n = 20 peixes por tanque). A temperatura da água foi mantida a 21,0 ± 0,2 °C; a salinidade a 33,7 ± 0,6 ppm; e o oxigénio dissolvido a 98,6 ± 1,3 % de saturação. O pH da água e os compostos azotados foram monitorizados diariamente e ajustados quando necessário.

No final do ensaio de alimentação, todos os peixes permaneceram 24 horas em jejum e três peixes de cada tanque foram eutanasiados. Estes peixes foram pesados e as amostras de fígado individuais foram congeladas de imediato em azoto líquido e armazenadas a -80 °C para posterior avaliação da resposta antioxidante. Os restantes peixes de cada tanque foram pesados para calcular os índices de desempenho de

crescimento e a taxa de conversão de alimento. Posteriormente, os restantes peixes foram expostos ao ensaio de desafio, que consistiu numa sequência de estímulos indutores de stress. Seis horas após o início do desafio as amostras de fígado foram recolhidas e congeladas imediatamente em azoto líquido e armazenadas a -80 °C, para posterior avaliação da resposta antioxidante. O estado oxidativo dos juvenis foi avaliado medindo a peroxidação lipídica (LPO), o estado antioxidante total (TAS), a atividade da catalase (CAT) e o conteúdo total de glutationa (tGSH) nas amostras de fígado individuais, antes e depois do ensaio de desafio. As diferenças estatísticas entre os tratamentos e a análise da variação delta dos indicadores entre pré e pós-stress foram calculadas pelo teste de análise de variância de uma via, usando o software IBM SPSS versão 29.

Os resultados mostraram que o desempenho de crescimento dos peixes não foi negativamente afetado pelas dietas suplementadas com microalga. A suplementação dietética com extrato de *Tetraselmis chui* não teve efeito significativo nos indicadores de desempenho de crescimento nem nas taxas de sobrevivência. No final do ensaio de alimentação, também não foram observadas diferenças significativas entre os tratamentos nos marcadores de stress oxidativo. Após o desafio, os peixes alimentados com a dieta LOW apresentaram níveis de TAS significativamente mais elevados em relação aos peixes alimentados com dietas CTRL ou HIGH, enquanto que os níveis de LPO, CAT e tGSH não apresentaram diferenças significativas entre tratamentos. No entanto, a variação dos níveis de LPO entre os eventos de pré e pós-stress foi significativamente menor nos peixes alimentados com as dietas LOW ou HIGH em comparação com os peixes CTRL. Em conjunto, estes resultados demonstram a existência de efeitos benéficos nos juvenis alimentados com a dieta LOW, protegendo os peixes contra o stress oxidativo associado ao desafio. Esta observação sugere que a suplementação com o extrato de microalga mitigou os efeitos do stress nos juvenis de dourada, dado o papel protetor das moléculas bioativas presentes na *Tetraselmis chui*.

Em conclusão, o presente estudo demonstrou que sob exposição a eventos de stress, os peixes alimentados com dietas suplementadas com 0.06 % extrato de *Tetraselmis chui* apresentaram variações de LPO menores, assim como um estado antioxidante total (TAS) superior aos peixes alimentados com a dieta CTRL. Assim, podemos concluir que estes peixes se tornaram mais resilientes aos danos oxidativos causados pelos eventos de stress.

Termos chave: *Sparus aurata*, rações funcionais, estado redox, microalgas, aquacultura.

Index

List of Figures

List of Tables

Abbreviations list

A: absorbance

ABTS: 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)

AECOSAN: Spanish Agency for Consumer Affairs, Food Safety and Nutrition

ANOVA: Analysis of Variance

BHT: butylated hydroxytoluene

BSA: Bovine Serum Albumin

CAT: catalase activity

cm: centimetre

CTRL: control diet (commercial-like diet)

DHA: Docosahexaenoic acid

DTNB: sulfhydryl reagent 5,5'-dithio-bis(2-nitrobenzoic acid)

EPA: Eicosapentaenoic acid

EPPO: experimental aquaculture research station

FAO: Food and Agriculture Organization of the United Nations

FCR: Feed Conversion Ratio

FEAP: Federation of European Aquaculture Producers

g: gram

g: relative centrifugal force

GBIF: Global Biodiversity Information Facility

GR: glutathione reductase

GSH: reduced glutathione

GSH-Px: glutathione peroxidase

GSSG: oxidized glutathione

h: hour

HIGH: control diet supplemented with 0.12 % *T. chui* extract

HSP: heat shock protein

Hz: Hertz

(H_2O_2): hydrogen peroxide

IBW: initial body weight

IU: international units

kg: kilogram

L: litre

l: pathlength of the light

(L•): lipid radical

(LOO•): lipid peroxyl radical

(LOOH): lipid hydroperoxides

LOW: control diet supplemented with 0.06 % *T. chui* extract

LPO: lipid peroxidation

M: molar

m^3: meter cubed

mg: milligram

mL: millilitre

mM: millimolar

mmol: millimole

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

nm: nanometres

n: number of observations

(O_2•−): superoxide anion

($_1O_2$): singlet oxygen

(OH•): hydroxyl radical

P: p-value

ppt: parts per thousand

PUFAs: polyunsaturated fatty acids

RAS: recirculating aquaculture system

RGR: relative growth rate

ROS: reactive oxygen species

S1: first sampling event

S2: second sampling event

SD: standard deviation

SOD: superoxide dismutase

TAS: total antioxidant status

TBA: Thiobarbituric acid

TBARS: Thiobarbituric acid-reactive substances

TCA: Trichloroacetic acid

tGSH: total glutathione content

TNB: 5'-thio-2-nitrobenzoic acid

UV: Ultra-violet

ε: extinction coefficient

µL: microliter

µM: micromolar

µmol: micromole

°C: degrees Celsius

1. Introduction

1.1. Current status of Aquaculture

Aquaculture is the most rapidly expanding sector in food production, fulfilling nearly 50 % of the world's demand for seafood. Recent data reveals that total global production of aquatic animals reached a record of 185.4 million tonnes in 2022 (FAO, 2024). Global aquaculture production surpassed capture fisheries for the first time, contributing 51 % to the total global production of aquatic animals, while capture fisheries accounted for 49 % (Figure 1.1) (FAO, 2024). The production of farmed aquatic animal species increased 6.7 million tonnes in 2022 compared to 2020. This growth was largely driven by Asia, which accounted for 87.9 % of the increase, significantly outpacing the other continents. This increase by species group was primarily due to finfish, which contributed 58.1 % of the increase, followed by crustaceans (24.6 %) and molluscs (15.6 %) (FAO, 2024).

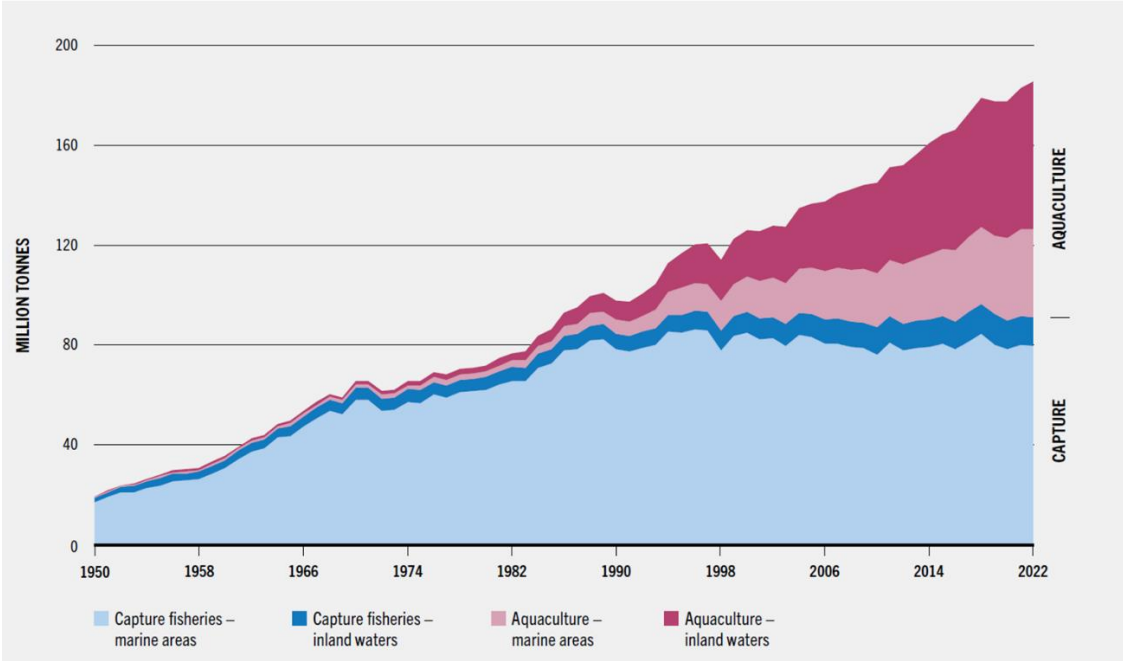

Figure 1. 1 – World capture fisheries and aquaculture production of aquatic animals from 1950 to 2022. *Source: FAO* **(FAO, 2024)**.

On the worldwide scale, meeting the demand for increased availability of aquatic animal foods will require a higher production, particularly for a human population projected to reach 9.7 billion by 2050 (FAO, 2024). In fact, by 2032 the aquaculture sector

is expected to account for 60 % of the aquatic animal foods for human consumption, as illustrated in Figure 1.2 (FAO, 2024).

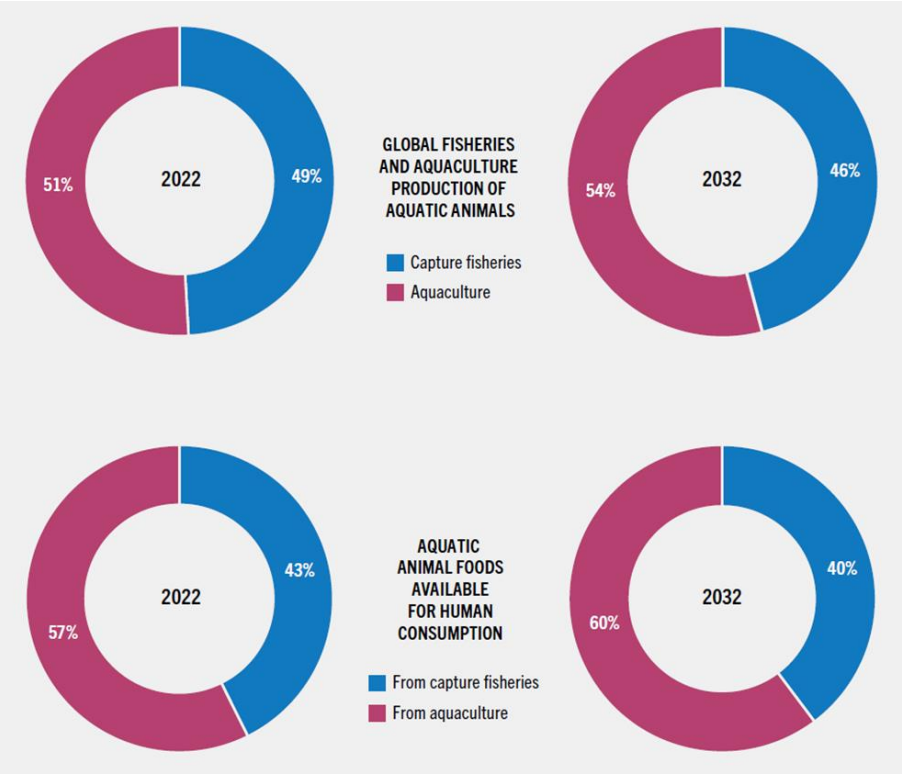

Figure 1. 2 – Increasing role of aquaculture. *Source: FAO* **(FAO, 2024)**.

The total European production of fish by aquaculture in 2022, reached approximately three million tonnes with Norway on the lead (FAO, 2024). Aquaculture production in Norway is primarily focused on Atlantic salmon (*Salmo salar*), which represents 58 % of Europe's total fish production. From this production, 27 % corresponds to the Mediterranean region (Croatia, Cyprus, France, Greece, Italy, Malta, Portugal, Spain and Turkey) (FEAP, 2023). Turkey and Greece rank as the second and fourth largest fish producers in Europe, mainly farming marine species such as gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) (FEAP, 2023).

1.2. Gilthead seabream

1.2.1. Species characteristics

The gilthead seabream (*Sparus aurata* Linnaeus, 1758) is a marine carnivorous teleost species belonging to the Sparidae family. It is characterized by an oval-shaped and laterally compressed body, with a predominant silvery-gray color. The common name for this species comes from the golden band located at the inter-orbital region of the head. A large black spot is observed at the origin of the lateral line which extends to the upper margin of the operculum (Figure 1.3). The dorsal fin is of a blue-gray color with a median black line and the caudal fin is edged in black at the fork and tips (Moretti et al., 1999; Pavlidis et al., 2011).

Figure 1. 3 – Gilthead seabream (*Sparus aurata* Linnaeus, 1758).

Gilthead seabream can be found in temperate, subtropical, and tropical coastal waters, as well as in brackish inshore waters. This species is commonly distributed along the Eastern Atlantic coast, from Great Britain to Senegal, in the Mediterranean Sea, to a lesser extent along the southeastern Mediterranean coast, and rarely seen in the Black Sea (Pavlidis et al., 2011).

As a demersal species, it frequently inhabits seagrass meadows with sandy or rocky substrates. Adjusting its feeding habits according to the available trophic resources, this species is an opportunistic feeder, with a diet consisting mainly of bivalves, small teleosts, gastropods, crustaceans and, occasionally, algae (Pavlidis et al., 2011; Pita et al., 2002; Wassef & Eisawy, 1985). They are protandrous hermaphrodites, becoming sexually mature males at 2 years of age and transitioning into females at 3 years of age, when they reach more than 30 cm in length (Chaoui et al., 2006; Mehanna, 2007).

1.2.2. Gilthead seabream production

In the Mediterranean region, the major contributors of seafood production include Egypt, France, Greece, Italy, Spain and Turkey (FAO, 2022). The primary cultivated marine fish species in this area are gilthead seabream and European seabass (Moretti et al., 2005). Gilthead seabream was traditionally farmed in coastal lagoons and saltwater ponds of the Mediterranean. In the early 1980s, large-scale fry production became feasible, and by the 1990s, there was significant enhancement in the intensive production of commercial-sized fish in cages or ponds. In recent years, gilthead seabream has experienced a steady increase in production (Colloca & Cesari, 2023; Moretti et al., 1999, 2005).

This marine fish species is one of the most produced in Southern Europe. In 2022, Greece and Turkey accounted for 84 % of the total Southern European production of gilthead seabream with Greece producing approximately 30 % (72 700 tonnes) and Turkey producing approximately 54.6 % (134 000 tonnes), respectively (FEAP, 2023). While gilthead seabream is primarily cultivated in cages, both inshore and offshore, particularly in the Mediterranean, various other production systems are used. These include onshore extensive earth ponds and lagoons, as well as intensive land-based concrete tanks equipped with a flow-through water supply and recirculating aquaculture systems (RAS) (Mozes et al., 2011).

The aquaculture sector, in its efforts to meet the increasing demand for seafood, must prioritize animal management to mitigate stress, increase robustness and decrease conditions for disease susceptibility. The welfare and overall production yield of fish in aquaculture are highly affected by physiological stress, posing a major concern (Monteiro et al., 2021).

1.3. Redox status

Fish, like all aerobic organisms, rely on oxygen intake for fulfilling various metabolic functions, yet are also naturally susceptible to the risks associated to oxidative stress (Davies, 2000). The continuous formation of reactive oxygen species (ROS) is inherent to the metabolism among aerobic organism (Davies, 2000; Halliwell & Gutteridge, 2015). One of the main endogenous sources of reactive oxygen species (ROS) is the mitochondrial electron-transport chain. This transport-chain supplies energy to the

cell, in the form of ATP through oxidative phosphorylation. In this process, molecular oxygen (O_2) serves as the terminal electron acceptor and eventually reduced to water (Nelson & Cox, 2005). However, electron leaks occurring during this process react directly with molecular oxygen, forming ROS which are highly unstable/radical molecules. The partial reduction of oxygen forms, either by energy transfer or by electron transfer reactions, lead to the production of highly unstable by-products (Figure 1.4) such as superoxide anion (O_2•−), singlet oxygen ($_1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•) (Halliwell & Gutteridge, 2015; Kurutas, 2015).

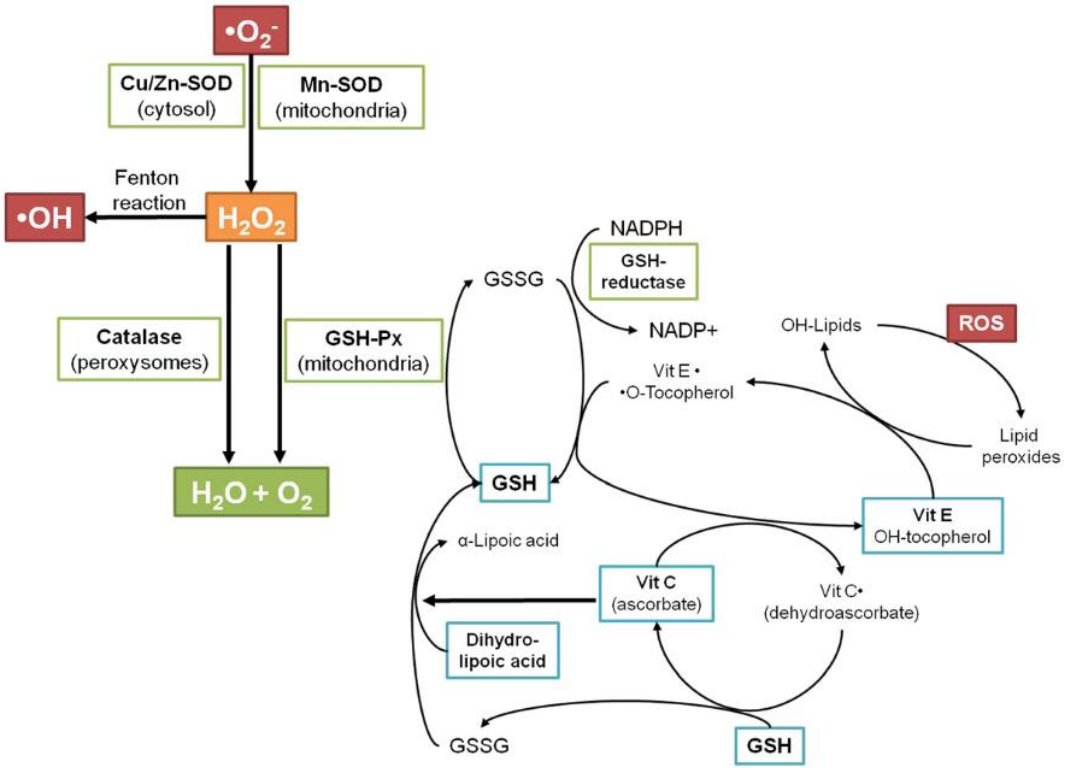

Figure 1. 4 – Antioxidant defenses in the organism. *Source: Kurutas (2015).*

During oxygen metabolism, the resulting ROS can destabilize the redox homeostasis, that is the equilibrium between the production and removal of ROS (Halliwell & Gutteridge, 2015). Various cellular functions, which rely on this balance, will become compromised due to a cumulative oxidative damage of macromolecules of great biological importance such as DNA, proteins and lipids (Halliwell & Gutteridge, 2015). Consequently, this destabilizes cellular membrane structure and function, leading to an overall state of oxidative stress and impairment of the organism's health (Halliwell & Gutteridge, 2015).

The main cause of this cellular damage is lipid peroxidation (LPO), which consists in an autocatalytic process occurring in three main steps: initiation, propagation and termination. Initiation involves the interaction between a prooxidant like OH• with a fatty acid to extract a hydrogen atom, forming an unstable lipid radical (L•). During propagation, L• quickly reacts with oxygen thus forming a lipid peroxyl radical (LOO•), which in turn extracts a hydrogen atom from another lipid molecule, generating a new L• and forming a chain reaction (Ayala et al., 2014). This chain ends when LOO• reacts with other peroxyl radicals or extracts a hydrogen atom from antioxidants, forming non-radical products such as lipid hydroperoxides (LOOH). These lipid hydroperoxides, upon undergoing reductive degradation, are converted to secondary products that may either diminish or enhance cytotoxic potential (Mourente et al., 2007; Sargent et al., 2003). Malonyldialdehyde (MDA), one of the final products of LPO is commonly used as a marker for oxidative stress and is typically assayed with thiobarbituric acid (Bird & Draper, 1984; Halliwell & Chirico, 1993). The damage resulting from LPO is linked to reduced membrane fluidity, altered membrane permeability, and the inactivation of membrane-bound enzymes (Sargent et al., 2003).

In a physiological context, the purpose of antioxidant compounds is to keep reactive ROS concentrations within a cell, at a level low enough to prevent potential damage (Seifried et al., 2007). As a countermeasure to the effects of ROS, aerobic organisms developed both enzymatic and nonenzymatic antioxidant defense systems (Figure 1.4). The enzymatic defense comprises superoxide dismutase, catalase, and glutathione peroxidase (Seifried et al., 2007). The different forms of superoxide dismutase, cytosolic copper/zinc-dependent and mitochondrial manganese-dependent forms, catalyze the dismutation of superoxide radicals into hydrogen peroxide which are consequently broken down by peroxisomal and mitochondrial catalase into molecular oxygen and water (Figure 1.4). Additionally, hydrogen peroxide may be degraded by cytosolic and mitochondrial GSH-Px using glutathione (GSH) as a co-substrate (Halliwell & Gutteridge, 2015).

Glutathione, synthesized in the cytosol, exists in both reduced (GSH) and oxidized (glutathione disulfide; GSSG) forms. It can be transported across cell membranes, as part of a complex interorgan transport network. The NADPH-dependent glutathione reductase (GR) converts GSSG to GSH, while GSH-Px catalyzes the reverse reaction (Wu et al.,

2004). GSH plays a crucial role in various cellular processes, effectively scavenging free radicals and other reactive oxygen species, participating in enzymatic reactions, and converting vitamin E and C into their active forms (Figure 1.4) (Wu et al., 2004). The main role of this group of enzymes consists in metabolizing a wide range of xenobiotics and endogenous compounds. These enzymes are involved in both the transport and neutralization of bilirubin, the end product of heme catabolism, and plays a key role in detoxifying lipid peroxides (Halliwell & Gutteridge, 2015).

Alternatively, the antioxidant defense can be nonenzymatic, in which case glutathione, thiols, vitamin C, vitamin E, phytochemicals such as isoflavones, polyphenols, and flavonoids are involved (Kurutas, 2015; Seifried et al., 2007). The critical role of antioxidant compounds in cellular responses to oxidative stress underscores the importance of exploring functional feed additives with antioxidant properties. Incorporating bioactive compounds into fish feeds has demonstrated positive effects on stress resistance, growth, and survival in various studies (Hossain et al., 2023).

1.4. Enhancing fish resilience through functional nutrition

The overall stress experienced by fish is from cumulative and interactive consequences of multiple stressors such as changes of physical and chemical factors in the aquatic environment that may create favourable conditions for higher disease susceptibility (Schreck & Tort, 2016; Varsamos et al., 2006). These may include water temperature, pH, dissolved oxygen, ammonia and nitrite, as well as, common harvesting practices like crowding, size sorting, and transportation.

When exposed to stressful events, fish often do not eat (Conde-Sieira et al., 2018). As such, feeding fish beforehand with supplemented diets containing functional additives, with antioxidant properties, can help improve resilience. Among these additives are vitamins, natural pigments, and amino acids, which have demonstrated positive effects including stress resistance, enhanced growth and survival (Hossain et al., 2023). Several studies, aiming at mitigation of oxidative stress, were carried out with different aquaculture species. Pacific white leg shrimp (*Litopenaeus vannamei*), fed supplemented diets with vitamins C and E presented a better redox status and higher survival rate (Ebadi et al., 2021). A higher growth and survival rates were observed in Nile tilapia

(*Oreochromis niloticus*) juveniles, after dietary supplementation with β-carotene and phycocyanin (Hassaan et al., 2021). Gilthead seabream fed diets supplemented with glutamine presented an increased catalase activity, together with reduced glutathione and total glutathione contents (Coutinho et al., 2016). Additionally, yellowfin seabream (*Acanthopagrus latus*) fed diets supplemented with taurine showed increased growth rates resulting from increased activities in both catalase and superoxide dismutase, together with increased GSH content and decreased lipid peroxidation (Dehghani et al., 2020). These findings suggest that supplementing feeds with antioxidant-rich sources yields promising outcomes for the production sector.

Natural products present potential for promoting development, enhancing antioxidant and immune responses, hence increasing robustness, while decreasing disease susceptibility in aquatic animals (Dawood et al., 2018; De Jesus Raposo et al., 2015; Kim, 2015b). The marine environment is a rich source of natural products such as polysaccharides, oligosaccharides, peptides, vitamins, minerals, fatty acids, sterols, carotenoids, and phenolic compounds (Romano et al., 2017). Recent research has particularly focused on marine-derived compounds extracted from microalgae, macroalgae, shellfish, fungus, sponges and corals. These compounds present a broad spectrum of biological activity (Barzkar et al., 2019; Kim, 2015a).

1.5. Microalgae

Microalgae encompass a diverse community of microscopic organisms, ranging from unicellular to multicellular, prokaryotic or eukaryotic organisms. Most of these organisms rely on photosynthesis to produce biomass and oxygen through the use of sunlight as the energy source and carbon dioxide as the source of inorganic carbon (Borowitzka, 2018; Chapman, 2013). As generalists, microalgae can grow in various types of terrestrial, marine and freshwater ecosystems (Little et al., 2021). Microalgae are well known for their high ecological value, high nutritional content and several bioactive compounds of pharmaceutical interest. However, they remain among the least exploited organisms, with only an estimated 5 % to 10 % of species having undergone analysis for their chemical content (Guedes et al., 2011).

Under standard growth conditions, microalgae are characterized by elevated protein content, ranging from 25 to 50 % of dry weight, 10 % to 30 % lipids and 5 to 40 % carbohydrates (Ventura et al., 2017). They are also a source of essential vitamins (A,

B_1, B_2, B_6, B_{12}, C, E, nicotinate, biotin, folic acid and pantothenic acid) and natural pigments (Spolaore et al., 2006). Consequently, microalgae serve as a rich source of various important biochemicals, including antioxidants in the form of pigments such as β-carotene, astaxanthin, and lutein; natural dyes; polyunsaturated fatty acids (PUFAs) from both the ω3 and ω6 families; peptides; and minerals (phosphorus, zinc, iron, calcium, selenium, magnesium). Even though these compounds have valuable applications across various industrial sectors, the extent of their production depends on the species, strain, and the conditions under which algae are cultivated (Moreno-Garcia et al., 2017; Spolaore et al., 2006; Vaz et al., 2016). In several studies, microalgae have been shown to be a viable source for the production of a wide range of high-value products in sectors such as cosmetics, feeds and human nutrition, pharmaceuticals and pollution-prevention (Koyande et al., 2019; Sathasivam et al., 2019).

1.5.1. Microalgae for Functional Feeds

Microalgae, among various marine sources of functional compounds, are emerging as a viable alternative for fish feed supplementation. They serve as antioxidant sources to mitigate stress and its adverse effects on the health of fish species (Ma & Hu, 2023). The supplementation of dietary microalgae in feeds for the aquaculture sector, has proven to be advantageous as is the case of Nile tilapia (Abdel-Tawwab et al., 2022), rainbow trout (*Oncorhynchus mykiss*) (Chen et al., 2021) and gilthead seabream (Jorge et al., 2019; Reyes-Becerril et al., 2013). Various microalgae species were tested as feed supplements, however less than twenty have been widely applied in aquaculture (Bahi et al., 2023). The most common include the genera: *Chlorella*, *Isochrysis*, *Nannochloropsis*, *Pavlova*, *Phaeodactylum*, *Tetraselmis*, *Scenedesmus*, *Skeletonema* and *Schizochytrium* sp. The impacts on fish include an enhanced antioxidant capacity, immune response, stress and disease resistance, antibacterial properties and stimulation of gut function (Bahi et al., 2023).

Meagre (*Argyrosomus regius*) fed a diet with *Nannochloropsis gaditana* extract supplemented at 1 % showed a decrease in oxidative stress and inflammatory responses after an acute handling stress event (Monteiro et al., 2021). Nile tilapia fed with 1 % supplemented whole *Arthrospira platensis* diet showed higher reduced glutathione and superoxide dismutase, and lower lipid peroxidation after an infection challenge (Mahmoud et al., 2018). Rainbow trout, fed a diet supplemented with 5 % whole

Chlorella sorokiniana had the highest glutathione peroxidase activity after an infection challenge. Moreover, dietary supplementation with either 5 % or 10 % *C. sorokiniana*, decreased lipid peroxidation (Chen et al., 2022)

Nonetheless, it is worth mentioning that microalgal supplementation in aquafeeds can bring limitations at inclusion levels above 10 % (Ahmad et al., 2020), particularly for what concerns the diet digestibility (Niccolai et al., 2019; Paterson et al., 2023). Recent studies with Atlantic salmon (Tibbetts et al., 2017) and Nile tilapia (Teuling et al., 2019), have shown that the digestibility of microalgae, increases upon cell wall rupture. A study involving Nile tilapia, assessed the potential of diets supplemented with *Chlorella pyrenoidosa* extract to reduce the effects of a 24-h stress test. The group fed with 0.27 % supplementation showed the highest activity of glutathione peroxidase (Peng et al., 2020).

1.5.2. Microalgal species: *Tetraselmis chui*

Tetraselmis chui Butcher (1959), is one of the main species of marine microalgae employed in aquaculture, mainly due to its nutritional value, fast growth rate and capability of withstanding broad ranges of pH, salinity, and temperature (Guedes & Malcata, 2012; Khatoon et al., 2021; Rahman et al., 2017; Úbeda-Mínguez et al., 2015). *T. chui* is a single-celled flagellated chlorophyte, of the Prasinophyceae class and Chlorodendraceae family (GBIF, 2023). Initially found off the coast of Great Britain in 1959, it has since been identified in various locations worldwide (Mantecón et al., 2019; Rahman et al., 2017). The dimensions of *T. chui* can range from 10 – 25 μm in length, 7 – 20 μm in width, and 6.5 – 18 μm in thickness, featuring a slightly flattened oval cell shape, with four flagella emerging near the apex for motility. (Khatoon et al., 2021; Rafay et al., 2020).

In 2014, *T. chui* was approved in the European Union as a novel food ingredient by the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) (Hurtado et al., 2017). This novel food meets the acceptance criteria outlined in Regulation (EC) Nº 258/1997 on novel foods and novel food ingredients (Hurtado et al., 2017; Torzillo et al., 2021). As a dietary source, *T. chui* shows great potential, containing high levels of protein (35 – 40 %), carbohydrates (30 – 35 %) and lipids (5 – 10 %) (Mantecón et al., 2019). Furthermore, this species is also rich in long-chain polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Mantecón et al., 2019). *T. chui* is also a source of bioactive

compounds, which include α-tocopherol (vitamin E) and carotenoids (fucoxanthin and β-carotene) with antioxidant properties essential for providing cellular protection against oxidative damage (Bonilla-Ahumada et al., 2018; Khatoon et al., 2021).

Several studies reported beneficial antioxidant effects when *Tetraselmis* genera were supplemented in aquafeeds. White-leg shrimp fed a diet supplemented with 50% *T. chui* presented a higher resistance after a salinity stress (Rahman et al., 2017). Nile tilapia fed a diet containing 1.5 % of *T. suecica* presented higher catalase, glutathione peroxidase and superoxide dismutase activities and survival after an infection challenge (Abdel-Tawwab et al., 2023).

2. Main objectives

This study aimed to assess the effect of microalgae (*Tetraselmis chui*) extracts as functional dietary supplements to enhance the resilience of gilthead seabream juveniles under stressful events.

3. Material and Methods

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 under project "PRR: Pacto Bioeconomia Azul – Vertical Algae – Subproject FEEDS (SP 5)". CCMAR facilities and their staff are certified to house and conduct experiments with live animals (licensed by the 'Direção Geral de Alimentação e Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

3.1. Rearing conditions

Gilthead seabream juveniles, with an average weight of 18.54 ± 2.48 g, were obtained from the experimental aquaculture research station (EPPO) (Olhão, Portugal) and transferred to the Centre of Marine Sciences of Algarve (CCMAR) facilities (University of Algarve, Faro, Portugal). Upon arrival, the fish were distributed among nine 100 L cylindrical tanks, at an initial density of 3.7 kg m^{-3} (n = 20 fish per tank). The tanks were in a recirculating aquaculture system (RAS) equipped with a mechanical filter, a trickling biological filter, an UV sterilizer and a protein skimmer coupled to an ozone sterilizer, under natural photoperiod (September). The seawater inlet was kept at 174 L h^{-1}. Water parameters (mean ± SD) were maintained as follow: temperature of 21.0 ± 0.2 °C; salinity of 33.7 ± 0.6 ppt; dissolved oxygen in water of 98.6 ± 1.3 % saturation. Water pH was monitored daily and adjusted when necessary to 8.0, and nitrogenous compounds (ammonium and nitrite) were kept below toxic limits. Fish mortality was monitored and recorded daily.

3.2. Experimental design

The experimental design consisted in two stages: Feeding, followed by Challenge, as represented in Figure 3.1.

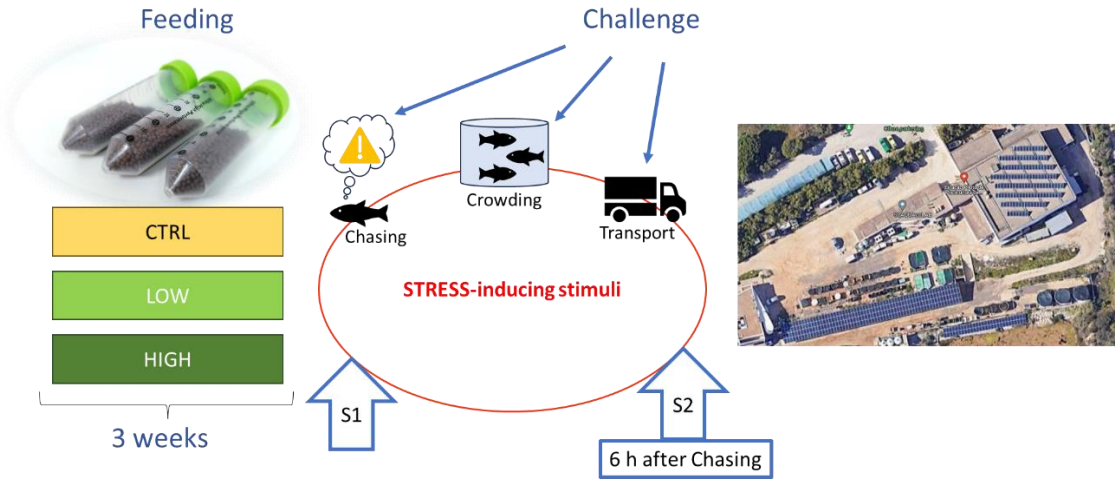

Figure 3. 1 – The experimental design diagram illustrating two stages of the trial: Feeding and Challenge. S1 and S2 represent the two sampling events.

3.2.1. Stage 1: Feeding trial

The trial was conducted for a three-week period at the CCMAR experimental facilities. Triplicate tanks were randomly assigned to one of the dietary treatments (Figure 3.1): CTRL – commercial-like diet; LOW – commercial-like diet supplemented with 0.06 % of *T. chui* extract; and HIGH – commercial-like diet supplemented with 0.12 % of *T. chui* extract. Fish were fed with the corresponding experimental diet (three meals per day: 09h30, 12h00, 16h30) with 2 % of fish biomass, which was adjusted based on remaining feed and fish feeding behaviour.

3.2.2. Stage 2: Challenge trial

After three weeks of feeding the experimental diets, at the end of the nutritional trial, fish were subjected to various stress-inducing stimuli, comprising "Chasing", "Crowding" and "Transport" events (Figure 3.1). "Chasing" consisted in dipping a fish net into each tank and chasing all schools of fish for 20 sec; "Crowding" was performed by transferring the fish to a confined space (transport bag: plastic bag filled with oxygen saturated seawater), increasing density approximately 13 times; and "Transport", which consisted of transporting the fish to the EPPO facilities (Figure 3.1).

3.3. Sampling

At the end of the Feeding trial (sampling point S1), all fish were fasted for a 24h-period, after which, three fish per tank (n = 9 fish per treatment) were euthanised with an overdose of anaesthetic (1 mL L^{-1} of 2-phenoxyethanol, Sigma-Aldrich, USA), followed by severing of spinal cord. Fish were individually weighed, and liver tissue was sampled. Liver samples were snap-frozen in liquid nitrogen and stored at -80 °C for subsequent physiological analysis of liver antioxidant status. After, fish from each tank were bulk weighed to calculate the growth performance indices and feed conversion ratio. Six hours after the beginning of the stress event, at the start of the Challenge trial (sampling point S2), fish liver samples were collected, snap-frozen in liquid nitrogen and stored at -80 °C for subsequent evaluation of the fish antioxidant physiological response (Figure 3.1).

3.4. Key performance indicators

The fish growth performance and feed utilisation were calculated as follows:

Weight gain (% IBW) = $\frac{Weight\ gain\ (\mathrm{g})}{initial\ Biomass\ (\mathrm{g})} \times 100$,

where Weight gain (g) = $(final\ Biomass - initial\ Biomass) + Dead\ fish\ weight$

Relative growth rate (RGR, % day^{-1}) = $(e^{g} - 1) \times 100$,

in which $g = \frac{ln_{final\ body\ weight} - ln_{initial\ body\ weight}}{days}$

Feed conversion ratio (FCR) = $\frac{apparent\ feed\ intake\ (\mathrm{g})}{wet\ weight\ gain\ (\mathrm{g})}$

3.5. Oxidative status

The antioxidant status of the fish was assessed by measuring lipid peroxidation (LPO), total antioxidant status (TAS), catalase activity (CAT) and total glutathione content (tGSH) in individual liver samples (n = 3 per tank, n = 9 per treatment). All determinations were performed spectrophotometrically, in 96-well flat-bottom microplates, with a temperature-controlled microplate reader (Synergy 4 Biotek, VT, USA) with the software (Gen5™ version 1.08, USA).

3.5.1 Sample homogenization

The liver samples were homogenized with potassium phosphate buffer (0.1 M, pH 7.4) using a ball mill (MM 500 Control, Retsch, Germany). Each weighed sample was placed in a safe-lock microtube with one ceramic sphere. After the samples were subjected to tissue lysis with two cycles of 24 Hz, for 30 sec. From the resulting homogenate, an aliquot was taken and preserved with 4 % butylated hydroxytoluene (BHT, Sigma-Aldrich) in methanol, for the determination of endogenous LPO. The remaining homogenate was centrifuged (Centrifuge 5804 R, Eppendorf, Germany) for 20 min, at 10 000 x *g*, 4 ºC and the post-mitochondrial supernatant was used to determine protein quantification, TAS, CAT and tGSH in each liver sample. After processing, all samples were kept at -80 ºC until analysis.

3.5.2 Protein quantification

The protein quantification of the samples was determined according to the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as standard. Protein standards were prepared by diluting a stock solution of BSA at 1 mg/mL in 0.1 M potassium phosphate buffer at pH 7.4. Samples were diluted to ensure absorbance values within the linear range of the standard curve (0.1 to 0.8 mg/mL of BSA). Each standard and sample were pipetted in triplicate into the microplate, and the Bradford reagent (Sigma-Aldrich) was added following the manufacturer's instructions. The samples were incubated in the dark for 10 min. Absorbance readings were obtained spectrophotometrically at 595 nm. The obtained results were used to create a standard curve by plotting the net absorbance at 595 nm against the protein standard concentrations to determine the protein concentration in the samples.

3.5.3 Lipid peroxidation

The lipid peroxidation was determined spectrophotometrically by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm as described by Bird & Draper, (1984). Briefly, samples were initially deproteinized by adding a solution of 100% Trichloroacetic acid (TCA) while on ice, followed by vortexing. A solution of 0.73% Thiobarbituric acid (TBA) was added, and the samples were mixed using a vortex. Subsequently, the samples were incubated at 100 ºC for 1 h and allowed to cool at room

temperature in the dark. The samples were then centrifuged for 5 min at 12 000 x *g* and the absorbance of a 200 µL aliquot was read in the spectrophotometer. The concentration of MDA was calculated by the Beer-Lambert law (A = ε x c x l) by using the extinction coefficient value (ε) of MDA (156 000 M^{-1} cm^{-1}); the pathlength of the light (l) for 200 µL (0.6 cm); and the absorbance value (A) of each sample. The results were expressed in nmol TBARS per mg of protein.

3.5.4 Catalase activity

The catalase activity was determined by measuring the decomposition of the substrate hydrogen peroxide H_2O_2 (ε = 40 M^{-1} cm^{-1}) at 240 nm for 2 min at 30 sec interval, according to Claiborne (1985) and adapted to microplates. The samples were diluted for a target protein value of 0.7 mg/mL with potassium phosphate buffer solution (0.1 M, pH 7.4) and pipetted in triplicate into 96-well flat-bottom UV light microplates. The results were expressed in IU per mg of protein (1 international unit is defined as the amount of catalase required to decompose 1 µM of H_2O_2 per min).

3.5.5 Total antioxidant status

Total antioxidant status was assessed using coloured 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^+$). The colour change resulting from the reduction of $ABTS^+$ to its colourless original molecule (ABTS) was measured as a change in absorbance at 660 nm. The reaction rate was calibrated with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solution, an analog of vitamin E which is a potent antioxidant (Erel, 2004). Trolox standards were prepared by serially diluting a 2 mM stock solution in 30 mM potassium phosphate buffer at pH 7.4. The samples were diluted to ensure absorbance values were within the linear range of the standard curve (0 to 1.0 mM of Trolox). Standards and samples were pipetted in triplicate into the microplates. Upon adding acetate buffer (0.4 M, pH 5.8) to every well, the first absorbance values were obtained at 660 nm. Following this step, colored ($ABTS^+$) working solution was added to each well and the samples were incubated for 5 min. Subsequently, a second reading of absorbance at 660 nm was taken. The obtained results were used to create a standard curve by plotting the net absorbance at 660 nm (Read 2 – Read 1) against the Trolox standard concentrations to determine the concentration in the samples. Results were expressed in mmol Trolox equivalent per mg protein.

3.5.6 Total glutathione content

Total glutathione content was assessed by measuring the rate of 5'-thio-2-nitrobenzoic acid (TNB) formation, measurable at 412 nm according to Baker et al., (1990). The spectrophotometric assay method for glutathione (GSH) involves oxidation of GSH by the sulfhydryl reagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma-Aldrich) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB), measured at 412 nm for 3 min. The glutathione disulfide (GSSG) formed can be recycled to GSH by glutathione reductase (GR) in the presence of NADPH. GSH standards were prepared with a 10 mM stock solution in 0.2 M potassium phosphate buffer at pH 6.5. The samples were diluted to ensure absorbance values were within the linear range of the standard curve (0 to 100 μM of GSH). Standards and samples were pipetted in triplicate into the microplates. Upon adding the reaction buffer (a solution of 6mM NADPH, 4mM DTNB and 15.2 μL/mL GR in sodium-potassium phosphate buffer (0.2 M, pH 8.0)) to each well, the absorbance values were obtained. Results were expressed in mmol GSH per mg protein.

3.6. Data and statistical analysis

All data are presented as means ± standard deviation (SD). Prior to the analysis of the growth performance indicators, the data expressed as a percentage underwent an arcsine square root transformation (Ennos, 2012) and was examined for normality of distribution, resorting to the Shapiro-Wilk test, and the homogeneity of variance with the Levene's test. Differences among groups and the analysis of delta variation between pre- and post-stress indicators (Xavier et al., 2021) were performed by a one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, or by a Kruskal-Wallis followed by Bonferroni's correction for multiple tests, when the assumptions for the one-way ANOVA were not verified. All statistical differences were considered significant at $P < 0{\cdot}05$. The statistical analyses were performed using the IBM SPSS software version 29.

4. Results

4.1 Growth performance indicators

At the end of the Feeding trial, fish growth performance was not negatively impacted by the algal-supplemented diets ($p > 0.05$, Table 4.1). Similarly, no significant differences were found on final biomass among fish fed the experimental diets. Fish biomass increased 60 %, from an average of 366.8 ± 4.9 g to 576.4 ± 8.6 g. The relative growth rate (RGR) remained mostly unchanged among fish fed the experimental diets and no significant differences were found among fish fed the different diets. Although the feed conversion ratio (FCR) was higher in fish fed the CTRL diet (0.9) compared to fish fed the supplemented diets (0.8 in both LOW and HIGH), the dietary treatments did not significantly affect the FCR. The experimental diets had no effect on the survival of the gilthead seabream juveniles.

Table 4. 1 – Key performance indicators of gilthead seabream (*Sparus aurata*) juveniles fed the experimental diets for three weeks.

Key performance indicators	CTRL	LOW	HIGH
Final biomass (g)	559.2 ± 22.2	583.3 ± 18.0	586.9 ± 5.6
Weight Gain (% IBW)	56.1 ± 3.2	58.6 ± 3.9	58.1 ± 1.4
RGR (% day^{-1})	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.0
FCR	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.0

Initial biomass = 366.8 ± 8.5 g; Values are presented as means ± standard deviation (n = 3 per dietary treatment). Absence of superscript letters indicate no significant differences among the dietary treatments ($p > 0{\cdot}05$).

4.2 Antioxidant status

4.2.1 Feeding trial

At the end of the Feeding trial, no significant differences were observed in any of the antioxidant physiological markers among the dietary treatments (Table 4.2). The algal-supplemented diets had no influence on the fish hepatic LPO levels. The CAT activity (51.0 ± 21.7 IU per mg of protein), TAS levels (121.9 ± 30.4 mmol Trolox per mg of protein) and tGSH content (69.6 ± 16.3 mmol GSH per mg of protein) showed no significant differences among dietary treatments ($p > 0{\cdot}05$).

Table 4. 2 – Antioxidant physiological markers on liver samples of gilthead seabream juveniles fed different experimental diets at the end of the Feeding trial.

	Experimental diets		
Antioxidant markers	**CTRL**	**LOW**	**HIGH**
LPO (nmol TBARS/mg protein)	0.005 ± 0.001	0.004 ± 0.001	0.005 ± 0.001
CAT (IU/mg protein)	37.4 ± 16.5	61.0 ± 17.1	53.0 ± 25.4
TAS (mmol Trolox/mg protein)	133.1 ± 24.4	109.8 ± 16.1	122.7 ± 42.8
tGSH (mmol GSH/mg protein)	75.8 ± 24.0	66.2 ± 7.2	66.6 ± 13.0

Values are presented as means ± standard deviation (n = 9 per dietary treatment). Absence of superscript letters indicate no significant differences among the dietary treatments ($p > 0{\cdot}05$).

4.2.2 Challenge trial

The dietary treatments affected fish total antioxidant status, after the challenge trial (Table 4.3). Fish fed the LOW diet presented a higher TAS when compared to HIGH and CTRL fed fish ($p < 0.05$). The experimental diets had no effect on the LPO levels of the gilthead seabream juveniles. The CAT activity and tGSH content showed similar values between dietary treatments, around 69.5 ± 24.1 IU per mg of protein and 51.1 ± 11.6 mmol GSH per mg of protein, respectively ($p > 0{\cdot}05$).

Table 4. 3 – Antioxidant physiological markers on liver samples of gilthead seabream juveniles fed the experimental diets after the Challenge trial.

	Experimental diets		
Antioxidant markers	**CTRL**	**LOW**	**HIGH**
LPO (nmol TBARS/mg protein)	0.007 ± 0.003	0.004 ± 0.001	0.005 ± 0.002
CAT (IU/mg protein)	79.4 ± 19.2	63.7 ± 23.5	66.6 ± 28.0
TAS (mmol Trolox/mg protein)	85.2 ± 29.0 ^{b}	118.5 ± 18.3 ^{a}	86.2 ± 14.3 ^{b}
tGSH (mmol GSH/mg protein)	48.1 ± 14.5	56.6 ± 10.0	48.4 ± 9.3

Values are presented as means ± standard deviation (n = 9 per dietary treatment). Significant differences among diets present different superscript letters ($p < 0{\cdot}05$). Absence of superscript letters indicate no significant differences among the dietary treatments ($p > 0{\cdot}05$).

4.2.3 Effect of stress events

The LPO delta variation between pre- and post-stress events showed significant differences among the dietary treatments (Figure 4.1a). A higher LPO variation was observed in the CTRL treatment compared to the fish fed the algal-supplemented diets (LOW and HIGH) between the pre- and post-stress events ($p < 0.05$). Despite an observed tendency to a decrease in the variation of CAT between pre- and post-stress events in fish fed LOW and HIGH diets, no significant differences were observed in catalase activity (Figure 4.1b). The variation of TAS and tGSH content between pre- and post-stress presented no significant differences among dietary treatments (Figure 4.1c and 4.1d).

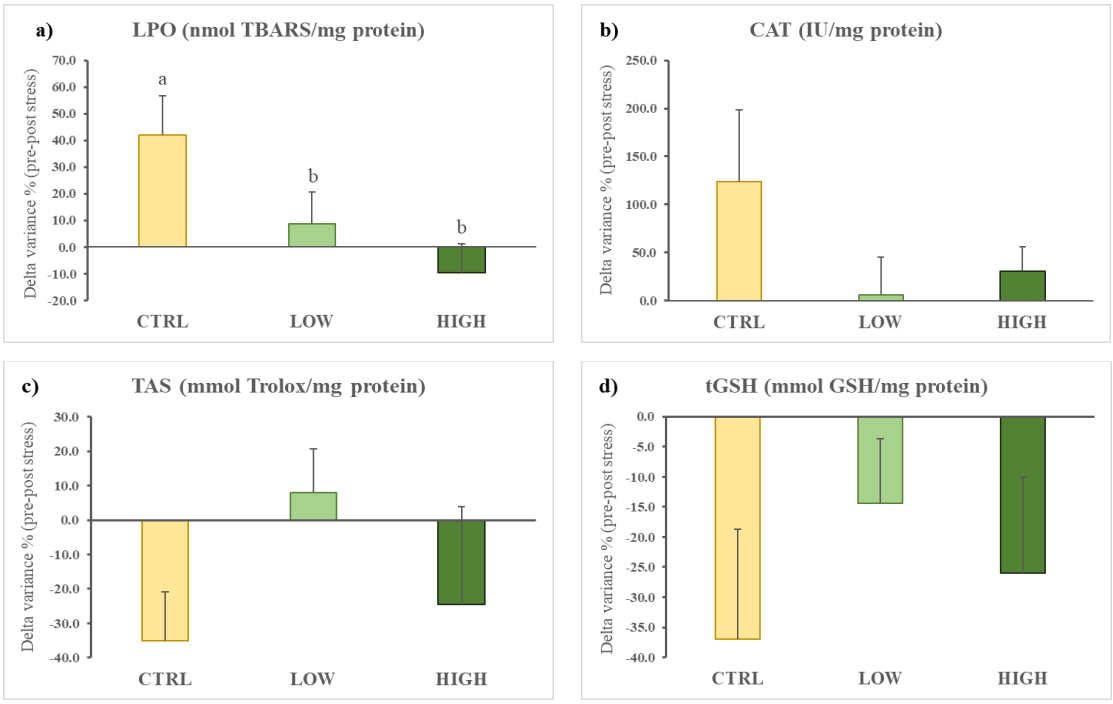

Figure 4. 1 – Delta variation (%) of the response of oxidative stress-related biomarkers (**a**) lipid peroxidation (LPO), (**b**) catalase activity (CAT), (**c**) total antioxidant status (TAS), and (**d**) total glutathione content (tGSH) from Feeding (pre-stress) to Challenge (post-stress) of gilthead seabream juveniles fed with different diets (CTRL, LOW and HIGH). Values presented are mean ± standard deviation ($n = 3$ per dietary treatment). Significant differences between dietary treatments present different superscript letters ($p < 0{\cdot}05$).

5. Discussion

Fish in aquaculture face stress from physical and chemical changes, including common practices such as crowding, size sorting, and transportation, which can increase disease susceptibility and reduced feeding (Conde-Sieira et al., 2018; Schreck & Tort, 2016). Preventive feeding with supplemented diets containing antioxidant additives can enhance fish resilience. Natural products derived from marine compounds have shown great potential in improving antioxidant and immune responses, thereby increasing robustness and reducing disease susceptibility (Dawood et al., 2018; Kim, 2015b). Recent research highlights the benefits of such compounds from microalgae, like *Tetraselmis chui*, recognized for its potential as a dietary source (Mantecón et al., 2019) and a source of bioactive compounds (Bonilla-Ahumada et al., 2018; Khatoon et al., 2021). The present study aimed to assess the effect of dietary microalgal extracts on enhancing the resilience of gilthead seabream juveniles to a stress challenge. The gilthead seabream juveniles were fed diets supplemented with different amounts of *T. chui* extract (0 %, 0.06 % and 0.12 %, respectively for CTRL, LOW and HIGH dietary treatments).

In the current study, the Feeding trial aimed to evaluate whether the experimental diets had any negative impacts on fish growth performance. The results showed that dietary supplementation with *T. chui* extract had no significant impact on weight gain, relative growth rate or feed conversion ratio. The fish promptly accepted the experimental diets and no significant difference in survival rates were observed among the dietary treatments. These findings indicate that *T. chui* supplementation had no detrimental effects on fish survival or growth performance. Similar results were obtained in Nile tilapia fed spirulina (*Arthrospira platensis*)-supplemented diets (Mahmoud et al., 2018) and in Nile tilapia fed diets supplemented with *Chlorella pyrenoidosa* (Peng et al., 2020). In meagre fed *Fucus vesiculosus*-supplemented diets, despite no significant differences observed in fish growth performance, the feed efficiency was significantly higher (Monteiro et al., 2021), which was not observed in the present study, as FCR is not significantly different among dietary treatments. In contrast, results obtained in Nile tilapia fed *Tetraselmis suecica*-supplemented diets showed that final weight and weight gain were significantly higher in fish fed diets with 1.5 % *Tetraselmis suecica* supplementation when compared to fish fed no supplemented diet (Abdel-Tawwab et al.,

2023). Likewise, a study performed in rainbow trout fed 5 % *Chlorella sorokiniana*-supplemented diet revealed that fish had a higher weight gain compared to control fed fish (Chen et al., 2022). These studies show that algal-supplemented diets do not adversely impact fish growth performance, although some variation of performance indicators may occur depending on the inclusion level and fish species.

After the Feeding trial no significant differences were observed in any of the antioxidant markers evaluated in the gilthead seabream juveniles fed the experimental diets. These findings suggest that the dietary inclusion of *Tetraselmis chui* extract in the experimental diets had no major effect on fish antioxidant status after 3 weeks feeding. Similar observations were reported in Nile tilapia fed diets with 5 – 10 % *Nannochloropsis oculata* meal, where no significant differences were observed in the antioxidant biomarkers among dietary treatments (Salem et al., 2022). In contrast, in European seabass fed algal-supplemented diets, significant differences were observed in fish fed the algae blended diet (*Nannochloropsis oceanica* and *Gracilaria gracilis*) where tGSH and GSH-Px were significantly lower compared to control fed fish (Batista et al., 2020).

After the Challenge trial, the TAS levels were highest for the fish fed the LOW diet, compared to the HIGH and CTRL fed fish. Since the beneficial effect is only observed in fish fed the LOW diet, this could suggest that the LOW treatment is more adequate than the HIGH treatment, as the HIGH treatment does not seem to counteract the effects of the stress events. In other words, the beneficial effect observed in the LOW treatment becomes absent when the extract concentration increases two-fold. At this point, it is uncertain as to why this happens, underscoring the need for further experimentation to confirm and explain this effect. Instead, the LOW treatment showed an antioxidant effect, thus contributing to higher TAS levels. Different results were reported in pacu (*Piaractus mesopotamicus*) juveniles fed diets with 2 – 6 % spirulina (*Arthrospira platensis*) meal (Carneiro et al., 2022). After a 24 h exposure to ammonia, no significant differences were observed in TAS levels of pacu juveniles across the various dietary treatments. Additionally, both activities of CAT and SOD were significantly higher, along with significantly lower LPO levels in fish fed diets with 6 % spirulina compared to fish fed a control diet. In a similar manner, *Litopenaeus vannamei* fed *Haematococcus pluvialis*-supplemented diets showed no significant differences in TAS levels nor in the activities of either SOD or GSH-Px compared to the control group

after exposure to an acute salinity stress. Simultaneously, LPO levels were significantly lower with the dietary supplementation of these microalgae, particularly in shrimp fed 0.17 % and 0.33 % supplemented diets compared to control fed shrimp (Xie et al., 2018). *T. chui* is rich in polyunsaturated fatty acids, particularly EPA and DHA, and serves as a natural source of vitamin E and carotenoids (Bonilla-Ahumada et al., 2018; Khatoon et al., 2021; Mantecón et al., 2019). These bioactive molecules may contribute to the antioxidant properties of *Tetraselmis chui*. The findings reported in both these studies, could be related to the microalgal species used, the inclusion of either whole microalgae or microalgal extract, the dose of microalgal supplementation and the animal model used.

The tendencies of the antioxidant markers related to oxidative stress, suggest that fish fed either LOW or HIGH diets, benefited from the supplementation. From the end of the Feeding trial to the end of the Challenge trial, the only significant result observed was in LPO, wherein the fish fed the CTRL diet increased lipid peroxidation significantly more than fish fed the supplemented diets (LOW and HIGH). The decreased lipid peroxidation observed in LOW and HIGH treatments is consistent with the lower variation observed in CAT activity before and after the Challenge event, although not significant. On the other hand, the observation in the variation of both TAS and tGSH levels do not show significant differences among the dietary treatments before and after the Challenge event. In a trial with rainbow trout fed *Chlorella sorokiniana*-supplemented diets, the antioxidant-related parameters were assessed 8 h after LPS injection (Chen et al., 2022). Fish fed the control diet had a significant increase in LPO levels between the pre- and post-LPS challenge, whereas fish fed the diets containing 5 % or 10 % *Chlorella sorokiniana* showed no significant changes during this period. While GSH-Px activity did not change significantly among dietary treatments, SOD activity decreased significantly in all dietary treatments during this period. In a similar study where gibel carp (*Carassius auratus gibelio*) were fed diets containing varying levels of spirulina as a fishmeal replacement, no significant differences in either SOD activity nor in LPO levels were observed among the dietary treatments before and after the challenge (Cao et al., 2018). Once again, the antioxidant response observed in the challenge studies could be explained by the different microalgal species used, the inclusion of either whole microalgae or microalgal extract, the dose of microalgal supplementation and the animal model used.

In the current study, the beneficial effects are mostly observed in the LOW treatment. Fish fed the LOW diet not only showed less variation in LPO levels before and

after the challenge, but also had significantly higher total antioxidant capacity 6 h after the stress events. The HIGH fish showed either no beneficial effects or only minimal effects. In this study, two concentrations of *T. chui* extract (0.06% and 0.12%) were compared to a control diet (not supplemented) to evaluate their effects in the antioxidant status of gilthead seabream juveniles. Given the obtained results with two supplemented dietary treatments, one being twice as supplemented as the other, future studies could explore the effects of other dosages of *T. chui* extract as there is the possibility that the optimal dose could fall within this interval of supplementation. Analysing other biomarkers, such as heat shock proteins (HSPs), could also offer deeper insights into the physiological responses to *T. chui* supplementation. In response to various forms of cellular stress such as hyperthermia, oxidative damage, physical injury or exposure to chemical agents the production of HSPs is significantly upregulated. This coordinated increase in HSP expression is known as the heat shock response (HSR), a universal adaptive mechanism observed across organisms from bacteria to mammals. It enables survival and adaptation to a broad spectrum of environmental stressors (Kalmar & Greensmith, 2009). Their role is crucial in protecting unaffected cells from protein misfolding or aggregation and assisting in the recovery from stress or injury, by aiding the removal of abnormal proteins (Miller & Fort, 2018).

The present study contributes significantly to the knowledge gap in aquaculture nutrition, specifically regarding the use of *T. chui* extracts in diets. These findings provide valuable insights into the potential benefits of microalgae supplementation to improve fish resilience.

6. Conclusion

This study set out to explore the effect of dietary *Tetraselmis chui* extract as a supplement to enhance the resilience of fish juveniles under stress conditions. This was achieved through an analysis of oxidative stress-related biomarkers. Overall, the study supports that *Tetraselmis chui* extract has beneficial effects as a feed additive for gilthead seabream juveniles. The supplemented diets improved antioxidant status and supressed oxidative stress in the fish juveniles, where the LOW diet seemed to be the most adequate of the two supplemented diets.

The findings herein suggest that *T. chui* has the potential to boost the antioxidant response, thus protecting gilthead seabream from important stressors commonly encountered in aquaculture practices. Furthermore, this study shows that *T. chui* extract can be useful as an additive to develop efficient and sustainable feeds, enhancing the productivity and welfare of farmed gilthead seabream. Ensuring that fish can manage stress effectively will likely become a major focus in future aquaculture practices, and supplementing diets with bioactive compounds seems a promising strategy to attain resilience.

7. References

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