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Cannabidiol and microbubble aeration enhance stress resilience and physiological homeostasis in European sea bass: a multi-tissue integrative approach

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Introduction: Animal welfare has become an increasingly important issue in the aquaculture sector, and improving it is essential for maintaining the health of farmed fish. Therefore, the aim of the present study was to evaluate the potential effect of dietary cannabidiol (CBD) supplementation (0.001%) environmental enrichment (EE) through modified aeration or their combination over 5 weeks to assess their suitability as strategies for improving welfare in aquaculture.
Methods: Methods: After this period, fish were subjected to a confinement stress challenge. Welfare indicators such as survival, selected stress and immunological parameters, lipid mediators, and the expression of several stress-response and appetite-regulating related genes in different brain regions (including telencephalon, hypothalamus, and pituitary gland) were analysed in European sea bass.
Results: The results showed that survival rates were significantly higher in all welfare-promoting groups; EE improved production parameters and increased dissolved oxygen levels; CBD treatments did not negatively affect growth performance, reduced plasma cortisol during both acute and adaptive stress responses, enhanced basal serum lysozyme activity, and promoted a faster post-stress stabilization; and the combined treatment supported a synergistic interaction between EE and CBD, in which lipid mediators suggested modulations of inflammatory responses under stress and helped to mitigate the effects of the stress response-related genes and a potential involvement in appetite regulation and energy balance strategies.
Discussion: These results highlight the potential of microbubble curtain enrichment and CBD for improving welfare of juvenile sea bass, enhancing growth, stress resilience, and physiological regulation, with the combined treatment showing the strongest and most effective improvements.

KEYWORDS
cannabidiol, environmental enrichment, microbubble curtain, stress, welfare

1 Introduction

Animal welfare has become an increasingly important consideration in aquaculture, particularly in the context of intensified production systems. In this context, the concepts of welfare and stress are closely interconnected within the stressotope scenario, as reduced stress levels are generally associated with improved welfare conditions in fish (Balasch and Tort, 2019). Stress responses are therefore widely used as indicators of animal welfare, since chronic stress can impair immune function, leading to increased susceptibility to disease and higher mortality rates (Barreto et al., 2022). Recent studies have also shown that teleost, similarly to higher vertebrates, presents complex cognitive, behavioral and affective capacities, enabling them to experience pain and suffering (Franks et al., 2021). Among the strategies proposed to improve welfare in aquaculture, environmental enrichment (EE) has gained increasing attention from the scientific community. EE involves increasing the structural or sensory complexity of captive environments to reduce the development of aberrant or maladaptive traits in teleost reared under stimulus-deprived conditions (Näslund and Johnsson, 2016). Several studies on aquaculture species have demonstrated that EE, including improvements in rearing conditions through complexity enrichment (Magnoni et al., 2025) can provide multiple benefits, including reduced aggressive and abnormal behaviors, improved growth performance, lower cortisol levels, enhanced neurogenesis and brain plasticity, improved learning and memory and increased survival rates (Nuñez Velazquez, 2019; Zhang et al., 2020; Arechavala-Lopez et al., 2022; Alimuddin et al., 2023).

From a different perspective, the use of adaptogens has recently emerged as an important strategy to improve welfare in farmed fish. Adaptogens are synthetic or plant-derived compounds that have the ability to increase the stability of an organism in the face of stress (Todorova et al., 2021). The adaptogen cannabidiol (CBD) is a phytocannabinoid derived from the herbaceous plant *Cannabis sativa*, which is reported to possess antipsychotic, anxiolytic, and anti-inflammatory properties (Izzo et al., 2009; Hudson et al., 2019), without showing psychoactive effects (Mortuza et al., 2023). Exogenous cannabinoids exert their effects through the endocannabinoid system (eCB) (Boggs et al., 2018) which plays a key role in the neuroendocrine modulation of the stress response, where it is involved in the maintenance of physiological homeostasis by regulating cognitive and physical processes, such as pain response, appetite, respiratory and cardiovascular function (Alteba et al., 2016). Furthermore, it participates in the recovery of physiological homeostasis following stress exposure (Henson et al., 2021). CBD is a practical tool to reduce stress in livestock, mainly through oral administration in oils or feed, helping the modulation of stress and inflammatory responses during management procedures such as transport or weaning (Fallahi et al., 2022; Meyer et al., 2022). In aquaculture, emerging evidence indicates that CBD may act as a modulator of both acute and chronic stress, exerting anxiolytic-like effects, suggesting its potential as a functional tool to enhance welfare in aquaculture systems (Mortuza et al., 2023; Jones et al., 2026).

Despite being one of the most widely farmed marine species in Europe, the European sea bass (*Dicentrarchus labrax*) is consistently described as highly sensitive to stress, particularly when compared with other commonly cultured Mediterranean fish species, and this physiological vulnerability is considered a major bottleneck for its intensive production systems (Samaras et al., 2018). However, despite growing research on functional dietary strategies aimed at mitigating stress in aquaculture species, including nutritional interventions with antioxidant and immunomodulatory properties (Torrecillas et al., 2024), there is currently no published research evaluating CBD in this species. This highlights a clear knowledge gap regarding the potential application of CBD for stress modulation and welfare improvement in this intensively farmed fish.

Although CBD has been shown to modulate stress-related behaviors under environmentally challenging conditions in fish, and EE is well established as a welfare-enhancing strategy in aquaculture, no previous studies have investigated the combined effects of these nutritional and environmental interventions in farmed fish. Assessing both strategies together may provide complementary insights into welfare-related physiological responses by simultaneously considering nutritional and environmental aspect of fish rearing. Therefore, the aim of the present study was to evaluate the potential effects of a CBD-based adaptogen as a feed additive, occupational enrichment implemented through modifications in tank aeration, and the combination of both strategies on the welfare of juvenile European sea bass.

2 Materials and methods

2.1 Ethical considerations

The protocols used were in accordance with the European Union Directive (2010/63/EU) and Spanish legislation (RD 53/2013) on animal experimentation, and the experiment was approved by the University of Las Palmas de Gran Canaria Animal Experimentation Ethics Committee (REF: OEBA-ULPGC-32/2023).

2.2 Experimental design

In this study 4 treatments were tested in triplicate (n= 12 tanks) using CBD as an adaptogen and a "microbubble curtain" as an EE (described below). For this, treatment 1 was used as a control without CBD supplementation and without EE (-CBD/-EE); treatment 2 included CBD in the diet at 0.001%, and no EE (+CBD/-EE); treatment 3 consisted of aeration with microbubble curtain as occupational enrichment with no CBD supplementation (-CBD/+EE) and treatment 4 combining both CBD and microbubble curtain (+CBD/+EE). The microbubble curtain consisted of a circular rubber aerator positioned at the bottom of the tank using weights attached to the structure to prevent movement (Figure 1). To generate the microbubbles in the EE system, a 12.5 mm diameter porous soaker hose (OREWORK, Ourense, Spain), with an approximate pore size of 50-100 µm, was connected to a 60-Hz air pump with a maximum airflow capacity of 250 m^3/h (4.167 L/min). Aeration was maintained continuously throughout the experiment for the EE treatment. The adaptogen came in a liquid form of pure,

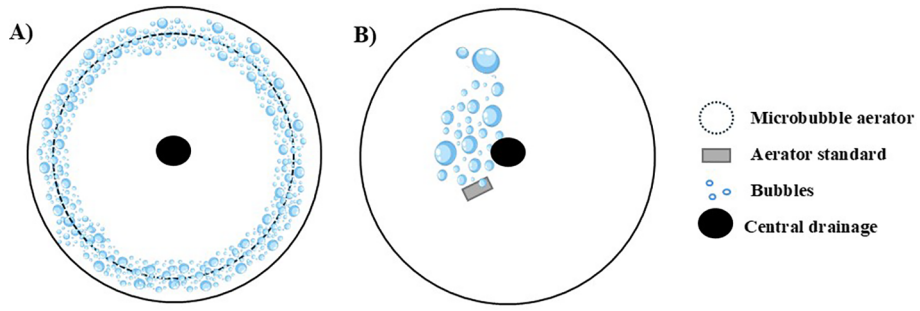

FIGURE 1
Environmental complexity in experimental tanks of juveniles European sea bass under enriched **(A)** and non-enriched **(B)** microbubbles curtain.

unprocessed, odorless, amber-colored hemp oil and was obtained from a commercial source (Alchemy CBD Oil 40%-Full Spectrum-MCT, CBD Alchemy, Castelfranco Emilia, Italy). The commercial CBD oil preparation contained 4000 mg per 10 mL. Final dietary concentration of 0.001% pure CBD per kg of feed was used in this experiment, according to previous studies on fish (Mortuza et al., 2021). CBD extract was incorporated in the diet following the protocol described by Juries (2021). Briefly, CBD was added to coconut oil (Guinama S.L.U., Valencia, Spain) at a ratio of 1:500 and gradually mixed with the pellets. The treatments that did not contain CBD oil were also mixed with coconut oil only. The mixture was then left at room temperature for at least 10 min.

2.3 Fish husbandry and feeding procedures

A 38-day feeding trial was conducted on juvenile European sea bass (*Dicentrarchus labrax*) (n=420) with an initial body weight of 105.06 ± 2.17 g (mean ± SD). Triplicate groups of fish, produced in the aquaculture facilities at ULPGC, were randomly distributed among twelve 500-L cylindroconical tanks at a density of 35 fish per tank (initial biomass density: 7.35 kg/m^3) and acclimated at 20.8 ± 0.17 °C, 12:12 light:dark photoperiod and a flow-through system with a water renewal rate of one tank volume per hour. The distribution and behavior of the fish were monitored for 2 min once a day during the feeding period using video recordings by trained observers. These observations focused on predefined behavioral categories, such as feeding and swimming activity and spatial distribution. The fish were hand-fed a commercial extruded L-4 Optibass 2P European sea bass feed from Skretting (Stavanger, Norway) at a daily ration of 3.5% of the initial biomass, calculated from the individual body weight of the fish, three times a day, 6 days a week. Uneaten feed was collected and dried once daily to calculate the feed conversion ratio (FCR). Growth performance parameters were estimated as follows:

Weight gain, WG (g) = final weight – initial weight; Specific growth rate, SGR (%/day) = (ln final mean weight - ln initial mean weight)/days x 100; Feed conversion ratio, FCR = dry feed intake (g)/weight gain (g); Condition factor (K) = (Weight (g) x 100)/ length$(cm)^3$.

2.4 Stress challenge test

At the end of the feeding trial, fish were fasted for 24 h before the final sampling. Then, 54 fish per treatment were randomly selected and subjected to a challenge test. The stress trial was conducted in the same experimental tanks used during the feeding trial (n=12). Within each tank, three 20-L submerged cages at a stocking density of 49kg/m^3 were used, each containing six fish assigned to one of the three post-confinement sampling times (2, 24 and 168 h). Thus, each treatment included three replicate cages for each sampling time, corresponding to 18 fish per sampling time and 54 fish per treatment. Basal samples (0 h; 18 fish per treatment) were collected prior to confinement. After the stress challenge, head kidney and brain samples, including telencephalon, hypothalamus, and pituitary, and blood samples were obtained. For individual blood samples, blood was collected from the caudal vein using 1 mL syringes after sedation with anesthetic (0.02 mL/L of clove oil) and 0.5 mL was collected for plasma and 0.5 mL for serum. Subsequently, the animals were euthanized with an overdose of clove oil (0.5 mL/L diluted in alcohol 100% 1:2), according to the protocol described by Torrecillas et al. (2024), to obtain pooled samples from 6 individuals per tissue from the different brain regions aforementioned, which were stored directly in TRI Reagent (Sigma-Aldrich, St Louis, MO, USA) and from the head kidney, which were stored directly in RNAlater™. During the confinement stress challenge, fish mortality was recorded for a graphical representation of the survival rate using the Kaplan-Meier curve.

2.5 Plasma cortisol

For the determination of plasma cortisol concentration, 0.5 mL aliquots of each blood sample from six fish per tank and sampling point (72 per treatment) were collected in 1.5 mL Eppendorf tubes, which were previously heparinized. Immediately after collection, the blood was centrifuged at 3.000 x g for 5 min at 4 °C to obtain plasma samples. The plasma was transferred to a new Eppendorf tube and stored at -80 °C until analysis. These samples were processed at Animal Lab, S.L. (Las Palmas de Gran Canaria, Canary Islands, Spain) using an Access Cortisol assay kit (Beckman Coulter, Inc., Brea, CA, USA). All samples were analyzed in duplicate.

2.6 Serum lysozyme activity

For the quantification of serum lysozyme activity, 0.5 mL aliquots of each blood sample from six fish per tank and sampling point (72 fish per treatment) were collected in 1.5 mL Eppendorf

tubes. The blood was stored at 4°C for 24 h to allow blood coagulation and then centrifuged at 5.000 x g for 3 min at 4°C to obtain serum samples. The serum was transferred to a new Eppendorf tube and stored at -80 °C until analysis. Lysozyme activity was determined according to the method described by Ellis (1988) using *Micrococcus lysodeikticus* (0.2mg/mL) in sodium phosphate buffer (0.05M, pH= 6.3). A newly prepared 40.000 U/mL egg white lysozyme (Sigma-Aldrich, St. Louis, MO, USA) was serially diluted in phosphate buffer to generate the calibration curve. Absorbance was measured every 5 min during 1 h at 450 nm using SpectraMax® Mini (Molecular Devices, California, USA) after adding 200 µL of *M. lysodeikticus* solution and 10 µL of serum sample to each well. Lysozyme activity was calculated using the absorbance recorded at 15 min, as this time point showed the best linearity of the standard curve. All samples were analyzed in duplicated. Lysozyme activity was calculated considering one unit of lysozyme as the quantity of enzyme needed for reducing absorbance 0.001 per milliliter and per minute. Protein quantification was performed to standardize lysozyme activity; the protein content of the samples was determined by the methodology described by Bradford (1976) using bovine serum albumin (BSA) as a standard. Final lysozyme activity was expressed as U/mg protein.

2.7 Lipid mediators analysis

Lipid mediators analyses were conducted as described by Campos-Sánchez et al. (Campos-Sánchez et al., 2022). at the Institute of Aquaculture, University of Stirling. Briefly, head kidney samples were weighed into polypropylene tubes, adding antioxidant solution (BHT, EDTA) and internal standard solution. The samples were extracted using cold methanol, then homogenized and incubated on ice before being centrifuged at 20.000 x g for 10 min at 4 °C. The supernatant was dried under nitrogen, reconstituted in a 90:10 (v/v) water:methanol mixture, acidified with acetic acid, and loaded onto a SepPak tC18 SPE column. The columns were conditioned with methanol and water and washed with water and hexane. The lipid mediators were eluted with methyl formate, dried under nitrogen, resuspended in a water:methanol solution, centrifuged at 13.000 x g for 2 min, and transferred to vials for analysis. Quantification was performed by UPLC-MS/MS using a Waters Xevo TQ-S. All samples were analyzed in duplicated. The lipid mediators analyzed were linoleic acid derivatives (LA) 18:2 n-6: 13-Hydroxyoctadecatrienoic acid (13-HOTrE), 9-Hydroxyoctadecadienoic acid (9-HODE), 13-Hydroxyoctadecadienoic acid (13-HODE), 15-Hydroxyeicosatrienoic acid (15-HETrE); arachidonic acid derivatives (ARA), 20:4 n-6: 5-Hydroxyeicosatetraenoic acid (5-HETE), 12-Hydroxyeicosatetraenoic acid (12-HETE), 15-Hydroxyeicosatetraenoic acid (15-HETE), 5,15-Dihydroxyeicosa-6,8,11,13-tetraenoic acid (5,15-diHETE), Prostaglandin D2 (PGD2), 8-iso-prostaglandin E2 (PGE2); eicosapentaenoic acid derivatives (EPA), 20:5 n-3: 5-Hydroxyeicosapentaenoic acid (5-HEPE), 12-Hydroxyeicosapentaenoic acid (12-HEPE), 5-Hydroxyeicosapentaenoic acid (15-HEPE); docosahexaenoic acid derivatives (DHA), 22:6 n-3: 14-Hydroxydocosahexaenoic acid (14-HDHA), 17-Hydroxydocosahexaenoic acid (17-HDHA), Protectin D1.

2.8 RNA extraction and gene expression analysis

To assess the relative expression of stress-response and appetite-regulating genes, real-time quantitative PCR (RT-qPCR) analysis was carried out as previously described (Torrecillas et al., 2021). Briefly, total RNA was extracted from the telencephalon, hypothalamus, and pituitary of juvenile sea bass stored in TRI-Reagent (Sigma-Aldrich, St. Louis, MO, USA) using the Omega commercial kit "E.Z.N.A. Total RNA Kit I" (Omega bio-tek, Norcoss, GA, USA). The quantity and quality of RNA was checked using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Then, the commercial *Kit iScriptTM cDNA Synthesis* (Bio-Rad, California, USA) was used to obtain complementary DNA (cDNA) by RNA retrotranscription using the BIORAD T100™ thermal cycler (Bio-Rad, Hercules, CA, USA) in 23 µL reaction containing 2 µL of total RNA. The samples were then diluted to 1:10 in Milli-Q water and stored at -20°C. Real Time-qPCR was carried out in a final volume of 25 µL, containing 2 µL of cDNA (100 ng), 12 µL of iTAQ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), and 500 nM of each primer using a C1000 Touch™ CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) under the following conditions: 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, 30 s at annealing temperature, and 1 cycle of 5 s from 55°C to 95°C. All reactions were carried out in duplicate for each template cDNA, and blank control reactions were carried out using water in place of the cDNA. Relative gene expression was measured using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001) and using *β-actin* as housekeeping gene. The primer sequences used are listed in Table 1.

2.9 Statistical analysis

The tank constituted the experimental unit in this experiment, considering that the treatments were not applied individually to each fish and, therefore, were not independent replicates. Consequently, the sample size was defined as the number of tanks per treatment (n=3), with a total of 35 fish per tank. Furthermore, to minimize the number of fish and ensure a valid statistical design, statistical power analyses were conducted to guarantee the robustness of the experimental design using G*Power v. 3.1.9.7 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The data of the present study are shown as mean ± standard deviation (SD). Statistical treatment of the data was performed with IBM SPSS Statistics v26 software (IBM Corp. Armonk, NY, United States). The results of the experiment were analyzed using Two-way ANOVA tests to evaluate the effects of CBD, EE and their interaction. A One-way ANOVA was performance to analyze differences among treatments, followed by the Tukey *post-hoc* range test, considering significant differences when $p < 0.05$ for growth parameters, non-specific immune parameters, lipid mediators and stress response parameters. The Kaplan-Meier survival curve was used to analyze the survival rate. The normality of the experimental data was tested with the Shapiro-Wilk test and the homogeneity of the variances was tested with the Levene's test.

TABLE 1 Sequences of primers used to evaluate gene expression by qPCR in European sea bass (*Dicentrarchus labrax*) brain.

Gene		Nucleotide sequence (5′-3′)	Access number	Reference
agrp1	Fw	TCTGCTGCGTCTCTCTTCTT	HE660086 [1]	(Martins et al., 2022)
	Rv	TCTCTCCAGGTCAGACAGGT		
agrp2	Fw	GGGCAGAGGACACAAAGAAA	HE660087 [1]	(Martins et al., 2022)
	Rv	TGTGACTTTCCTGTGGTGGA		
cart2	Fw	CCGAACCTGACCAGCGAGAA	MZ441181 [1]	(Martins et al., 2022)
	Rv	GCTCCCCGACATCACACGTT		
cnr1	Fw	GCAGTTGGACAATAGCCTCG	XM_051409468 [1]	
	Rv	CGTGACGGCCAATTAGACAG		
cnr2	Fw	GTGATCCTTACCCACACCCA	XM_051407832 [1]	
	Rv	ACACTTCCCAACCTCACACA		
crf	Fw	AACCCAAAACTCCCAGCAG	JF274994.1 [1]	(Azeredo et al., 2022)
	Rv	TGTTCCCAACTTTCCCTTGT		
gr2	Fw	GACGCAGACCTCCACTACATTC	AY619996.1 [1]	(Sadoul et al., 2018)
	Rv	GCCGTTCATACTCTCAACCAC		
htr1aβ	Fw	GGAGCGTAAAACGGTGAAAA	DLAgn_00119560 [2]	(Azeredo et al., 2022)
	Rv	TGGGGTTGAGGAGAGAGTTG		
mc4r	Fw	ATGAACACCACAGAGGCTCA	FM253127 [1]	(Sánchez et al., 2009)
	Rv	ATAGCATCCTGTGGACGAGT		
npy	Fw	ACTCAGCCCTGAGACACTACA	AJ005378 [1]	(Martins et al., 2022)
	Rv	ACTGTGGAAGCTGGTCTGTG		
pomc	Fw	TCTTCCTCCTCCTCTCCACA	AY691808.1 [1]	(Azeredo et al., 2022)
	Rv	CGCCTTCTCATCTCTTCAGG		
β-actin	Fw	ATGTGGATCAGCAAGCAGG	AJ537421 [1]	(El Aamri et al., 2015)
	Rv	AGAAATGTGTGGTGTGGTCG		

Sequences were obtained from NCBI GenBank[1] and dicLab v1.0c[2] databases. *agrp1*: agouti-related protein 1; *agrp2*: agouti-related protein 2; *cart2*: cocaine-and-amphetamine-regulated transcript; *cnr1*: cannabinoid receptor 1; *cnr2*: cannabinoid receptor 2; *crf*: corticotropin release factor *gr2*: glucocorticoid receptor; *hrt1aβ*: serotonin receptor 1aβ; *mc4r*: melanocortin 4 receptor; *npy*: neuropeptide Y; *pomc*: proopiomelanocortin.

3 Results

3.1 Growth performance

After 5 weeks of starting the trial, the use of EE induced a significantly higher final weight ($p = 0.05$). The combined application of EE and the adaptogen significantly increased both the SGR ($p = 0.009$) and WG ($p = 0.005$) compared with the adaptogen treatment in the absence of EE. In addition, EE significantly reduced FCR ($p = 0.022$). In contrast, the condition factor did not differ significantly among treatments (Table 2).

3.2 Water oxygenation and behavioral observations

During the experimental period, the EE treatments resulted in significantly higher dissolved oxygen (DO) concentrations ($p = 0.003$) (Table 3). Qualitative observations during the feeding trial suggested differences in behavioral patterns according to environmental complexity. Fish reared in microbubble curtain tanks appeared to exhibit a more homogeneous distribution and reduced erratic movements. In contrast, fish subjected to standard aeration congregated near the aerator and showed limited swimming activity (Figure 2).

3.3 Survival after stress challenge

At the end of the confinement stress challenge, the control group showed the highest mortality rate, which was significantly ($p = 0.04$) greater than that observed in other treatments. Nevertheless, no significant differences ($p > 0.05$) were detected among the +CBD/-EE, -CBD/+EE, and +CBD/+EE treatments (Figure 3).

3.4 Plasma cortisol concentrations

The application of the different welfare-improving strategies resulted in significantly lower basal concentrations of plasma cortisol ($p = 0.005$). At the initial sampling point (0 h), fish in the control group exhibited significantly higher plasma cortisol levels than those in all other treatments ($p < 0.001$). At 2 h post-stress, plasma cortisol levels decreased in the control group, although they remained significantly higher than those observed in the EE treatment group. Similar cortisol

TABLE 2 Growth performance and feed utilization of European sea bass (*Dicentrarchus labrax*) juveniles after 38 days of the feeding trial.

Parameter	CONTROL	+CBD/-EE	-CBD/+EE	+CBD/+EE	Two-way ANOVA		
					CBD	EE	CBD*EE
Initial weight (g)	105.24 ± 8.73	105.62 ± 7.34	104.22 ± 8.04	105.06 ± 7.96	ns	ns	ns
Final weight (g)	162.95 ± 20.52^{ab}	159.71 ± 20.10^{b}	164.91 ± 17.87^{ab}	168.3 ± 18.82^{a}	ns	p=0.05	ns
WG (g)	57.69 ± 4.01^{ab}	54.09 ± 2.71^{b}	60.69 ± 1.38^{a}	63.15 ± 0.42^{a}	ns	p=0.005	ns
SGR (%/day)	1.15 ± 0.07^{ab}	1.09 ± 0.05^{b}	1.21 ± 0.05^{ab}	1.24 ± 0.01^{a}	ns	p=0.009	ns
FCR	1.69 ± 0.17^{a}	1.78 ± 0.08^{a}	1.59 ± 0.06^{a}	1.56 ± 0.02^{a}	ns	p=0.022	ns
K-Factor	1.33 ± 0.02	1.36 ± 0.02	1.34 ± 0.02	1.36 ± 0.04	ns	ns	ns

Two-way ANOVA, $p < 0.05$; diet and EE as fixed factors. Different letters (mean ± SD) denote significant differences ($p < 0.05$) between different experimental strategies analyzed with Tukey *post-hoc* range test. ns, not significant. CBD, cannabidiol; EE, environmental enrichment with microbubbles.

TABLE 3 Dissolved oxygen concentrations (mg/L) in experimental tanks of European sea bass (*Dicentrarchus labrax*) juveniles after 38 days of the experimental phase.

CONTROL	+CBD/-EE	-CBD/+EE	+CBD/+EE	Two-way ANOVA		
				CBD	EE	CBD*EE
6.92 ± 0.54^{b}	7.02 ± 0.46^{ab}	7.29 ± 0.48^{a}	7.24 ± 0.49^{ab}	ns	p=0.003	ns

Two-way ANOVA, $p < 0.05$; diet and EE as fixed factors. Different letters (mean ± SD) denote significant differences ($p < 0.05$) between different experimental strategies analyzed with Tukey *post-hoc* range test. ns, not significant; CBD, cannabidiol; EE, environmental enrichment with microbubbles.

levels were maintained in the EE groups. At 24 h post-stress, fish receiving the CBD treatment showed significantly lower concentrations of circulating plasma cortisol. At the final sampling point (168 h), the treatments including the adaptogen (Treatments 2 and 4) exhibited significantly lower concentrations of circulating plasma cortisol than the other treatments ($p < 0.001$). Two-way ANOVA revealed significant interactions between diet and EE at 0 ($p = 0.005$), 2 ($p = 0.023$), and 24 h ($p= 0.042$), whereas significant dietary effects were detected at 168 h compared to EE (Figure 4).

3.5 Serum lysozyme activity

The +CBD/-EE treatment induced a significantly higher basal concentration of lysozyme activity compared with the other treatments ($p = 0.029$). Nevertheless, control and +CBD/-EE groups showed significantly higher levels 2 h after stress. No significant differences were observed among the groups at 24 and 168 h after the stress. According to the Two-way ANOVA, significant effects of EE were found at 0 h ($p = 0.001$) and 2 h ($p < 0.001$) (Figure 5).

3.6 Lipid inflammatory mediators

Significant differences ($p < 0.05$) in levels of 15-HEPE were observed at the 0 h sampling point, with CBD-fed fish showing significantly higher levels. After 2 h of confinement, significant differences ($p < 0.05$) in the relative levels of several lipid inflammatory mediators were observed. Fish from +CBD/+EE treatment showed significantly higher ($p < 0.05$) 15-HETrE, 12-HETE, 15-HETE, PGD2, 12-HEPE and 14-HDHA levels, whereas fish from the control treatment (-CBD/-EE) exhibited significantly higher 9-HODE levels. After 24 h of stress exposure, -CBD/+EE and +CBD/+EE showed higher levels of 15-HETE and -CBD/+EE exhibited more expression in 15-HEPE than the control treatment (-CBD/-EE). No significant differences ($p > 0.05$) were found at 168 h. Two-way ANOVA analysis revealed significant effects of diet at 2 h for 12-HEPE and 14-HDHA and after 24 h in 12-HETE. Significant EE effects were detected at 0 h for protectin D1 ($p = 0.019$) and after 168 h 17-HDHA ($p = 0.05$). Significant interactions between diet and EE were observed at 0 h for 5-HETE ($p = 0.04$), 15-HEPE ($p =$

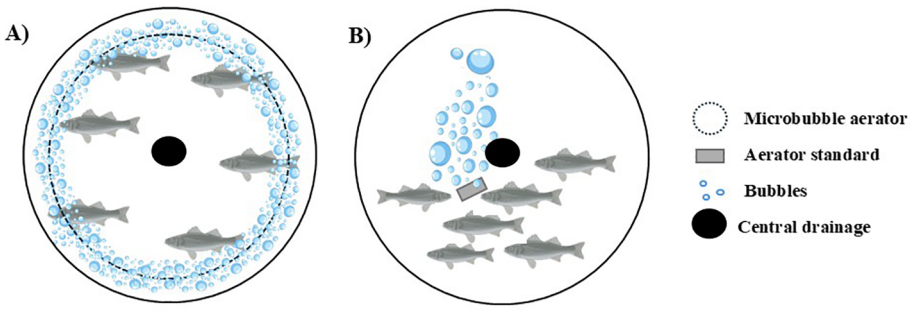

FIGURE 2
Behavioral patterns in experimental tanks of juveniles European sea bass under enriched **(A)** and non-enriched **(B)** microbubbles curtain.

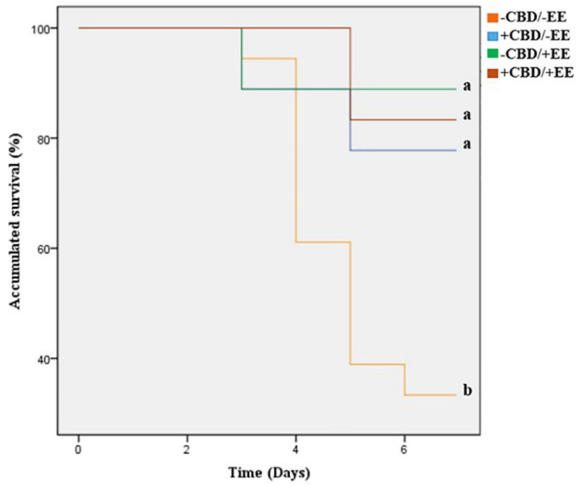

FIGURE 3
Kaplan-Meier survival curve for juvenile European sea bass (*Dicentrarchus labrax*) after 7-day confinement stress challenge. Different letters denote significant ($p < 0.05$) differences. CBD, cannabidiol; EE, environmental enrichment with microbubbles.

0.031) and 17-HDHA ($p = 0.019$); after 2 h for 9-HODE ($p = 0.043$), 13-HODE ($p = 0.032$), 5-HETE ($p = 0.049$), 5,15-diHETE ($p = 0.047$), 15-HETrE ($p = 0.009$) and 17-HDHA ($p = 0.006$) and at 24 h for 15-HETE ($p = 0.008$) and 15-HEPE ($p = 0.005$) (Figure 6).

3.7 Relative gene expression of stress response-related genes in brain

3.7.1 Gene expression in telencephalon

3.7.1.1 Relative expression of stress-response related genes

No significant differences ($p > 0.05$) in the relative expression of targeted genes were found at the 0 h sampling point in the telencephalon of the fish exposed to any of the treatments. After 2 h of confinement stress, the relative expression of *crf* was significantly higher ($p < 0.05$) in fish from +CBD/-EE treatment. In addition, *pomc* expression was significantly higher ($p < 0.05$) in fish from −CBD/+EE treatment compared with fish from the control and treatment 2 groups (−CBD/−EE; +CBD/−EE). The relative expression of *gr2* was significantly higher ($p < 0.05$) in -CBD/+EE and +CBD/+EE treatment after 24 h of the stress test. According to Two-way ANOVA analysis, differences in EE were found at 24 h for *gr2* ($p = 0.017$) expression and at 2 h significant interactions between diet and EE were found for *gr2* (0.021), *crf* ($p = 0.012$), *pomc* ($p = 0.016$) and *hrt1aβ* ($p = 0.035$) gene expression (Figure 7).

3.7.1.2 Relative expression of appetite-regulating related genes

No significant differences ($p > 0.05$) in the relative expression of the targeted genes were observed at the 0 h sampling point in the telencephalon among treatments. After 2 h of confinement stress, fish from +CBD/−EE treatment exhibited significantly higher ($p < 0.05$) expression of *agrp1* and *cnr2*. After 24 h, the relative expression of *agrp2*, *npy* and *mc4r* were significantly ($p < 0.05$) higher in +CBD/+EE treatment, whereas *cnr2* expression was significantly higher ($p < 0.05$) in -CBD/+EE and +CBD/+EE. Two-way ANOVA analysis revealed significant effects of EE at 0h for *cnr2* ($p = 0.038$) and at 24 h for *cnr2* ($p < 0.001$) and *mc4r* ($p < 0.001$). Significant dietary effects were detected at 2 h for *npy* ($p < 0.001$) and *agrp2* ($p = 0.015$) and at 24 h for *npy* ($p = 0.002$). Significant interactions between diet and EE were observed at 2 h for *agrp1* ($p = 0.005$), *cnr2* ($p = 0.002$), and *mc4r* ($p = 0.018$); at 24 h for *agrp2* ($p = 0.024$); and at 168 h for *agrp1*, *cnr1*, *cnr2*, and *mc4r* expression (Figure 7).

3.7.2 Gene expression in hypothalamus

3.7.2.1 Relative expression of stress-response related genes

No significant differences ($p > 0.05$) in the relative expression of the studied genes were found in the hypothalamus at the 0h sampling point among the different treatments. After 24 h of confinement

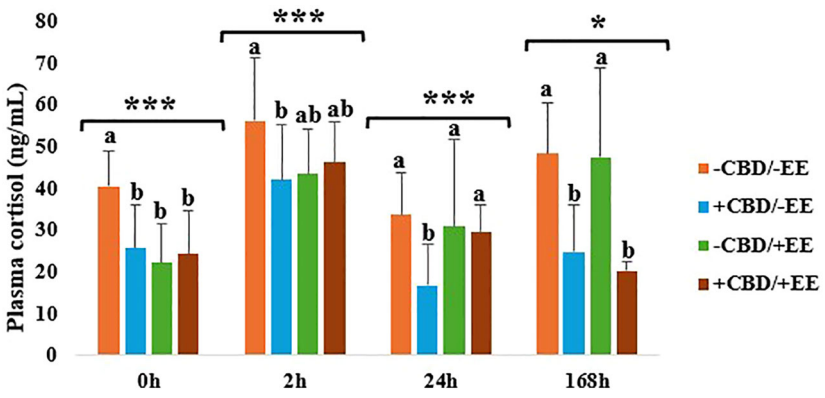

FIGURE 4
Circulating plasma cortisol concentration expressed in ng/mL of European sea bass (*Dicentrarchus labrax*) juveniles at different sampling stress-confinement time points. Different symbols denote significant differences ($p < 0.05$) analyzed with Two-way ANOVA, CBD (*) and EE (**) as combined factors (***). Different letters denote significant differences ($p < 0.05$) analyzed with One-way ANOVA followed *post-hoc* Tukey range test in a given sampling point. CBD, cannabidiol; EE, environmental enrichment with microbubbles.

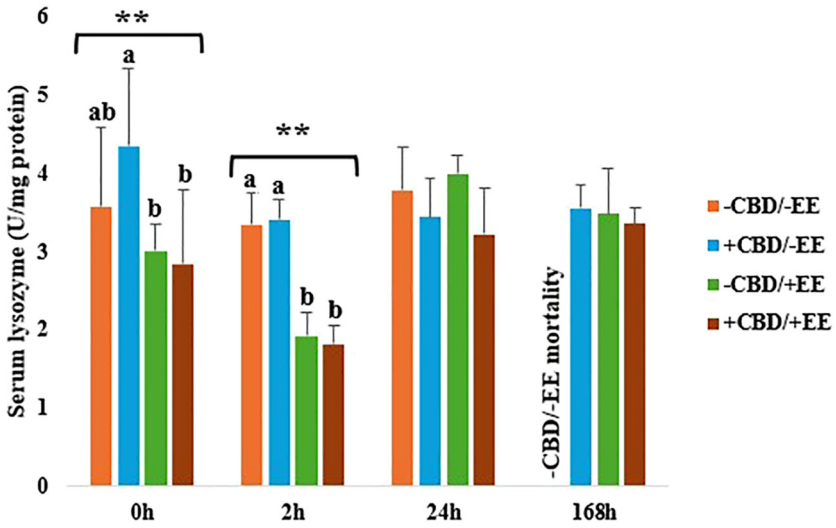

FIGURE 5
Serum lysozyme concentration expressed in U/mg of protein in European sea bass (*Dicentrarchus labrax*) juveniles at different sampling stress-confinement time points. Different symbols denote significant differences ($p < 0.05$) analyzed with Two-way ANOVA, CBD (*) and EE (**) as combined factors (***). Different letters denote significant differences ($p < 0.05$) analyzed with One-way ANOVA followed *post-hoc* Tukey range test in a given sampling point. CBD, cannabidiol; EE, environmental enrichment with microbubbles.

stress, the relative expression of *crf* was significantly higher ($p < 0.05$) in fish from the control treatment (-CBD/-EE). After 168 h of stress challenge, the relative expression of *gr2*, *crf* and *hrt1aβ* were also significantly higher ($p < 0.05$) in the control group (-CBD/-EE). Two-way ANOVA revealed significant effects of EE on *crf* ($p = 0.018$) expression at 24 h. In addition, significant interactions between diet and EE were detected for *gr2* ($p = 0.018$), *crf* ($p = 0.029$), *pomc* ($p = 0.017$), and *hrt1aβ* ($p = 0.021$) expression at 2 and 168 h (Figure 8).

3.7.2.2 Relative expression of appetite-regulating related genes

Significant differences ($p < 0.05$) in the relative expression of several appetite-regulating genes were observed in the hypothalamus at the 0 h sampling point. Fish from −CBD/+EE treatment showed significantly higher *agrp2* expression, whereas fish from +CBD/+EE treatment exhibited significantly higher *cnr1* expression compared with the control treatment (−CBD/−EE). After 2 h of confinement stress, the relative expression of *npy* and *cart2* was significantly higher in -CBD/+EE, whereas *mc4r* expression was significantly higher in +CBD/+EE treatment. After 24 h of stress exposure, *mc4r* expression remained significantly higher in +CBD/+EE treatment. Two-way ANOVA indicated significant effects of EE on *cnr1* ($p = 0.006$) expression at 0 h. Furthermore, significant interactions between diet and EE were observed for *agrp1, agrp2, cart2, cnr2, npy*, and *mc4r* across the different sampling time points (Figure 8).

3.7.3 Gene expression in pituitary gland

3.7.3.1 Relative expression of stress-response related genes

Significant differences ($p < 0.05$) in the relative expression of stress-related genes were found in the pituitary gland at the 0 h sampling point. Fish from -CBD/+EE treatment showed significantly higher *pomc* expression, compared to fish from +CBD/+EE ($p < 0.05$). Similarly, *hrt1aβ* expression was significantly higher in fish from -CBD/+EE treatment ($p < 0.05$). After 168 h of confinement stress, the relative expression of *gr2, crf*, and *hrt1aβ* was significantly higher ($p < 0.05$) in −CBD/+EE treatment. According to the Two-way ANOVA, differences in diet were found at 0 h and 168 h for *hrt1aβ* ($p = 0.044$) and *crf* ($p = 0.032$), respectively; in EE at 168 h for *gr2* ($p = 0.037$) and *hrt1aβ* ($p = 0.042$) and significant interactions between diet and EE for *pomc* ($p = 0.026$) expression at 0 h (Figure 9).

3.7.3.2 Relative expression of appetite-regulating related genes

No significant differences ($p > 0.05$) were found in the relative expression of the studied genes at 0 h in the pituitary gland of the fish exposed to any of the treatments. After 2 h of confinement stress, *cnr1* expression was significantly higher ($p < 0.05$) in fish from −CBD/+EE treatment, whereas *mc4r* expression was significantly higher in the control treatment (−CBD/−EE). After 24 h of stress exposure, both *cnr1* and *mc4r* expression levels were significantly higher ($p < 0.05$) in the control group. Two-way ANOVA revealed differences in diet were found at 2 h for *cnr1* ($p = 0.005$), *cnr2* ($p = 0.042$), *mc4r* ($p = 0.006$) and *npy* ($p = 0.045$) and at 168 h for *cnr1* ($p = 0.02$); in the EE at 2 h for *agrp2* ($p = 0.013$) and significant interactions between diet and EE for *cnr1* ($p = 0.026$) at 24 h and for *mc4r* ($p = 0.022$) and *npy* ($p = 0.048$) at 168 h (Figure 9).

4 Discussion

The utilization of the microbubble curtain system as EE improved the growth performance and feed efficiency in juvenile

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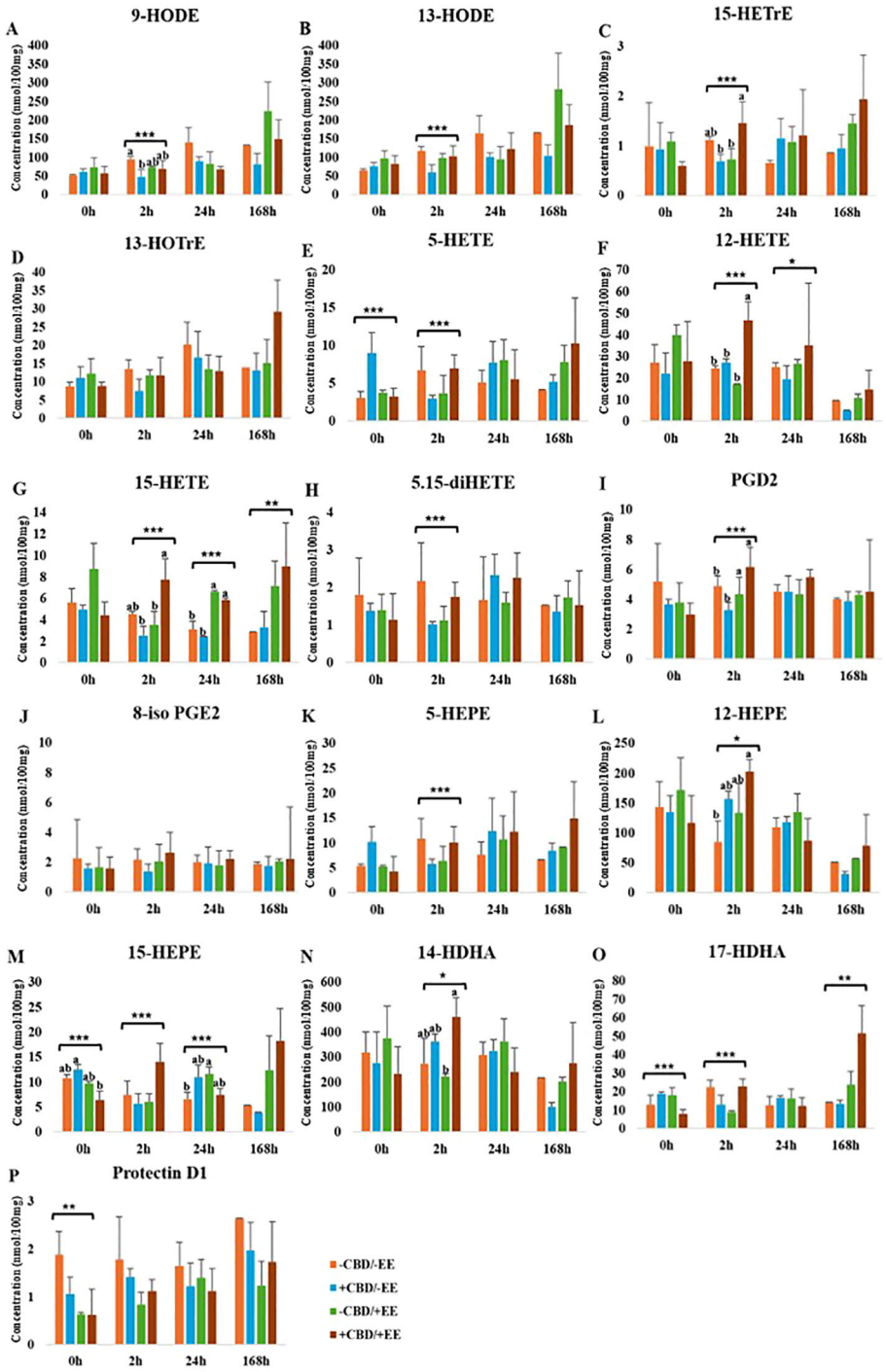

FIGURE 6
Concentrations of lipid inflammatory mediators expressed in nmol/100mg in the head kidney of European sea bass (*Dicentrarchus labrax*) juveniles during the stress challenge test. Linoleic acid derivatives (LA) 18:2 n-6 **(A–D)**; arachidonic acid derivatives (ARA), 20:4 n-6 **(E–J)**; eicosapentaenoic acid derivatives (EPA), 20:5 n-3 **(K–M)**; docosahexaenoic acid derivatives (DHA), 22:6 n-3 **(N–P)**. Different symbols denote significant differences ($p<0.05$) analyzed with Two-way ANOVA, CBD (*) and EE (**) as combined factors (***). Different letters denote significant differences ($p<0.05$) analyzed with One-way ANOVA followed *post-hoc* Tukey range test in a given sampling point; 13-Hydroxyoctadecatrienoic acid (13-HOTrE); 9-Hydroxyoctadecadienoic acid (9-HODE); 13-Hydroxyoctadecadienoic acid (13-HODE); 5-Hydroxyeicosapentaenoic acid (5-HEPE); 12-Hydroxyeicosapentaenoic acid (12-HEPE); 5-Hydroxyeicosapentaenoic acid (15-HEPE); 5-Hydroxyeicosatetraenoic acid (5-HETE); 12-Hydroxyeicosatetraenoic acid (12-HETE); 15-Hydroxyeicosatetraenoic acid (15-HETE); 15-Hydroxyeicosatrienoic acid (15-HETrE); 5,15-Dihydroxyeicosa-6,8,11,13-tetraenoic acid (5,15-diHETE); 14-Hydroxydocosahexaenoic acid (14-HDHA); 17-Hydroxydocosahexaenoic acid (17-HDHA); Prostaglandin D2 (PGD2); 8-iso-prostaglandin E2 (PGE2); CBD, cannabidiol; EE, environmental enrichment with microbubbles.

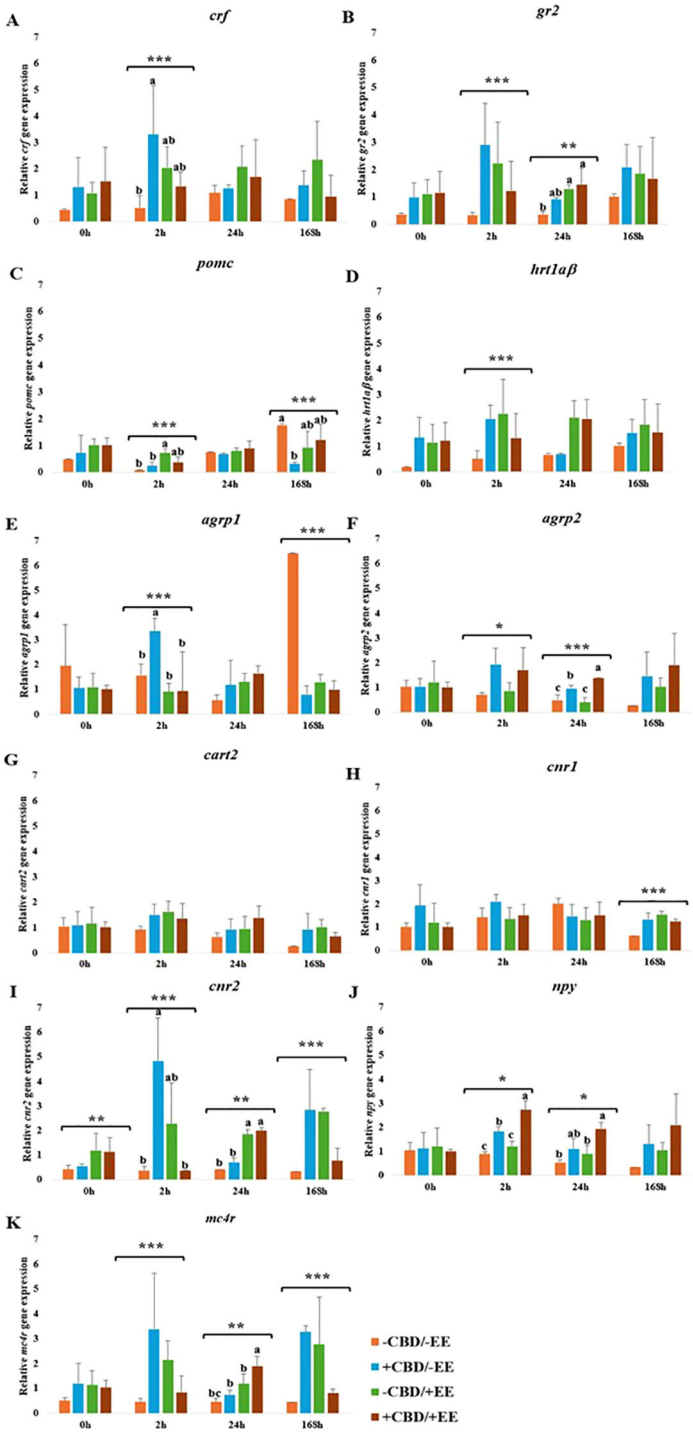

FIGURE 7
Telencephalon relative gene expression in European sea bass (*Dicentrarchus labrax*) during the stress challenge test. Stress-response related genes **(A–D)**; appetite-regulating related genes **(E–K)**. Different symbols denote significant differences ($p<0.05$) analyzed with Two-way ANOVA, CBD (*) and EE (**) as combined factors (***). Different letters denote significant differences ($p < 0.05$) analyzed with One-way ANOVA followed *post-hoc* Tukey range test in a given sampling point. *crf*, corticotropin release factor; *gr2*, glucocorticoid receptor; *pomc*, proopiomelanocortin; *hrt1aβ*, serotonin receptor 1aβ; *agrp1*, agouti-related protein 1; *agrp2*, agouti-related protein 2; *cart2*, cocaine-and-amphetamine-regulated transcript; *cnr1*, cannabinoid receptor 1; *cnr2*, cannabinoid receptor 2; *npy*, neuropeptide Y; *mc4r*, melanocortin 4 receptor; CBD, cannabidiol; EE, environmental enrichment with microbubbles.

European sea bass and significantly increased the DO levels in the experimental tanks. Likewise, EE has been reported to promote growth and higher DO levels in different aquaculture farmed species like red tilapia (*Oreochromis* sp.), whiteleg shrimp (*Litopenaeus vannamei*) and striped catfish (*Pangasianodon hypophthalmus*) (Lim et al., 2021; Heriyati et al., 2022; Subhan et al., 2022). In parallel, EE has also been shown to improve growth-related parameters in rainbow trout (*Oncorhynchus mykiss*), juvenile gilthead sea

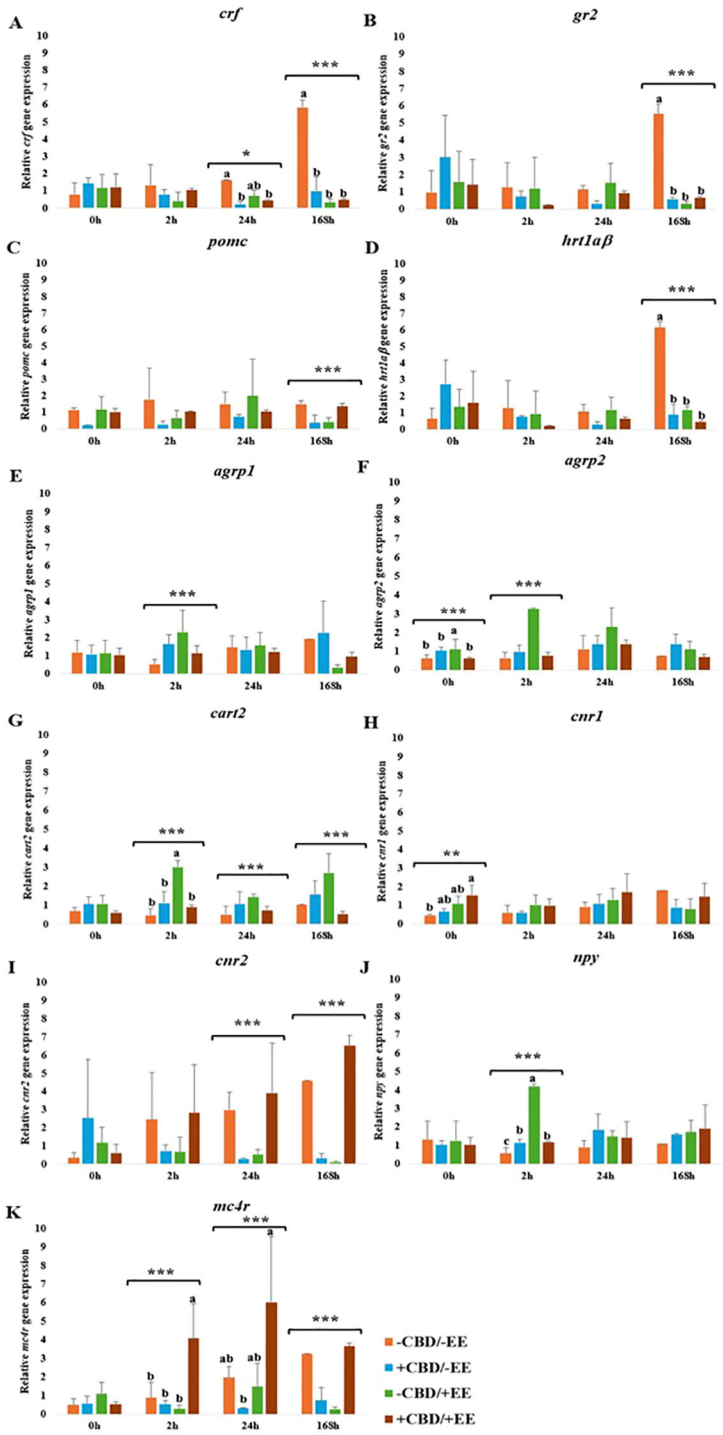

FIGURE 8
Hypothalamus relative gene expression in European sea bass (*Dicentrarchus labrax*) during the stress challenge test. Stress-response related genes **(A–D)**; appetite-regulating related genes **(E–K)**. Different symbols denote significant differences ($p < 0.05$) analyzed with Two-way ANOVA, CBD (*) and EE (**) as combined factors (***). Different letters denote significant differences ($p < 0.05$) analyzed with One-way ANOVA followed *post-hoc* Tukey range test in a given sampling point. *crf*, corticotropin release factor; *gr2*, glucocorticoid receptor; *pomc*, proopiomelanocortin; *hrt1aβ*, serotonin receptor 1aβ; *agrp1*, agouti-related protein 1; *agrp2*, agouti-related protein 2; *cart2*, cocaine-and-amphetamine-regulated transcript; *cnr1*, cannabinoid receptor 1; *cnr2*, cannabinoid receptor 2; *npy*, neuropeptide Y; *mc4r*, melanocortin 4 receptor; CBD, cannabidiol; EE, environmental enrichment with microbubbles.

bream (*Sparus aurata*) and in African catfish (*Clarias gariepinus*) (Batzina et al., 2014; M. Voorhees et al., 2020; Brunet et al., 2022; Ojelade et al., 2022). These findings suggest that the beneficial effects observed in this study were likely mediated by a combination of improved water quality and greater environmental complexity. By contrast, dietary CBD did not significantly affect growth performance, which is consistent with previous reports in Nile tilapia (*Oreochromis niloticus*) and mammalian models where CBD

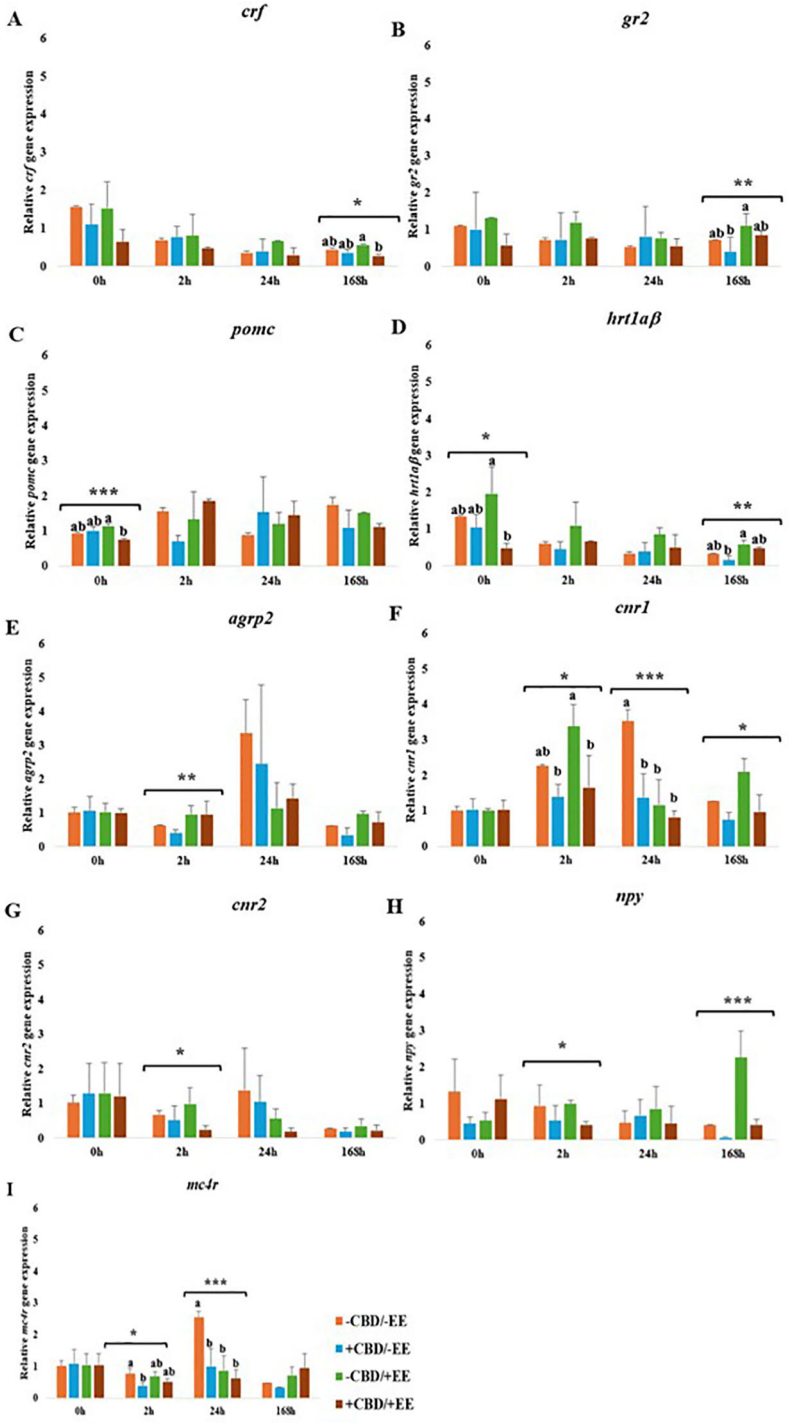

FIGURE 9
Pituitary gland relative gene expression in European sea bass (*Dicentrarchus labrax*) during the stress challenge test. Stress-response related genes **(A–D)**; appetite-regulating related genes **(E–I)**. Different symbols denote significant differences ($p < 0.05$) analyzed with One-way ANOVA followed Two-way ANOVA, CBD (*) and EE (**) as combined factors (***). Different letters denote significant differences ($p < 0.05$) analyzed with *post-hoc* Tukey range test in a given sampling point. *crf*, corticotropin release factor; *gr2*, glucocorticoid receptor; *pomc*, proopiomelanocortin; *hrt1aβ*, serotonin receptor 1aβ; *agrp2*, agouti-related protein 2; *cnr1*, cannabinoid receptor 1; *cnr2*, cannabinoid receptor 2; *npy*, neuropeptide Y; *mc4r*, melanocortin 4 receptor; CBD, cannabidiol; EE, environmental enrichment with microbubbles.

supplementation did not alter feed intake or body weight development (Rajesh et al., 2010; Watt et al., 2020; Juries, 2021; Camargo-dos-Santos et al., 2022).

EE with microbubble curtains suggested differences in positional behavior between EE and non-EE fish. From a broad perspective, fish subjected to EE appeared to be more uniformly distributed throughout the tank and a greater range of movement, while increased feeding activity was also observed. These observations coincided with the growth parameters in EE treatments. Similarly, in Atlantic cod (*Gadus morhua*), Atlantic salmon

(*Salmo salar*), rainbow trout and sea bream, the use of EE resulted in improvements in cognitive learning capacity, changes in swimming activity, in the distribution of fish within the tank and in gregarious behavior (Salvanes and Braithwaite, 2005; Villamizar et al., 2011; Zimmermann et al., 2012; Johansson et al., 2014; Arechavala-Lopez et al., 2020; Güller et al., 2020), suggesting effects on fish welfare.

During the confinement stress challenge, all welfare-promoting strategies increased survival compared with the control group. This result indicates that fish reared under adaptogen or EE with microbubbles developed a greater capacity to cope with subsequent stress. Similar benefits of EE on survival have been reported in several aquatic species, including brown trout (*Salmo trutta*) and Atlantic salmon fingerling exposed to *Flavobacterium columnare* (Räihä et al., 2019) and improved post-vaccination performance in the Mahseer fish (*Tor soro*) (Alimuddin et al., 2023). However, evidence for CBD remains limited and sometimes inconsistent. Dietary supplementation showed no effect on survival in juvenile large yellow croaker (*Larimichthys crocea)* (Wang et al., 2023), whereas waterborne exposure improved zebrafish (*Danio rerio*) survival (Pandelides et al., 2020). Beneficial effects have also been described in mammalian disease models, where CBD increased survival in mice with cerebral malaria and in rats with Dravet syndrome (Campos et al., 2015; Patra et al., 2020). Taken together, our results support the idea that both EE and adaptogen use can enhance the robustness of European sea bass.

Stress analysis strongly supports this conclusion. Fish exposed to CBD, EE, or both showed lower basal concentrations of circulating plasma cortisol than controls. This indicates an improved physiological state before the stress challenge. EE has previously been linked to a reduced cortisol response in Chinook Salmon *(Oncorhynchus tshawytscha)* and black rockfish (*Sebastes schlegelii)* (Cogliati et al., 2019; Zhang et al., 2020). Similarly, CBD also resulted in lower basal cortisol levels in Nile tilapia (Camargo-dos-Santos et al., 2022). In the present study, the +CBD/−EE treatment showed the lowest cortisol concentrations during the acute phase (2 and 24 h), suggesting a rapid anti-stress or anxiolytic effect of CBD. Comparable responses have been described in dogs, where CBD reduced stress-associated measures during separation and transport challenges (Hunt et al., 2023; Flint et al., 2024). Under prolonged confinement, CBD-fed fish also maintained lower cortisol levels after 7 days, indicating that supplementation may facilitate recovery or reduce sustained activation of the hypothalamus-pituitary-interrenal (HPI) axis. Not all studies have found similar outcomes, since Mortuza et al (Mortuza et al., 2021, 2023) reported that CBD was unable to mitigate the negative effect of cortisol under chronic stress conditions in Nile tilapia. Such discrepancies are likely related to differences in species sensitivity, dose, supplementation duration, and the stress model used. Nevertheless, further research is still needed to establish optimal CBD dosages for different applications across aquaculture species.

Innate immune response biomarkers indicated different patterns depending on the treatments and experimental conditions. Under basal conditions, CBD-fed fish showed higher lysozyme activity. After 2 h of confinement, the control and CBD treatment exhibited the strongest lysozyme response. The subsequent stabilization of lysozyme levels in CBD-supplemented groups suggests that the adaptogen might prevent a prolonged, potentially exhaustive inflammatory response. This could facilitate a faster return to homeostasis. Besides, since chronic elevation of cortisol can suppress immune functions, the lower endocrine stress response observed in treated fish could be associated with the improved humoral defense found in the present study (Tort, 2011). Similarly, supplementation with *Cannabis* leaves in silver barb (*Barbonymus gonionotus*) infected with Southeast Asian liver fluke (*Opisthorchis viverrini*) resulted in increased lysozyme activity and disease resistance (Senasri et al., 2025). In contrast, in mahseer fish and rainbow trout, EE did not significantly affect lysozyme activity, although EE increased survival rates and decreased cortisol levels (Brunet et al., 2022; Alimuddin et al., 2023; Subramani et al., 2025).

Lipid inflammatory mediator analyses further suggest that these treatments modulated inflammatory regulation. In the present study, the +CBD/−EE group showed lower PGD2 levels, whereas the +CBD/+EE treatment exhibited higher levels of 12-HETE and 15-HETE. In fish, PGD2 participates in the control and resolution of inflammation through modulation of reactive oxygen species and cytokine pathways (Gómez-Abellán et al., 2015). HETE derivatives are recognized as bioactive mediators involved in leukocyte recruitment and inflammatory signaling. The higher expression of these lipid mediators in the combined group suggests that CBD and EE may synergistically influence inflammatory response pathways under stress. Welfare-promoting treatments also increased the levels of 12-HEPE and 15-HEPE, metabolites often associated with EPA-derived anti-inflammatory pathways. Similar shifts in LOX-derived metabolites have been described after cannabinoid exposure in human immune cells (Peltner et al., 2023) and after EPA+DHA supplementation in humans (McDaniel et al., 2011). EE and CBD-dependent interactions seem to induce a selective reprogramming of oxylipin metabolism, characterized by increased levels of 15-HETE and 15-HEPE and decreased levels of 15-HETrE. Although the precise physiological roles of these compounds in European sea bass remain to be clarified, the overall pattern suggests that CBD and EE may contribute to the modulation of inflammatory response during stress. In contrast, the higher 9-HODE levels detected in non-welfare-promoting groups are noteworthy, as oxidized linoleic acid derivatives have been associated in other vertebrates with oxidative stress and inflammatory damage (Sule et al., 2024) and CBD has been demonstrated to promote the production of anti-inflammatory eicosanoids via 15-LOX, suggesting that the changes in 9-HODE may be associated with alterations in lipoxygenase pathway (Morris et al., 2025). Overall, these changes suggest that there are treatment-related alterations in lipid signaling that are linked to the regulation of inflammation and the response to oxidative stress. The distinct mediator profiles indicate that CBD and EE have different effects on lipid-related pathways during stress.

Analyses of gene expression in the brain revealed tissue-specific modulation of neuroendocrine pathways related to stress perception, behavioral regulation, and feeding control, indicating changes in the molecular regulation of these physiological processes.

In the telencephalon, welfare-promoting treatments modified the expression of genes such as *gr2*, *crf*, *cnr2*, *htr1aβ*, and

proopiomelanocortin (*pomc)*. This region is functionally linked to limbic-like processes and monoaminergic regulation in teleosts (Sokolowski and Corbin, 2012). Although circulating cortisol was lower in treated fish, receptor dynamics do not necessarily mirror hormone concentrations. Previous work in European sea bass showed lower glucocorticoid receptor expression despite elevated cortisol under crowding stress (Terova et al., 2005), suggesting additional post-transcriptional or compensatory mechanisms (Vijayan et al., 2003). Modulation of cannabinoid and serotonergic pathways is particularly relevant because CBD has been shown to regulate these systems in rodents. These effects have often been associated with reduced anxiety-like responses (Viudez-Martínez et al., 2018; Gasparyan et al., 2021a, 2021b). Additionally, EE has also been found to influence these pathways (Koh et al., 2007; Campos et al., 2012). In parallel, the adaptogen treatments (+CBD/-EE; +CBD/+EE) influenced several appetite-regulating genes. Dietary interventions during early development in several teleost species enhance growth, feed efficiency and immune function (Wu et al., 2016; Zou et al., 2017; Xu et al., 2021; Feng et al., 2022; Ghalwash et al., 2022). These effects have been associated with the central upregulation of orexigenic pathways and suppression of anorexigenic signals. MC4R regulates feeding behavior, energy homeostasis and stress responses in teleosts (Tsalafouta et al., 2017; Hou and Wen, 2022). Therefore, *mc4r* upregulation found in the present study in welfare promoting treatments may reflect improved homeostatic regulation.

The hypothalamus acts as the main integrator of the HPI axis, coordinating the endocrine and metabolic responses required for coping with adverse conditions. It also exhibits complementary responses consistent with attenuation of central stress signaling (Gorissen and Flik, 2016). In our study, several stress-related genes, including *crf*, *gr2*, *hrt1aβ* and *pomc* were generally lower in the fish receiving adaptogen or EE during the adaptive phase. Since hypothalamic CRF directly stimulates pituitary ACTH release and subsequent cortisol secretion (Schreck et al., 2016), the lower *crf* expression observed in CBD-fed fish may be associated with reduced plasma cortisol concentrations. Similar inhibitory effects of CBD on stress-axis genes have been reported in mammalian confinement and anxiety models (Viudez-Martínez et al., 2018; Gasparyan et al., 2021a). Conversely, the use of EE in rodents modulated stress-response genes (Costa et al., 2021). In terms of appetite-regulating expression, welfare-promoting treatments induced treatment- and time-dependent changes in *agrp1*, *agrp2*, and *npy*, with upregulation observed in several cases. Dietary strategies may also modify the molecular control of feeding-related neuroendocrine circuits (Yao et al., 2018; Greene et al., 2024). Environmental complexity has been shown to modulate hypothalamic neuroendocrine activity by upregulating feeding behavior expression in response to stressors. This consistent with enhanced adaptive regulation of stress-related signaling pathways in rodents (Hendriksen et al., 2012; Varman and Rajan, 2015).

The pituitary results reinforce this perspective. As a central endocrine organ of the HPI axis, the pituitary directly controls ACTH secretion and therefore cortisol production (Schreck et al., 2016). The combined treatment downregulated several stress-associated genes, including *pomc*, *crf*, *cnr1*, *gr2* and *htr1aβ*. POMC is the precursor of ACTH, and its lower expression may provide a mechanistic explanation for the reduced plasma cortisol levels observed in the treated fish. Comparable effects of CBD on hypothalamic-pituitary stress pathways have been described in mammalian studies (Navarrete et al., 2018; Viudez-Martínez et al., 2018; Gasparyan et al., 2021a). In parallel, the pituitary expression patterns of appetite-regulating genes were broadly consistent with those observed in other brain regions, further suggesting a coordinated neuroendocrine response to the welfare-promoting treatments.

Overall, the welfare-promoting treatments elicited a coordinated neuroendocrine response across the brain. In the telencephalon, treatment effects were detected in genes related to stress regulation, cannabinoid and serotonergic signaling, and appetite control. In the hypothalamus, several stress-axis and appetite-related genes were modulated, suggesting that welfare-promoting conditions may affect both stress and metabolic control. In the pituitary gland, combined treatment downregulated key stress-associated genes. The pattern across brain regions is consistent with a shift toward lower HPI-axis activity and a more stable neuroendocrine state under welfare-promoting conditions in European sea bass.

5 Conclusions

Taken together, these findings indicate that microbubble curtain-based EE and dietary CBD may enhance the robustness of juvenile European sea bass by enhancing growth performance during the rearing trial and increasing survival and physiological response to confinement stress. The coordinated modulation of circulating plasma cortisol, innate immune activity, lipid mediator profiles, and brain gene expression suggests that both strategies act on interconnected endocrine and immune pathways involved in stress adaptation and processes involved in the physiological response to stress. Notably, the combined treatment generally elicited the most consistent responses, supporting the complementary interaction between environmental complexity and CBD supplementation. Although the underlying mechanisms require further elucidation, these results provide evidence that integrating nutritional and environmental interventions may be a valuable approach to promote welfare and sustainable production in the sea bass aquaculture industry.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: AccedaCRIS: https://accedacris.ulpgc.es/.

Ethics statement

The animal study was approved by the European Union Directive (2010/63/EU) and Spanish legislation (RD 53/2013) on animal experimentation, and the experiment was approved by the University of Las Palmas de Gran Canaria Animal Experimentation Ethics Committee (REF: OEBA-ULPGC-32/2023). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

VR: Methodology, Writing – original draft, Data curation, Investigation, Formal analysis, Software, Validation, Writing – review & editing. ST: Writing – review & editing, Supervision, Conceptualization, Investigation, Visualization, Validation. LM-A: Writing – review & editing, Methodology. FA: Writing – review & editing. RB: Methodology, Writing – review & editing. MB: Writing – review & editing. DM: Visualization, Validation, Project administration, Methodology, Writing – review & editing, Investigation, Writing – original draft, Conceptualization, Supervision, Funding acquisition. ÁF-M: Supervision, Conceptualization, Validation, Project administration, Investigation, Writing – review & editing, Methodology, Funding acquisition, Writing – original draft, Visualization.

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Conflict of interest

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