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Dietary Supplementation of *Ulva* sp. Water Extract Improves Growth and Resistance to Hypoxia Stress in Zebrafish (*Danio rerio*)

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Abstract

Dissolved oxygen depletion in intensive aquaculture systems poses a threat to fish immunity and survival. *Ulva* sp. contains bioactive polysaccharides, particularly ulvan, which have demonstrated antioxidant and immunostimulatory properties. This study investigated the effects of *Ulva* sp. water extract supplementation at various doses on growth performance and hypoxia stress resistance in zebrafish (*Danio rerio*). Before feeding trials, toxicity was assessed using *Artemia* sp. bioassay at concentrations of 125,000; 62,500; 31,250; 15,625; and 7,813 ppm to determine the LC_{50} value. A completely randomized design (CRD) consisting of five dietary treatments (0%, 2.5%, 5%, 10%, and 20% of the LC_{50} value) with three replicates per treatment was employed. Fish were fed the experimental diets twice daily for 30 days and subsequently subjected to hypoxia stress tests in sealed airtight aquaria. The LC_{50} value of *Ulva* sp. water extract was 44,977.92 ppm, indicating non-toxicity. Dietary supplementation significantly enhanced growth performance, with the highest absolute weight gain recorded in fish receiving 20% of the LC_{50} supplementation level (167 ± 0.02 mg; $p < 0.05$). Supplementation also improved resistance to hypoxia stress, as evidenced by significantly higher survival rates in the 10% and 20% LC_{50} treatments (57%) compared with the control group ($p < 0.05$). However, survival during the 30-day feeding period did not differ significantly among treatments ($p > 0.05$). Overall, the results suggest the potential of *Ulva* sp. water extract as a functional feed additive, while further research is required to determine optimal supplementation levels and feeding duration for enhanced aquaculture performance.

1. Introduction

Intensive aquaculture systems have significantly contributed to global food production, yet high-density farming practices frequently compromise dissolved oxygen (DO) levels within rearing environments (Son *et al.*, 2025; Yousefi *et al.*, 2018). Hypoxic conditions impose serious physiological consequences on fish, including oxidative stress, immune suppression, and elevated pathogen susceptibility, ultimately leading to impaired growth and increased mortality. Dietary supplementation with functional feed additives therefore represents a practical and sustainable strategy to enhance fish resilience under suboptimal dissolved oxygen conditions (Suratno and Putra, 2021).

Ulva *sp.*, a Chlorophyta macroalga abundant in shallow coastal waters, contains diverse bioactive compounds, including polyphenols, dietary fiber, and sulphated polysaccharides (Pawestri and Arsyi, 2025). Its primary polysaccharide constituent, *Ulva* sp., exhibits well-documented immunomodulatory and antioxidant activities, including macrophage activation, lymphocyte proliferation, and enhanced phagocytic function across multiple aquatic species (Premarathna *et al.*, 2024; Ramadhan *et al.*, 2022). These biological properties position *Ulva* sp. water extract as a promising candidate for functional aquaculture feed formulations.

Prior studies have examined *Ulva* sp. supplementation predominantly using whole-powder or ethanolic extract formulations under standard rearing conditions (Rama Nisha P. *et al*., 2014; Pappou *et al*., 2024). However, the specific efficacy of *Ulva* sp. water extract as a dietary supplement in fish subjected to acute hypoxia stress remains largely unexplored. This gap is particularly relevant given that aqueous extraction is more practically scalable and the hypoxic challenge more directly reflects conditions encountered in intensive aquaculture settings.

Before incorporation into feed, the safety evaluation of natural extracts is essential. *Artemia* sp. bioassays are widely adopted for this purpose due to the organism's sensitivity to foreign compounds, ecological relevance, and practical ease of culture (Charoeythornkhajhornchai *et al*., 2023). Zebrafish (*Danio rerio*) were subsequently selected as the experimental vertebrate model, given their genetic homology with other vertebrates, short life cycle, high fecundity, and established utility in nutritional and toxicological research (Adhish and Manjubala, 2023; Lawrence *et al.*, 2012; Qualhato *et al.*, 2024).

Therefore, this study investigates the effects of *Ulva* sp. water extract incorporated as a dietary supplement on growth performance and hypoxia stress resistance in zebrafish (*Danio rerio*). By extending previous immunostimulant characterization of *Ulva*-based supplements to an in vivo hypoxic challenge model, this work aims to provide preliminary evidence on the biological efficacy of aqueous *Ulva* extract as a functional feed additive, which may support further applied research on macroalgae-based nutritional interventions in aquaculture systems.

2. Material and methods

2.1 Water Extract Preparation of *Ulva* sp.

Ulva sp. was collected from Sadranan Beach Gunung Kidul, Yogyakarta, Indonesia, cleaned, sun-dried, and cut into small fragments for extraction. *Artemia* sp. cysts (Supreme Plus) and zebrafish (*Danio rerio*) were obtained commercially. Aqueous extraction was perfomed as described by Wang and Chen (2016) with modifications. Ten grams of dried *Ulva* sp. were dissolved in 250 mL of distilled water (1:25 w/v), acidified to pH 3 with diluted HCl (Sunaryo *et al.*, 2024), and heated at 70°C for 3 hours under continuous stirring. The filtrate was concentrated to 50 mL, stored at −20°C, and diluted to a stock solution of 250,000 ppm at a 1:3 ratio with distilled water.

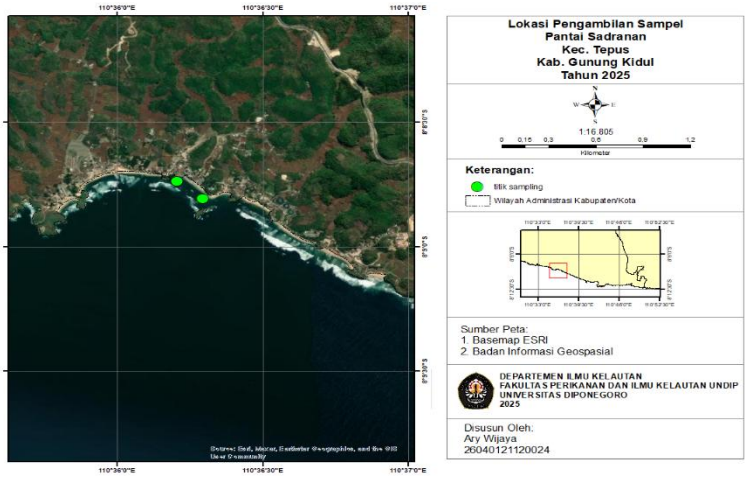

Figure 1.Sampling Location of *Ulva* sp.

2.2 Toxicity Bioassay

Artemia sp. cysts (1 g) were hatched in 1 liter of sterilized seawater under continuous aeration and adequate illumination for 48 hours (Aqiila *et al*., 2017). Toxicity was assessed using the Brine Shrimp Lethality Test (BSLT) following Sunaryo *et al.* (2024). Freshly hatched *Artemia* sp. nauplii (20 individuals per vial) were exposed to extract concentrations of 7,813; 15,625; 31,250; 62,500; and 125,000 ppm for 24 hours in triplicate (Aqiila *et al*., 2017). Mortality was recorded at 15 and 30 minutes, one hour, up to 24 hours. LC_{50} was determined by probit analysis of percentage mortality data (Lestari *et al*., 2019).

2.3 Dietary Supplementation Formulation

Supplementation doses were formulated as %w/w ratios of extract volume to commercial pellet mass, with the highest dose derived from the rounded LC_{50} value, following Hariati *et al.* (2024) with modifications. Four treatment levels were established at 2.5% (P1), 5% (P2), 10% (P3), and 20% (P4) of the LC_{50} value, alongside an unsupplemented control (P0). Feed batches of 10 g were prepared every 10 days by uniformly mixing the designated extract volume with commercial pellets on a petri dish, then air-dried and stored until use.

2.4 Feeding Trial and Growth Assessment

Twenty zebrafish per aquarium were acclimated for a 7 to 30 days feeding trial under a completely randomized design (CRD) with 4 treatment groups and 3 replicates (Prama *et al*., 2014). Fish were fed supplemented diets at 4% biomass twice daily (Yudiati *et al*., 2020), with aquaria cleaned by siphoning each afternoon concurrent with partial water changes (Setyati *et al*., 2017). Water quality was monitored weekly and maintained at temperature 28.74 ± 1.6°C, pH 7.93 ± 0.26, and dissolved oxygen 4.87 ± 0.31 mg L^{-1} (Khan and Alhewarini, 2018). Fish were weighed at the start (W_0) and end (Wt) of the trial. Growth and survival were calculated as follows (Situmorang *et al.*, 2021):

$$Survival\ Rate\ (SR) = \frac{Final\ number\ of\ fish}{Initial\ number\ of\ fish}\ x\ 100\%$$

$$Absolute\ Weight\ Gain\ (AWG) = W_t - W_0$$

$$Specific\ Growth\ rate\ (SGR) = \frac{(\ln W_t - ln\ W_0)}{rearing\ period}\ x\ 100\%$$

2.5 Hypoxia Stress Test

Following a 24-hour fasting period after the feeding trial, ten zebrafish per treatment were transferred into 3-liter aquaria containing 500 mL of freshwater without aeration. Aquaria were sealed airtight with plastic wrap to restrict gas exchange (Yudiati *et al.*, 2020). Observations commenced at t_0 and continued at 12-hour intervals until 50% mortality was recorded per aquarium.

2.6 Statistical Analysis

All data were analyzed using one-way ANOVA in SPSS version 23 (Hasan *et al.*, 2023). Normality and homogeneity of variance were verified before ANOVA was performed. Where significant differences were detected ($p \leq 0.05$), Duncan's Multiple Range Test was applied for post-hoc comparisons among treatment groups. Graphical outputs were generated using Microsoft Excel 2021.

3. Results

3.1 Toxicity of *Ulva* sp. Water Extract

Mortality of *Artemia* sp. increased proportionally with extract concentration across all tested levels (Table 1), indicating a clear dose-dependent response. Probit analysis yielded an LC_{50}-24h value of 44,977.92 ppm, which exceeds the 1,000 ppm threshold for non-toxic classification (Meyer *et al.*, 1982). The linear regression curve produced a coefficient of determination ($R^2 = 0.9486$), confirming a strong and reliable relationship between extract concentration and *Artemia* sp. mortality. Based on the LC_{50} value, supplementation doses were calculated and rounded to 3.5 mL of stock solution, corresponding to %w/w formulations of 0%, 0.4375%, 0.875%, 1.75%, and 3.5% for the control, P1, P2, P3, and P4 treatments, respectively.

Table 1. Probit analysis of LC_{50}-24h of *Ulva* sp. water extract against *Artemia* sp.

Concentration (ppm)	Log Concentration	Total Animals	Mortality (n)	Mortality (%)	Probit Value
7,813	3.89	60	4	6.7	3.52
15,625	4.19	60	8	13.0	3.87
31,250	4.49	60	15	25.0	4.33
62,500	4.80	60	35	58.3	5.20
125,000	5.10	60	54	90.0	6.28
LC_{50} = 44,977.92					

3.2 Survival Rate during Feeding Trial

Survival rate of zebrafish over the 30 days feeding trial showed no statistically significant differences among treatment groups ($p > 0.05$). All supplemented groups recorded higher survival percentages relative to the unsupplemented control, with the highest survival rate observed in fish receiving 3.5 mg of *Ulva* sp. supplementation (P4) and the lowest in the control group (Figure 2). These results are consistent with the non-toxic classification of *Ulva* sp. water extract established in the toxicity bioassay.

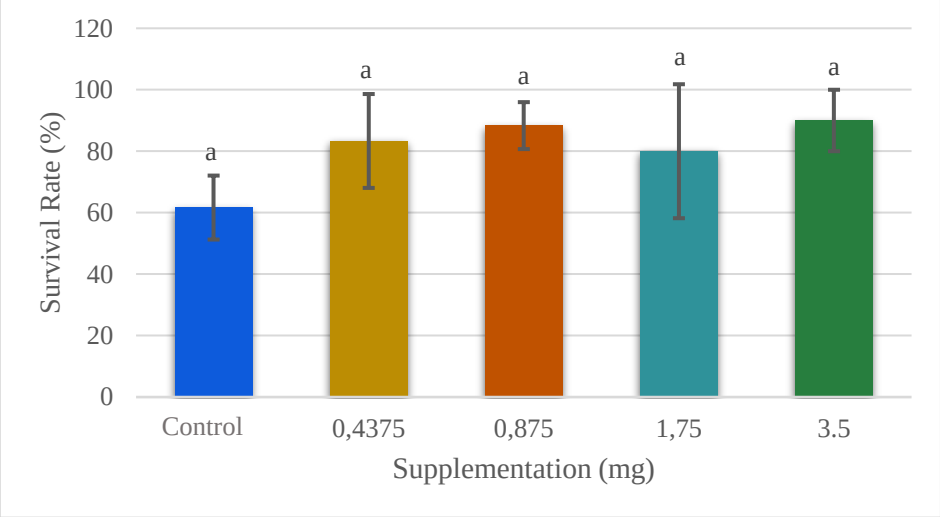

Figure 2. Survival rate (%) of zebrafish (*Danio rerio*) during the feeding trial. Values are presented as mean ± SD. P0–P4 represent dietary supplementation levels of 0, 0.4375, 0.875, 1.75, and 3.5 mg of *Ulva* sp. water extract, respectively. Similar letters indicate no significant differences among treatments ($p > 0.05$).

3.3 Growth Performance

Absolute weight gain (AWG) differed significantly among treatment groups ($p < 0.05$), with P4 recording the highest value (0.167 ± 0.02 g) and the control the lowest (0.105 ± 0.01 g) (Figure 3). In contrast, specific growth rate (SGR) showed no statistically significant differences among treatments ($p > 0.05$). P1 and P4 recorded the highest SGR values at 1.54% per day, while the control recorded the lowest at 1.23% per day (Figure 4). Despite the absence of statistical significance, all supplemented groups consistently outperformed the control in both growth parameters.

3.4 Hypoxia Stress Tolerance

Zebrafish survival under acute hypoxic conditions was monitored over 84 hours, at which point the control group was the first to reach 50% cumulative mortality (Figure 5). Survival rate at 84 hours showed no statistically significant differences among treatment groups ($p > 0.05$); however, all supplemented groups maintained higher survival percentages compared to the control throughout the observation period. The highest survival rates were recorded in P3 and P4 treatments, while the control recorded the lowest, suggesting a dose-related trend in hypoxia tolerance among zebrafish fed *Ulva* sp. water extract-supplemented diets.

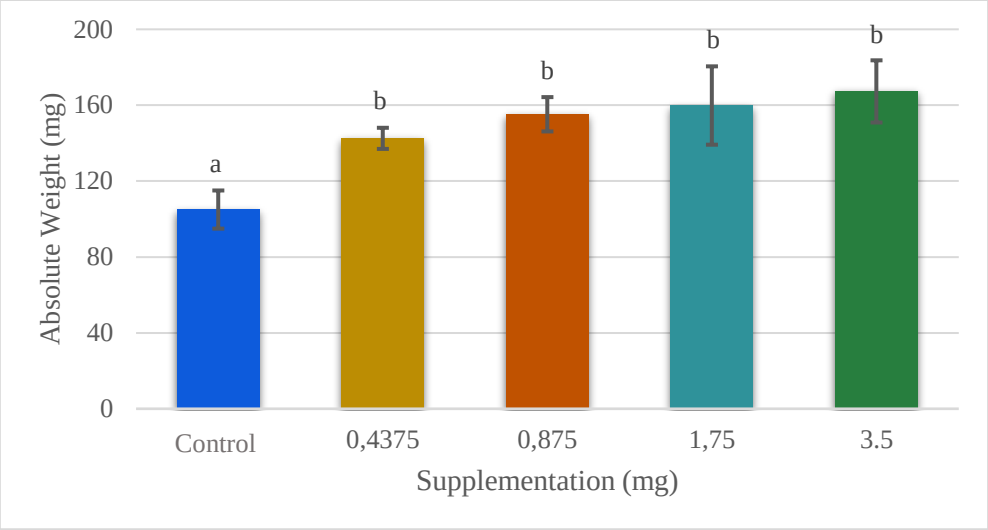

Figure 3. Absolute weight gain of zebrafish (*Danio rerio*) after 30 days of rearing under different dietary supplementation treatments. Values are presented as mean ± SD. P0–P4 represent dietary supplementation levels of 0, 0.4375, 0.875, 1.75, and 3.5 mg of *Ulva* sp. water extract, respectively. Different letters (a, b) indicate statistically significant differences among treatments ($p < 0.05$).

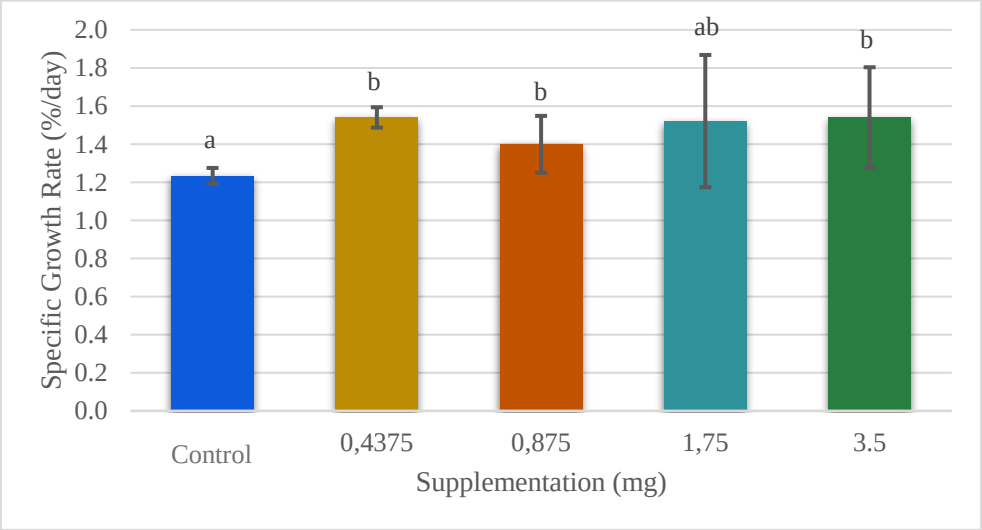

Figure 4. Specific growth rate of zebrafish (*Danio rerio*) after 30 days of rearing under different dietary supplementation treatments. Values are presented as mean ± SD. P0–P4 represent dietary supplementation levels of 0, 0.4375, 0.875, 1.75, and 3.5 mg of *Ulva* sp. water extract, respectively. Different letters (a, b) indicate statistically significant differences among treatments ($p < 0.05$).

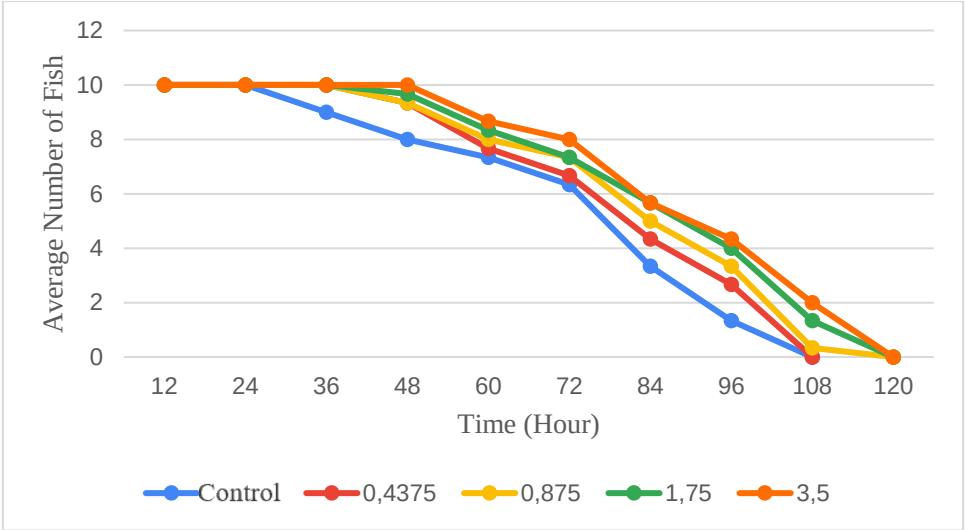

Figure 5. Cumulative mortality of zebrafish (*Danio rerio*) during the hypoxia stress test following the 30-day feeding trial. P0–P4 represent dietary supplementation levels of 0, 0.4375, 0.875, 1.75, and 3.5 mg of *Ulva* sp. water extract, respectively.

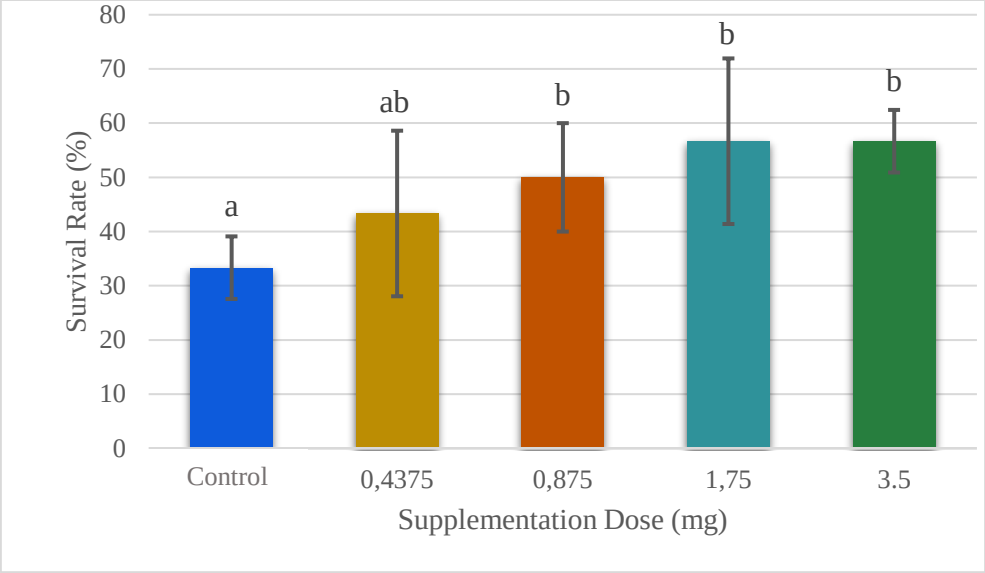

Figure 6. Survival rate of zebrafish (*Danio rerio*) at 84 hours of hypoxia stress test. Values are presented as mean ± SD. P0–P4 represent dietary supplementation levels of 0, 0.4375, 0.875, 1.75, and 3.5 mg of *Ulva* sp. water extract, respectively. Different letters (a, b) indicate statistically significant differences among treatments ($p < 0.05$).

4. Discussion

The *Artemia* sp. bioassay yielded an LC_{50} value of 44,977.92 ppm for *Ulva* sp. water extract, classifying it as non-toxic according to the threshold established by Meyer *et al.* (1982). The strong coefficient of determination (R^2 = 0.9486) confirms that extract concentration exerted a dominant and reliable influence on *Artemia* sp. mortality, lending statistical credibility to the derived LC_{50} value as a basis for subsequent dose formulation. This outcome is consistent with Sunaryo *et al*. (2024), who reported a comparable non-toxic classification for *Ulva* sp. aqueous extract under similar extraction conditions, collectively reinforcing the safety profile of this macroalga for application in aquatic feed systems. From a practical standpoint, the non-toxic classification supports the feasibility of incorporating *Ulva* sp. water extract into aquaculture feed without posing acute risk to target organisms.

Dietary supplementation with *Ulva* sp. water extract produced a statistically siginifcant effect on absolute weight gain ($p < 0.05$), while specific growth rate and survival remained non-significant across treatment groups ($p > 0.05$). Despite the absence of significance in growth rate, all supplemented groups consistently outperformed the control, indicating that the extract contributed nutritional value to zebrafish growth rather than exerting a direct pharmacological effect at the doses tested. This pattern is consistent with the established role of water-soluble polysaccharides in enhancing digestive enzyme activity and stimulating intestinal microbiota (Savari *et al*., 2019; Mohan *et al*., 2016), which collectively improve nutrient utilization efficiency. These findings align with Akbary and Aminikhoei (2018) and Pezeshk *et al.* (2018), who demonstrated improved growth parameters in fish supplemented with Ulva-derived polysaccharides, suggesting that the growth-promoting potential of *Ulva* sp. water extract warrants further dose optimization in future studies.

Survival rate during the 30-day feeding trial remained comparable across all groups, a result that is both consistent with the extract's non-toxic classification and reflective of adequate water quality management throughout the experimental period. Zebrafish are eurythermal organisms with broad environmental tolerance, having been recorded across temperatures of 12-39°C across diverse natural habitats (Souza *et al.*, 2025; Omelda and Vazquez, 2011), which partly explains their comparable survival regardless of treatment. Mortalities recorded during the trial were therefore more likely attributable to individual variation in health status or genetic background rather than to supplementation effects. This implies that survival rate alone may not be a sufficiently sensitive indicator for detecting the immunostimulant effects of *Ulva* sp. extract under standard rearing conditions, and that stress-challenge models offer greater discriminatory power for this purpose.

The oxygen shock test revealed no statistically significant difference in hypoxia tolerance among treatment groups ($p > 0.05$); however, zebrafish fed supplemented diets consistently exhibited higher survival rates under acute hypoxic conditions compared to the control, suggesting a biologically meaningful trend. Prolonged dissolved oxygen depletion triggers cellular hypoxia, impairs mitochondrial ATP synthesis, and elevates reactive oxygen species (ROS), ultimately precipitating oxidative stress, immune suppression, and apoptosis (Mayasari, 2017; Purnamasari *et al.*, 2024). The comparatively greater survival observed in supplemented groups implies that sulfated polysaccharides in *Ulva* sp. water extract may partially attenuate these cascading effects through antioxidant and immunostimulant mechanisms. This interpretation is supported by Qi *et al.* (2005), who demonstrated antioxidant activity of *Ulva pertusa* sulfated polysaccharides against hydroxyl and superoxide radicals, and by Bourguiba *et al*. (2017), who reported enhanced superoxide dismutase activity in zebrafish embryos treated with *Ulva rigida* extract. These findings collectively indicate that *Ulva* sp. water extract holds genuine biological potential as a hypoxia-mitigating dietary additive, though the effect magnitude requires amplification through methodological refinement.

The lack of statistical significance in hypoxia tolerance outcomes likely reflects the constraints of the current experimental design rather than an inherent limitation of the extract. Thirty days of supplementation at the doses tested may not have been sufficient to induce measurable immunological conditioning, particularly given that polysaccharide bioactivity is highly sensitive to molecular weight, sulfate group content, and extraction method (Chen *et al*., 2021; Qi *et al*., 2005). The absence of gill histological data and immune gene expression analysis further limits the depth of mechanistic interpretation that can be drawn from

survival-based endpoints alone. Nevertheless, the higher survival rates recorded in supplemented groups during the hypoxia challenge point to a genuine biological effect that deserves further exploration. Future studies would benefit from longer supplementation periods, higher dose levels, and the inclusion of gill histology and immune response markers to better understand how *Ulva* sp. water extract may support fish health under low dissolved oxygen conditions in aquaculture conditions.

5. Conclusions

This study provides preliminary evidence that *Ulva* sp. water extract is non-toxic to *Artemia* sp., with an LC_{50} value well above the toxic threshold, supporting its safe application as a dietary supplement in aquaculture feed formulations. Dietary supplementation influenced absolute weight gain in zebrafish, though effects on specific growth rate, survival, and hypoxia tolerance remained non-significant, with supplemented groups nonetheless consistently outperforming the control. These findings suggest the possible immunostimulant and antioxidant capacity of the extract, with practical relevance to nature-based functional feed development in intensive aquaculture systems. Further studies involving optimized dosage regimens, extended rearing periods, and molecular immune profiling are necessary to clarify its biological mechanisms under hypoxic conditions.

Ethics approval

No permits were required.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

AW contributed to data collection and curation, conducted the investigation, performed formal analysis, and prepared the original draft of the manuscript. EY contributed to the conceptualization of the study, supervised the overall research process, and provided research resources. SS contributed to the conceptualization of the study, data validation, and provided scientific supervision. NA contributed to the provision of research resources and methodology development. MSR and MSRD contributed to formal analysis, writing review, and editing. All authors contributed to manuscript revision and approved the final version.

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Declaration of competing Interest

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

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