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Laminaria digitata Supplementation as a Climate-Smart Strategy to Counteract the Interactive Effects of Marine Heatwaves and Disease Outbreaks in Farmed Gilthead Seabream (*Sparus aurata*)

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Abstract

Extreme weather events, such as marine heatwaves (MHWs), pose serious threats to the aquaculture sector, facilitating the occurrence of disease outbreaks and compromising farmed animals' welfare and survival. Hence, finding eco-innovative strategies to improve animal immunocompetence is essential to assure aquaculture's sustainability and resilience in a rapidly changing ocean. This study evaluated the immunostimulatory potential of *Laminaria digitata* powder (0.3% and 1.5%) and extract (0.3%) in juvenile gilthead seabream (*Sparus aurata*) exposed to a *Vibrio harveyi* outbreak during a Category III MHW event (T = 25.7 °C). Overall, *L. digitata* supplementation did not significantly affect fish immunocompetence under optimal rearing conditions (T = 21.4 °C; no infection), nor did it induce any adverse effects. However, both the powder (1.5%) and extract (0.3%) forms of *L. digitata* supplementation effectively mitigated the negative impacts prompted by the MHW and

Vibrio harveyi infection—evidenced by improvements in fish health indicators, hematological parameters, leukocyte viability, granulocyte proportions, and reductions in peroxidase activity and immunoglobulin M levels. From an economic standpoint, supplementation with 1.5% *L. digitata* powder emerged as the most promising strategy, offering a practical balance between effectiveness and affordability for large-scale applications. These findings highlight the potential of *L. digitata* as an immunostimulatory aquafeed supplement, with promising benefits for fish health and resilience under adverse rearing conditions.

Keywords: aquaculture; disease outbreaks; extreme weather events; functional feeds; macroalgae; hematological parameters; immune response; leukocyte viability; macroalgae extracts; vibriosis

1. Introduction

Marine ecosystems are increasingly experiencing the profound impacts of climate change, with the intensification of extreme weather events—particularly marine heatwaves (MHWs)—emerging as one of the most critical environmental challenges [1]. MHWs are prolonged periods of unusually high sea surface temperatures, and their frequency, intensity, and duration have increased significantly in recent decades [1,2]. These events are projected to become even more frequent and severe, increasing the threat to marine biodiversity and ecosystem stability [1,2]. The ecological consequences of MHWs are far-reaching, disrupting the delicate balance of marine ecosystems by altering species distributions, transforming food web dynamics, and significantly compromising marine organisms' physiological and immune responses [2]. These disturbances jeopardize natural marine ecosystems and have profound implications for industries that depend on wild and farmed marine resources.

Aquaculture's rapid expansion is driven by increasing market demand, evolving global trade dynamics, and changing consumer preferences for safe, nutritious, and high-quality seafood products [3]. However, despite significant technological advances to support this growth, aquaculture remains highly vulnerable to environmental factors, particularly in outdoor systems, which are directly exposed to fluctuating conditions, such as in temperature, salinity, and water quality [4,5]. Among these, MHWs have emerged as a critical stressor, affecting farmed species' health, welfare, and productivity [5]. This is especially concerning for commercially valuable species, such as gilthead seabream (*Sparus aurata*), the top most produced finfish in the Mediterranean region [6,7]. Acute and intense environmental fluctuations, such as those occurring during MHW events, induce physiological stress in farmed fish and create favorable conditions for pathogen growth and transmission. Indeed, elevated seawater temperatures have been associated with opportunistic pathogens' proliferation and increased virulence [8,9]. Among the pathogens of concern is *Vibrio harveyi*, a Gram-negative, bioluminescent marine bacterium that thrives in warmer waters and a well-known causative agent of vibriosis in marine species [10]. In aquaculture systems, vibriosis is particularly harmful, leading to high levels of morbidity and mortality in farmed species, resulting in substantial economic losses for aquaculture producers [11,12]. The interaction between MHWs and bacterial infections, such as vibriosis, further compromises the health of farmed fish, increasing their vulnerability and susceptibility to disease outbreaks [6]. This complex interplay between pathogens and environmental factors highlights the urgent need to effectively address disease management in aquaculture to ensure the industry's sustainable growth and resilience and to safeguard animal welfare. In their efforts to manage disease outbreaks, aquaculture producers still rely on a limited number of government-approved antibiotics

and chemotherapeutics [13–15]. Yet, this chemical-based practice raises several ecological and public health concerns, as the introduction of pollutants into marine ecosystems contributes to the emergence of drug-resistant pathogens, further exacerbating the issue of antimicrobial resistance [14–16]. Furthermore, using antimicrobials poses additional risks, as drug residues can accumulate in farmed fish tissues, raising concerns about consumer safety and the overall quality of the aquaculture products [16,17].

In recent years, there has been growing interest in using probiotics and immunostimulants as alternative strategies to control disease outbreaks in aquaculture [16–18]. These approaches have particularly focused on plant-based products, such as herbs, seaweed, spices, and commercial plant products. Seaweed, in particular, has emerged as a promising feed ingredient due to its natural abundance of bioactive compounds that are known for their anti-inflammatory, immunomodulatory, antioxidative, and antibacterial properties [19–21]. Previous studies have demonstrated numerous benefits of incorporating seaweed into fish diets, particularly in modulating metabolism and digestion, and in boosting immune and antioxidative responses [21]. One example is the brown macroalga *Laminaria digitata*, which has been shown to significantly improve fish immunity [22,23] and metabolic functions [24], particularly under optimal growth conditions. The beneficial effects of *L. digitata* are largely attributed to its diverse profile of bioactive compounds, since this seaweed is known to be a source of numerous functional compounds, including polyphenols (such as phenolic acids, phlorotannins, flavonoids, and halogenated derivatives), carotenoids (e.g., fucoxanthin and β-carotene), and polysaccharides (e.g., alginates, fucoidan, laminarin, and mannitol) [25–29]. These properties make *L. digitata* a valuable dietary supplement in aquaculture, offering potential benefits for fish health and farm productivity.

However, despite the promising findings acquired so far, the effects of seaweed supplementation on the immune responses of fish exposed to environmental stressors—such as MHWs and pathogens—remain poorly understood.

Within this context, the present study aimed to investigate the effects of MHWs on the immune responses of gilthead seabream (*S. aurata*), both in isolation and in combination with exposure to a *V. harveyi* outbreak. Additionally, the study sought to assess the efficacy of *L. digitata* powder and extracts as a dietary supplement to enhance the immunocompetence of gilthead seabream in response to these environmental and microbiological stressors. To this end, a multifaceted assessment was conducted, combining blood cell identification, counts, and viability through flow cytometry, hematological, and humoral immune parameters.

2. Materials and Methods

2.1. Seaweed Extract and Experimental Diets

L. digitata was harvested by boat at an approximate depth of 5–10 m during mid to high tide in the Parc Marin d'Iroise, a conservation area off the west coast of Brittany (France) using "Scoubidou" technology [30]. Subsequently, the seaweed was transported to the Algaia factory in Lannilis, where it was selected and dried using a bench-top fluid-bed dryer (TG200; Retsch, Haan, Germany). Dried seaweed samples were ground, sieved (0.75 mm stainless steel sieve), and stored in sealed plastic bags at room temperature in the dark.

The *L. digitata* extract was prepared by subcritical water extraction (SWE) using a 400 mL Parr Reactor (Series 4560 high-pressure mini-reactors; Parr Instrument Company, Moline, IL, USA) equipped with a Parr Reactor Controller (Series 4848, Parr Instrument Company, Moline, IL, USA). Extraction was performed at 40 bar, 180 °C, with an extraction time of 50 min and using a 30:1 liquid-to-solid ratio. During extraction, the sample was agitated with a four-blade impeller at 200 rpm. After the SWE, the extracts were

filtered through Whatman n° 1 paper, centrifuged (Sigma 3-30KS; Sigma, Schnelldorf, Germany) at 8000 rpm for 5 min, and frozen at −80 °C for subsequent lyophilization (Cryodos −80 model; Telstar, Barcelona, Spain). Afterwards, samples were stored at 4 °C until use.

Both the dried seaweed powder and extract were sent to a company specializing in fish feed production (SPAROS Lda, Olhão, Portugal), which formulated four experimental diets of similar nutritional composition (detailed feed compositions can be found in Appendix A, Table A1): (i) a commercial control diet without seaweed supplementation (0%; CTR); (ii) a diet supplemented with 0.3% *L. digitata* powder (P 0.3%); (iii) a diet supplemented with 1.5% *L. digitata* powder (P 1.5%); and (iv) a diet supplemented with 0.3% *L. digitata* extract (EXT 0.3%).

The powdered ingredients were first blended and milled (<200 µm) using a micropulverizer hammer mill (SH1; Hosokawa-Alpine, Augsburg, Germany). Afterwards, oils were incorporated into the powder blends, which were humidified with 25% water and agglomerated through a low-shear and low-temperature extrusion process (ITALPLAST, Italy). The resulting 2.0 mm pellets were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4 h at 55 °C. The diets were then packaged, sealed, and transported to the research facility.

2.2. Modulation of Marine Heatwaves

Sea surface temperature (SST) data from Mediterranean coastal areas were obtained from the Climate Data Record (CDR) of the National Oceanic and Atmospheric Administration (NOAA), specifically focusing on representative locations where *S. aurata* farms are settled, i.e., in Greece and Turkey (coordinates 38°33′10.9″ N 25°02′41.4″ E and 38°06′12.0″ N 25°03′53.0″ E). The data set covered a period of 30 years (1993–2023).

The intensity, onset and offset rates, as well as the duration of MHW events were analyzed using the R software version 4.4.2 (R Core Team, Vienna, Austria), more specifically, employing the "heatwaveR" package [31], and following standardized MHW definitions [32]. In addition, the "heatwaveR" package was used to obtain the Yearly Temperature Model (YTM), which represents the average daily temperature values expected on each day of the year. This model allowed for the determination of temperature thresholds necessary for classifying MHW events. In our study, the threshold for MHW categorization was set at 1.424 °C above the YTM baseline. A baseline temperature of 21.4 °C was selected based on the average SST expected on June 16th, near the onset of summer. A Category III MHW event was simulated, in which the peak of MHW temperature reached 25.7 °C. This peak was determined by adding three times the threshold value to the control temperature (21.4 °C + (3 × 1.424 °C)). The simulated temperature rise occurred at a rate of 0.5 °C per day over a 10-day period, reflecting the onset phase of the MHW. Once the peak temperature was reached, it was maintained for a plateau period of 7 days (Figure 1).

2.3. Vibrio harveyi Bacterial Culture

For the bacterial challenge, a *V. harveyi* pathogenic strain isolated from moribund fish during a disease outbreak at a commercial farm and provided by the Aquaculture Research Station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) was used. The strain was initially cryopreserved in 25% (*v*/*v*) glycerol at −80 °C and then cultured on Tryptic Soy Agar (TSA, Sigma-Aldrich, Deutschland, Germany) at 24 °C for 48 h with continuous shaking (160–180 rpm) to promote exponential growth. Bacterial cells were then collected by centrifugation (3000 rpm, 10 min) and resuspended in sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; Sigma-Aldrich, St. Louis, MO, USA). To determine the

bacterial concentration, serial dilutions of the suspension were plated on TSA, followed by incubation at 24 °C for 48 h. Colony-forming units (CFU) were counted to confirm the bacterial concentration. The bacterial suspension was adjusted to an optical density (OD) of 1 at 600 nm, corresponding to an estimated 1×10^9 CFU mL^{-1} concentration. Finally, a bacterial inoculum suspension was prepared under sterile conditions, taking as reference the sub-lethal concentration previously determined (i.e., final concentration: 4.9×10^8 CFU mL^{-1}) for this specific strain during pilot LC50 trials with *S. aurata*.

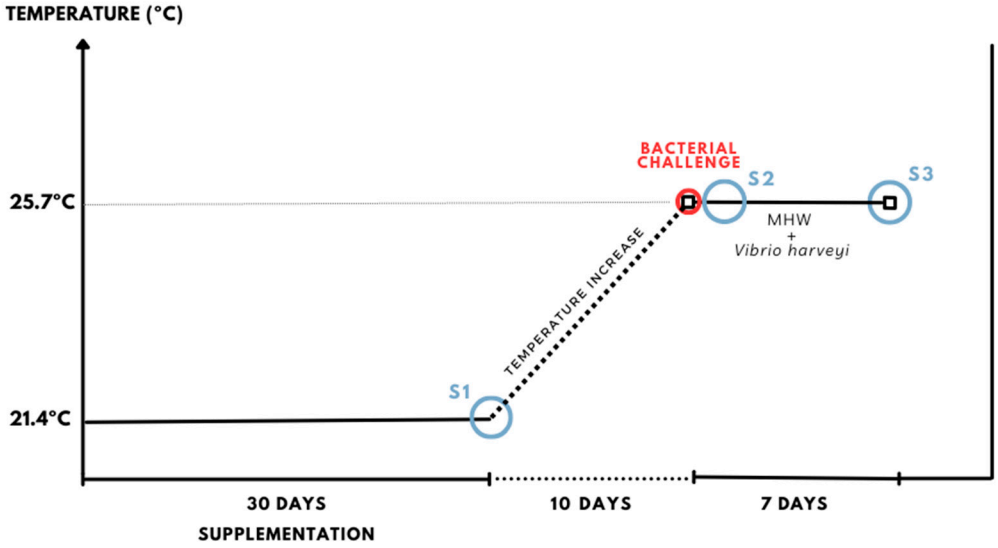


Figure 1. Experimental timeline of supplementation, marine heatwave exposure, and pathogen (*V. harveyi*) challenge. Abbreviations: MHW (marine heatwave); S1 (first sampling point; after 30 days of supplementation under optimal conditions. No MHW or bacterial challenge was applied at this experimental stage.); S2 (second sampling point; 24 h after exposure to the bacterial pathogen *V. harveyi* under MHW conditions); S3 (third and final sampling point; 7 days after the *V. harveyi* challenge and MHW exposure).

2.4. Sparus aurata and Acclimation Period

Specimens of *S. aurata* were reared until the juvenile stage (weight (W): 29.7 ± 4.9 g; total length (TL): 12.2 ± 0.6 cm; mean ± SD, $n = 243$) at EPPO-IPMA (Olhão, Portugal) under routine hatchery conditions and transported to IPMA's Live Marine Organisms Laboratory (LABVIVOS) in Algés, Portugal. Subsequently, the fish were equally distributed into quarantine tanks (660 L total capacity each), where they were maintained for 3 weeks under the following abiotic conditions, similar to those registered at the rearing site: (i) temperature: 21.4 ± 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; (v) photoperiod: 14 h light/10 h dark. The seawater temperature set during acclimation as well as during the control treatment (21.4 °C) is in accordance with the average values in Mediterranean coastal areas (where fish farms are settled), obtained upon modulation of the MHW (see Section 2.2). Throughout the quarantine period, the animals were hand-fed twice a day with a commercial control diet, provided at 2% of their average body weight (bw). The proximate composition of this diet was adjusted to meet the specific nutritional requirements of juvenile *S. aurata*.

2.5. Experimental Design and Fish-Rearing Conditions

Following a 3-week acclimation period, fish were randomly distributed into 27 rectangular glass tanks (200 L each), housed within independent recirculating aquaculture systems (RASs). The experimental design (Appendix A, Figure A1) encompassed three stages (S1, S2, and S3):

- **S1 (prophylactic supplementation phase)**: For 30 days, fish were hand-fed twice daily (2% bw) with experimental diets under optimal temperature conditions (21.4 °C; Figure 1). Each treatment consisted of 27 fish divided into three replicates (9 fish/tank; Appendix A, Figure A1). The dietary treatments were as follows: (i) CTR (control): non-supplemented commercial diet for *S. aurata* juveniles; (ii) P 0.3%: diet supplemented with 0.3% *L. digitata* powder; (iii) P 1.5%: diet supplemented with 1.5% *L. digitata* powder; and iv) EXT 0.3%: diet supplemented with 0.3% *L. digitata* extract. After the 30-day supplementation period, 6 fish per treatment (2 fish/tank) were sampled (indicated by the blue tanks and "scissors" symbol in Appendix A, Figure A1). An equal number of fish were removed from the other treatments to ensure population density consistency.
- **S2 (temperature-increase ramp and bacterial challenge)**: A gradual MHW ramp was initiated over 10 days (0.5 °C/day; Figure 1), except for the CTR group, which was kept at optimal conditions throughout the experiment. The feeding regime and diets remained the same as in S1. At this stage, each treatment consisted of 21 fish (7 fish/tank; Appendix A, Figure A1). Once the temperature reached 25.7 °C, the treatments CTRHW PAT, P 0.3% HW PAT, P 1.5% HW PAT, and EXT 0.3% HW PAT were challenged with *V. harveyi* (Figure 1). Anesthetized fish were intraperitoneally injected with 100 µL of a bacterial suspension (4.9×10^8 CFU mL^{-1}). The other groups (CTR, CTRHW, P 0.3% HW, P 1.5% HW, and EXT 0.3% HW) were injected with 100 µL of sterile PBS. At 24 h post-challenge, 6 fish per treatment (2 fish/tank) from the pathogen-challenged groups and the CTRHW group were sampled (represented by the blue tanks and "scissors" symbol in Appendix A, Figure A1). An equal number of fish were removed from the other treatments to maintain a consistent population density.
- **S3 (exposure to MHW and pathogen)**: During a 7-day period, fish were exposed to either an MHW (25.7 °C) or a combination of an MHW and *V. harveyi* challenge (Figure 1). The feeding regime and diets remained the same as in S1 and S2. Each treatment consisted of 15 fish (5 fish/tank; Appendix A, Figure A1). After the 7-day MHW exposure, 6 fish per treatment (2 fish/tank) were sampled for further analysis (Appendix A, Figure A1).

To maintain the seawater quality and to keep abiotic parameters at the defined levels, each system was equipped with protein skimmers (Tornado 120; Mantis), physical filtration, consisting of a filter bag (400 µm; TMC Iberia, Lisbon, Portugal), filter sponge, and glass wool; biological filtration (Bio Balls 1.5″ Aquarium Pond Filter; TMC Iberia, Portugal), an ultraviolet water sterilizer (ClearUVC-36; EHEIM, Deizisau, Germany), and submerged air stones. The temperature was controlled by submerged digital heaters (300W, V2Therm Digital Heaters; TMC Iberia, Portugal) and automated cooling systems (Foshan Weinuo Refrigeration Equipment Co., Ltd., Foshan, China), both connected to a computerized control system (ProfiLux 3 Outdoor; GHL, Bonn, Germany). This system continuously monitored the temperature via individual sensors (PT 1000; GHL, Germany) and adjusted it every 3 s. The temperature was measured daily with a thermometer (TFX 430; Ebro Electronic, Ingolstadt, Germany), while other abiotic parameters (pH, salinity, and dissolved oxygen) were measured with a multi-parameter device (Multi 3420 SET G; WTW, Weilheim, Germany) connected to a digital pH electrode (SenTix® 940; WTW, Germany), a digital conductivity cell (TetraCon® 925; WTW, Germany), and an optical dissolved oxygen sensor (FDO® 925; WTW, Germany). Seawater parameters were adjusted as necessary to maintain dissolved oxygen at 7.2 ± 0.2 mg L^{-1}, the salinity at 35.0 ± 0.5 ‰, and the pH at 8.0 ± 0.1 units. A photoperiod of 14 h light/10 h dark was maintained throughout the experiment. Total ammonia (NH_3, NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) levels were monitored weekly

using colorimetric test kits (Salifert, Duiven, The Netherlands), and kept below detectable limits (except for nitrates, which were kept at <50 mg L^{-1}).

2.6. Fish Sampling Procedures

Three sampling points were performed: (i) S1, after a 30-day prophylactic supplementation period; (ii) S2, following a 10-day temperature-increase ramp and 24 h after bacterial challenge; (iii) S3, after a 7-day exposure to either an MHW or a combination of MHW and bacterial challenge (Figure 1). Prior to each sampling point, fish were fasted for 24 h. Six fish per treatment (i.e., two fish from each replicate tank) were randomly collected and anesthetized by immersion in an overdosed solution of tricaine methanesulfonate (2 g L^{-1} of MS-222; Acros Organics, Geel, Belgium) and buffered with sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, USA). Once anesthetized, the fish were weighed and measured, and peripheral blood was collected from the caudal vein using heparinized syringes. A portion of the blood was used for hematological analyses, while the remain portion was transferred into microtubes (1.5 mL) containing 20 µL of heparin (3000 U mL^{-1} in 0.9% NaCl; Sigma-Aldrich, USA). The blood was then centrifuged at 10,200 rpm for 10 min at 4 °C (AccuSpin Micro 17R Centrifuge; Fisher Scientific, Schwerte, Germany) to isolate the plasma and stored at −80 °C for later analysis of innate humoral parameters. Subsequently, the fish were euthanized via a cervical cut, and head kidney samples were aseptically collected for leukocyte isolation. Spleen samples were also collected for DNA extraction and pathogen detection via PCR amplification.

2.7. Confirmation of Vibrio harveyi Infection

2.7.1. DNA Extraction

Disruption of spleen samples (pool of 3 spleens/treatment) was performed using a TissueLyser II (Qiagen, Venlo, The Netherlands) for 40 s at a frequency of 30 s^{-1} 3 times in 400 µL of Tris-EDTA buffer (pH 7.5). DNA was extracted using a Maxwell RSC Tissue DNA kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA yield and purity were determined by measuring the absorbance at 260 nm and 280 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were diluted to a final concentration of 50 ng μL^{-1} for the PCR reaction.

2.7.2. Pathogen Detection Using PCR Amplification

Specific primers for PCR were designed to amplify a 316 bp fragment of the *V. harveyi* gyrB gene. The primers' specificity was previously determined by PCR amplification of extracted DNA from other species of the genus *Vibrio* and other bacterial genera, including *Photobacterium*, *Tenancibaculum*, *Pseudomonas*, and *Aeromonas*.

The PCR reaction consisted of 1 µL of DNA sample (50 ng), 10 µL of PCRBio Taq Mix Red DNA Polymerase (PCR Biosystems, London, UK), 1 µL of forward primer GAGCGCGAAGATGGTATCTC (0.4 µM), 1 µL of reverse primer CAACCGCTGACTTCACTTCA (0.4 µM), and 12 µL of nuclease-free water. PCR reactions were carried out in a thermal cycler (Biometra Trio, AnalytikJena, Jena, Germany) using the following conditions: an initial polymerase activation of 95 °C for 5 min, followed by 35 cycles of a denaturation step at 95 °C for 30 s, an annealing step for 30 s at 57 °C, and an extension step at 72 °C for 30 s, and a final extension step of 72 °C for 10 min, followed by storage at 4 °C. Nuclease-free water was used as the negative control, and DNA extracted from a pure colony of *V. harveyi* was used as the positive control. A 10 µL aliquot of a molecular marker (ladder VI, NZytech, Lisbon, Portugal) and 10 µL of each PCR product were then subjected to electrophoresis in a 1.5% (*w*/*v*) agarose gel, and the images were collected using Chemidoc XRS+ (Bio-Rad, Algés, Portugal) with Image Lab software (Bio-Rad).

2.8. Hematological Parameters

Red blood cell (RBC) and white blood cell (WBC) counts were performed using a Neubauer hemocytometer with fresh blood diluted in phenol-red-free Hank's balanced salt solution (HBSS; Sigma-Aldrich, Germany).

The hemoglobin (Hb) concentration was determined using a colorimetric detection kit (Arbor Assays, Ann Arbor, MI, USA) based on the cyanmethemoglobin method [33].

The hematocrit (Ht) was measured by drawing blood into microhematocrit tubes (Marienfeld Superior, Lauda-Königshofen, Germany) by capillary action, sealing one end with wax, and centrifuging the tubes at 10,200 rpm for 5 min (AccuSpin Micro 17R; Thermo Fisher Scientific, USA). After centrifugation, the length of the packed red blood cell column was measured.

The mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were calculated as follows:

$$\mathrm{MCH}(\mathrm{pgcell}^{-1}) = \left(\frac{\mathrm{Hb}}{\mathrm{RBC}}\right) \times 10 \quad (1)$$

$$\mathrm{MCV}(\mu\mathrm{m}^3) = \left(\frac{\mathrm{Ht}}{\mathrm{RBC}}\right) \times 10 \quad (2)$$

$$\mathrm{MCHC}(\mathrm{g\ 100\ mL}^{-1}) = \left(\frac{\mathrm{Hb}}{\mathrm{Ht}}\right) \times 100 \quad (3)$$

2.9. Head Kidney Leucocyte Isolation and Flow Cytometry Analysis

Head kidney leukocytes were isolated under sterile conditions from six fish per experimental condition, following the Percoll-purification-based protocol described in detail by Marmelo et al. [34].

Leukocyte viability was assessed using the LIVE/DEAD™ Fixable Dead Cell Stain Kit (Life Technologies Europe, Bleiswijk, The Netherlands).

All cytometric cell measurements were performed with an Attune NTx flow cytometer (ThermoFisher Scientific, Waltham, MA, USA) and analyzed with FlowJoTM v10.9.1 software (BD Life Sciences, Franklin Lakes, NJ, USA). Flow cytometer performances were checked daily using quality controls (Performance Test Beads; ThermoFisher Scientific). Cell cytograms were analyzed from a minimum of 10,000 events recorded at an acquired rate of 200 events/second. Partial differentiation of leukocyte subpopulations was performed based on the forward- and side-scatter parameters, as described by Marmelo et al. [34]. Three cell populations were established: (i) lymphocytes, (ii) monocytes, and (iii) granulocytes (Figure 2C). The threshold that allows live and dead cells to be distinguished was established based on the levels of dye-staining leukocytes subjected to a mild heat shock (Figure 2D).

2.10. Innate Humoral Parameters

Plasma samples were used to assess the humoral immunological parameters, following well-established protocols, i.e., total antiprotease activity (or trypsin activity inhibition) was determined according to Hanif et al. [35], immunoglobulin M was measured by the indirect enzyme-linked immunosorbent assay (ELISA)-based protocol described by Cuesta et al. [36], and peroxidase activity was assessed according to Quade and Roth [37]. All minor modifications to the aforementioned methods are described in detail in Marmelo et al. [22].

2.11. Statistical Analysis

Statistical analyses were conducted at a significance level of 0.05 using STATISTICA™ software (Version 7.0, StatSoft Inc., Tulsa, OK, USA). Prior to analysis, all data were tested

for normality using the Kolmogorov–Smirnov test and for homogeneity of variances with Levene's test. Data meeting these assumptions were analyzed using a one-way ANOVA. When significant differences were detected, Tukey's HSD post hoc test was applied to identify significant differences (p-value < 0.05).

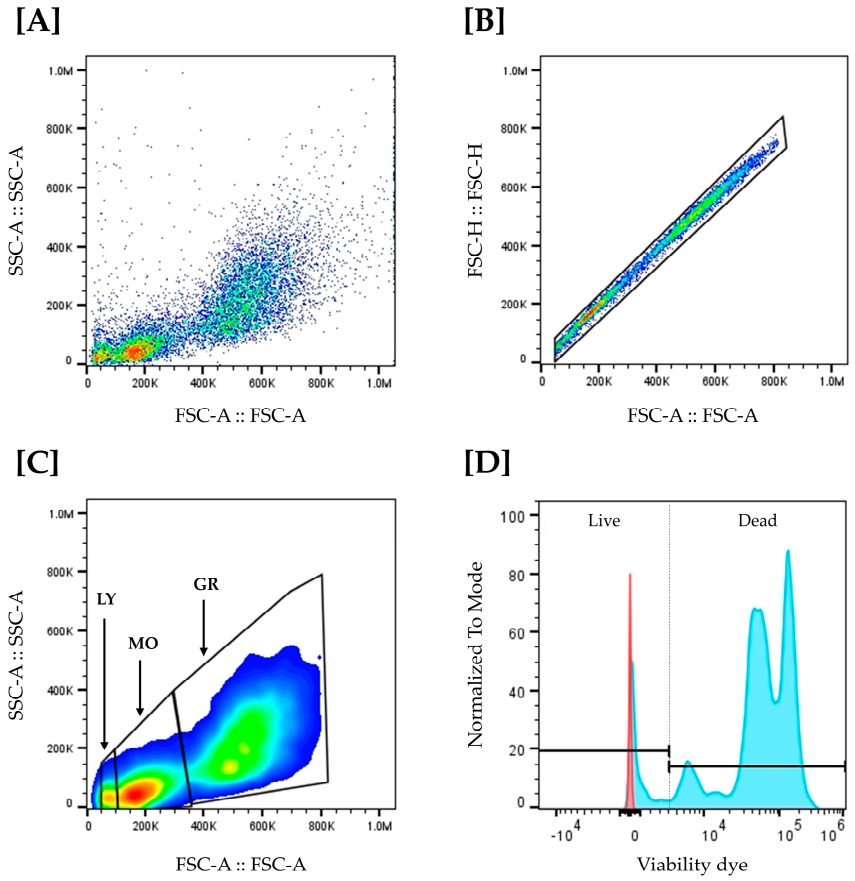


Figure 2. Flow cytometry gating strategy used to determine the leukocyte subpopulation distribution in the whole leukocyte suspension. (**A**) is a representative graph of the Forward Scatter (FSC)-A and Side Scatter (SSC)-A profile of collected events; (**B**) shows the multiplet exclusion based on the definition of the singlet region, using the linearity between FSC-A and FSC-H, which measure the area and height of the signal, respectively; (**C**) represents the estimation of leukocyte subpopulations based on FSC-A/SSC-A, after multiplet exclusion, i.e., lymphocytes (LY), monocytes (MO), and granulocytes (GR); (**D**) is a histogram showing the Live/Dead Viability dye staining. To set the threshold to distinguish between live and dead cells, leukocytes were subjected to a mild heat shock (50 °C, 7 min) and then stained with a viability dye. Positive high-staining-intensity cells (++) are the dead cells, and low-staining positive cells (+−) are the live cells.

3. Results

3.1. Confirmation of Vibrio harveyi Infection

PCR analysis confirmed the presence of *V. harveyi* at sampling point S2 (24 h post-challenge under MHW conditions) across all the treatment groups (infected tanks), as evidenced by the distinct fluorescent band size (Figure 3A). Notably, fish supplemented with the 0.3% *L. digitata* extract (EXT0.3% HW PAT) at S2 exhibited a faint band, suggesting a lower spleen bacterial load compared with fish from other treatments. *V. harveyi* was not detected at sampling points S1 (before MHW and bacterial challenge) and S3 (7 days post-challenge under MHW conditions) or in non-infected tanks at S2, indicating that this bacterium was either absent or present in levels below the detection limit.

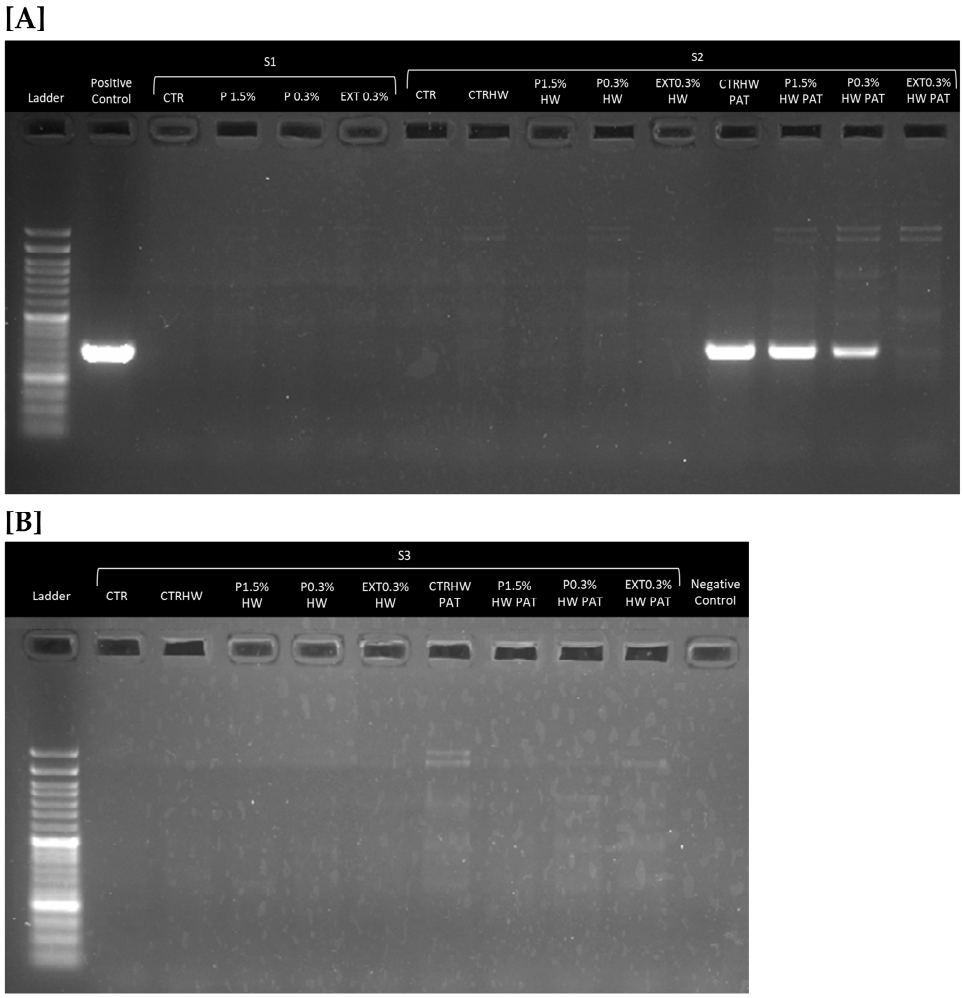


Figure 3. *V. harveyi* detection by PCR in fish spleen samples taken after treatment under different conditions. (**A**,**B**) *Ladder*, DNA ladder VI (50–1500 base pairs; Nzytech); S1, sampling point before exposure to MHW and *V. harveyi* challenge; S2, sampling point at 24 h post-*V. harveyi* challenge under MHW conditions; S3, sampling point at 7 days post-*V. harveyi* challenge and MHW exposure; Positive control, sample with a positive control for *V. harveyi*; Negative control, sample containing water instead of *V. harveyi*; CTR, commercial control diet; P 0.3%, diet supplemented with 0.3% *L. digitata* powder; P 1.5%, diet supplemented with 1.5% *L. digitata* powder; EXT 0.3%, diet supplemented with 0.3% *L. digitata* extract; HW, heatwave; PAT, pathogen.

3.2. Mortality Rates After Vibrio harveyi Challenge

Figure 4 presents the cumulative mortality of gilthead seabream over a 7-day period of MHW following challenge with *V. harveyi*. No mortality was observed in non-infected tanks/treatments, regardless of temperature conditions (i.e., CTR, CTRHW, P0.3% HW, P1.5% HW, and EXT0.3% HW treatments). The treatments fed with the commercial diet (i.e., non-supplemented aquafeed; CTRHW PAT) and supplemented with 0.3% *L. digitata* powder (P0.3% HW PAT) exhibited the highest mortality rates (32.5% and 30.0%, respectively). The treatments with 1.5% *L. digitata* powder (P1.5% HW PAT) and 0.3% *L. digitata* extract (EXT0.3% HW PAT) showed significantly lower mortality rates (i.e., 7.3% and 7.7%, respectively).

3.3. Hematological Parameters

3.3.1. Effects of Seaweed Supplementation in Non-Infected Fish Under Optimal (S1) and Marine Heatwave (S3) Conditions

Table 1 shows the hematological parameters after 30 days of supplementation under optimal conditions (21.4 °C; S1) and following 7 days of exposure to an MHW (25.7 °C; S3).

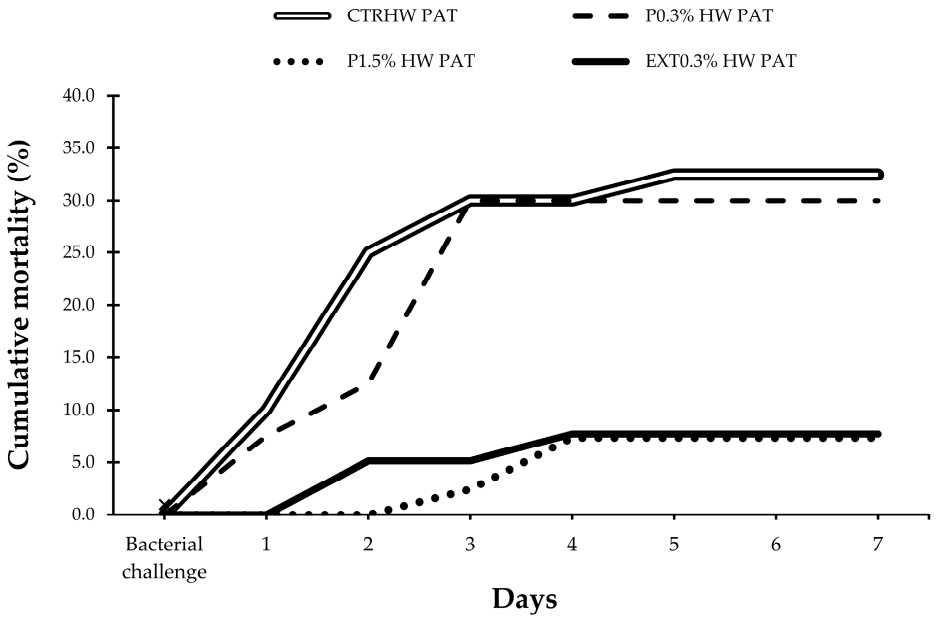


Figure 4. Cumulative mortality (%) of gilthead seabream (*S. aurata*) over the 7-day plateau period in the exposure to an MHW and following intraperitoneal injection of *V. harveyi* (CTRHW PAT, P0.3% HW PAT, P1.5% HW PAT, and EXT0.3% HW PAT) (n = 21).

Table 1. Hematological parameters in non-infected gilthead seabream (*S. aurata*) supplemented with *L. digitata* under optimal (S1; 21.4 °C) and MHW conditions (S3; 25.7 °C). The table shows (i) the effects of supplementation under optimal conditions (comparisons between control (CTR) and supplemented groups P 0.3%, P 1.5%, and EXT 0.3% at S1); (ii) the effects of the MHW without supplementation (comparison between CTR and CTRHW at S3); and (iii) the effects of supplementation under MHW conditions (comparisons between CTRHW and P 0.3% HW, P 1.5% HW, and EXT 0.3% HW at S3). Parameters include red blood cells (RBCs; $\times 10^6$ μL^{-1}), white blood cells (WBCs; $\times 10^5$ μL^{-1}), mean corpuscular volume (MCV; μm^3), mean corpuscular hemoglobin (MCH; pg $cell^{-1}$), mean corpuscular hemoglobin concentration (MCHC; g 100 mL^{-1}), hemoglobin (Hb; g dL^{-1}), and hematocrit (Ht; %) (n = 6 per treatment). Data are presented as the fold-change relative to the respective control group, with statistical significance ($p < 0.05$) indicated where applicable (n.s. = not significant; ↑ X-fold = increase; ↓ X-fold = decrease). A detailed overview of the results of the hematological parameters can be found in Table S1 in the Supplementary Material file.

	Effect of Supplementation (No MHW)			**Effect of MHW (No Supplementation)**			**Effect of Supplementation Under MHW Conditions**		
	Comparisons Against CTR at S1 (Fold-Change)			**Comparisons Against CTR at S3 (Fold-Change)**			**Comparisons Against CTRHW at S3 (Fold-Change)**		
RBCs ($\times 10^6 \mu L^{-1}$)	**S1**	P 0.3%	n.s.	**S3**	CTRHW	n.s.	**S3**	P 0.3% HW	n.s.
		P 1.5%	n.s.					P 1.5% HW	n.s.
		EXT 0.3%	n.s.					EXT 0.3% HW	n.s.
WBCs ($\times 10^5 \mu L^{-1}$)	**S1**	P 0.3%	n.s.	**S3**	CTRHW	n.s.	**S3**	P 0.3% HW	n.s.
		P 1.5%	n.s.					P 1.5% HW	n.s.
		EXT 0.3%	n.s.					EXT 0.3% HW	n.s.
MCV (μm^3)	**S1**	P 0.3%	↑ 0.5-fold	**S3**	CTRHW	n.s.	**S3**	P 0.3% HW	n.s.
		P 1.5%	↑ 0.4-fold					P 1.5% HW	n.s.
		EXT 0.3%	↑ 0.4-fold					EXT 0.3% HW	n.s.
MCH (pg $cell^{-1}$)	**S1**	P 0.3%	↑ 0.4-fold	**S3**	CTRHW	↓ 0.3-fold	**S3**	P 0.3% HW	↑ 0.3-fold
		P 1.5%	n.s.					P 1.5% HW	n.s.
		EXT 0.3%	n.s.					EXT 0.3% HW	↑ 0.4-fold

Table 1. *Cont.*

	Effect of Supplementation (No MHW)			Effect of MHW (No Supplementation)			Effect of Supplementation Under MHW Conditions		
MCHC (g 100 mL^{-1})	**S1**	P 0.3%	n.s.	**S3**	CTRHW	n.s.	**S3**	P 0.3% HW	↑ 0.2-fold
		P 1.5%	n.s.					P 1.5% HW	↑ 0.2-fold
		EXT 0.3%	n.s.					EXT 0.3% HW	n.s.
Hb (g dL^{-1})	**S1**	P 0.3%	n.s.	**S3**	CTRHW	n.s.	**S3**	P 0.3% HW	↑ 0.4-fold
		P 1.5%	n.s.					P 1.5% HW	↑ 0.3-fold
		EXT 0.3%	n.s.					EXT 0.3% HW	↑ 0.2-fold
Ht (%)	**S1**	P 0.3%	n.s.	**S3**	CTRHW	n.s.	**S3**	P 0.3% HW	n.s.
		P 1.5%	n.s.					P 1.5% HW	n.s.
		EXT 0.3%	n.s.					EXT 0.3% HW	n.s.

Abbreviations: S1, sampling point 1—after 30 days of supplementation; S3, sampling point 3—7 days after the plateau phase of the marine heatwave; MHW, marine heatwave; CTR, commercial control diet under optimal conditions; P 0.3%, diet containing 0.3% *L. digitata* powder under optimal conditions; P 1.5%, diet containing 1.5% *L. digitata* powder under optimal conditions; EXT 0.3%, diet containing 0.3% *L. digitata* extract under optimal conditions; CTRHW, commercial control diet and exposed to heatwave; P 0.3% HW, diet containing 0.3% *L. digitata* powder and exposed to heatwave; P 1.5% HW, diet containing 1.5% *L. digitata* powder and exposed to heatwave; EXT 0.3% HW, diet containing 0.3% *L. digitata* extract and exposed to heatwave.

No significant differences were found in the Ht and RBC, and WBC counts between the treatments ($p > 0.05$), indicating that these parameters were unaffected by either *L. digitata* supplementation or acute thermal stress. However, significant differences in MCV, MCH, MCHC, and Hb levels were observed after supplementation. Fish fed with *L. digitata*-supplemented diets showed higher MCV values, particularly with 0.3% powder (P 0.3%; 0.5-fold increase compared with CTR; $p < 0.001$), while diets with 1.5% powder (P 1.5%) and 0.3% extract (EXT 0.3%) showed a 0.4-fold increase ($p < 0.01$). No differences in MCV were observed after exposure to MHW ($p > 0.05$).

Under optimal conditions, the P 0.3% powder diet led to the highest MCH (33.5 ± 1.8 pg cell^{-1}, a 0.4-fold increase vs. CTR; $p < 0.01$) and Hb levels (5.8 ± 0.8 g dL^{-1}) but the lowest MCHC (13.1 ± 0.7 g 100 mL^{-1}) compared with the other supplementation levels.

Upon exposure to an MHW (S3), similar patterns were observed in non-infected fish supplemented with *L. digitata*, exhibiting, overall, increased MCH, MCHC, and Hb levels under MHW conditions. The CTRHW treatment showed the lowest MCH (20.9 ± 1.1 pg cell^{-1}, a 0.3-fold decrease vs. CTR; $p < 0.001$), and the lowest Hb level (4.5 ± 0.2 g dL^{-1}; $p > 0.05$). The MCH increased 0.4-fold with the 0.3% extract diet (EXT 0.3% HW; $p < 0.001$) and 0.3-fold with the 0.3% powder (P 0.3% HW; $p < 0.01$), while the MCHC increased 0.2-fold with P 0.3% HW and P 1.5% HW compared with CTRHW ($p < 0.05$). Hb levels increased 0.4-fold with P 0.3% HW, 0.3-fold with P 1.5% HW, and 0.2-fold with EXT 0.3% HW compared with CTRHW ($p < 0.05$).

3.3.2. Effect of Seaweed Supplementation on Fish Responses upon 24 h (S2) and 7 Days (S3) of *Vibrio harveyi* Challenge

Table 2 shows the hematological parameters 24 h (S2) and 7 days (S3) after *V. harveyi* challenge during MHW exposure (25.7 °C).

Comparing CTRHW vs. CTRHWPAT 24 h post-challenge (S2), there was a significant increase in the Ht (0.7-fold; $p < 0.01$) and a decrease in the MCH and MCHC (0.3-fold; $p < 0.01$), with no differences in RBC, WBC, MCV, or Hb ($p > 0.05$) levels. After 7 days of bacterial challenge (S3), WBC (0.8-fold; $p < 0.001$), MCHC (0.2-fold; $p < 0.01$), and Hb levels (0.2-fold; $p < 0.01$) decreased, while the MCV (0.8-fold; $p < 0.001$) and MCH (0.4-fold; $p < 0.001$) increased. No differences were found in the RBC counts or Ht ($p > 0.05$).

Table 2. Hematological parameters of gilthead seabream (*S. aurata*) following exposure to *V. harveyi* and MHW (25.7 °C) conditions. Data are shown for 24 h (S2) and 7 days (S3) after bacterial challenge. The table presents (i) the effect of *V. harveyi* under MHW conditions (comparisons between CTRHW and CTRHW PAT at S2 and S3); (ii) the effect of dietary supplementation under MHW and *V. harveyi* conditions (comparisons between CTRHW PAT and P 0.3% HW PAT, P 1.5% HW PAT, and EXT 0.3% HW PAT at S2 and S3). Parameters include red blood cells (RBCs; $\times 10^6$ μL^{-1}), white blood cells (WBCs; $\times 10^5$ μL^{-1}), mean corpuscular volume (MCV; μm^3), mean corpuscular hemoglobin (MCH; pg $cell^{-1}$), mean corpuscular hemoglobin concentration (MCHC; g 100 mL^{-1}), hemoglobin (Hb; g dL^{-1}), and hematocrit (Ht; %) (n = 6 per treatment). Data are presented as the fold-change relative to the respective control group, with statistical significance ($p < 0.05$) indicated where applicable (n.s. = not significant; ↑ X-fold = increase; ↓ X-fold = decrease). A detailed overview of the results of the hematological parameters can be found in Table S2 in the Supplementary Material file.

	Effect of *V. harveyi* Under MHW Conditions (No Supplementation)			**Effect of Supplementation Under MHW and *V. harveyi* Conditions**					
	Comparisons Against CTRHW(Fold-Change)			**Comparisons Against CTRHW PAT at S2 (Fold-Change)**			**Comparisons Against CTRHW PAT at S3 (Fold-Change)**		
RBCs ($\times 10^6 \mu L^{-1}$)	**S2**	CTRHW PAT	n.s.	**S2**	P 0.3% HW PAT	n.s.	**S3**	P 0.3% HW PAT	n.s.
					P 1.5% HW PAT	n.s.		P 1.5% HW PAT	n.s.
	S3	CTRHW PAT	n.s.		EXT 0.3% HW PAT	n.s.		EXT 0.3% HW PAT	n.s.
WBCs ($\times 10^5 \mu L^{-1}$)	**S2**	CTRHW PAT	n.s.	**S2**	P 0.3% HW PAT	n.s.	**S3**	P 0.3% HW PAT	↑ 3.4-fold
					P 1.5% HW PAT	n.s.		P 1.5% HW PAT	n.s.
	S3	CTRHW PAT	↓ 0.8-fold		EXT 0.3% HW PAT	n.s.		EXT 0.3% HW PAT	↑ 2.3-fold
MCV (μm^3)	**S2**	CTRHW PAT	n.s.	**S2**	P 0.3% HW PAT	n.s.	**S3**	P 0.3% HW PAT	↓ 0.5-fold
					P 1.5% HW PAT	n.s.		P 1.5% HW PAT	n.s.
	S3	CTRHW PAT	↑ 0.8-fold		EXT 0.3% HW PAT	n.s.		EXT 0.3% HW PAT	n.s.
MCH (pg $cell^{-1}$)	**S2**	CTRHW PAT	↓ 0.3-fold	**S2**	P 0.3% HW PAT	↑ 0.5-fold	**S3**	P 0.3% HW PAT	↓ 0.3-fold
					P 1.5% HW PAT	↑ 0.5-fold		P 1.5% HW PAT	n.s.
	S3	CTRHW PAT	↑ 0.4-fold		EXT 0.3% HW PAT	↑ 0.5-fold		EXT 0.3% HW PAT	n.s.
MCHC (g 100 mL^{-1})	**S2**	CTRHW PAT	↓ 0.3-fold	**S2**	P 0.3% HW PAT	n.s.	**S3**	P 0.3% HW PAT	↑ 0.2-fold
					P 1.5% HW PAT	↑ 0.4-fold		P 1.5% HW PAT	n.s.
	S3	CTRHW PAT	↓ 0.2-fold		EXT 0.3% HW PAT	n.s.		EXT 0.3% HW PAT	n.s.
Hb (g dL^{-1})	**S2**	CTRHW PAT	n.s.	**S2**	P 0.3% HW PAT	↑ 0.4-fold	**S3**	P 0.3% HW PAT	↑ 0.2-fold
					P 1.5% HW PAT	↑ 0.4-fold		P 1.5% HW PAT	↑ 0.2-fold
	S3	CTRHW PAT	↓ 0.2-fold		EXT 0.3% HW PAT	n.s.		EXT 0.3% HW PAT	↑ 0.2-fold
Ht (%)	**S2**	CTRHW PAT	↑ 0.7-fold	**S2**	P 0.3% HW PAT	n.s.	**S3**	P 0.3% HW PAT	n.s.
					P 1.5% HW PAT	n.s.		P 1.5% HW PAT	n.s.
	S3	CTRHW PAT	n.s.		EXT 0.3% HW PAT	n.s.		EXT 0.3% HW PAT	n.s.

Abbreviations: MHW, marine heatwave; S2, sampling point 2—24 h after *V. harveyi* challenge, during MHW exposure; S3, sampling point 3—7 days after *V. harveyi* challenge, during MHW exposure; CTRHW, commercial control diet exposed to heatwave; CTRHW PAT, commercial control diet exposed to heatwave and pathogen; P 0.3% HW PAT, diet containing 0.3% *L. digitata* powder exposed to heatwave and pathogen; P 1.5% HW PAT, diet containing 1.5% *L. digitata* powder exposed to heatwave and pathogen; EXT 0.3% HW PAT, diet containing 0.3% *L. digitata* extract exposed to heatwave and pathogen.

Seaweed supplementation did not significantly affect RBC, WBC, MCV, or Ht values at 24 h post-challenge. However, the MCH increased across all the supplemented diets (0.5-fold; $p < 0.01$), and the MCHC increased explicitly in the P 1.5% HW PAT diet (0.4-fold; $p < 0.001$). Hb levels were also significantly higher in the P 0.3% HW PAT and P 1.5% HW PAT treatment groups (0.4-fold; $p < 0.01$). However, after 7 days, the effects of supplementation were more pronounced, especially in the diet with 0.3% *L. digitata* powder. In this treatment, MCV (0.5-fold; $p < 0.001$) and MCH (0.3-fold; $p < 0.001$) decreased, while WBC (3.4-fold; $p < 0.001$), MCHC, and Hb (0.2-fold; $p < 0.05$) levels increased. Notably, Hb levels increased across all the supplemented diets ($p < 0.05$). No differences were observed in the RBC count or Ht ($p > 0.05$).

3.4. Leukocyte Abundance and Viability

3.4.1. Effects of Seaweed Supplementation on Non-Infected Fish Under Optimal Conditions (S1)

Figure 5 shows the abundance and viability of total leukocytes and their subpopulations in the head kidney of gilthead seabream after 30 days of supplementation under optimal conditions (21.4 °C).

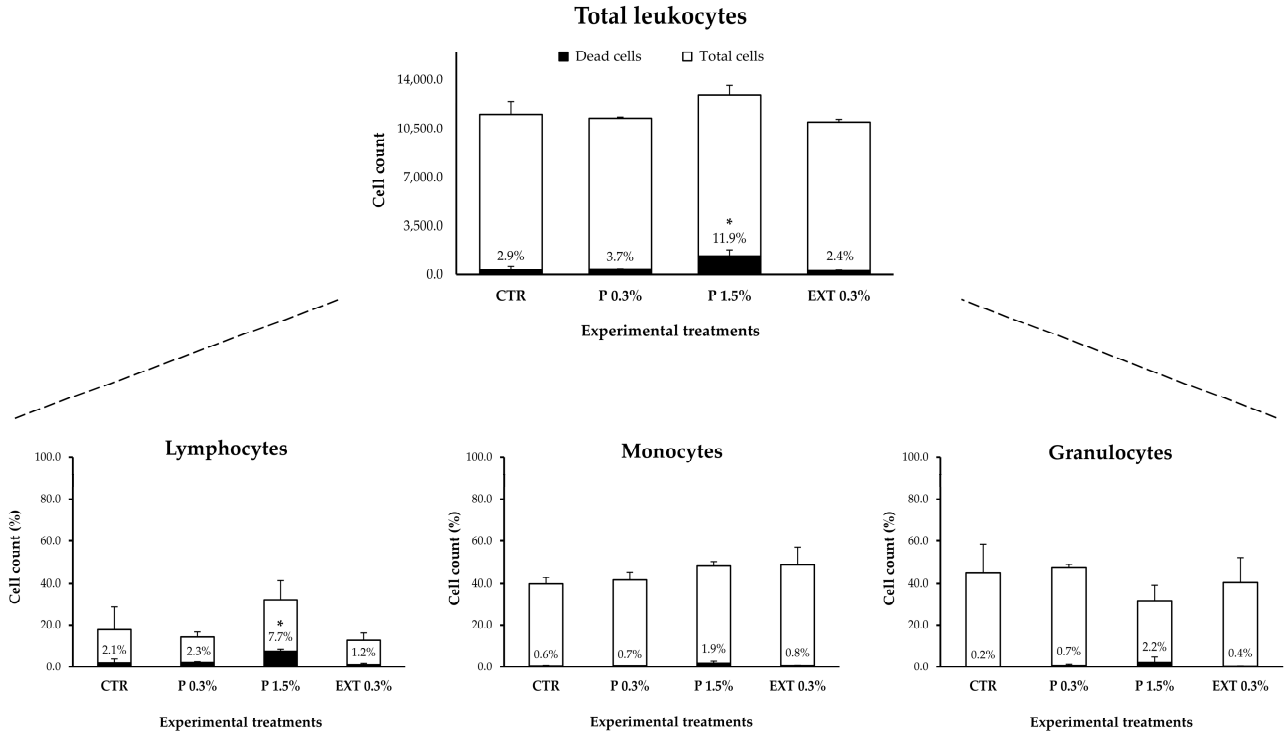


Figure 5. Abundance (white bars) and viability (black bars) of gilthead seabream head kidney total leukocytes (**top panel**) and subpopulations (lymphocytes, monocytes, and granulocytes; **bottom panel**) after 30 days of supplementation under optimal conditions (21.4 °C). Each set of data comprises 6 biological replicates and is represented as the mean ± SD. Significant differences relative to the control are represented by asterisks (*; $p < 0.01$). Abbreviations: CTR, commercial control diet under optimal conditions; P 0.3%, diet containing 0.3% *L. digitata* powder under optimal conditions; P 1.5%, diet containing 1.5% *L. digitata* powder under optimal conditions; EXT 0.3%, diet containing 0.3% *L. digitata* extract under optimal conditions.

There were no significant differences in the percentage of cell mortality across the treatments, except for the diet with 1.5% *L. digitata* powder (P 1.5%), which showed 11.9% mortality (a 3.1-fold increase compared with CTR; $p < 0.01$), where lymphocytes were the

subpopulation with the highest mortality (7.7%). Monocytes and granulocytes did not show notable differences in mortality rates across the treatments ($p > 0.05$).

3.4.2. Effects of Seaweed Supplementation on Non-Infected Fish Under Marine Heatwave Conditions (S3)

Figure 6 illustrates the abundance and viability of total leukocytes and their subpopulations in the head kidney of gilthead seabream after 7 days of exposure to an MHW (25.7 °C).

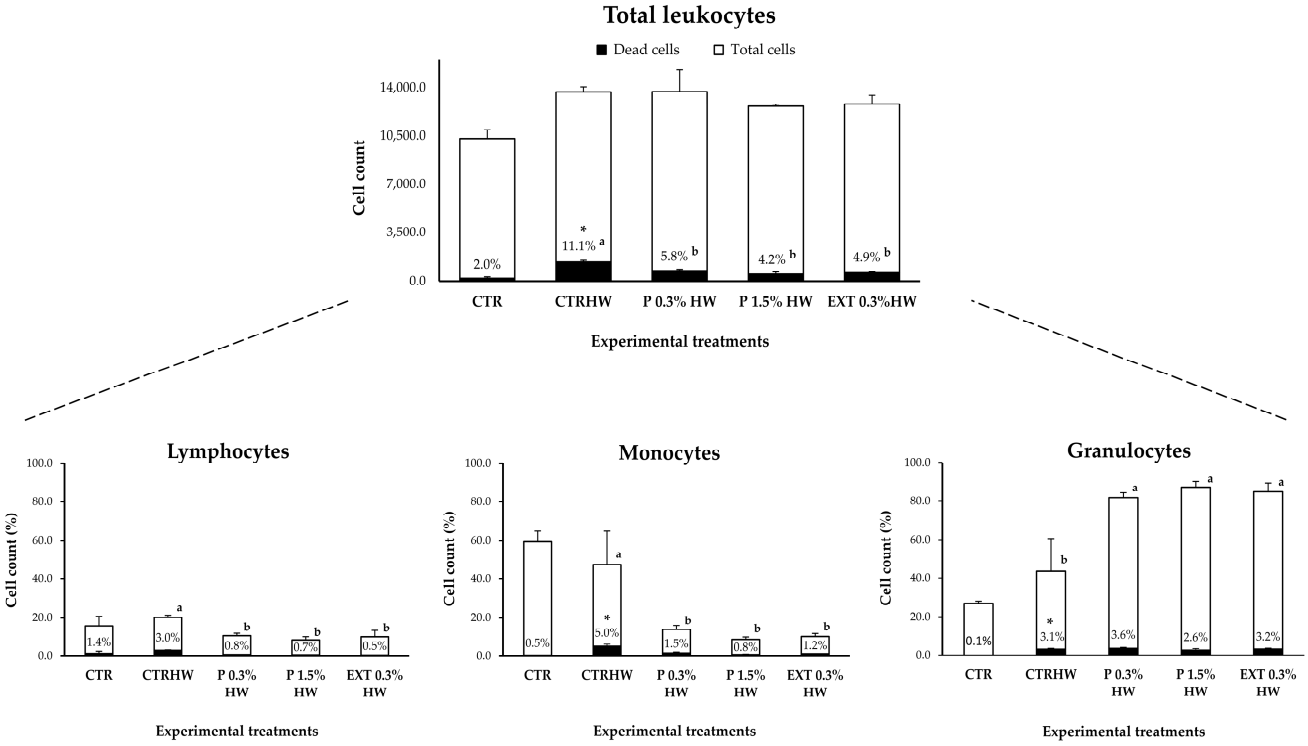


Figure 6. Abundance (white bars) and viability (black bars) of gilthead seabream head kidney total leukocytes (**top panel**) and subpopulations (lymphocytes, monocytes and granulocytes; **bottom panel**) after 7 days of exposure to an MHW (25.7 °C). Each set of data comprises 6 biological replicates and is represented as the mean ± SD. Significant differences between CTR and CTRHW are indicated by an asterisk (*; $p < 0.001$). Different lowercase letters indicate significant differences among treatment groups exposed to an MHW ($p < 0.01$). Abbreviations: CTR, commercial control diet under optimal conditions; CTRHW, commercial control diet and exposed to heatwave; P 0.3% HW, diet containing 0.3% *L. digitata* powder and exposed to heatwave; P 1.5% HW, diet containing 1.5% *L. digitata* powder and exposed to heatwave; EXT 0.3% HW, diet containing 0.3% *L. digitata* extract and exposed to heatwave.

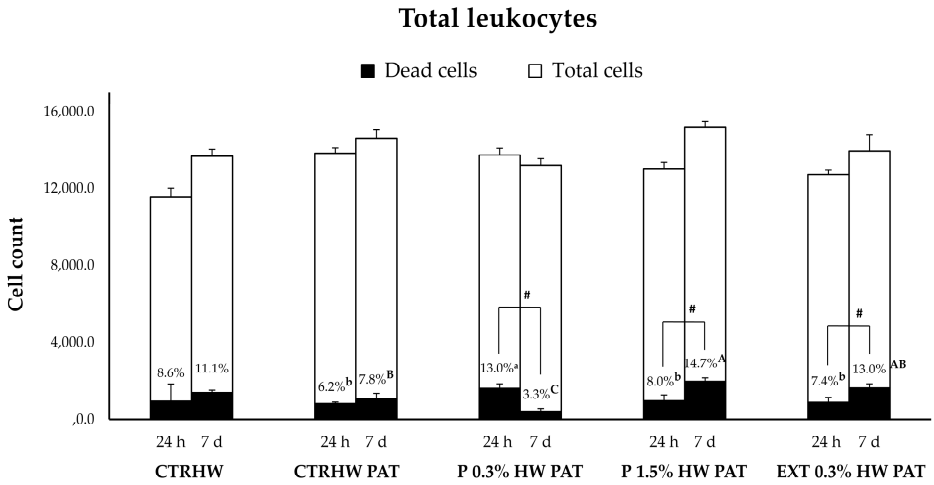
Exposure to the MHW significantly increased total leukocyte mortality (CTR vs. CTRHW; 4.4-fold; $p < 0.001$). However, the inclusion of *L. digitata* powder (P0.3% HW or P1.5% HW) or extract (EXT0.3% HW) significantly reduced leukocyte mortality in all the treatment groups ($p < 0.01$), with no significant differences between the different supplemented diets.

Dietary supplementation with *L. digitata* led to a significant increase in the proportion of granulocytes in all the treatment groups, accompanied by a decrease in the proportions of lymphocytes and monocytes. Despite these changes in leukocyte subpopulations, no significant differences in cell viability were observed within these populations across the supplemented diets. The only notable differences in cell mortality were observed after

MHW exposure in the CTRHW treatment, which showed increased monocyte (5.0%) and granulocyte (3.1%) mortality.

3.4.3. Effect of Seaweed Supplementation on Fish Responses upon 24 h (S2) and 7 Days (S3) of *Vibrio harveyi* Challenge

Figure 7 shows the abundance and viability of total leukocytes and their subpopulations in the head kidney of gilthead seabream after 24 h and 7 days of exposure to *V. harveyi* under MHW (25.7 °C) conditions.



		Lymphocytes (%)		Monocytes (%)		Granulocytes (%)	
		In relation to total cells	Dead	In relation to total cells	Dead	In relation to total cells	Dead
24 h	**CTRHW**	20.3 ± 5.2	6.6 ± 6.6	50.8 ± 8.8	1.4 ± 0.9	28.9 ± 12.3	0.6 ± 0.4
	CTRHW PAT	24.0 ± 2.3^{a}	2.8 ± 1.2^{a}	54.8 ± 2.1^{a}	2.5 ± 0.7	21.2 ± 4.3^{b}	0.9 ± 0.3^{b}
	P0.3% HW PAT	10.3 ± 0.9^{b}	1.9 ± 0.5^{ab}	11.7 ± 0.6^{b}	3.9 ± 0.5	78.0 ± 1.3^{a}	7.2 ± 1.1^{a}
	P1.5% HW PAT	13.1 ± 1.3^{b}	1.4 ± 0.2^{ab}	13.3 ± 2.5^{b}	3.4 ± 1.6	73.6 ± 3.7^{a}	3.2 ± 1.0^{b}
	EXT0.3% HW PAT	10.5 ± 1.5^{b}	1.0 ± 0.2^{b}	13.6 ± 1.2^{b}	2.5 ± 0.1	75.9 ± 2.1^{a}	3.9 ± 1.8^{b}
7 days	**CTRHW**	17.2 ± 1.2	3.0 ± 0.2	42.3 ± 17.6	5.0 ± 1.0	40.5 ± 17.2	3.1 ± 0.5
	CTRHW PAT	21.2 ± 1.3^{A*}	2.0 ± 0.5^{A*}	12.5 ± 2.6*	2.2 ± 0.7^{AB*}	66.3 ± 2.6^{B}	3.6 ± 1.1^{B}
	P0.3% HW PAT	9.8 ± 0.9^{B}	0.5 ± 0.3^{B}	11.1 ± 1.7	0.9 ± 0.1^{B}	79.1 ± 1.1^{A}	1.9 ± 0.9^{B}
	P1.5% HW PAT	15.5 ± 3.5^{AB}	2.6 ± 0.6^{A}	12.1 ± 3.7	3.2 ± 0.8^{A}	72.4 ± 7.1^{AB}	8.9 ± 0.7^{A}
	EXT0.3% HW PAT	10.9 ± 3.5^{B}	1.9 ± 0.6^{A}	12.2 ± 1.6	3.5 ± 0.6^{A}	76.9 ± 5.0^{AB}	7.6 ± 1.7^{A}

Figure 7. Abundance (white bars) and viability (black bars) of gilthead seabream head kidney total leukocytes (**top panel**) and subpopulations (lymphocytes, monocytes and granulocytes; **bottom panel**) after 24 h and 7 days of challenge with *V. harveyi* and during exposure to an MHW (25.7 °C). Each set of data comprises 6 biological replicates and is represented as the mean ± SD. Significant differences between CTRHW and CTRHW PAT are indicated by an asterisk (*; $p < 0.05$). Different lowercase letters indicate significant differences among treatments at 24 h post-challenge, while uppercase letters indicate significant differences between treatments at 7 days post-challenge ($p < 0.05$). Differences between the same treatment at 24 h and 7 days are indicated by the symbol # ($p < 0.01$). Abbreviations: CTRHW, commercial control diet and exposed to heatwave; CTRHW PAT, commercial control diet and exposed to heatwave and pathogen; P 0.3% HW PAT, diet containing 0.3% *L. digitata* powder and exposed to heatwave and pathogen; P 1.5% HW PAT, diet containing 1.5% *L. digitata* powder and exposed to heatwave and pathogen; EXT 0.3% HW PAT, diet containing 0.3% *L. digitata* extract and exposed to heatwave and pathogen.

No significant differences in total leukocyte viability were observed between the control treatments (CTRHW and CTRHW PAT) at either 24 h or 7 days ($p > 0.05$), indicating that the pathogen challenge did not significantly affect leukocyte viability. At 24 h, the P0.3% HW PAT treatment resulted in the highest percentage of dead leukocytes (13.0%), which was significantly higher than the P1.5% HW PAT (8.0%) and EXT0.3% HW PAT (7.4%) treatments ($p < 0.05$). However, after 7 days, leukocyte mortality decreased significantly

to 3.3% in the P0.3% HW PAT treatment group ($p < 0.05$), while mortality increased in the P1.5% HW PAT (14.7%) and EXT0.3% HW PAT (13.0%) treatment groups.

Regarding leukocyte subpopulations, no significant differences were found between CTRHW and CTRHW PAT, except for lymphocytes (0.2-fold increase) and monocytes (0.7-fold decrease) after 7 days ($p < 0.05$). Dietary supplementation with *L. digitata* (both powder and extract) significantly changed the leukocyte profile, with a marked increase in the proportion of granulocytes and a decrease in monocytes and lymphocytes compared with the CTRHW PAT treatment ($p < 0.05$). At 24 h, the P0.3% HW PAT treatment group showed the highest granulocyte mortality (7.2 ± 1.1%, $p < 0.05$). However, after 7 days, this treatment not only showed the lowest percentage of granulocyte death (1.9 ± 0.9%) but also the lowest mortality of lymphocytes (0.5 ± 0.3%) and monocytes (0.9 ± 0.1%).

3.5. Innate Humoral Parameters

3.5.1. Effects of Seaweed Supplementation on Non-Infected Fish Under Optimal (S1) and Marine Heatwave (S3) Conditions

Figure 8 represents the fish immune humoral responses after 30 days of supplementation under optimal conditions (21.4 °C) and following 7 days of exposure to an MHW (25.7 °C).

After 30 days, no significant differences were found in antiprotease activity (AP), immunoglobulin M (IgM) levels, or peroxidase activity (POD) across all diets ($p > 0.05$), indicating that *L. digitata* supplementation had no major impact under optimal conditions.

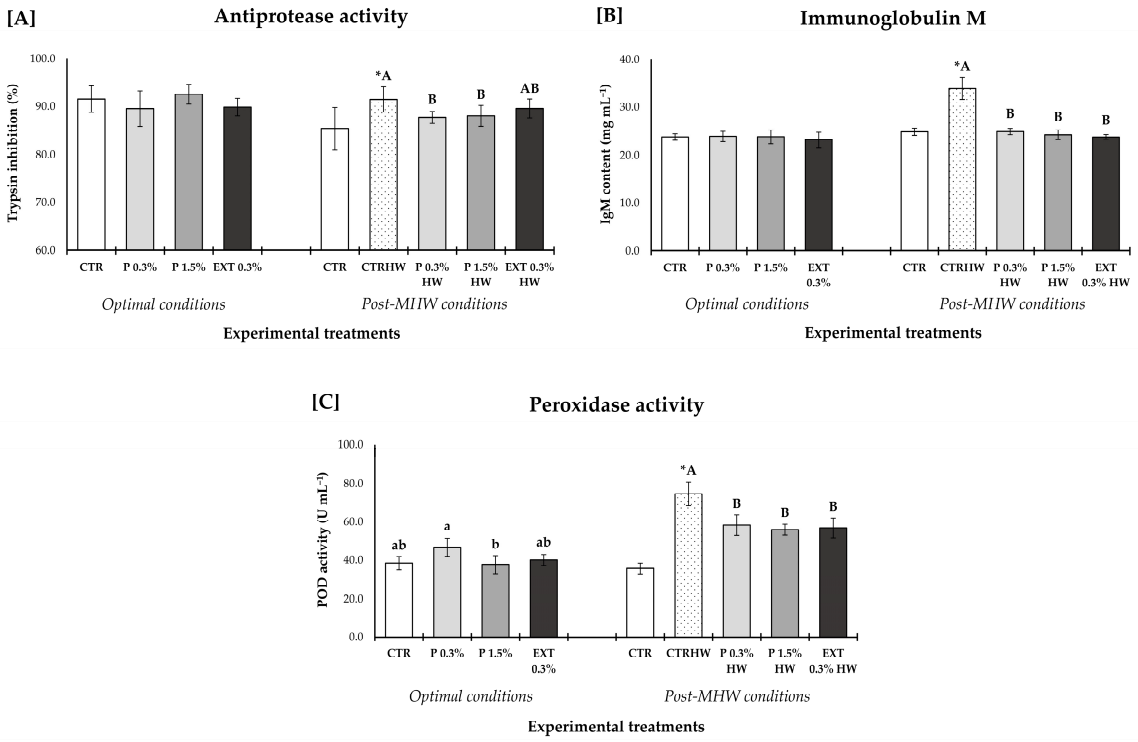


Figure 8. Plasma immune parameters of gilthead seabream (*S. aurata*) after 30 days of supplementation under optimal conditions (21.4 °C) and after 7 days of exposure to an MHW (25.7 °C). (**A**) Antiprotease activity (expressed as % trypsin inhibition), (**B**) immunoglobulin M (mg mL^{-1}), and (**C**) peroxidase activity (U mL^{-1}) (mean ± SD; $n = 6$). Different lowercase letters indicate significant differences among treatments after 30 days of supplementation, while uppercase letters indicate significant differences between treatments after 7 days of MHW exposure ($p < 0.05$). Significant differences between CTR and CTRHW are indicated by an asterisk (*; $p < 0.05$). Abbreviations: MHW, marine heatwave; CTR, commercial control diet under optimal conditions; P 0.3%, diet containing 0.3% *L. digitata* powder under optimal conditions; P 1.5%, diet containing 0.3% *L. digitata* powder

under optimal conditions; EXT 0.3%, diet containing 0.3% *L. digitata* extract under optimal conditions; CTRHW, commercial control diet and exposed to heatwave; P 0.3% HW, diet containing 0.3% *L. digitata* powder and exposed to heatwave; P 1.5% HW, diet containing 1.5% *L. digitata* powder and exposed to heatwave; EXT 0.3% HW, diet containing 0.3% *L. digitata* extract and exposed to heatwave.

After MHW exposure, the control diet (CTRHW) resulted in a significant increase in AP (0.1-fold; $p < 0.05$), POD (1.1-fold; $p < 0.001$), and IgM (0.4-fold; $p < 0.001$), suggesting an immune response to heat stress. However, *L. digitata* supplementation appeared to mitigate these stress responses since fish fed with supplemented diets (both powder and extract) showed lower levels of immune parameters, which suggests a protective effect of the macroalga against heat-induced stress.

3.5.2. Effect of Seaweed Supplementation on Fish Responses to *Vibrio harveyi* upon 24 h (S2) and 7 Days (S3) of Challenge

Figure 9 shows the humoral immune responses of fish exposed to an MHW (25.7 °C) at 24 h and 7 days after a challenge with *V. harveyi*.

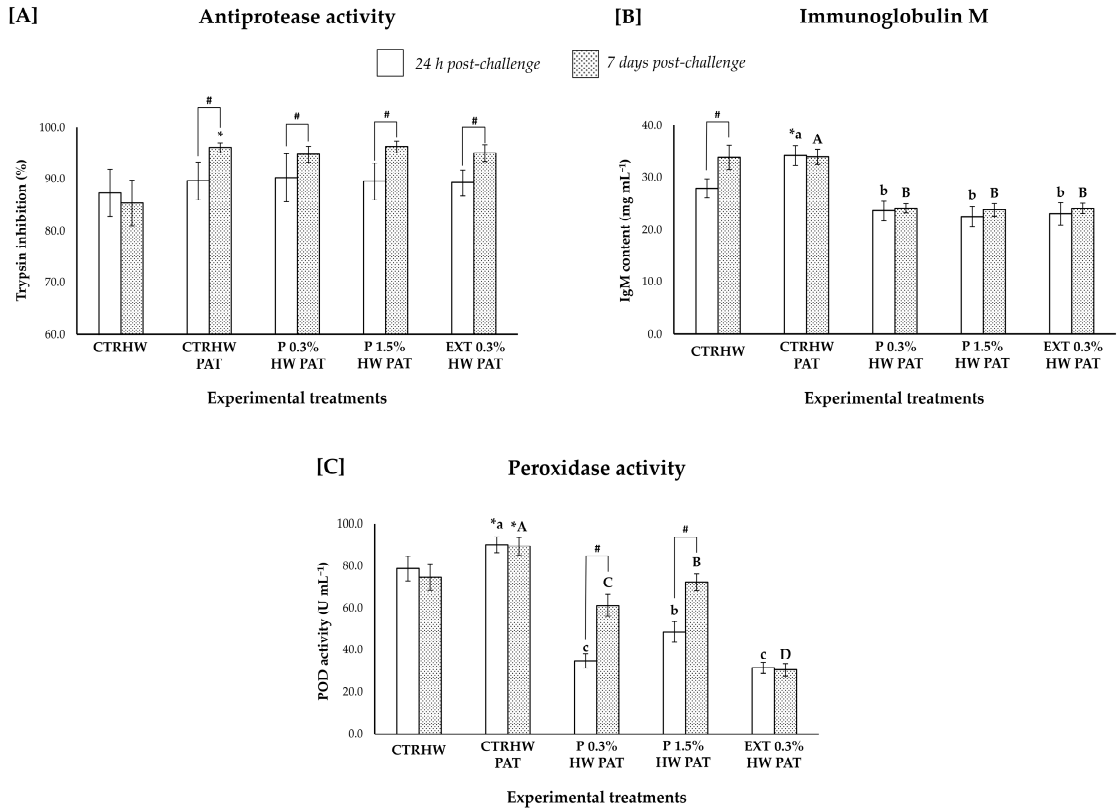


Figure 9. Plasma immune parameters of gilthead seabream (*S. aurata*) 24 h and 7 days after challenge with *V. harveyi* under an MHW (25.7 °C) exposure. (**A**) Antiprotease activity (expressed as % trypsin inhibition), (**B**) immunoglobulin M (mg mL^{-1}), and (**C**) peroxidase activity (U mL^{-1}) (mean ± SD; $n = 6$). Different lowercase letters indicate significant differences among treatments at 24 h post-challenge, while uppercase letters indicate significant differences between treatments at 7 days post-challenge ($p < 0.05$). Significant differences between CTRHW and CTRHW PAT are indicated by an asterisk (*; $p < 0.05$). Differences between the same treatment at 24 h and 7 days are indicated by the symbol # ($p < 0.05$). Abbreviations: CTRHW, commercial control diet and exposed to heatwave; CTRHW PAT, commercial control diet and exposed to heatwave and pathogen; P 0.3% HW PAT, diet containing 0.3% *L. digitata* powder and exposed to heatwave and pathogen; P 1.5% HW PAT, diet containing 1.5% *L. digitata* powder and exposed to heatwave and pathogen; EXT 0.3% HW PAT, diet containing 0.3% *L. digitata* extract and exposed to heatwave and pathogen.

At 24 h post-challenge, the CTRHW PAT treatment (fish fed the control diet exposed to MHW and pathogen) showed a significant increase in IgM levels (0.2-fold; $p < 0.001$) and POD activity (0.1-fold; $p < 0.05$) compared with CTRHW, indicating an immune response to the combined stressors. In contrast, fish fed *L. digitata*-supplemented diets showed significantly lower immune responses compared with CTRHW PAT, suggesting a protective effect of the macroalga. All supplemented treatment groups (P0.3% HW PAT, P1.5% HW PAT, EXT0.3% HW PAT) had reduced IgM levels (0.3-fold decrease; $p < 0.001$) compared with CTRHW PAT. Additionally, P0.3% HW PAT and EXT0.3% HW PAT showed a 0.7-fold decrease in POD activity ($p < 0.001$), while P1.5% HW PAT demonstrated a 0.5-fold reduction ($p < 0.001$). No significant differences in AP activity were observed between treatments ($p > 0.05$).

At 7 days post-challenge, the same effect and trends observed at 24 h persisted. The CTRHWPAT group continued to exhibit the highest levels of AP, IgM, and POD activity, indicating a prolonged immune response to the combined stressors. In contrast, the fish supplemented with *L. digitata* (P0.3% HW PAT, P1.5% HW PAT, EXT 0.3% HW PAT) showed consistently lower levels of IgM (0.3-fold decrease; $p < 0.001$) and POD activity (0.3-fold decrease in P0.3% HW PAT and P1.5% HW PAT and a 0.7-fold decrease in EXT 0.3% HW PAT; $p < 0.001$).

In some treatment groups, the AP activity (CTRHW PAT, P0.3% HW PAT, P1.5% HW PAT, and EXT0.3% HW PAT) and POD activity (P0.3% HW PAT and P1.5% HW PAT) showed a significant increase between 24 h and 7 days post-challenge ($p < 0.05$), indicating that the immune system remained active beyond the acute phase. In contrast, IgM levels did not show significant changes between 24 h and 7 days in the pathogen-exposed treatments ($p > 0.05$).

4. Discussion

The following sections will discuss the use of *L. digitata* (both in powder and extract forms) as a functional feed ingredient for juvenile gilthead seabream (*S. aurata*), focusing on three key aspects: (i) its immunostimulatory efficacy under optimal fish-rearing conditions, (ii) its immunostimulatory efficacy in the face of an MHW event, and (iii) its immunostimulatory efficacy in the face of a *V. harveyi* outbreak prompted by an MHW event.

4.1. Effects of Laminaria digitata Supplementation Under Optimal Conditions

Our findings indicate that including *L. digitata* in fish diets did not yield significant improvements in the performance and immune responses of *S. aurata* under ideal rearing conditions.

The fact that the hematological parameters of the supplemented fish did not differ significantly from those of the control group can be considered an encouraging outcome, as it confirms that *L. digitata* does not induce adverse effects in marine carnivorous fish. This is a crucial factor when introducing novel functional ingredients into aquaculture feeds. Many novel ingredients, though promising, can sometimes trigger toxicity or immune disturbances, which could impair fish health and growth performance. As such, previous studies have demonstrated that including macroalgae in aquafeeds does not always yield positive results, as excessive amounts can negatively affect growth rates and the feed efficiency [38–40]. Fish responses to seaweed in the diet are influenced by various factors, including the species of macroalgae, fish species, dosage, and the duration of the feeding trial [39].

Under optimal rearing conditions, significant differences were only observed in the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) between fish fed the 0.3% *L. digitata* powder (P0.3%) diet and the control group (CTR; commercial

diet). The notably higher MCV and MCH levels in the P0.3% treatment group suggests that fish undergoing this treatment had larger red blood cells, which are consequently capable of carrying more hemoglobin (MCH). However, this physiological change does not necessarily translate into an enhanced oxygen-carrying capacity. Larger cells, while capable of holding more hemoglobin, have a lower surface-area-to-volume ratio, which can slow oxygen diffusion across the cell membrane [41]. As a result, this could potentially limit the fish's ability to perform sustained aerobic activities, particularly during stressful conditions or periods of high activity [41]. Thus, this adaptation may compromise the fish's overall metabolic efficiency rather than enhancing its performance. Despite these hematological changes, no significant alterations were noted in the fish immune humoral parameters or overall health status. However, an important observation was the increase in leukocyte mortality in fish fed the 1.5% *L. digitata* powder (P1.5%) diet, with lymphocytes being the most affected cell subpopulation. Lymphocytes play a key role in adaptive immunity, as they are involved in antibody production (B-lymphocytes) and directly target and destroy infected or abnormal cells (T-lymphocytes) [42]. A decline in the viability of these critical cell populations suggests that higher concentrations of *L. digitata* may impair the immune function, leaving fish more susceptible to infections and diminishing their resilience against environmental stressors. *L. digitata* is rich in bioactive compounds, including polysaccharides, omega-3 fatty acids, peptides, vitamins, carotenoids, minerals, phenolics, and laminarin [23]. While these compounds are known for their beneficial properties, excessive amounts can induce oxidative stress or disrupt normal immune function, ultimately leading to cellular damage [23]. This aligns with previous studies, which reported that higher doses of *L. digitata* (3% and 6%, respectively) did not worsen but also did not improve immune or antioxidative responses, nor did they enhance metabolic or digestive performance in juvenile gilthead seabream [22,24]. These findings emphasize the importance of using lower inclusion levels of macroalgae in aquafeed formulations. Although *L. digitata* supplementation at the tested levels (0.3% powder, 1.5% powder, and 0.3% extract) and under optimal conditions did not yield any positive or negative effects that we could claim to be considerable, its potential benefits may become more evident under environmental stress or pathogenic challenges.

4.2. Effects of Laminaria digitata Supplementation Under Marine Heatwave Conditions

In contrast to the results observed under optimal rearing conditions, the simulated marine heatwave scenario induced more pronounced physiological and immune responses in the fish, allowing a more precise assessment of the potential of *L. digitata* to mitigate the effects of thermal stress.

Fish fed with the commercial diet and exposed to the simulated marine heatwave (CTRHW) showed lower mean corpuscular hemoglobin (MCH) and hemoglobin (Hb) levels, indicating that the marine heatwave negatively affected the oxygen-carrying capacity of their red blood cells. This reduction in MCH suggests that their red blood cells contained less hemoglobin, potentially impairing the fish's ability to maintain efficient aerobic metabolism under heat stress. However, supplementing the diets with *L. digitata*—in powder form (P0.3% HW and P1.5% HW) and extract form (EXT0.3% HW)—effectively reversed this decline. Fish fed with the enriched diets recovered their MCH and Hb levels, reaching values comparable to those from fish fed the commercial diet under optimal temperature conditions. Furthermore, the mean corpuscular hemoglobin concentration (MCHC) also increased with the biofortified diets, indicating a higher concentration of hemoglobin per unit volume of red blood cells, which would improve the oxygen transport efficiency [43]. These results highlight that *L. digitata* supplementation mitigates the adverse effects of heat stress on oxygen transport.

In terms of immune cell populations, exposure to the marine heatwave increased leukocyte mortality (CTRHW treatment), particularly affecting monocytes and granulocytes. Monocytes are essential components of the innate immune system, recognized for their ability to perform phagocytosis and non-specific cytotoxic activities [42]. Meanwhile, granulocytes (including neutrophils, eosinophils, and basophils) form the first line of defense in the immune system, quickly responding to infections and inflammatory signals [44]. Both monocytes and granulocytes are essential not only for protecting fish from pathogens but also to ensure a rapid and effective immune response to fight infections, promote tissue repair, and maintain overall immune homeostasis [42]. The loss of viability of these critical immune cells, particularly under heat stress, suggests that the fish immune system was compromised and, thus, that the animals were more susceptible to infection and less able to cope with environmental challenges. A promising finding emerged from the fact that all treatments supplemented with *L. digitata* (P0.3% HW, P1.5% HW, and EXT0.3% HW) showed a significant reduction in leukocyte mortality, indicating that the inclusion of *L. digitata* mitigated the harmful effects of MHW. Yet, notably, no specific dose and/or inclusion form proved to be more effective in this aspect.

Exposure to an MHW also resulted in higher levels of antiprotease (AP) and peroxidase (POD) activities, as well as increased immunoglobulin M (IgM) levels. This up-regulation of immune humoral parameters suggests an adaptive physiological response to counteract heat stress. Each immune parameter—AP, POD, and IgM—plays a distinct but complementary role in the immune response, supporting the fish's ability to fight off infections and to cope with environmental challenges. The increase in AP, POD, and IgM levels observed in the heatwave-exposed control treatment (CTRHW) indicates that the immune system is on high alert, possibly as a precaution against the immunosuppression associated with thermal stress. Hence, this compensatory mechanism has probably paved the way for an improved immune defense system and a readiness to deal with the occurrence of concomitant stressors (e.g., pathogen infection, discussed later) [5].

L. digitata (whether in powder or extract form) significantly lowered AP, POD, and IgM levels compared with the non-supplemented treatment. This reduction suggests that *L. digitata* modulated the immune response, potentially preventing excessive immune activation and chronic inflammation that might otherwise compromise the overall health of the fish. As previously discussed, bioactive compounds in *L. digitata* are likely responsible for these beneficial effects, as these substances are known for their anti-inflammatory and antioxidative properties [25–29]. These results align with other studies that have demonstrated the benefits of macroalgae supplementation in similar contexts. For instance, *Asparagopsis taxiformis* has been reported to enhance immune responses [5] and to improve metabolic and digestive functions [45] in *Diplodus sargus* during marine heatwaves. Similarly, research by Kamunde et al. [46] revealed that the dietary inclusion of seaweed meal derived from *Laminaria* sp. enhanced the growth, antioxidative capacity, and resilience to temperature stress in Atlantic salmon (*Salmo salar*).

4.3. Effects of Laminaria digitata Supplementation on the Interaction Between Marine Heatwaves and Vibrio harveyi Outbreaks

Previous studies have highlighted the benefits of macroalgae in enhancing resistance to specific pathogens [47], but, to the best of the authors' knowledge, this is the first study assessing the potential of *L. digitata* to mitigate the effects of disease outbreaks under sub-optimal rearing conditions (i.e., when an MHW strikes). The results clearly show that *L. digitata* supplementation attenuates, to some extent, the adverse effects prompted by both *V. harveyi* infection and MHW, suggesting its potential as a functional ingredient in a climate-smart context.

After 24 h of pathogen challenge, reductions in the mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were observed, suggesting that, at this early-stage, fish red blood cells were less efficient at transporting oxygen [43]. Conversely, the increase in the hematocrit (Ht), defined as the proportion of red blood cells in the blood, suggests a compensatory response to enhance oxygen delivery [48]. This response is commonly seen in fish under thermal stress, where the body attempts to maintain an adequate oxygen supply despite cellular impairment [48]. The inclusion of *L. digitata* in the diets, particularly in the 1.5% powder form (P1.5%HW PAT), improved multiple hematological parameters, including MCH, MCHC, and hemoglobin (Hb) levels. These improvements suggest that *L. digitata* preserves red blood cell function, enhancing the fish oxygen-carrying capacity and potentially contributing to better resilience under simultaneous thermal- and pathogen-induced stressors.

After 7 days of exposure, the detrimental effects of the MHW and *V. harveyi* stressors became more evident, as did the protective effects of *L. digitata* supplementation. Prolonged exposure exacerbated the immune suppression and altered the blood cell morphology, as evidenced by a significant decrease in white blood cell (WBC) counts. Under normal circumstances, an increase in WBC counts indicates a robust immune response as the organism mobilizes these cells to fight infection. However, the simultaneous stress of an MHW and pathogen exposure appears to have overwhelmed the fish's immune system, potentially leading to a state of immune exhaustion characterized by a reduced capacity to respond effectively to challenges. Such exhaustion increases the fish's susceptibility to infections and, in severe cases, can result in higher mortality rates [49].

Indeed, higher mortality rates were observed in infected fish exposed to an MHW. Following bacterial infection, both the control (CTRHW PAT) and the 0.3% *L. digitata* powder (P0.3%HW PAT) treatment groups experienced an acute and rapid increase in mortality within the first few days. Mortality in the P0.3%HW PAT group stabilized by day 3 (30.0%), while that in the CTRHW PAT group stabilized by day 5 (32.5%). These high mortality rates suggest that the fish were unable to effectively control the infection (developing septicemia), possibly due to an early onset of immune exhaustion caused by the combined stressors. In contrast, fish fed with 1.5% *L. digitata* powder (P1.5%HW PAT) or 0.3% extract (EXT0.3%HW PAT) showed a resilient response, evidencing slower mortality rates (7.3% and 7.7%, respectively) that stabilized by day 4. These results again highlight the protective effects of *L. digitata* and suggest that a minimum dose of this macroalga may be needed to boost fish immunity when multiple stress factors occur. This, therefore, highlights that higher doses of *L. digitata* powder (1.5%) or the extract form (0.3%) reduces mortality and provides more durable immune support, allowing the fish to manage the infection more effectively during its critical early life stages.

Recent studies have explored the pathology, pathogenesis, and virulence of vibriosis in several fish species [50]. A previous pathogenicity study showed significant species-specific differences in mortality rates [51]. *S. aurata* infection with *V. harveyi* at 10^7 CFU mL^{-1} led to a relatively low mortality rate (25%). In contrast, the same bacterial dose caused a much higher mortality rate (95%) in European seabass (*Dicentrarchus labrax*) [51]. The virulence of *V. harveyi* can be influenced by multiple factors, including host susceptibility, bacterial load, and environmental conditions [50]. Our study, alongside the previous research of Firmino et al. [51], highlights key patterns in the infection and clearance of *V. harveyi* in gilthead seabream. Both studies showed that mortality peaked within the first few days post-inoculation, with no further mortality after the fourth day. This pattern suggests an acute phase where the fish either succumb to or manage to control the infection. Interestingly, the fact that fish supplemented with 0.3% *L. digitata* extract (EXT0.3% HW PAT) showed a weaker *V. harveyi* PCR band at the 24 h mark is indicative of a lower bacterial load

in this treatment compared with the other ones, thus suggesting that bioactive compounds in *L. digitata*, which are typically more concentrated in extracts, may have strengthened the fish's immune defenses against pathogens. Firmino et al. [51] also observed that fish exposed to *V. harveyi* could clear the pathogen from their bodies if they survived the initial infection, suggesting that immune mechanisms responsible for clearing the pathogen are likely associated with innate immune effectors. Our PCR results also align with the arguments presented by Firmino et al. [51], as the undetectable presence of *V. harveyi* 7 days after infection supports the idea that innate immunity is key to defending against this pathogen, and that the spleen is a primary organ for pathogen clearance. These findings support the need for further research to clarify how specific bioactive compounds can enhance immune defenses and pathogen clearance, particularly under environmental stressors. Other studies have also provided significant evidence of the potential of dietary approaches for strengthening fish immune function. For instance, Harikrishnan et al. [52] observed that kelp grouper (*Epinephelus bruneus*), a marine ray-finned fish, supplemented with *Pueraria thunbergiana* (Fabaceae) extract showed improved immunity and increased survival against *V. harveyi*. Similarly, Rashidah et al. [53] found that *Polygonum chinense* (Polygonaceae) extract enhanced survival rates and immune responses in Asian seabass (*Lates calcarifer*), and Abdel-Razek et al. [54] demonstrated that ethanolic extracts of *Ulva* sp. (Chlorophyta) and garlic (*Allium sativum*) significantly reduced mortality in white-leg shrimp (*Litopenaeus vannamei*) infected with *V. harveyi*. The fact that all the aforementioned studies were conducted under optimal rearing conditions reinforces the need for further research to validate these nutrition-based strategies under sub-optimal and multi-stressor contexts.

Supplementation with *L. digitata* powder or extract also significantly modulated the viability and dynamics of immune cell subpopulations, as after 24 h of pathogen challenge, both the 1.5% *L. digitata* powder (P1.5%HW PAT) and 0.3% extract (EXT0.3% HW PAT) treatment groups showed a reduction in cell mortality, indicating an effective and rapid immune response. The fact that after 7 days of pathogen infection, supplementation with 0.3% *L. digitata* powder led to the lowest leukocyte mortality rate (only 3.3%) somewhat contradicts the trends observed in the other endpoints (i.e., improved immune defenses upon supplementation with 1.5% powder or 0.3% extracts) and, thus, point to the importance of conducting holistic multi-biomarker approaches to disclose the functional potential of alternative aquafeed supplements, such as seaweed.

Both the powder and extract forms of the macroalga increased the proportion of granulocytes, suggesting an enhanced ability to frame a quick immune response. However, while granulocytes became the most common immune cell type, they also showed the highest rate of mortality/apoptosis (programmed cell death). This observation may initially seem counterintuitive, as it could suggest immune cell depletion and weakening immune defenses. However, this process is important since granulocyte apoptosis is usually observed in response to an infection, leading to the removal of intracellular microorganisms, the release of microbicidal components, and an increase in anti-inflammatory responses [55,56]. Thus, increased granulocyte mortality may represent an important advantage in controlling pathogen proliferation. Similar findings were reported by Santos et al. [57], who demonstrated that extracellular extracts from *Bacillus* spp. possess immunomodulatory properties, effectively enhancing the immune response of gilthead seabream against pathogens such as *Vibrio anguillarum* and *Edwardsiella tarda*. Fuentes-Appelgren et al. [58] also observed an increase in granulocytes after feeding zebrafish (*Danio rerio*) with diets containing soybean components.

Regarding innate humoral parameters, IgM and POD activity increased at 24 h and 7 days post-exposure, indicating an adaptive physiological response to the MHW and

pathogen challenge (see Section 4.2 for further explanation). However, dietary supplementation with *L. digitata* positively modulated these immune responses by reducing POD and IgM levels. Among the treatments, the 0.3% extract diet (EXT0.3%HW PAT) proved to be the most advantageous, achieving the most significant reduction in POD activity across all groups.

All in all, from a purely research perspective (i.e., overlooking the associated production costs), the 0.3% extract emerged as the most effective dose for counteracting diseases under adverse thermal conditions. Yet, it is important to consider the practical implications of this nutritional strategy in terms of raw material availability and the time and costs required to produce seaweed extracts at an industrial scale. For example, our study showed that the production of *L. digitata* extract had a yield of only 17.4%, meaning that 5.75 g of powdered macroalga is required to produce only 1 g of extract. At an industrial scale, this low yield can pose a significant cost challenge, which may not be justified given that the 1.5% powder also delivered strong immune-boosting effects. Thus, while the 0.3% extract demonstrates high efficacy, the 1.5% powder formulation may offer a more cost-effective solution for large-scale aquafeed production, providing robust immune support without the high expenses associated with extracts. This balance between dose-effectiveness and practical/economic constraints underscores the importance of validating innovative nutrition-based strategies in different production scenarios, as research in this direction will pave the way for sustainable and climate-smart aquaculture practices that do not need to entirely rely on chemically-based disease management approaches and that can, therefore, contribute to the long-term economic sustainability of the aquaculture sector.

5. Conclusions

This study provides important insights into the dual threats of marine heatwaves and *V. harveyi* outbreaks on the immune responses of gilthead seabream (*S. aurata*). Our findings show that MHWs significantly compromise the immune system of farmed fish, intensifying the severity of infections when coupled with bacterial exposure.

While *L. digitata* supplementation under optimal conditions did not yield significant overall improvements (nor did it elicit any adverse outcomes), its true potential became evident when juvenile gilthead seabream faced environmental stressors. Both powder (1.5%) and extract (0.3%) forms of *L. digitata* effectively mitigated the harmful impacts of MHWs and bacterial infections, though the 0.3% extract was ultimately the most effective in enhancing the seabream's immune response. However, from an economic perspective, the 1.5% powder formulation should be preferred, as it offers a practical balance between effectiveness and affordability for large-scale applications.

Overall, these results highlight *L. digitata* as a valuable functional aquafeed ingredient that can be successfully used in a climate-smart context to strengthen the immunocompetence and resilience of farmed marine carnivorous fish against vibriosis. This represents a major step forward in aquaculture nutrition, with tangible impacts on animal welfare, environmental, and economic standpoints.

Future studies should aim to explore the effects of other climate-related stressors (e.g., hypoxia and ocean acidification) across different marine fish species. This is essential to better anticipate disease dynamics under changing environmental conditions and to validate sustainable and effective prophylactic strategies, such as the targeted use of functional aquafeeds, during the most vulnerable seasons, rearing periods, and species life stages.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments12070226/s1, Table S1. Hematological parameters of gilthead seabream (*Sparus aurata*) after 30 days of supplementation under optimal conditions (S1, 21.4 °C) and after 7 days of exposure to a marine heatwave (MHW; S3; 25.7 °C); Table S2. Hematological parameters of gilthead seabream (*Sparus aurata*) 24 h (S2), and 7 days (S3) after challenge with *Vibrio harveyi* under marine heatwave exposure (MHW; 25.7 °C).

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Institutional Review Board Statement: Fish experiments and handling were approved by IPMA's Animal Welfare and Ethics Body (ORBEA, LABVIVOS-002-AquaClimAdapt), which is overseen by the Directorate-General for Food and Veterinary (DGAV), under the ethical clearance number 20596/25-S. The study was performed by accredited scientists (EU functions A and B), in strict accordance with the ARRIVE (Animal research: reporting of in vivo experiments) guidelines and the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). All procedures complied with EU Directive 2010/63 and Portuguese legislation (Decree-Law nr. 113/2013).

Data Availability Statement: Data are contained within the article or Supplementary Materials.

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Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	Analysis of variance
AP	Antiprotease activity
bw	Body weight
CTR	Control
EXT0.3%	Diet with 0.3% extract
Hb	Hemoglobin
Ht	Hematocrit
IgM	Immunoglobulin M
MCH	Mean corpuscular hemoglobin

MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MHW	Marine heatwave
p	*p*-value
P0.3%	Diet with 0.3% powder
P1.5%	Diet with 1.5% powder
PCR	Polymerase chain reaction
POD	Peroxidase activity
RBC	Red blood cells
SD	Standard deviation
WBC	White blood cells

Appendix A

Appendix A.1 Detailed Feed Composition of the Experimental Diets

Table A1. Ingredient composition (%) and proximate analysis (%DM; dry matter) of the experimental diets used to feed *Sparus aurata* juveniles. Diets: CTR—Control (no seaweed); P 0.3%—0.3% *L. digitata* powder; P 1.5%—1.5% *L. digitata* powder; EXT 0.3%—0.3% *L. digitata* extract.

Ingredients (%)	**CTR**	**P 0.3%**	**P 1.5%**	**EXT 0.3%**
Fishmeal Super Prime [1]	17.0	17.0	17.0	17.0
Fish protein hydrolysate [2]	2.0	2.0	2.0	2.0
Poultry meal [3]	7.0	7.0	7.0	7.0
Soy protein concentrate [4]	6.0	6.0	6.0	6.0
Wheat gluten [5]	10.0	10.0	10.0	10.0
Corn gluten meal [6]	11.0	11.0	11.0	11.0
Soybean meal (Hipro) [7]	12.8	12.8	12.8	12.8
Wheat meal [8]	12.0	11.7	10.5	11.7
Whole peas [9]	6.0	6.0	6.0	6.0
Vitamin and mineral premix [10]	1.0	1.0	1.0	1.0
Choline chloride 50% [11]	0.2	0.2	0.2	0.2
Monosodium phosphate [12]	1.0	1.0	1.0	1.0
Fish oil [13]	6.0	6.0	6.0	6.0
Salmon oil [14]	8.0	8.0	8.0	8.0
Macroalga *Laminaria digitata* [15]	-	0.3	1.5	-
Macroalga Extract (*Laminaria digitata* [15])	-	-	-	0.3
Proximate composition (%)				
Crude protein, %DM	46.0	46.0	45.9	46.0
Crude fat, %DM	16.0	16.0	16.0	16.0
Fiber, %DM	1.4	1.4	1.4	1.4
Starch, %DM	13.5	13.3	12.5	13.3

Table A1. *Cont.*

Ingredients (%)	CTR	P 0.3%	P 1.5%	EXT 0.3%
Ash, %DM	7.4	7.4	7.7	7.4
Gross energy, MJ kg^{-1}	20.5	20.5	20.4	20.5

[1] Diamante: 66.3% crude protein (CP), 11.5% crude fat (CF); South America, Pesquera Diamante, Peru. [2] CPSP90: 82.6% CP, 9.6% CF; Sopropêche, France. [3] Poultry meal: 67.4% CP, 12.5% CF; SAVINOR UTS, Portugal. [4] Soycomil P: 62.2% CP, 0.7% CF; ADM, The Netherlands. [5] VITAL: 80.4% CP, 5.8% CF; Roquette, France. [6] Corn gluten meal: 61.2% CP, 5.2% CF; COPAM, Portugal. [7] Alphasoy 530: 52.9% CP, 2.6% CF, dehulled solvent extracted; ABNeo AS, Denmark. [8] Wheat meal: 11.7% CP, 1.6% CF; Molisur, Spain. [9] Whole peas: 19.6% CP, 2.2% CF; Ribeiro & Sousa Lda., Portugal. [10] Vitamin and mineral premix (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; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 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. [11] Choline chloride 50%; ORFFA, The Netherlands. [12] Monosodium phosphate: 25.8% P, 19.2% Na; OMNISAL GmbH, Germany. [13] Fish oil: 98.1% CF; Sopropêche, France. [14] Salmon oil: 98.3% CF; Sopropêche, France. [15] *Laminaria digitata*: Parc Marin d'Iroise, Brittany, France.

Appendix A.2 Experimental Design

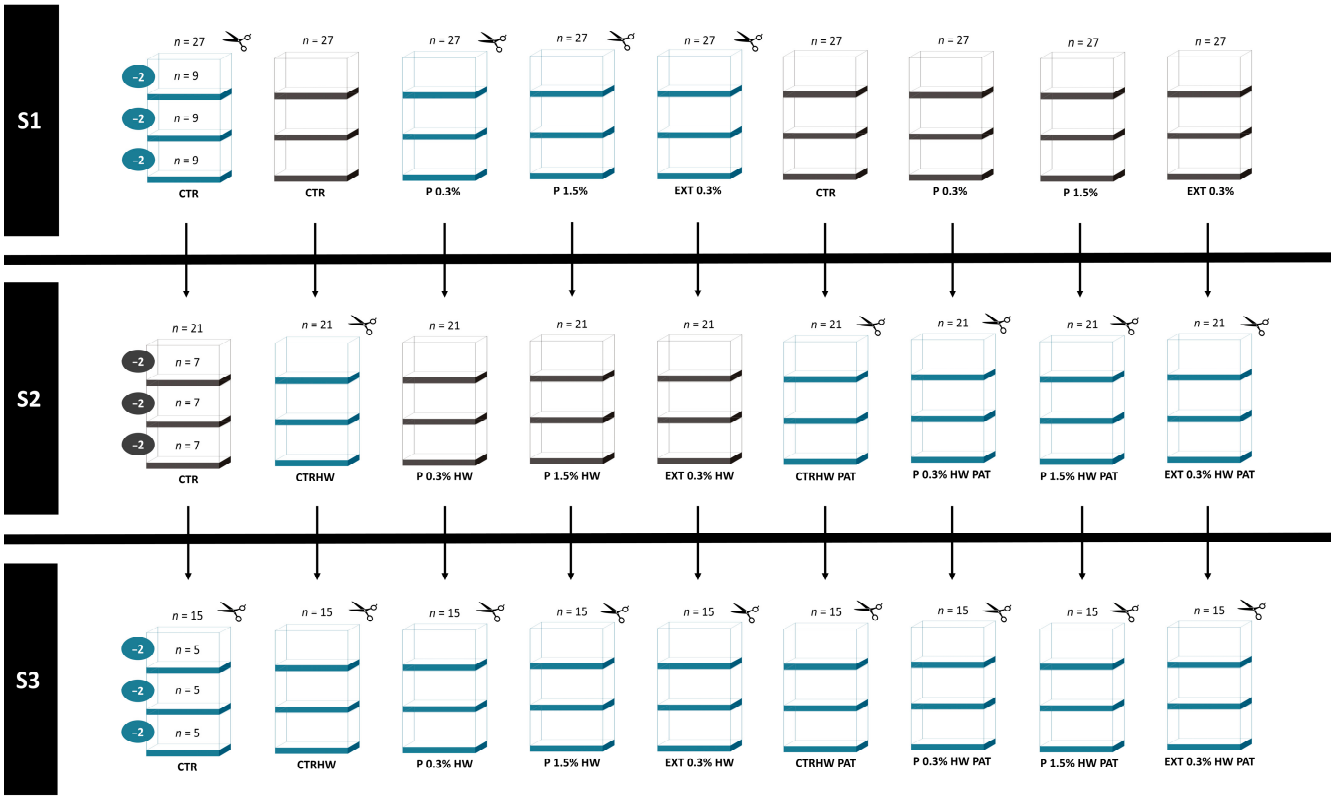

Figure A1. Schematic representation of the experimental design. The experiment was structured in three sequential stages (S1, S2, and S3). In each stage, fish were maintained in 200 L glass tanks distributed across independent recirculating aquaculture systems. Each arrow indicates the transition from one stage to the next. Blue-colored tanks and scissor icons denote the tanks from which the fish were sampled. **S1 (prophylactic supplementation phase):** Fish (n = 27 per treatment) were hand-fed twice daily with the experimental diets for 30 days at the optimal temperature (21.4 °C). Each treatment had three replicate tanks (9 fish per tank). The diet groups included the following: CTR (control), P 0.3% (0.3% *L. digitata* powder), P 1.5% (1.5% *L. digitata* powder), and EXT 0.3% (0.3% *L. digitata* extract). After 30 days, 2 fish per tank (6 fish per treatment) were sampled; **S2 (temperature-increase ramp and bacterial challenge):** Fish were subjected to a gradual marine heatwave (MHW) simulation over 10 days (0.5 °C/day), except for the CTR group, which remained

at 21.4 °C. The same diets and feeding regime were maintained. Each treatment included 21 fish (7 fish per tank). Once 25.7 °C was reached, groups labeled "PAT" were challenged with *Vibrio harveyi* (100 µL; 4.9 × 10^8 CFU mL^{-1} per fish), while others received sterile PBS. After 24 h, 2 fish per tank were sampled from the challenged groups and from the CTRHW group; **S3 (exposure to MHW and pathogen):** Fish were kept under MHW conditions (25.7 °C) for 7 days. Each treatment had 15 fish (5 per tank). At the end of this stage, 2 fish per tank (6 per treatment) were sampled.

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