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Research article

Eco-innovative aquafeeds biofortified with *Asparagopsis taxiformis* to improve the resilience of farmed white seabream (*Diplodus sargus*) to marine heatwave events

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ABSTRACT

Extreme weather events, like marine heatwaves (MHWs), are becoming more frequent and severe due to climate change, posing several challenges to marine ecosystems and their services. As disease outbreaks are often prompted by these acute phenomena, it is essential to develop eco-innovative strategies that can efficiently improve farmed fish resilience, especially under sub-optimal rearing conditions, thereby ensuring a sustainable aquaculture production.

This study aimed to unveil farmed juvenile white seabream (*Diplodus sargus*, 28.50 ± 1.10 g weight, $n = 150$) immune and antioxidant responses under a category II MHW in the Mediterranean Sea (+4 °C, 8 days of temperature increase plus 15 days of plateau at the peak temperature) and to investigate whether a 30 days period of prophylactic biofortification with

Abbreviations: ANOVA, analysis of variance; AP, antiprotease activity; AT, *Asparagopsis taxiformis*; bw, body weight; CAT, catalase activity; CTR, control; EF1α, elongation factor 1-alpha; ENAs, erythrocytes nuclear abnormalities; GPX1, glutathione peroxidase 1; GST, glutathione S-transferase activity; IgM, immunoglobulin M; K, Fulton's condition index; LPO, lipid peroxidation; Lys, lysozyme; MHWs, marine heatwaves; *p*, p-value; POD, peroxidase activity; *r*, Pearson correlation coefficient; RPL27, ribosomal protein L27; SD, standard deviation; SOD, superoxide dismutase activity; SSI, splenosomatic index; T-AOC, total antioxidant capacity; TCRβ, T-cell receptor beta; TL, total length; W, weight.

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Asparagopsis taxiformis (1.5 %, 3 % and 6 %) enhanced fish resilience to these extreme events. Several biomarkers from different organization levels (individual, cellular, biochemical and molecular) were assessed upon 30 days of biofortification (T30), exposure (after 8 days of temperature increase + 15 days at peak temperature, T53) and recovery (8 days of temperature decrease, T61) from the MHW.

Results showed that MHW negatively affected the fish physiological status and overall well-being, decreasing specific growth rate (SGR) and haematocrit (Ht) and increasing erythrocyte nuclear abnormalities (ENAs) and lipid peroxidation (LPO). These adverse effects were alleviated through biofortification with *A. taxiformis*. Seaweed inclusion at 1.5 % was the most effective dose to minimize the severity of MHW effects, significantly improving immune responses of *D. sargus* (i.e. increased levels of immunoglobulin M, peroxidase activity and lysozyme expression) and modulating antioxidant responses (i.e. decreased LPO, catalase and glutathione S-transferase activity). These findings confirm that *A. taxiformis* is a functional ingredient of added value to the aquaculture industry, as its inclusion in marine fish diets can beneficially modulate fish immunity and resilience under optimal and adverse rearing conditions.

1. Introduction

Climate change is a major global challenge of the 21st century, arising profound impacts on ecosystems, economies, and society [1]. The ocean plays a crucial role in regulating Earth's temperature by absorbing and storing most of the excess heat from the atmosphere [2]. Despite its circulation patterns and capacity to mitigate heat, the ocean is not exempt from climate change impacts. Rising sea temperatures, altered ocean currents, incidence and extent of hypoxic zones, sea level rise and ocean acidification are among the myriad consequences, posing significant challenges to marine ecosystems and the millions of people who depend on them for sustenance and livelihoods [1]. Among these changes, extreme weather events such as marine heatwaves (MHWs) have emerged as a particularly concerning phenomenon due to their hasty (and often unpredictable) nature and, thus, the far-reaching ecological and socioeconomic implications they raise [3]. Recent projections foresee that MHWs will become more frequent, intense, and long-lasting (especially in the summer and/or confined coastal areas where aquaculture facilities are usually settled), with seawater temperatures reaching up to +4 °C in some geographic areas, such as the Mediterranean Sea [4,5].

Aquaculture plays a crucial role in global food security by providing a vital protein source for millions of people worldwide [6,7]. Currently, it is one of the world's fastest-growing industries, supplying 56 % of aquatic food demand in 2020, contrasting to 6 % in the 1960s [7]. This remarkable expansion is propelled by market dynamics, trade, and evolving consumer preferences. Consumers are increasingly demanding not only quantity, but also safe, nutritious, and high-quality seafood products [8]. However, despite this exponential growth, aquaculture facilities, especially outdoor ones, face substantial challenges due to their susceptibility to environmental fluctuations, including temperature variations. MHWs represent a major challenge to aquaculture, amplifying stress levels among farmed species and compromising marine biota's resilience to simultaneous environmental stressors, such as chemical or microbiological contaminations [9,10]. As a result, seafood production and safety are compromised. MHWs can negatively affect aquaculture not only by increasing the frequency, distribution, and virulence of pathogens but also by altering host's physiological well-being due to the stress induced by sub-optimal abiotic conditions [11]. Despite the scientific knowledge acquired so far, the complexity of climate change-induced stressors (especially in what concerns extreme weather events) needs to be further explored, particularly their impacts on farmed fish immune and stress responses and resilience to diseases. This knowledge is essential since aquaculture facilities, characterized by their confined areas and high animal densities, are particularly vulnerable to disease outbreaks. Outbreaks of this nature often result in significant mortality rates and subsequent financial losses in production costs [12]. Given the importance of the aquaculture sector and the limited data regarding MHWs on more complex biological mechanisms, it is essential to understand the extent to which farmed animals will become more vulnerable to diseases. Apart from the undeniable need to identify the impacts of these extreme events in the aquaculture sector, it is also imperative to develop adaptive solutions to mitigate them. To this end, sustainably produced aquafeeds enriched with natural immunostimulant ingredients emerge as a promising avenue under the circular economy paradigm [13,14]. It is widely recognized that the nutritional adequacy of aquafeeds plays a crucial role in enhancing the overall performance and resilience of fish [15]. Seaweeds as feed ingredient are an integral part of this approach due to their natural richness in a panoply of bioactive compounds (e.g. alginates, carrageenans, agar, fucoidan, β-glucan, polyphenols, and carotenoids) [16,17] with well-known benefits (e.g. anti-inflammatory, immunomodulatory, antioxidant, and antibacterial properties) [18–20] for both human and animal health.

Asparagopsis taxiformis is a red macroalgae belonging to the *Asparagopsis* genus (*Bonnemaisoniaceae*, Rhodophyta), originally from Indo-Pacific coastal areas [21], that has recently been declared invasive in the Mediterranean and Atlantic areas, being recognized as one of the main biological threats to local biodiversity [22]. According to Mancuso et al. [23], the earliest reports of its presence in the Mediterranean Sea (from back in 1798–1801, in Alexandria, Egypt) suggest that its introduction resulted from trading operations and the opening of the Suez Canal. Furthermore, its presence was first detected in 2000 along the Italian coast (western Sicily). Studies on the detrimental effects of *A. taxiformis* in Mediterranean coastal ecosystems are still very scarce, but recent evidence highlights that its proliferation poses a considerable risk to native biodiversity by disrupting ecological balances and competing with indigenous species for resources [22]. However, this macroalga holds enormous biotechnology potential [22]. It can be commercially harvested from the wild, with potentially positive ecological outcomes if adequately managed, and can also be cultivated in both land-based and

ocean-based aquaculture systems [24]. *A. taxiformis* is rich in bioactive compounds with various biological activities, such as antioxidant [25,26], cytotoxic [25,27], antimicrobial [28,29], antiviral [30] and enzyme inhibition activities [22,27].

Several studies have explored the immunomodulatory potential of *A. taxiformis* and its extracts on various fish species, including mottled rabbitfish (*Siganus fuscescens*) [31,32], Atlantic salmon (*Salmo salar*) [33], convict surgeonfish (*Acanthurus triostegus*) [34], European seabass (*Dicentrarchus labrax*) [35], and gilthead seabream (*Sparus aurata*) [35]. However, none of them have tested the use of aquafeeds supplemented with *A. taxiformis* under adverse rearing conditions, such as during the occurrence of MHWs. Further research is, therefore, fundamental to validate the use of macroalgae in general and *A. taxiformis* in particular, as an eco-innovative adaptation strategy to overcome climate change impacts in the aquaculture sector. White seabream (*Diplodus sargus*) was used in the current study as a model species due to its considerable economic importance in aquaculture, particularly in the Mediterranean region [36], and because of the availability of specimens, ease of maintenance in captivity and suitability for experimental manipulation.

In this context, this study aimed to investigate, under laboratory conditions, the effects of a category II Mediterranean MHW on the immune and antioxidant responses of farmed juvenile fish (*D. sargus*) and to assess whether functional aquafeeds enriched with whole *A. taxiformis* constitute an efficient eco-friendly strategy to enhance *D. sargus* immunity and resilience to MHWs.

Table 1
Ingredient composition (%) and proximate analysis (%DM) of the experimental diets (CTR, 1.5-AT, 3-AT and 6-AT) used to feed *Diplodus sargus* juveniles.

Ingredients (%)	CTR	1.5-AT	3-AT	6-AT
Fishmeal Super Prime[a]	25.0	25.0	25.0	25.0
Fish protein concentrate[b]	2.0	2.0	2.0	2.0
Soy protein concentrate[c]	10.0	10.0	10.0	10.0
Pea protein concentrate[d]	3.0	3.0	3.0	3.0
Wheat gluten[e]	6.5	6.5	6.5	6.5
Corn gluten meal[f]	10.0	10.0	10.0	10.0
Soybean meal 44[g]	6.0	6.0	6.0	6.0
Rapeseed meal[h]	6.0	6.0	6.0	6.0
Wheat meal[i]	10.8	9.3	7.8	4.8
Faba beans (low tannins)[j]	6.0	6.0	6.0	6.0
Vitamin and mineral premix[k]	1.0	1.0	1.0	1.0
Choline chloride 50 %[l]	0.2	0.2	0.2	0.2
Monoammonium phosphate[m]	1.2	1.2	1.2	1.2
Fish oil[n]	5.0	5.0	5.0	5.0
Soybean oil[o]	7.3	7.3	7.3	7.3
Macroalga *Asparagopsis taxiformis*[p]	0	1.5	3.0	6.0
Dry matter, %DM	94.2	94.0	93.9	94.1
Crude protein, %DM	46.0	46.0	45.9	45.7
Crude fat, %DM	16.0	16.0	16.1	16.1
Fiber, %DM	1.8	1.9	2.0	2.1
Starch, %DM	13.7	12.8	11.8	9.9
Ash, %DM	6.8	7.1	7.4	8.0
Gross energy, MJ kg^{-1}	21.0	21.0	20.9	20.8

[a] Diamante: 66.3 % crude protein (CP), 11.5 % crude fat (CF), South America, Pesquera Diamante, Peru.
[b] CPSP90: 82.6 % CP, 9.6 % CF, Sopropêche, France.
[c] Soycomil P: 62.2 % CP, 0.7 % CF, ADM, The Netherlands.
[d] Lysamine GPS: 78.1 % CP, 8.3 % CF, Roquette, France.
[e] VITAL: 80.4 % CP, 5.8 % CF, Roquette, France.
[f] Corn gluten meal: 61.2 % CP, 5.2 % CF, COPAM, Portugal.
[g] Soybean meal 44: 43.8 % CP, 3.5 % CF, solvent extracted, Ribeiro & Sousa Lda., Portugal.
[h] Rapeseed meal: 34.3 % CP, 2.1 % CF, solvent extracted, Ribeiro & Sousa Lda., Portugal.
[i] Wheat meal: 11.7 % CP, 1.6 % CF, Molisur, Spain.
[j] Faba beans (low tannins): 24.5 % CP, 1.7 % CF, Ribeiro & Sousa Lda., Portugal.
[k] Vitamins (IU or mg kg^{-1} diet): DL-alphatocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotidin acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} 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 middlings. Premix Lda., Portugal.
[l] Choline chloride 50 %: ORFFA, The Netherlands.
[m] Windmill AQUAPHOS: 26 % phosphorus, ALIPHOS, The Netherlands.
[n] Fish oil: 98.1 % CF, 16 % EPA, 12 % DHA, Sopropêche, France.
[o] Soybean oil: 98.6 % CF, JC Coimbra, Portugal.
[p] Asparagopsis *taxiformis*: SeaExpert Company, Angústias dock, Faial Island, Azores, Portugal.

2. Material and methods

Ethical statement

The present study was conducted by researchers certified in animal experimentation (EU functions A and B). All procedures related to animal handling and sample collection were in strict compliance with the ARRIVE (Animal research: reporting of *in vivo* experiments) guidelines and followed ethical standards for the care and use of animals, always in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) and the Portuguese legislation for Laboratory Animal Science (EU Directive 2010/63; Decreto-Lei nº 113/2013). The research was approved by IPMA's Animal Welfare and Ethics Body (ORBEA), overseen by the National Authority for the use of live animals, also known as the Directorate-General for Food and Veterinary (DGAV).

2.1. Seaweed collection and experimental diets

The gametophyte life-stage of *A. taxiformis* was harvested by the company SeaExpert from rocky substrates between 3 and 5 m deep by scuba diving in Angústias dock, Faial Island, Azores, Portugal (38°31′45.0''N 28°37′09.0''W). Seaweeds were transported to the processing facility in a cool and dark environment and in proper boards to allow excess water to flow, and subsequently dried in the dark using a Black Block solar dryer (BBKW, Portugal) set at a maximum temperature of 40 °C for 2 days. Dried seaweeds were stored in pre-labelled dark plastic bags and sent to *a company specialised in fish feed productio*n (SPAROS Lda, Olhão, Portugal), where four diets with similar nutritional composition were prepared, considering the nutritional needs of juvenile white seabream (D. sargus) (detailed feed composition can be consulted in Table 1). Diets were produced with the same feed ingredients, and dried A. taxiformis was added to the basal diets at doses of 0 % (CTR), 1.5 % (1.5-AT), 3 % (3-AT) and 6 % (6-AT).

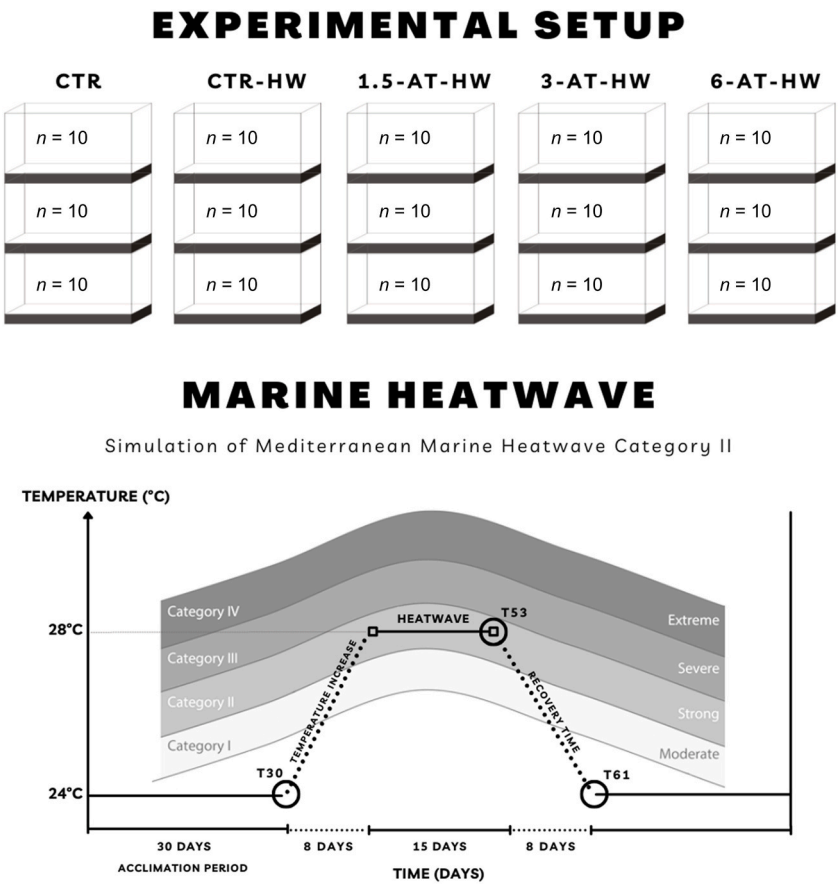

Fig. 1. Experimental setup and simulation of the category II Mediterranean heatwave. ***Abbreviations:*** CTR – Control (commercial diet, under optimal growth conditions); CTR-HW – Control + Heatwave (commercial diet, exposed to a heatwave); 1.5-AT-HW – 1.5 % *A. taxiformis* + Heatwave (diet containing 1.5 % *A. taxiformis*, exposed to a heatwave); 3-AT-HW – 3 % *A. taxiformis* + Heatwave (diet containing 3 % *A. taxiformis*, exposed to a heatwave); 6-AT-HW – 6 % *A. taxiformis* + Heatwave (diet containing 6 % *A. taxiformis*, exposed to a heatwave); Circles represent the sampling points: T30 – sampling after 30 days of exposure to the diets (biofortification period); T53 – sampling after 15 days of exposure to MHW peak temperature; T61 – sampling after upon recovery from MHW.

2.2. Fish rearing conditions

Specimens of *D. sargus* were reared until juvenile stage (28.50 ± 1.10 g weight; 12.2 ± 0.3 cm total length; mean ± standard deviation, $n = 150$) at the Aquaculture Research Station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) under routine hatchery conditions, and transported to IPMA's Live Marine Organisms Bioterium (LABVIVOS) in Algés (Portugal). Fish were equally distributed in two tanks (660 L total capacity each) and kept in quarantine for 3 weeks under abiotic conditions as close as possible to those they experience in their natural habitat: i) temperature: 24.0 ± 0.5 °C; ii) dissolved oxygen: 7.2 ± 0.2 mg L^{-1}; iii) salinity: 35.0 ± 0.5 ‰; iv) pH: 8.0 ± 0.1 units; v) photoperiod: 12h light/12h dark. The temperature was maintained at 24 °C for acclimatization/control purposes, as it represents the average surface seawater temperature in the Mediterranean region during summer [37]. During the quarantine period, all animals were hand-fed twice day with a high commercial control diet (2 % average body weight, bw) with a proximal composition adapted to the nutritional requirements of juvenile *D. sargus*.

2.4. Experimental design

After a 3-week acclimation period, fish were transferred to the experimental systems, being randomly distributed in 15 rectangular shaped glass tanks (200 L each, total volume), within independent recirculating aquaculture systems (RAS). Five treatments were considered in the experimental design (Fig. 1): i) Treatment 1 (Control, CTR) – Non-biofortified, i.e., animals fed with a commercial diet for *D. sargus* juveniles, while being exposed to optimal growth conditions, i.e., 24 °C, throughout the entire experimental period; ii) Treatment 2 (Control + Heatwave, CTR-HW) – Non-biofortified, i.e. animals fed with a commercial diet for *D. sargus* juveniles, exposed to 24 °C for 30 days, followed by the exposure to a category II Mediterranean marine heatwave, i.e., 28 °C, during 15 days; iii) Treatment 3 (1.5 % *A. taxiformis* + Heatwave, 1.5-AT-HW) – Animals fed with biofortified diet containing 1.5 % of dried *A. taxiformis*, exposed to 24 °C for 30 days, followed by the exposure to a category II Mediterranean marine heatwave, i.e., 28 °C, during 15 days; iv) Treatment 4 (3 % *A. taxiformis* + Heatwave, 3-AT-HW) – Animals fed with biofortified diet containing 3 % of dried *A. taxiformis*, exposed to 24 °C for 30 days, followed by the exposure to a category II Mediterranean marine heatwave, i.e., 28 °C, during 15 days; v) Treatment 5 (6 % *A. taxiformis* + Heatwave, 6-AT-HW) – Animals fed with biofortified diet containing 6 % of dried *A. taxiformis*, exposed to 24 °C for 30 days, followed by the exposure to a category II Mediterranean marine heatwave, i.e., 28 °C, during 15 days.

To ensure that seawater quality and abiotic parameters were maintained at adequate levels, each system was equipped with protein skimmers (Tornado 120, Mantis), physical filtration consisting of a filter bag (400 μm; TMC Iberia, Portugal), filter sponge and glass wool, biological filtration (Bio Balls 1.5″ Aquarium Pond Filter, TMC Iberia, Portugal), ultraviolet water sterilizer (ClearUVC-36, EHEIM, Germany), and submerged air stones. Temperature was regulated up and down as required by the experimental design by means of submerged digital heaters (300W, V^2Therm Digital Heaters, TMC Iberia, Portugal) and automatic water refrigeration systems (Foshan Weinuo Refrigeration Equipment Co., Ltd, China), both connected to a computerized control system (ProfiLux 3 Outdoor, GHL, Germany) which monitored (by means of individual temperature sensors, PT 1000, GHL, Germany) and adjusted the temperature in each tank every 3 s.

Temperature was daily measured with a thermometer (TFX 430, Ebro Electronic, Germany), while the other abiotic parameters (dissolved oxygen, salinity and pH) were measured with a multi-parameter device (Multi 3420 SET G, WTW, Germany), connected to an IDS (intelligent, digital sensor) digital conductivity cell (TetraCon® 925, WTW, Germany), an IDS DO optical sensor (FDO® 925, WTW, Germany) and an IDS pH-electrode (SenTix® 940, WTW, Germany). Seawater abiotic parameters were adjusted as necessary, with dissolved oxygen maintained at 7.2 ± 0.2 mg L^{-1}, salinity at 35.0 ± 0.5 ‰ and pH at 8.0 ± 0.1 units. Throughout the experiment a 12h light/12h dark photoperiod regime was set. Ammonia (NH_3/NH_4), nitrites (NO_2^-) and nitrates (NO_3^-) levels were measured on a weekly basis using colorimetric test kits (Salifert, The Netherlands) and maintained below detectable levels (except for nitrates which were kept <50 mg L^{-1}), by daily water renewals in each tank.

During the prophylactic biofortification period, fish were hand-fed twice daily (2 % bw) with the respective aquafeed in each treatment, while being exposed to 24 °C for 30 consecutive days (Fig. 1). After this period, the occurrence of a category II (strong) marine heatwave typically observed in the Mediterranean environment [3] was simulated by increasing the seawater temperature to 28 °C (i.e. ΔTemperature = +4 °C) in all treatments (+0.5 °C per day, i.e. 8 days of temperature ramp increase), except for treatment 1 (Control). The seawater temperature was maintained at this level for 15 days (heatwave "plateau") and then, decreased until 24 °C in all treatments (−0.5 °C per day, i.e., 8 days of temperature ramp decrease).

2.5. Fish sampling

Three sampling points were performed: (i) T30, i.e., after 30 days of prophylactic biofortification period; (ii) T53, i.e., after 15 days of exposure to the peak marine heatwave temperature (at the end of the "plateau" stage); (iii) T61, i.e., after post-heatwave recovery period.

Fish were fasted for 24 h prior to samplings. Nine fish per treatment (i.e., three fish from each replicate tank; $n = 9$ per treatment and sampling point) were randomly collected and anaesthetised by immersion in an overdosed tricaine methanesulfonate solution (2 g L^{-1} of MS-222, Acros Organics, Belgium), buffered with sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, USA), using a ratio of 1:2 of MS-222:Sodium bicarbonate to reduce fish stress [17]. Once the anaesthetic took effect, fish were weighted (g, weight, W) on an analytical scale (EMS 300-3, Kern & Sohn, Germany), measured (cm; total length, TL) and peripheral blood was collected from the caudal vein using heparinized syringes. A fraction of the fish blood was used for haematological analyses, while the remaining blood was transferred into a microtube (1.5 mL) with 20 μL of heparin (3000 U mL^{-1} in saline solution 0.9 % NaCl, Sigma-Aldrich, USA), centrifuged at

10,000×*g* for 10 min at 4 °C (Fisher Scientific AccuSpin Micro 17R Centrifuge, Germany) to isolate plasma [17], and then stored at −80 °C until assayed for innate humoral parameters. After this procedure, fish death was confirmed by cervical cut, and the animals were then dissected. The spleens were collected, weighed, and homogenized in ice-cold conditions with 1.0 mL of phosphate buffered saline pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; reagents from Sigma-Aldrich, USA), using an Ultra-Turrax® device (T25 digital, Ika, Germany). Homogenates were centrifuged in 1.5 mL microtubes at 10,000×*g* for 10 min at 4 °C, and the supernatants were transferred to new microtubes, immediately frozen and stored at −80 °C until further analyses. Head-kidneys were also aseptically collected for gene expression analysis. After collection, head-kidneys were stored in RNA later (Sigma-Aldrich, USA; with a proportion of 1/10 *w/v*) at 4 °C for the first 24 h and then transferred to −20 °C until processing.

2.6. Body condition indexes and growth parameters

The Fulton's condition index (K) was directly calculated from the biometric data according to the equation [38]:

$$\mathbf{K} = \frac{\mathbf{Total\ weight\ (g)}}{\mathbf{Total\ Lenght^3\ (cm)}} \times \mathbf{100}$$

To determine the condition of the spleen and assess its relative size compared to the total body weight of the fish, the splenosomatic index (SSI) was calculated using the following equation:

$$\mathbf{SSI} = \frac{\mathbf{Spleen\ weight\ (g)}}{\mathbf{Total\ weight\ (g)}} \times \mathbf{100}$$

The weight gain rate (WGR), specific growth rate (SGR), survival ratio (SR) and feed conversion ratio (FCR) were determined using the following formulas:

$$\mathbf{WGR}\ (\%) = \frac{\mathbf{Final\ body\ weight\ (g) - Initial\ body\ weight\ (g)}}{\mathbf{Initial\ body\ weight\ (g)}} \times \mathbf{100}$$

$$\mathbf{SGR}\ \left(\%\ \boldsymbol{day^{-1}}\right) = \frac{\mathbf{[ln\ (Final\ body\ weight) - ln\ (Initial\ body\ weight)]}}{\mathrm{Days}} \times \mathbf{100}$$

$$\mathbf{SR}\ (\%) = \frac{\mathbf{Final\ number\ of\ fish}}{\mathbf{Initial\ number\ of\ fish}} \times \mathbf{100}$$

$$\mathbf{FCR} = \frac{\mathbf{Amount\ of\ feed\ given}}{\mathbf{Fish\ weight\ gained}}$$

2.7. Haematological parameters

A portion of the peripheral blood was used to determine the haematocrit (Ht). Blood was collected by capillary action into a microhaematocrit tube (Marienfeld, Germany) and one end sealed with wax plates (Marienfeld, Germany). The tubes were placed on a haematocrit rotor (Thermo Fisher Scientific, USA) and centrifuged (accuSpin Micro 17R, Thermo Fisher Scientific, USA) at 10,000×*g* for 5 min at room temperature. After centrifugation, the tubes were placed on a reading graph and the percentage of each component (i.e., erythrocytes, leucocytes, and plasma) was registered.

Fish blood smears were prepared by applying a drop of blood to pre-cleaned glass microscope slides ($n = 3$ individuals per treatment, i.e., one smear per individual; VWR, USA) and spread by capillarity. The smears were then air dried overnight at room temperature and fixed in 95 % methanol (Sigma-Aldrich, USA) for a few seconds, according to the protocol of Kaplow and Ladd [39]. After 24 h, the fixed blood smears were stained using the Hemacolor staining reagent (Hemacolor® Rapid staining of blood smear, Sigma-Aldrich, USA), following the manufacturer's instructions and mounted with Entellan® medium (Sigma-Aldrich, USA). The microscope used for analysis was the optical LEICA DM2500 (Germany), and a minimum of 500 cells per slide were meticulously counted and categorized as either erythrocytes or leucocytes. Erythrocyte nuclear abnormalities (ENAs) were identified according to the criteria of Carrasco et al. [40].

2.8. Biochemical analyses

For biochemical biomarkers, plasma and spleen samples ($n = 6$ per treatment and sampling point) were analysed, at least, in triplicate, using 96-well microplates (Greiner Bio-one, Austria) and a Multiskan GO 1510 microplate reader (ThermoFisher Scientific, USA). All reagents and standards were of pro analysis (or superior) grade. The procedures are extensively described in the supplementary materials (see Supplementary Table S1).

2.8.1. Innate humoral parameters

The following non-specific humoral immunological parameters were assessed in plasma samples: total antiprotease (AP), total peroxidase (POD) and immunoglobulin M (IgM).

Total antiprotease activity was determined by a method developed by Ellis [41] and modified by Hanif et al. [42] with minor adaptations. Peroxidase activity was determined according to Quade and Roth [43], also with minor modifications. Plasma immunoglobulin M was measured according to the protocol of Cuesta et al. [44], with some modifications. Detailed descriptions of these three methods and the specific modifications can be found in Marmelo et al. [17].

2.8.2. Oxidative stress biomarkers

Spleen samples were used to determine antioxidant biomarkers: total antioxidant capacity (T-AOC), lipid peroxidation (LPO), catalase (CAT) activity, glutathione S-transferase (GST) activity and superoxide dismutase (SOD) activity.

Total antioxidant capacity was determined according to the method described by Kambayashi et al. [45] and lipid peroxidation was determined using a 96-well microplates protocol adapted from the method of Uchiyama and Mihara [46]. Catalase activity was determined following the method of Johansson and Borg [47], glutathione S-transferase activity was determined according to the method of Habig et al. [48] and superoxide dismutase activity was determined following the methodology described by Sun et al. [49]. All these three methods were adapted to 96-well microplates [17]. The results (except for SOD, % inhibition) were normalized according to the protein content of the samples (i.e., expressed in mg of total protein), determined through the Bradford method [50].

2.9. Gene expression studies

2.9.1. RNA isolation, cDNA synthesis and real-time PCR

Head-kidney samples (~30 mg) were stored in RNA later (2 mL), and placed in RNA TRItidy G™ (PanReac AppliChem, Germany) for total RNA extraction according to the manufacturer's instructions. RNA integrity was assessed by 1.5 % agarose gel electrophoresis, through analysis of ribosomal RNA 18S and 28S fragments, and RNA quantity was determined using NanoDrop DS-11 FX spectrophotometer (DeNovix, USA). Total RNA (1000 ng) was reverse-transcribed for 1 h at 37 °C using the SuperScript™ IV First-Strand Synthesis System (Invitrogen, Lithuania), following the manufacturer's protocol. Quantitative analysis of glutathione peroxidase 1 (*GPX1*), superoxide dismutase 1 (*SOD1*), lysozyme (*Lyz*), and T-cell receptor beta (*TCRβ*) gene expression in head-kidney tissues was performed using quantitative real-time polymerase chain reaction (qRT-PCR). All qRT-PCR reactions were performed in triplicates using SensiFAST (BioPortugal, Portugal), 0.25 μM of gene-specific primers (Table 2) and 10 ng of cDNA. PCR amplification was as follows: an initial denaturation step of 1 min at 95 °C and 40 cycles of amplification (5 s at 95 °C and 10 s at 65 °C). Efficiency of amplification was above 95 % for all primer sets. Levels of gene expression were calculated using the ΔΔCt comparative method described by Livak and Schmittgen [51] and normalized using housekeeping genes elongation factor 1-alpha (*EF1α*) and ribosomal protein L27 (*RPL27*) because of their abundance and Ct values consistency among treatments, whose suitability was evaluated using Normfinder and BestKeeper algorithms as suggested by Spiegelaere et al. [52].

2.10. Data analysis

Data was presented as mean ± standard deviation. Significant differences between treatments in body condition indexes, as well as, haematology results, and biomarker levels were assessed using a one-way nested-ANOVA analysis (with time, T30, T53 and T61, nested in treatment). Data were Log transformed, whenever necessary, to comply with the assumptions of normality (Kolmogorov–Smirnov's test) and homogeneity of variances (Levene's test) required for this analysis. Subsequently, the post-hoc Tukey HSD test was carried out to identify significant differences. Significant correlations between biomarker levels and body condition indexes (K and SSI), as well as T-AOC and LPO between the other oxidative stress biomarkers (CAT, SOD and GST), were determined by means of Pearson's correlation analysis. All statistical analyses were performed at a significance level of 0.05 using STATISTICA™ software (Version 7.0, StatSoft Inc., USA). Statistical analysis for gene expression was performed using a one-way ANOVA analysis, followed by a Dunnet Post test in GraphPad Prism (v10.2.4).

Table 2
Primers Forward (Fw) and Reverse (Rv) sequences used in this study.

Acession ID	Primer Name		Sequence 5' – 3'	PCR product (bp)
CAEMYA010002102	*TCRβ*	Fw	GCCAAATGCAGCCTCAGGAA	316
		Rv	CAAGACAAATTAACAAGCGAGTGAGCAGTA	
CAEMYA010000748	*Lys*	Fw	TGCCTCAGCGAAAACAGCTG	459
		Rv	GAGCCGACATCAACATTTGC	
CAEMYA010000071	*GPX1*	Fw	GAAGGTGGATGTGAATGGAAAAGATG	129
		Rv	CTGACGGGACTCCAAATGATGG	
CAEMYA010000025	*SOD1*	Fw	CAGTAGGACCGGCATGATTTTTACCA	308
		Rv	TGAAGCTCACAGGAGAAATCAAAGGA	
CAEMYA010000126	*RPL27*	Fw	TCCTTTCCTGTTAGCGGTGG	425
		Rv	CCACTTGTTCTTGCCCGTCT	
CAEMYA010000230	*EF1α*	Fw	CACCCCTTACCCCCATCCTG	148
		Rv	GCACTGTCGTGTGATTCAGGT	

Abbreviations: *TCRβ:* T-cell receptor beta; *Lys:* lysozyme; *GPX1:* glutathione peroxidase 1; *SOD1:* superoxide dismutase 1; *RPL27:* ribosomal protein L27; *EF1α:* elongation factor 1-alpha; bp: base pairs.

3. Results

3.1. Body condition indexes and growth parameters

Body condition indexes and growth parameters are presented in Table 3.

There were no statistically significant differences between treatments ($p > 0.05$) in terms of TL, W, K, SSI, WGR, FCR and SR. SGR was significantly higher during the first 30 days of biofortification (T30) across all treatments ($p < 0.05$). However, after the heatwave (T53), a decrease in SGR values was observed, with no significant differences registered between treatments or remaining times (T53 *vs* T61; $p > 0.05$).

A positive correlation was found between total fish weight and length ($r = 0.861$; $p < 0.001$), and between K and SSI, ($r = 0.317$; $p < 0.001$; Table 4).

3.2. Haematological profile

The haematological profile was assessed in *D. sargus* juveniles fed aquafeeds supplemented with different levels of *A. taxiformis* and exposed to a marine heatwave. The results indicate that neither the experimental feeds (at 1.5 %, 3 % or 6 % of inclusion level) nor the MHW had a significant effect on the percentages of erythrocytes and leucocytes (see Table 5; $p > 0.05$). Erythrocytes percentages remained consistently high at over 94.0 % of total cells, and leucocyte percentages remained below 6.0 % of total cells (Table 5). However, notable differences were observed in the percentages of erythrocyte nuclear abnormalities (ENAs) between treatments ($p < 0.05$). As shown in Table 5, CTR treatment consistently showed the lowest percentage of ENAs (below 5 %), regardless of the duration of the experiment (30, 53 or 61 days; $p < 0.05$). The inclusion of the macroalga *A. taxiformis* (T30) resulted in an increase in the number of ENAs compared to the control aquafeed ($p < 0.05$), with no significant differences related to the level of macroalga inclusion (1.5 %, 3 % or 6 %; $p > 0.05$; Table 5). However, this panorama changed completely after the heatwave exposure (T53). Following the heatwave (T53), juvenile *D. sargus* showed a significant increase ($p < 0.05$) in the number of ENAs, with the highest percentages observed in the CTR-HW treatment (fish fed the commercial diet and exposed to the heatwave; 20.9 ± 1.7 %, Table 5) and the 6-AT-HW treatment (fish fed a feed supplemented with 6 % *A. taxiformis* and exposed to the heatwave; 22.6 ± 1.8 %, Table 5). The

Table 3
Growth and body metrics in *Diplodus sargus* after 30 days (T30), 53 days (T53), and 61 days (T61) of feeding trial (mean ± SD; $n = 9$).

	TL (cm)	W (g)	K_{index}	SSI_{index}	WGR (%)	SGR (% day^{-1})	FCR	SR (%)
30 days (biofortification period)								
Control	14.29 ± 0.87	50.34 ± 7.21	1.73 ± 0.22	0.04 ± 0.01	75.10 ± 12.32	1.86 ± 0.24*	0.92 ± 0.53	100
1.5 % *A. taxiformis*	14.57 ± 0.77	53.48 ± 7.87	1.73 ± 0.20	0.05 ± 0.02	71.97 ± 17.79	1.79 ± 0.37*	0.77 ± 0.32	100
3 % *A. taxiformis*	14.66 ± 0.84	57.71 ± 9.17	1.83 ± 0.13	0.06 ± 0.04	87.26 ± 19.87	2.08 ± 0.36*	0.64 ± 0.20	100
6 % *A. taxiformis*	14.58 ± 0.85	53.53 ± 8.27	1.73 ± 0.19	0.05 ± 0.02	71.62 ± 19.62	1.78 ± 0.38*	0.76 ± 0.28	100
53 days (post-heatwave)								
Control	15.19 ± 0.84	63.07 ± 8.64	1.80 ± 0.18	0.05 ± 0.01	93.59 ± 15.20	1.34 ± 0.16#	0.92 ± 0.24	100
Control + Heatwave	14.43 ± 0.78	53.80 ± 8.82	1.78 ± 0.06	0.04 ± 0.00	70.77 ± 18.46	1.00 ± 0.21#	1.35 ± 0.53	100
1.5 % *A. taxiformis* + Heatwave	14.74 ± 0.87	56.41 ± 8.76	1.75 ± 0.15	0.04 ± 0.01	82.83 ± 25.12	1.12 ± 0.27#	1.22 ± 0.54	100
3 % *A. taxiformis* + Heatwave	14.86 ± 0.68	55.60 ± 7.17	1.69 ± 0.08	0.04 ± 0.01	81.21 ± 13.70	1.12 ± 0.15#	1.19 ± 0.32	100
6 % *A. taxiformis* + Heatwave	14.96 ± 0.66	57.03 ± 8.75	1.70 ± 0.16	0.05 ± 0.01	82.51 ± 13.55	1.13 ± 0.14#	1.14 ± 0.33	100
61 days (post-recovery time)								
Control	14.64 ± 0.99	57.81 ± 10.67	1.83 ± 0.14	0.04 ± 0.01	81.95 ± 24.55	0.97 ± 0.23#	1.37 ± 0.59	100
Control + Heatwave	15.08 ± 0.85	58.53 ± 9.13	1.70 ± 0.14	0.05 ± 0.02	92.39 ± 30.60	1.05 ± 0.30#	1.38 ± 0.90	100
1.5 % *A. taxiformis* + Heatwave	15.16 ± 0.52	59.15 ± 7.80	1.69 ± 0.14	0.04 ± 0.01	93.73 ± 22.54	1.07 ± 0.19#	1.21 ± 0.35	100
3 % *A. taxiformis* + Heatwave	14.97 ± 0.67	58.56 ± 6.32	1.74 ± 0.10	0.06 ± 0.01	93.62 ± 16.49	1.08 ± 0.15#	1.21 ± 0.30	100
6 % *A. taxiformis* + Heatwave	15.18 ± 0.92	57.60 ± 10.94	1.63 ± 0.09	0.03 ± 0.01	80.53 ± 17.19	0.96 ± 0.16#	1.34 ± 0.47	100

Total length (TL; cm), weight (W; g), Fulton's condition index (K), splenosomatic index (SSI), weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR) and survival ratio (SR). In each column, different letters indicate significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (*, #) indicate significant differences ($p < 0.05$) between sampling days (T30, T53 and T61) for the same treatment. The absence of letters or symbols indicates no statistical differences ($p > 0.05$).

Table 4
Pearson correlation coefficients (*r*) between fish (*Diplodus sargus*) morphometry, body condition indexes and biomarker responses.

Treatments used in the analysis	Variables	*r*
All (T30+T53+T61)	**W x TL**	0.861**
	K x SSI	0.317**
All (T30+T53+T61)	**K x CAT**	0.323*
	K x SOD	0.265*
	K x GST	0.224
	K x LPO	−0.101
	K x T-AOC	−0.101
	K x IgM	−0.254
	K x AP	0.209
	K x POD	−0.091
All (T30+T53+T61)	**SSI x CAT**	0.560**
	SSI x SOD	0.394**
	SSI x GST	0.320*
	SSI x LPO	−0.162
	SSI x T-AOC	−0.101
	SSI x IgM	−0.161
	SSI x AP	−0.092
	SSI x POD	−0.172
All (T30+T53+T61)	**T-AOC x CAT**	0.604**
	T-AOC x SOD	−0.173
	T-AOC x GST	0.517**
All (T30+T53+T61)	**LPO x CAT**	−0.168
	LPO x SOD	−0.356*
	LPO x GST	−0.132

Abbreviations: AP – antiprotease activity; CAT – catalase activity; GST – glutathione S-transferase activity; IgM - Immunoglobulin M; K - Fulton's condition index; LPO – lipid peroxidation; POD – peroxidase activity; SOD – superoxide dismutase activity; SSI - splenosomatic index; T-AOC – total antioxidant capacity; TL – total length; W – weight.
In each column, asterisks indicate significant correlations between variables (*$p < 0.05$; **$p < 0.001$).

Table 5
Haematological parameters of *Diplodus sargus* juveniles after 30 days (T30, biofortification period), 53 days (T53, post-heatwave) and 61 days (T61, post-recovery time) of feeding trial (mean ± SD; $n = 9$).

	Erythrocytes (%)	Leucocytes (%)	ENAs (%)	Haematocrit (%)
30 days (biofortification period)				
Control	96.0 ± 0.8	4.0 ± 0.8	4.3 ± 1.2^{b}	39.9 ± 1.4
1.5 % *A. taxiformis*	95.6 ± 0.3	4.4 ± 0.3	$8.6 \pm 0.3^{a*}$	40.4 ± 4.1*
3 % *A. taxiformis*	95.4 ± 0.2	4.6 ± 0.2	$8.1 \pm 0.4^{a*}$	39.0 ± 2.9*
6 % *A. taxiformis*	95.4 ± 0.4	4.6 ± 0.4	$8.1 \pm 0.5^{a*}$	$31.8 \pm 5.9^{*\#}$
53 days (post-heatwave)				
Control	95.7 ± 0.1	4.3 ± 0.1	5.0 ± 0.6^{d}	37.6 ± 0.5^{a}
Control + Heatwave	95.2 ± 0.9	4.8 ± 0.9	20.9 ± 1.7^{ab}	$23.9 \pm 4.6^{b*}$
1.5 % *A. taxiformis* + Heatwave	95.1 ± 1.8	4.9 ± 1.8	$16.0 \pm 1.5^{c\#}$	$25.4 \pm 2.9^{b\#}$
3 % *A. taxiformis* + Heatwave	94.7 ± 1.4	5.3 ± 1.4	$19.1 \pm 1.3^{bc\#}$	$23.6 \pm 5.2^{b\#}$
6 % *A. taxiformis* + Heatwave	95.7 ± 0.6	4.3 ± 0.6	$22.6 \pm 1.8^{a\#}$	$24.5 \pm 1.3^{b\#}$
61 days (post-recovery time)				
Control	95.9 ± 0.7	4.1 ± 0.7	4.2 ± 0.6^{c}	38.3 ± 2.4
Control + Heatwave	95.1 ± 0.2	4.9 ± 0.2	21.0 ± 1.7^{a}	$34.1 \pm 1.5^{\#}$
1.5 % *A. taxiformis* + Heatwave	94.6 ± 0.5	5.4 ± 0.5	$16.2 \pm 1.2^{b\#}$	$35.4 \pm 2.8^{*\#}$
3 % *A. taxiformis* + Heatwave	94.5 ± 0.6	5.5 ± 0.6	$18.9 \pm 1.2^{ab\#}$	37.5 ± 3.2*
6 % *A. taxiformis* + Heatwave	94.9 ± 0.2	5.1 ± 0.2	$20.2 \pm 0.3^{a\#}$	36.3 ± 4.1*

In each column, different letters indicate significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (*, #) indicate significant differences ($p < 0.05$) between sampling days (T30, T53 and T61) for the same treatment. The absence of letters or symbols indicates no statistical differences ($p > 0.05$).

treatments supplemented with 1.5 % (1.5-AT-HW) and 3 % (3-AT-HW) *A. taxiformis* showed statistically lower percentages of ENAs compared to the other treatments exposed to the heatwave, with values of 16.0 ± 1.5 % and 19.1 ± 1.3 %, respectively (Table 5). Following the recovery period (T61), the ENAs percentages of all treatments did not differ significantly from the percentages obtained after the heatwave (T53).

After 30 days of biofortification (T30), there were no significant differences in Ht among the different levels of *A. taxiformis* inclusion (1.5 %, 3 % and 6 %; $p > 0.05$), with values hovering around 40 % of red blood cells (Table 5). However, exposure to the MHW (T53) resulted in a reduction in haematocrit values of approximately 40 % compared to the levels obtained during the biofortification

period (T30; $p < 0.05$). After the recovery period (T61), Ht values recovered and approached the levels observed before the heatwave exposure (T30, Table 5).

3.3. Immune humoral parameters

Fish immune humoral responses are presented in Fig. 2. The inclusion of whole *A. taxiformis* in the aquafeeds resulted in an increase in IgM levels (Fig. 2-A) and a decrease in POD activity (Fig. 2-C) after 30 days of biofortification, compared to the control treatment ($p < 0.05$). It is noteworthy that no significant differences ($p > 0.05$) were observed between the different percentages of inclusion of the macroalga at this time (30 days), indicating a consistent response regardless of biofortification dose. At day 53 there was an increase in IgM content (1.5-fold increase, $p < 0.05$; Fig. 2-A) and POD activity (1.2-fold increase, $p < 0.05$; Fig. 2-C) in the non-biofortified treatment exposed to the heatwave (CTR-HW) compared to the control treatment under optimal temperature conditions (CTR). Nevertheless, the IgM content was significantly higher in fish fed with 1.5 % of *A. taxiformis* (1.5-AT-HW) and 3 % of *A. taxiformis* (3-AT-HW), compared to the remaining treatments ($p < 0.05$; Fig. 2-A). POD activity was also higher in fish fed with 1.5 % of *A. taxiformis* (1.5-AT-HW; $p < 0.05$), however the treatments with 3 % and 6 % of *A. taxiformis* resulted in lower POD activity compared to the control treatment exposed to a heatwave (CTR-HW), with a decrease of 0.9-fold and 0.6-fold, respectively ($p < 0.05$; Fig. 2-C). Following the recovery period (61 days), overall IgM and POD activity levels exhibited a similar trend as observed at the 30-day mark, which was before the heatwave. However, there was a slight increase in IgM levels in the 1.5-AT-HW treatment (Fig. 2-A), and the POD activity values remained higher than those observed at 30 days (Fig. 2-C). The antiprotease activity showed no significant differences between the treatments, whether exposed to the heatwave or not ($p > 0.05$; Fig. 2-B).

No significant correlations were found between Fulton's condition index (K) or splenosomatic index (SSI) and the immune humoral parameters (IgM, AP, and POD; $p > 0.05$; Table 4).

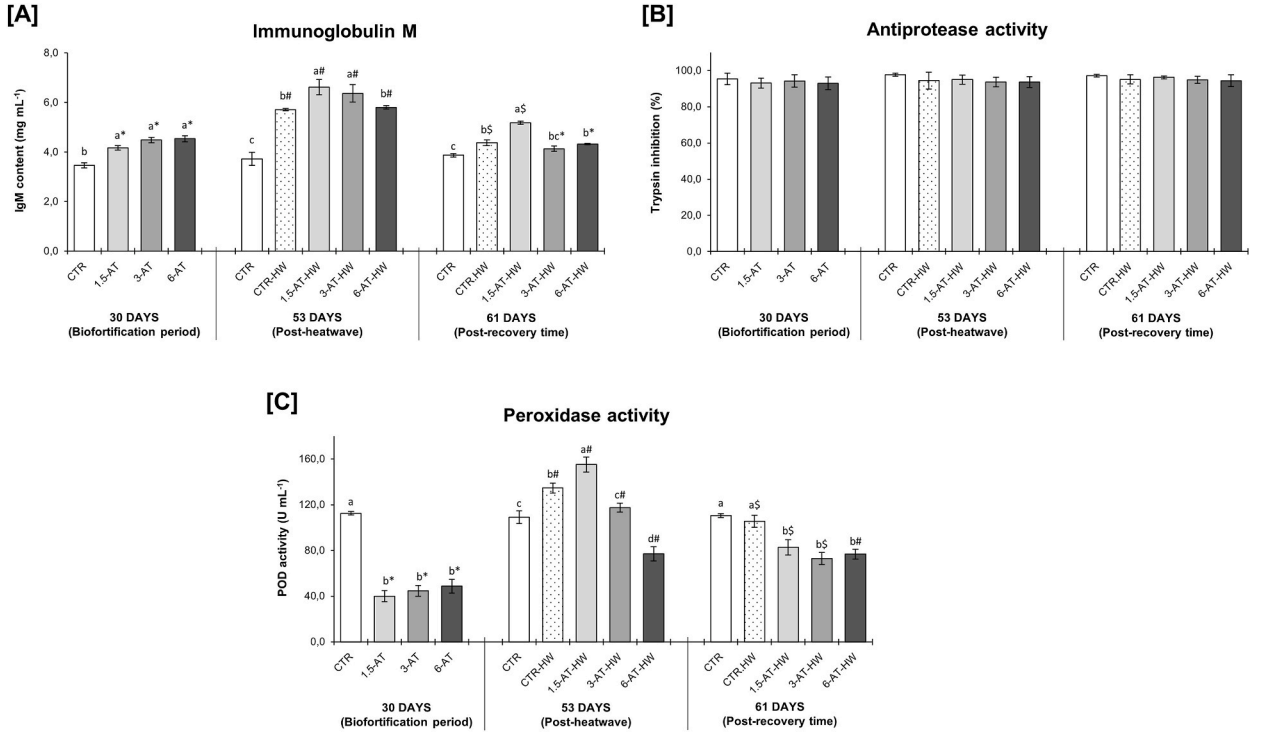

Fig. 2. Plasma immune parameters of *Diplodus sargus* juveniles after 30 days (T30, biofortification period), 53 days (T53, post-heatwave) and 61 days (T61, post-recovery time) of feeding trial: immunoglobulin M (**A**, mg mL^{-1}), antiprotease activity (**B**, expressed as % trypsin inhibition) and peroxidase activity (**C**, U mL^{-1}) (mean ± SD; $n = 6$). Different lowercase letters indicate significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (*, #, $) indicate significant differences ($p < 0.05$) between sampling days (T30, T53 and T61) for the same treatment. The absence of letters or symbols indicates no statistical differences ($p > 0.05$). ***Abbreviations:*** CTR – Control (commercial diet, under optimal growth conditions); 1.5-AT – diet containing 1.5 % *A. taxiformis*, under optimal growth conditions; 3-AT – diet containing 3 % *A. taxiformis*, under optimal growth conditions; 6-AT – diet containing 6 % *A. taxiformis*, under optimal growth conditions; CTR-HW – Control + Heatwave (commercial diet, exposed to a heatwave); 1.5-AT-HW – 1.5 % *A. taxiformis* + Heatwave (diet containing 1.5 % *A. taxiformis*, exposed to a heatwave); 3-AT-HW – 3 % *A. taxiformis* + Heatwave (diet containing 3 % *A. taxiformis*, exposed to a heatwave); 6-AT-HW – 6 % *A. taxiformis* + Heatwave (diet containing 6 % *A. taxiformis*, exposed to a heatwave).

3.4. Oxidative stress biomarkers

Oxidative stress biomarkers are presented in Fig. 3. The inclusion of the macroalga *A. taxiformis* in the aquafeeds significantly increased CAT, GST and T-AOC levels compared to the control (CTR) treatment after 30 days of biofortification (T30, biofortification period), regardless of the inclusion level used ($p < 0.05$; Fig. 3). Among the three inclusion levels (1.5 %, 3 %, and 6 % of *A. taxiformis*), CAT exhibited its highest activity in the 3-AT treatment (a 2.8-fold increase compared to CTR; $p < 0.05$; Fig. 3-B), whereas GST (Fig. 3-C) and T-AOC (Fig. 3-E) reached their highest levels in the 3-AT (2.4-fold and 1.5-fold increase, respectively; $p < 0.05$) and 6-AT (2.4-fold and 1.6-fold increase, respectively; $p < 0.05$) treatments. Moreover, during the 30-day biofortification period, LPO exhibited significant differences (1.2-fold increase) in the 3-AT treatment compared to the control group (CTR; $p < 0.05$; Fig. 3-A). When exposed

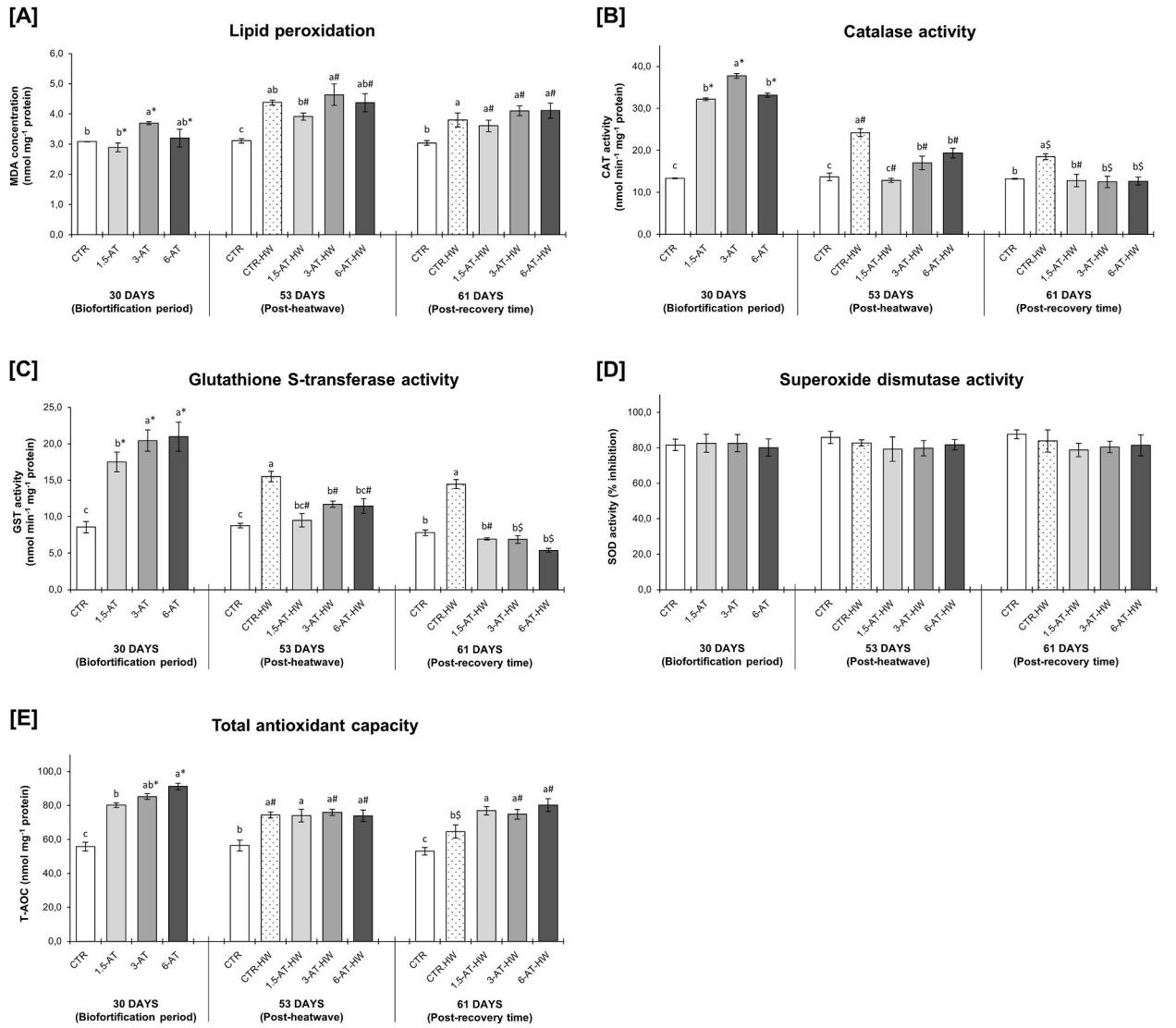

Fig. 3. Oxidative stress biomarkers in the spleen of *Diplodus sargus* juveniles after 30 days (T30, biofortification period), 53 days (T53, post-heatwave) and 61 days (T61, post-recovery time) of feeding trial: lipid peroxidation (**A**, expressed as MDA concentration, nmol mg^{-1} protein), catalase activity (**B**, nmol min^{-1} mg^{-1} protein), glutathione S-transferase activity (**C**, nmol min^{-1} mg^{-1} protein), superoxide dismutase activity (**D**, % inhibition) and total antioxidant capacity (**E**, nmol mg^{-1} protein) (mean ± SD; $n = 6$). Different lowercase letters indicate significant differences ($p < 0.05$) between treatments on the same sampling day. Different symbols (*, #, $) indicate significant differences ($p < 0.05$) between sampling days (T30, T53 and T61) for the same treatment. The absence of letters or symbols indicates no statistical differences ($p > 0.05$). ***Abbreviations:*** CTR – Control (commercial diet, under optimal growth conditions); 1.5-AT – diet containing 1.5 % *A. taxiformis*, under optimal growth conditions; 3-AT – diet containing 3 % *A. taxiformis*, under optimal growth conditions; 6-AT – diet containing 6 % *A. taxiformis*, under optimal growth conditions; CTR-HW – Control + Heatwave (commercial diet, exposed to a heatwave); 1.5-AT-HW – 1.5 % *A. taxiformis* + Heatwave (diet containing 1.5 % *A. taxiformis*, exposed to a heatwave); 3-AT-HW – 3 % *A. taxiformis* + Heatwave (diet containing 3 % *A. taxiformis*, exposed to a heatwave); 6-AT-HW – 6 % *A. taxiformis* + Heatwave (diet containing 6 % *A. taxiformis*, exposed to a heatwave).

to a marine heatwave (T53, post-heatwave), LPO levels significantly increased in all treatments compared to the unexposed CTR, with a 1.5-fold increase observed in 3-AT-HW, a 1.4-fold increase in both CTR-HW and 6-AT-HW, and the smallest increase of 1.3-fold in 1.5-AT-HW ($p < 0.05$; Fig. 3-A). The same pattern in LPO observed at T53 persisted after the recovery period (T61), with no significant differences between the post-heatwave and post-recovery periods ($p > 0.05$; Fig. 3-A). Following the marine heatwave (T53), CAT and GST activities increased in CTR-HW (~1.8-fold), 3-AT-HW (~1.3-fold), and 6-AT-HW (~1.3-fold) treatments ($p < 0.05$), while the 1.5-AT-HW treatment showed no significant differences compared to the unexposed control (CTR; $p > 0.05$; Fig. 3-B and 3-C). After the recovery period (T61), a significant decrease in CAT and GST activities were observed in 3-AT-HW and 6-AT-HW treatments ($p < 0.05$; Fig. 3-B and 3-C). T-AOC increased in all treatments (1.3-fold; $p < 0.05$; Fig. 3-E) after the MHW (T53) and remained stable until the recovery period (T61), except for the CTR-HW which decreased slightly compared to T53 ($p < 0.05$; Fig. 3-E). It is noteworthy that these three biomarkers, CAT, GST, and T-AOC, consistently exhibited lower values in T53 and T61 than those observed at T30. SOD activity did not differ significantly between treatments or when exposed to the MHW ($p > 0.05$; Fig. 3-D). Fulton's condition index (K) was significantly and positively correlated with CAT ($r = 0.323$ and $p < 0.05$) and SOD ($r = 0.265$ and $p < 0.05$), and splenosomatic index (SSI) was significantly and positively correlated with CAT ($r = 0.560$ and $p < 0.001$), SOD ($r = 0.394$ and $p < 0.001$) and GST ($r = 0.320$ and $p < 0.05$; Table 4). CAT and GST activities were positively correlated with T-AOC ($r = 0.604$ and $p < 0.001$, and $r = 0.517$ and $p < 0.001$, respectively), and LPO levels were negatively correlated with SOD ($r = -0.356$ and $p < 0.05$; Table 4).

3.5. Gene expression of immune and stress related genes

Gene expression of immune and stress biomarkers is presented in Fig. 4. Regarding *TCRβ*, the inclusion of different percentages of the macroalga *A. taxiformis* in the aquafeeds in the recovery period (T61) significantly decreased *TCRβ* expression levels in the 1.5%-AT condition (0.1-fold decrease; $p < 0.01$). Interestingly, the % of inclusion of *A. taxiformis* in the feeds led to an increase in *TCRβ* expression levels in a dose-dependent manner (not significant when compared to CTR-HW condition; $p > 0.05$; Fig. 4-A). No expression of *Lyz* was observed in the CTR and CTR-HW conditions (Fig. 4-B). The inclusion of *A. taxiformis* displayed a significant increase in *Lyz* gene expression in all biofortified feeds, with the highest effect observed in the 1.5%-AT treatment ($p < 0.01$; Fig. 4-B). Regarding *SOD1* expression no significant differences were observed between biofortified feeds, regardless of the inclusion level used ($p > 0.05$; Fig. 4-C) and the control condition. Concerning *GPX1* expression, all conditions with *A. taxiformis* displayed a decrease in *GPX1* expression when compared to CTR-HW ($p < 0.05$; Fig. 4-D), being similar to CTR levels (before heatwave exposure).

4. Discussion

The results obtained in this study highlight the dynamic nature of farmed fish physiology in response to fluctuating dietary and environmental parameters, offering pathways for further exploration of their adaptive mechanisms and ways to modulate possible detrimental effects. To the best of the authors' knowledge, this is the first study to investigate the effects of incorporating a macroalga, namely *A. taxiformis*, into aquafeeds under marine heatwave (MHW) conditions. Our research is pioneering in this area, as there are currently no other studies on this topic. Interpreting the present results in an integrated way and drawing comparisons with previous studies was, therefore, a challenging task. Still, it should be noted that, by shedding light on the interaction between macroalgal inclusion in aquafeeds and fish responses during MHWs events, this study addresses a significant gap in the literature and opens avenues for future research and development in this novel field.

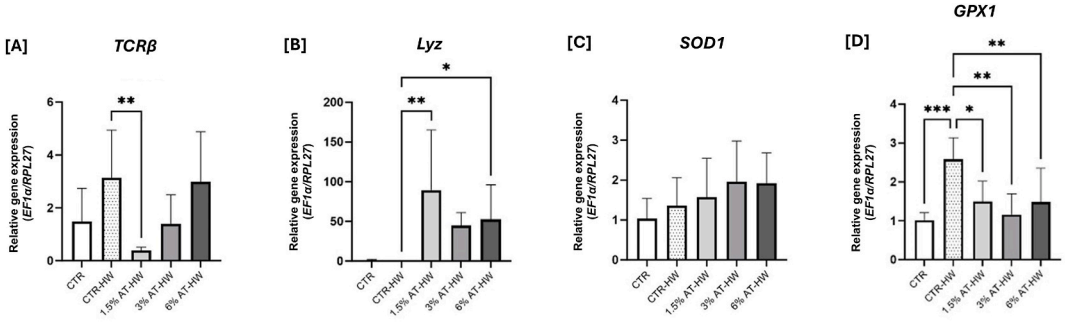

Fig. 4. Relative gene expression of immune (*TCRβ* and *Lyz*) and stress (*SOD1* and *GPX1*) related genes in the head-kidney of *Diplodus sargus* juveniles after 61 days (T61, post-recovery time) of feeding trial: (**A**) expression of T-cell receptor beta (*TCRβ*), (**B**) expression of lysozyme (*Lyz*), (**C**) expression of superoxide dismutase 1 (*SOD1*) and (**D**) expression of glutathione peroxidase 1 (*GPX1*). Transcript levels were determined by quantitative real-time PCR (qPCR) and normalized using the elongation factor 1-alpha (*EF1α*) and ribosomal protein L27 (*RPL27*) as housekeeping genes. Data are expressed as mean ± SD ($n = 3$ per treatment). Different *p*-values indicate significant differences among treatments (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). ***Abbreviations:*** CTR – Control (commercial diet, under optimal growth conditions); CTR-HW – Control + Heatwave (commercial diet, exposed to a heatwave); 1.5-AT-HW – 1.5 % *A. taxiformis* + Heatwave (diet containing 1.5 % *A. taxiformis*, exposed to a heatwave); 3-AT-HW – 3 % *A. taxiformis* + Heatwave (diet containing 3 % *A. taxiformis*, exposed to a heatwave); 6-AT-HW – 6 % *A. taxiformis* + Heatwave (diet containing 6 % *A. taxiformis*, exposed to a heatwave).

4.1. Responses at the whole organism level

Fish body condition indices and growth parameters are valuable indicators of health and welfare. Fish that remain in good condition are more adaptable to environmental challenges and associated stressors. This adaptability is attributed to their higher likelihood of having a robust and effective immune system, which not only contributes to their resilience to infections and diseases that are more prevalent in warmer conditions, but also facilitates physiological and behavioural adaptation to unusual abiotic factors [53].

This study showed that biofortification with *A. taxiformis* did not promote significant changes (regardless of inclusion percentages) in juvenile *D. sargus* at the individual level, as no significant differences in K, SSI and WGR were observed across treatments. It should also be noted that the K_{index} results were consistently high (>1), indicating that the fish were well-nourished throughout the trial. This suggests that the inclusion of *A. taxiformis* in the aquafeed did not affect the growth of juvenile *D. sargus*, meaning that the macroalga was efficiently digested and its nutritional benefits were readily available to the fish. These results are in line with previous studies highlighting the potential of various red macroalgae species as beneficial ingredients in aquafeeds to promote fish health and growth [33,54–58]. However, it is essential to recognise that different macroalgae species, at different concentrations, as well as different fish species or environmental factors, may yield different responses. For example, a study conducted by Guerreiro et al. [59] tested two species of macroalgae – a red species, *Chondrus crispus* (Rhodophyta) at 5 %, and a green species, *Ulva lactuca* (Chlorophyta) at 5 %, as well as a combination of both at 2.5 %. Contrary to what was expected, the inclusion of the red macroalgae at the highest inclusion percentage (5 %) resulted in reduced growth and adverse effects on body composition and lipid metabolism of juvenile *Sparus aurata*. Similarly, Araújo et al. [60] tested the inclusion of *Gracilaria vermiculophylla* (Rhodophyta) in rainbow trout (*Oncorhynchus mykiss*) diets and found that no detrimental effects were observed on inclusions up to 5 %, but higher inclusion levels resulted in impaired growth. These observations suggest that the incorporation of seaweed at an appropriate level can either significantly improve or maintain growth performance in a similar stage to non-biofortified diets, while higher inclusion levels may adversely affect fish growth and overall health. These results highlight the importance of previously carrying out an optimization of macroalgae dose when formulating tailor-made functional aquafeeds.

In our study, *D. sargus* demonstrated a remarkable resilience, with a 100 % survival rate (SR) across all experimental conditions, even when exposed to simulated high seawater temperatures that reached 28 °C (equivalent to the average peak temperature of a category II Mediterranean MHW during summer). This resilience has been previously reported in the studies conducted by Madeira et al. [61], which attained a critical thermal maximum (CTMax) of 34 °C for this species and by Vinagre et al. [62], which revealed the species' low thermal sensitivity and minimal mortality when exposed to temperatures between 18 °C and 30 °C. This resilience reflects the wide geographic distribution of *D. sargus*, which includes tropical and equatorial waters without upwelling. Despite the thermal plasticity of this species (and the survival rate observed in the present study), our results pointed out that the exposure to a MHW triggered a stress response, affecting fish metabolism and overall health and well-being.

While most growth indicators, including K_{index}, SSI_{index}, and WGR, did not show significant differences between treatments, the specific growth rate (SGR) exhibited a decline post-exposure to the MHW. This decline in SGR suggests that elevated seawater temperatures played a role in slowing down fish growth, as SGR is an important coefficient used to quantify the percentage increase in fish weight per day. Elevated seawater temperatures can induce stress in fish, negatively impacting their metabolic processes and overall well-being. The consistent decrease in SGR across all treatments suggests that the high seawater temperatures imposed considerable stress on the fish, leading to a generalized response that affected growth, regardless of biofortification. Unlike some previous studies [63–65], which linked warmer temperatures to enhanced growth prompted by increased metabolic and feeding rates, our results show a different trend. It is important to note that these divergent results may be due to the higher temperature (28 °C) used in our study, which induces greater stress on fish and results in reduced growth. For example, Chen et al. [66] observed increased SGR between 18 °C and 21 °C in juvenile hybrid sturgeon (*Acipenser baerii*♀ x *Acipenser schrenckii*♂), but reported a decrease in SGR values between 24 °C and 30 °C. After 8 days of post-MHW (T61), SGR values showed no signs of recovery. Several factors could be associated with this result, including: (i) persistence of stress, i.e. the effects of thermal stress may continue to affect the fish after the heatwave has ended, as the 15-day duration of the heatwave may have resulted in metabolic effects that extend beyond the direct exposure period; (ii) gradual recovery, i.e. the fish may not have been able to recover immediately after the stress event, as recovery is often a gradual process that takes time as fish re-establish their metabolic balance and repair any tissue damage; and (iii) permanent damage, as severe thermal stress can sometimes result in irreversible damage to organisms, including cellular or structural damage that cannot be fully reversed. Recovery from a stressful event is a process influenced by many variables, including the species and its life history, the magnitude and duration of the stress, and the experimental/environmental conditions themselves [67].

In terms of FCR, during the 30-day biofortification period, the treatment supplemented with 3 % *A. taxiformis* showed the lowest FCR value (0.64 ± 0.20; Table 3), aligned with the faster growth (i.e. higher SGR), revealing higher efficiency in converting feed into growth. Upon exposure to a MHW (T53, 28 °C), FCR values slightly increased in all treatment groups, without significant differences observed. This indicates that the inclusion of the macroalga *A. taxiformis* did not affected the FCR values, even under elevated temperatures. This outcome aligns with previous studies where dietary seaweed showed no significant impact on FCR in various fish species, including seabass (*Dicentrarchus labrax*) [68], senegalese sole (*Solea senegalensis*) [69], and gilthead seabream (*Sparus aurata*) [17,70].

4.2. Responses at cellular and haematological levels

The assessment of *D. sargus* juveniles' haematological profile, particularly under varying levels of *A. taxiformis* supplementation and exposure to a MHW, provides a comprehensive insight into how these factors intrinsically influence the health and physiological

responses of fish. Our study revealed a remarkable stability in the proportions of erythrocytes and leucocytes, with erythrocytes consistently representing over 94 % of total cells. This underscores the exceptional oxygen-carrying capacity of fish, even under thermal stress, which is vital for meeting metabolic demands associated with growth, energy production, and overall physiological functions [71,72]. However, within the high percentages of erythrocytes in each treatment, notable differences emerged when assessing erythrocyte nuclear abnormalities (ENAs). Non-biofortified fish unexposed to the heatwave (CTR treatment) consistently maintained the lowest percentage of ENAs (below 5 %) throughout the experiment, illustrating the sustained health of the erythrocyte population. On the other hand, the inclusion of *A. taxiformis* in the aquafeeds under optimal conditions (T30, 24 °C) significantly increased the ENAs percentage, regardless of the inclusion level (1.5 %, 3 % or 6 %). This may be due to nutritional imbalances, as the incorporation of *A. taxiformis* may alter overall dietary composition, affecting essential nutrients that are essential for DNA synthesis and repair. The thermal stress induced by MHW, significantly increased the number of ENAs, most likely as a result of exacerbated fish stress and, thus, increased production of reactive oxygen species, leading to cellular damage [73]. Still, this effect seemed to be somewhat counteracted by 1.5 % and 3 % of supplementation with *A. taxiformis*. This suggests a potential protective effect or mitigation of heatwave-induced adverse effects on erythrocyte nuclei at lower *A. taxiformis* supplementation levels, but not at the highest (6 %). This finding confirms the beneficial effects promoted by *A. taxiformis* in a dose-dependent manner. After the recovery period (T61), the percentages of ENAs did not differ significantly from values observed after the heatwave (T53) across all treatments, indicating that the fish erythrocyte nuclear health did not fully recover from prior exposure to the MHW.

Leucocytes play a key role in regulating inflammatory responses and are at the forefront of identifying and combating pathogens, including viruses and bacteria [74,75]. The low leucocyte percentages observed in all treatments (always below 6 %) indicate that there were no signs of infection or inflammation, underlining the absence of an active immune response following exposure to the experimental aquafeeds, even in treatments subjected to heat stress. The low leucocyte percentages are consistent with the experimental design, as they reflect a state of immune quiescence, i.e., the experimental conditions successfully maintained a controlled and pathogen-free environment. This was a positive sign, indicating that the fish did not face additional and unexpected health challenges during the experimental study.

Haematological studies in teleosts have suggested that Ht values may be useful as a general indicator of fish health, as fish on iron-deficient diets or those showing anaemia all have reduced Ht values [76]. Our study showed that biofortification with *A. taxiformis* at optimal temperatures (T30, 24 °C) did not result in significant differences in Ht levels in *D. sargus* at different algae inclusion levels (1.5 %, 3 % and 6 %), consistently maintaining around 40 % of red blood cells (RBCs) in all treatments. This result is consistent with another study that also found no significant differences in Ht in rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with *Gracilaria pygmaea* (Rhodophyta) [77]. Ht levels are directly influenced by temperature, as RBCs are responsible for supplying oxygen (O_2) to the cells, and the O_2 demand increases as temperature rises [78]. In response to this higher O_2 demand, the body tends to compensate by increasing the number of RBCs in the blood [76]. Unexpectedly, exposure to MHW (T53, 28 °C) induced a significant decrease in haematocrit, potentially related with a lower blood viscosity or even the onset of anaemia [76,79–81]. Meanwhile, the Ht values recovered during the recovery period (T61) and approached the levels observed before exposure to the heatwave (T30). This recovery suggests a degree of physiological adaptability in juvenile *D. sargus*, demonstrating that fish were able to recover Ht levels following transient environmental stress.

4.3. Immune humoral and antioxidant responses

As for humoral immune responses, IgM is a very important antibody in both adaptive and innate immune responses, actively recognising and neutralising invading pathogens to enhance the fish's ability to control infections [82]. Peroxidases are enzymes with a crucial microbicidal role, using hydrogen peroxide (H_2O_2) as a co-substrate to generate highly reactive species. These reactive species play an important role in phagocytic activities, causing damage and effectively eliminating pathogens and microbial invaders [83,84]. Finally, antiproteases plays a key role in neutralising pathogen proteases by using various protease inhibitors, (e.g. α1-antitrypsin (AAT) and α2-macroglobulin (α2M)) that have anti-enzyme activity. By regulating protease activity, antiprotease ensures effective immune processes by maintaining tissue integrity and avoiding damage to surrounding tissues [85]. The combination of these three immune components strongly contributes to the fish's resistance to infection, thereby promoting overall health and well-being.

In our study, a significant increase in IgM content and a decrease in POD activity were observed in fish biofortified with *A. taxiformis* under optimal conditions (T30, 24 °C). These responses were consistent across different percentages of macroalga inclusion (1.5 %, 3 % and 6 %), indicating a concentration-independent effect on these two biomarkers. The increased content of IgM suggests a robust immune response, highlighting the positive role of the macroalga in enhancing the fish's immune defence. The decrease in POD activity may also indicate effective regulation of oxidative stress, contributing to the overall health of the fish. Our results are in line with previous studies that have already demonstrated the immunomodulatory roles of *A. taxiformis* in immune parameters in several aquatic species, such as orbicular batfish (*Platax orbicularis*) [86], atlantic salmon (*Salmo salar*) [33], mottled rabbitfish (*Siganus fuscescens*) [31], shrimp (*Penaeus monodon*) [87], seabass (*Dicentrarchus labrax*) [35] and gilthead seabream (*Sparus aurata*) [35]. A plausible explanation for the observed effects could be attributed to the presence of bioactive compounds in *A. taxiformis*. This macroalga is recognized as one of the most promising species to produce bioactive metabolites [35]. Previous studies have shown that the genus *Asparagopsis* is rich in polysaccharides (such as sulphated galactan derivatives and mannitol) and also a variety of halogenated compounds, including ketones (e.g., 1,1-dibromo-3-iodo-2-propanone, 1,3-dibromo-2-propanone and 1, 3-dibromo-1-chloro-2-propanone (±) form), carboxylic acids (e.g, dibromoacetic acid, bromochloroacetic acid and dibromoacrylic acid) and alkanes (e.g., bromoform and dibromochloromethane), which confer powerful antimicrobial and anti-inflammatory properties [88–90]. In fact, the study conducted by Marino et al. [35] demonstrated the antibacterial activity of the whole macroalga

A. taxiformis and its extracts against fish pathogenic bacteria in European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), including species like *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio alginolyticus*, *V. vulnificus*, *Photobacterium damselae* subsp. *damselae*, *P. damselae* subsp. *piscicida*, *V. harveyi* and *V. parahaemolyticus*. Although our study did not include challenge tests, the results obtained suggest that *A. taxiformis* is a promising functional ingredient in aquafeeds with immunomodulatory effects. This finding becomes even more relevant when considering the imminent changes in future atmospheric conditions driven by the effects of climate change. Exposure to MHW (T53, 28 °C) led to an increase in IgM levels and peroxidase activity in the control treatment (CTR-HW), indicating a natural immune response triggered by the environmental stress of high temperatures. Interestingly, fish biofortified with 1.5 % *A. taxiformis* (1.5-AT-HW) had significantly higher IgM levels and POD activity compared to other inclusion levels (0 %, 3 %, or 6 %). Thus, the results suggest that the 1.5 % *A. taxiformis* diet may have a more pronounced immunomodulatory effect during the heatwave, enhancing the immune response of the fish. Following the recovery period (T61), IgM and POD activity levels declined as expected, since the animals were no longer under the stress of the heatwave, and the immune system did not need to maintain heightened activity. However, the 1.5 % *A. taxiformis* treatment maintained higher IgM levels than the other ones. This may indicate that the 1.5 % *A. taxiformis* diet has a sustained effect on the fish's immune response, providing a lasting benefit even in the absence of stress. It is a remarkable finding that suggests the potential long-term effects of *A. taxiformis* supplementation on the immune system of juvenile *D. sargus*.

Antiprotease activity was not significantly affected by the incorporation of *A. taxiformis* in the aquafeeds or by the MHW induced stress. Considering that the main role of these enzymes is to neutralise proteases produced by pathogens [85], future studies aimed at exploring the immunostimulatory potential of *A. taxiformis* should include challenge tests with fish pathogens in the experimental design, as such an approach is likely to produce more pronounced responses.

A harmonious interplay between immune responses and antioxidant defence mechanisms is essential to safeguarding the overall health and resilience of fish. The antioxidant defence system, which is responsible for neutralising harmful reactive oxygen species, plays a pivotal role in supporting the immune system by maintaining a balanced and functional physiological system [91].

In our study, different oxidative stress biomarkers were analysed in spleen, as it is the major secondary lymphoid organ in fish, being involved in a wide range of immunological functions (e.g. filtration of blood-borne pathogens and antigens), haematopoietic and red blood cell clearance mechanisms [92,93]. Results showed that the inclusion of the macroalga *A. taxiformis* in the aquafeeds under optimal conditions (T30, 24 °C) significantly increased CAT, GST and T-AOC levels, while SOD activity remained unchanged in all treatments. An increase in the activity of antioxidant enzymes is usually an indication of a greater presence of radicals that need to be scavenged. The presence of radicals may be related to an adaptation process to the new aquafeeds composition, as the introduction of macroalga may have contributed to the ingestion of undesired compounds (e.g. heavy metals, as *A. taxiformis* is considered to be a good biosorbent for heavy metals) [94–96], which may induce oxidative stress and, consequently, increase antioxidant enzyme activities. In addition, *A. taxiformis* is recognized for its high content of antioxidant compounds, including polyphenols (e.g., phenolic acids, flavonoids, tannins and halogenated derivatives), carotenoids (e.g., lutein, α-carotene and zeaxanthin) and polysaccharides (e.g., alginates and carrageenan) [25,26,91,97]. Although these substances play a crucial role in mitigating the effects of free radicals and protecting cells from oxidative stress, excessive levels of antioxidants can also have adverse effects. In a simplified way, this finding can be related to the concept of a "dose-response" relationship, where an increased concentration of antioxidants may adversely affect the health and overall well-being of the fish. Paradoxically, an excess of antioxidants, instead of neutralising free radicals, may generate reactive oxygen species, thereby inducing oxidative stress. Additionally, the production of lipid peroxides (LPO) was higher at higher concentrations of macroalga (especially at 3 %), leading us to believe that lower doses of *A. taxiformis*, particularly at 1.5 % inclusion, may be better for maintaining the redox state and avoiding tissue damage.

Results also showed that exposure to MHW induced oxidative stress, as demonstrated by the increase in LPO levels in all treatments exposed to high temperatures (T53, 28 °C). However, it should be noted that the diet with 1.5 % *A. taxiformis* showed a lower lipid peroxidation, even in relation to the control (CTR-HW; although without significant differences), which corroborates the beneficial effect of this aquafeed seen so far. The antioxidant responses, represented by the increase in CAT and GST activities in non-biofortified fish exposed to MHW (CTR-HW), as well as the increase in T-AOC, reflect the organism's efforts to neutralise the harmful effects of oxidative stress under challenging conditions. However, the 1.5 % *A. taxiformis* diet contributed to a reduction in CAT and GST activity, bringing these enzymes closer to levels of the non-biofortified fish reared at the optimal temperature (CTR). This suggests a potential modulating role of 1.5 % *A. taxiformis* diet in the antioxidant response during thermal stress conditions. Thus, the presence of low levels of macroalga in the aquafeeds composition appears to attenuate oxidative stress, as evidenced by dual effect on the reduction in lipid peroxidation and in the activity of antioxidant enzymes. The effects promoted by MHW on fish spleen antioxidant responses persisted beyond the recovery period (T61, 24 °C), particularly in terms of LPO and T-AOC, as both patterns remained similar to those observed after the heatwave (T53, 28 °C). This suggests that the duration of the recovery phase might have been insufficient to fully mitigate the oxidative stress induced by the temperature rise. Extending the recovery period could provide further insight into whether LPO levels could, at least, return to baseline levels observed before the heatwave (T30, 24 °C). Interestingly, after the recovery period (T61, 24 °C), a significant decrease in CAT and GST activities was observed in the treatments supplemented with 3 % and 6 % of *A. taxiformis*. This decrease could indicate a transition from an acute stress response to a recovery phase characterized by a normalization of antioxidant enzyme levels at the end of the heat stress. However, it is important to note that CAT and GST levels were significantly lower than those observed before the marine heatwave (T30, 24 °C), which could be due to the factors explained above. Therefore, it is plausible to consider a possible adaptive response promoted by the new aquafeed formulations over time. As the fish adapt to the new dietary composition and environmental conditions, their antioxidant enzyme levels may stabilize or undergo further adjustments.

4.4. Responses at molecular level

The analysis of differentially expressed genes confirmed the aforementioned trends, evidencing a differential expression of immune and antioxidant-related genes upon biofortification with *A. taxiformis* and/or the exposure to the MHW. The T-cell receptor (*TCR*), composed by two different protein chains (an alpha (α) and a beta (β) chain), is a protein complex found on the surface of T cells, or T lymphocytes [98]. *TCRβ* was shown to be upregulated in tilapia upon bacterial infection, indicating the activation and proliferation of T cells, which play a vital role in the adaptive immune response in fish [99] and is therefore a biomarker of adaptive immune response [100]. Lysozyme (Lys) is an important part of the innate immune system and exhibits strong antimicrobial activities. It protects against infections, acts as a natural antibiotic, and enhances the efficacy of other antibiotics, while it also strengthens the immune system [101]. The significant down-regulation of *TCRβ* gene expression in fish biofortified with 1.5 % of *A. taxiformis* points to a potential inhibition of adaptive immunity. However, when looking at *Lyz* expression, we observed that this gene was not expressed in CTR and CTR-HW treatments, whereas the incorporation of *A. taxiformis* in the feeds up-regulated its expression. This indicates an activation of the innate immune response in the presence of that *A. taxiformis* supplementation, which is in agreement with our results showing higher IgM levels in 1.5%-AT-HW conditions. The differences in these two immune-related genes might be explained by the different immune pathways they represent (innate vs adaptive) and by the time that each one of these pathways takes to be activated – the innate immune system is the first line of defence and the adaptive immune system usually takes several days to mount a significant response, as several mechanisms like selection of specific receptors, cellular proliferation and protein synthesis are required for the activation of adaptive immunity [102].

Looking at the expression of genes related with the antioxidant response, we observed that, although not significant, the inclusion of *A. taxiformis* in *D. sargus* diets led to an increase in *SOD1* expression (and, therefore, the occurrence of oxidative stress) that was more obvious in higher percentages of *A. taxiformis*. In accordance with a better response to stress, *GPX1* expression was significantly reduced in fish biofortified with *A. taxiformis* compared to non-biofortified ones (CTR-HW). Noteworthy, our results also show a significant *GPX1* expression increase after the MHW event. A similar response in oxidative stress markers was previously observed in other teleost fishes in response to different stressors [103–105], particularly thermal stress inducing oxidative stress in teleost species [106–109]. Altogether, the presence of *A. taxiformis* in fish diets potentiated the antioxidant response and diminished lipid peroxidation after a heat-related stress event. Pérez-Sánchez et al. [110] also showed that fish fed with a blend of vegetable oils in replacement of fish oil had a quantitatively and qualitatively lower relative expression of genes related to stress response when compared with fish fed with fish oil proteins. These results indicate that fish might conveniently adjust the stress response to a specific nutritional background and risk of oxidative stress [111], which is also consistent with our findings in this work.

5. Conclusions

An understanding of the complex interplay between dietary supplementation and environmental stressors, such as marine heatwaves, is essential for the optimization of aquafeeds formulation and to ensure the health and resilience of farmed fish.

Our findings demonstrate that supplementing aquafeeds with *A. taxiformis* does not compromise the overall health or growth performance of *D. sargus* maintained under optimal conditions. When exposed to a MHW, the physiological functions and general well-being of the fish were adversely affected, as evidenced by a decrease in SGR and Ht levels, together with an increase in ENAs and LPO, indicative of heightened oxidative stress. Encouragingly, supplementation with *A. taxiformis*, especially at lower inclusion levels, effectively mitigated these adverse effects, demonstrating its immunomodulatory potential. Notably, at a 1.5 % inclusion level, *A. taxiformis* not only preserved the overall fish health and growth performance but also enhanced immune responses, as evidenced by increased levels of IgM levels, POD activity and lysozyme expression during MHW conditions. Furthermore, *A. taxiformis* supplementation modulated antioxidant responses by reducing LPO, CAT and GST activity.

The inclusion of *A. taxiformis* in aquafeeds showed a clear improvement in the fitness and immune-antioxidant status of fish reared under sub-optimal conditions, particularly during MHWs. Given the costs involved in using "tailor-made" functional feeds, it is advisable to employ *A. taxiformis* biofortification as a prophylactic treatment, primarily during periods when seawater temperatures are expected to rise abruptly and MHW events strike the most. This strategy can help ensure that fish resilience and welfare are optimized and ready to face environmental challenges such as extreme weather events and disease outbreaks, thus, minimizing animal mortality and the inherent economical losses. In addition, the findings support the need of future studies aimed at exploring the immunostimulatory potential of *A. taxiformis* by including challenge tests with fish pathogens in the experimental design, as such an approach is likely to produce more pronounced responses. By integrating scientific knowledge with practical solutions, we can work towards ensuring the long-term sustainability and viability of aquaculture as a critical component of global food production systems.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Isa Marmelo: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cátia Lourenço-Marques:** Writing – review & editing, Investigation. **Iris A.L. Silva:** Writing – review & editing, Investigation. **Florbela Soares:** Writing – review & editing. **Pedro Pousão-Ferreira:** Resources. **Leonardo Mata:** Writing – review & editing, Resources. **António Marques:** Writing – review & editing, Supervision, Resources. **Mário Sousa Diniz:** Writing – review & editing, Supervision. **Ana Luísa Maulvault:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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