



Effects of *Ulva lactuca* as a dietary supplement on growth, antioxidants and digestive enzymes of Nile tilapia (*Oreochromis niloticus*) under intermittent fasting and regular feeding regimes in the recirculating system

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Abstract

This study evaluated the effects of dietary supplementation with *Ulva lactuca* (5% = ULM5; 10% = ULM10) on Nile tilapia (*Oreochromis niloticus*) under prolonged intermittent fasting (1 week fasting/1 week feeding for 12 weeks). While fasting reduced growth and body composition parameters, protein content remained above 20% across all groups. ULM5 improved growth performance under normal feeding, whereas ULM10 enhanced antioxidant defense [$\uparrow$ SOD (superoxide dismutase), $\uparrow$ CAT (catalase), $\downarrow$ LPO (lipid peroxidation)], particularly during stress. Digestive enzyme activities declined during fasting but recovered in *U. lactuca*-fed groups. These findings suggest that *U. lactuca*, especially at 10%, may support physiological resilience and oxidative balance during nutritional stress, highlighting its potential as a functional feed additive in sustainable aquaculture.

Keywords *Ulva lactuca* · *Oreochromis niloticus* · Enzyme · Antioxidants · Feeding regime

Introduction

While the consumption of animal foods plays an important role in healthy nutrition, meeting the food needs of the rapidly growing world population continues to be one of the biggest problems of our day. In this context, aquaculture has become an indispensable sector to meet the animal protein needs of the growing population (İrkin 2020). Current estimates predict that the global population will reach 9.6 billion by 2050, emphasizing the critical role aquaculture products from marine and freshwater systems will play in addressing food security challenges (Calone et al. 2019). Tilapia fish, which has shown a rapid increase in production in recent years, has become one of

the important species and is recorded as the third most produced species in the world, especially in China (FAO 2020). However, in addition to the hunger problem that may be experienced worldwide, another important problem that threatens the life cycle is the limited water resources. With the increasing population, the efficient use of water resources has become a necessity. Freshwater resources need to be used effectively not only as drinking water but also for agriculture (Kargın and Bilgüven 2018). At this point, recirculation systems (RAS) in aquaculture offer opportunities to reduce water use, improve waste management and increase nutrient recycling. It is clear that these systems, which make intensive fish production compatible with environmental sustainability, will be preferred even more in aquaculture in the future.

Another key area shaping the future of aquaculture is the use of alternative raw materials in fish feeds, which is essential for maintaining competitiveness. Traditionally, plant-based cereals and oilseeds, which are cost-effective energy sources, have been utilized to partially replace fishmeal in aquafeeds (Hussain et al. 2024). While these materials are viable alternatives, their use can significantly influence the nutritional quality of aquaculture products. On the other

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hand, algae—an integral part of the aquatic food chain consumed by zooplankton and fish larvae—stand out as a highly efficient feed ingredient. Research indicates that at least 19 tropical and 34 edible-quality algae species can provide sufficient protein when incorporated into fish feeds (Wells et al. 2017). Moreover, algae inclusion, either directly or as a component of formulated feeds, serves as a versatile and natural nutrient source supporting fish health, reduces stress, and enhances overall welfare, offering sustainable benefits for aquaculture (Norambuena et al. 2015).

The aquatic environment is an important ecosystem that harbours many varieties and species of algae, each waiting to be explored to see if it can serve a new purpose. However, algae are an important group that will be a key part of sustainable aquaculture and will be present in the future to achieve sustainability. Macro- and microalgae have been used as dietary supplements to improve the health and nutritional performance of various farmed fish species (Güroy et al. 2011). Compared to microalgae, macroalgae are relatively poor sources of protein, but they can be considered as feed additives to promote growth, lipid mobilisation and improve meat quality (Nakagawa 1985; Wassef et al. 2001). *Ulva* species are important macroalgae used as dietary components for a wide range of fish species (Güroy et al. 2007). *Ulva lactuca*, also known as sea lettuce, has a cosmopolitan distribution in the littoral and sublittoral zones of coastal areas (Pushparaj et al. 2014; Mantri et al. 2020). *Ulva* species have the potential to be commercial products due to their taste, chemical composition and quality. They are known sources of industrially important biopolymers and their organic extracts have been found to have antibacterial, antifungal and antioxidant activities against cancer cell lines (Pushparaj et al. 2014). *Ulva lactuca* contains bioactive compounds with antibacterial potential such as alkaloids, flavonoids, tannins, steroids, saponins and anthraquinones (Umapoorani et al. 2016). In addition, sulphated polysaccharides, phenolics, terpenoids, lactones, sterols and fatty acids in its composition have been reported to be responsible for its antioxidant activity (El-Baky et al. 2008; Alghazeer et al. 2013; Putra et al. 2024).

Studies on the identification of alternative feed additives for aquaculture are increasing. Probiotics, prebiotics, synbiotics, organic acids and medicinal plants are among the alternative feed additives that have shown beneficial effects in aquaculture (NRC 2011). Seaweed or seaweed-derived ingredients are attracting increasing interest as alternative feed additives in fish feed formulations due to their high nutritional value and excellent versatility. Seaweeds have been widely used as functional feed additives to improve fish growth, nutrient utilisation, stress response, fish health and disease resistance and skin pigmentation in ornamental fish (Rouhani et al. 2022). However, an important consideration in the use of macroalgae in aquafeeds is their content of

anti-nutritional factors (ANFs), which may limit their nutritional benefits. Compounds such as phytic acid, oxalates, saponins, and tannins can interfere with protein digestibility, inhibit digestive enzymes, and reduce mineral bioavailability, ultimately impairing feed efficiency and fish growth (Holdt and Kraan 2011; Makkar et al. 2016). Additionally, the complex polysaccharide structure and high fiber content in some macroalgae, including *Ulva* spp., may reduce nutrient digestibility and increase gut transit time (Peixoto et al. 2016; Wells et al. 2017). Therefore, understanding the nutritional limitations of macroalgae and implementing pretreatments (e.g., enzymatic hydrolysis, fermentation) are essential to optimize their use as functional feed ingredients.

In commercial tilapia culture, short-term fasting (e.g., 1–2 days per week) is commonly practiced to reduce feed costs and improve feed efficiency (El-Araby et al. 2020). Controlled fasting has also been associated with improved immunity and compensatory growth responses in some fish species (Kim and Lovell 1995). However, prolonged or cyclical fasting may trigger metabolic stress, impair growth, and negatively affect organ development, digestive enzyme activities, and oxidative balance (Biswas et al. 2006; Furné et al. 2008). During fasting, fish mobilize energy reserves from liver and muscle tissues, which may compromise physiological functions if nutritional support is not provided. Hence, identifying dietary strategies and functional feed additives that can maintain metabolic homeostasis and enhance resilience under fasting-induced stress has become a research priority in sustainable aquaculture. Fish use tissue reserves such as liver and muscle to meet their energy requirements during fasting periods, which may affect their metabolic adaptation during this period (Furné et al. 2008). Therefore, the use of functional feed additives that can increase stress tolerance and maintain metabolic balance during starvation has gained importance. In this study, the effects of 5% and 10% *Ulva lactuca* supplementation in tilapia diets on growth performance, digestive and antioxidant enzyme activities, and amino acid profiles were evaluated under regular feeding and cyclical fasting conditions. Additionally, the possible protective effects of *U. lactuca* were addressed by correlating them with the physiological responses of fish, especially under stress conditions such as fasting.

Materials & methods

Fish source and acclimatization

The Nile tilapia (*Oreochromis niloticus*) were obtained from the Çukurova University Faculty of Fisheries Freshwater Products Research Station, transferred to the recirculation system in the Research and Application Center of Sinop

University, Fisheries Faculty (Sinop, Turkey) and kept for four weeks before the start of the experiment for acclimatization. Fish were stocked in a 3000-L round fiberglass tank in the recirculation system in an indoor facility during acclimation.

Experimental design and rearing conditions

After acclimatization, fish (mean weight $\sim 10.67 \pm 0.01$ g) were fasted for 24 h, weighed, and randomly assigned to 18 glass tanks (120 L each) using a completely randomized design. The experiment was structured into six dietary treatment groups with three replicates per group, totaling 360 fish (20 fish per tank). Distribution of replicates and number of fish per treatment group in the experimental design is shown in Table 1. Treatments were not blocked; replicates were distributed randomly across tanks within the recirculating system to minimize tank position bias. The fish used were mixed-sex juveniles, as sex-specific responses were not considered within the scope of this study. Individual body weights were recorded at the beginning and end of the 12-week trial using a digital scale with 0.01 g precision. All tanks were part of a thermoregulated indoor recirculating system supplied continuously with filtered freshwater to ensure uniform environmental conditions across all replicates.

Water quality and system maintenance

In the recirculation system water quality variables were measured daily; mean water temperature was 24.28 ± 0.56 °C, dissolved oxygen (DO) 5.78 ± 0.11 mg L⁻¹ and pH 7.32 ± 0.05 and did not change significantly between treatments during the experiment. In addition to temperature, dissolved oxygen, and pH, key RAS water quality indicators—including total ammonia nitrogen (TAN), nitrite (NO₂⁻), and nitrate (NO₃⁻)—were measured weekly using standard water quality test kits. These parameters were

strictly maintained within optimal ranges recommended for Nile tilapia culture to ensure a stable and healthy environment throughout the experimental period. The Nile tilapia was kept under a natural light regime, and aeration of tanks was facilitated with an air pump. During the experimental period, water was exchanged daily at a rate of about 10% of the total volume. Mechanical, chemical and biological filtration was provided thanks to the continuous water circulation and filter materials in the sump filter system.

Animal welfare monitoring

Throughout the experimental period, fish were monitored daily for signs of stress, abnormal behavior, and mortality. Water quality parameters were closely tracked to maintain optimal environmental conditions. Mortalities, if any, were recorded, and dead fish were promptly removed to prevent water quality deterioration. The health status of fish was assessed based on external appearance, swimming behavior, feed intake, and response to stimuli. Any individuals showing signs of severe distress or disease were humanely euthanized using an overdose of MS-222 in accordance with institutional ethical guidelines. These procedures were implemented to ensure compliance with animal welfare standards and minimize experimental stress.

Feeding regime

The purpose of adding *U. lactuca* to the feed was to determine the changes in fish in fasting and regular feeding situations. For this, the experiment was designed as 6 groups, three of which were fasting and regular feeding. Regular feeding groups were considered as control of the fasting groups. Fish in all groups were fed *ad libitum* by hand twice a day (at 09:00 AM and 3:30 PM) for 12 weeks. The feeding procedure was carried out carefully to be sure all fish took the food. Fish were fed with one of the following six feeding regimes:

Table 1 Distribution of replicates and number of fish per treatment group in the experimental design

Group Code	Feeding Regime	Diet Type	Number of Tanks (Replicates)	Fish per Tank	Total Fish per Group
Cfeeding	Regular feeding	Control (0% ULM)	3	20	60
Cfasting	1 wk fasting/1 wk feeding	Control (0% ULM)	3	20	60
ULM5feeding	Regular feeding	5% <i>Ulva lactuca</i>	3	20	60
ULM5fasting	1 wk fasting/1 wk feeding	5% <i>Ulva lactuca</i>	3	20	60
ULM10feeding	Regular feeding	10% <i>Ulva lactuca</i>	3	20	60
ULM10fasting	1 wk fasting/1 wk feeding	10% <i>Ulva lactuca</i>	3	20	60
Total			18 tanks		360 fish

ULM: *Ulva lactuca*, wk: week.

Each group consisted of 3 replicate tanks containing 20 fish per tank, totaling 360 Nile tilapia individuals.

1. Cfeeding: Regular feeding for 12 weeks (0% *U. lactuca*)
2. Cfasting: Alternating 1-week fasting, 1-week feeding (0% *U. lactuca*)
3. ULM5feeding: Regular feeding for 12 weeks (5% *U. lactuca*)
4. ULM5fasting: Alternating 1-week fasting, 1-week feeding (5% *U. lactuca*)
5. ULM10feeding: Regular feeding for 12 weeks (10% *U. lactuca*)
6. ULM10fasting: Alternating 1-week fasting, 1-week feeding (10% *U. lactuca*)

Diet formulation and composition

Three experimental diets were formulated to be isonitrogenous (39% crude protein) and isolipidic (8% crude lipid), in accordance with NRC (2011) recommendations for optimal growth of juvenile Nile tilapia. The crude protein content of the *U. lactuca* used in this study was determined to be 10.66% on a dry matter basis. A component balancing method was applied using Microsoft Excel, where the inclusion levels of different feed ingredients were adjusted based on their nutrient compositions to meet the targeted protein and lipid levels. This approach ensured that any observed differences in experimental outcomes were attributable to the tested ingredient rather than variations in macronutrient content. The diets included:

- C (Control): 0% *Ulva lactuca* meal
- ULM5: 5% *Ulva lactuca* meal
- ULM10: 10% *Ulva lactuca* meal

All ingredients were sourced from Sibal Black Sea Feed, a local commercial feed manufacturer. In the experimental diets, *U. lactuca* partially replaced soybean meal. The 5% and 10% inclusion levels were selected based on previous studies reporting positive outcomes at these rates (Wassef et al. 2001; Güroy et al. 2007). *Ulva lactuca* was chosen for its high glutamic and aspartic acid content and antioxidant properties. The diet formulations, along with their biochemical and amino acid compositions, are presented in Table 2.

Ulva lactuca collection and processing

Ulva lactuca was collected from rocky areas along the littoral zones of coastal regions and transported to the laboratory. Upon arrival, it was thoroughly rinsed under running water to remove any debris or contaminants and then dried in an oven at 50 °C. The drying temperature for *U. lactuca* (50 °C) is consistent with literature suggesting minimal degradation of antioxidant compounds at this temperature (Putra et al. 2024). The dried biomass was subsequently ground into a fine powder and stored under refrigeration until use. For

Table 2 Formulation (g kg⁻¹), proximate composition (%) and amino acid composition (g (100 g)⁻¹) of the experimental diets

	Experimental Diets		
	C	ULM5	ULM10
Fish meal	180	180	180
Extracted soybean meal	230	220	210
Corn protein	150	150	150
Wheat flour	274	234	194
<i>Ulva lactuca</i> meal	-	50	100
Rice bran	120	120	120
Fish oil	30	30	30
Dicalcium phosphate	10	10	10
Vitamin premix(*)	3	3	3
Mineral premix(*)	3	3	3
<i>Proximate composition (%)</i>			
Moisture	5.63	5.98	5.65
Protein	39.74	39.77	39.75
Lipid	8.84	9.19	8.65
Ash	6.58	7.36	8.37
NFE + Fibre ^a	39.21	37.70	37.58
Gross energy (kJ g ⁻¹) ^b	19.54	19.42	19.08
<i>Essential Amino Acids (EAA; g (100 g)⁻¹)</i>			
Arginine	1.91	0.76	1.28
Histidine	0.53	0.26	0.51
Isoleucine	1.17	0.51	0.81
Leucine	0.52	0.17	0.37
Lysine	0.68	0.24	0.47
Methionine	0.22	0.11	0.19
Phenylalanine	0.50	0.19	0.32
Threonine	6.38	2.96	4.53
Valine	1.50	0.64	1.12
<i>Non-Essential Amino Acids (NEAA; g (100 g)⁻¹)</i>			
Alanine	9.44	4.22	7.56
Glutamine	0.79	0.30	0.50
Glutamic Acid	37.56	15.63	26.33
Aspartic Acid	14.96	6.20	8.80
Cysteine	0.23	0.06	0.14
Serine	4.29	1.65	2.92
Tyrosine	0.62	0.32	0.48
Proline	5.33	2.87	4.60
ΣAmino acids (AA)	86.63	37.07	60.91
ΣEAA	13.41	5.83	9.59
ΣNEAA	73.22	31.24	51.32
ΣEAA/ΣNEAA	0.18	0.19	0.19

* Vitamin-mineral premix (mg kg⁻¹ premix): vitamin A, 210,000 IU; vitamin D₃, 35,000 IU; vitamin E, 7000 mg; vitamin K₃, 322 mg; vitamin B₁, 588 mg; vitamin B₂, 252 mg; vitamin B₆, 294 mg; vitamin B₁₂, 826 mcg; niacin, 1400 mg; biotin, 7583 mcg; 182 mg folic acid, pantothenic acid, 1722 mg; inositol, 17,220 mg; vitamin C, 933.31 mg; Calcium 1000 mg; Magnesium 600 mg; Potassium 450 mg; Zinc 90 mg; Manganese 12 mg; Copper 5 mg

^aNFE + Fibre = 100 - (%protein + %lipid + %ash + %moisture)

^bGross energy is calculated according to 23.6 kJ g⁻¹ protein, 39.5 kJ g⁻¹ lipid and 17 kJ g⁻¹ NFE

diet formulation, all dry ingredients were homogeneously blended using a laboratory food mixer. The mixture was moistened with tap water to form a malleable dough suitable for pellet formation. The wet feed was extruded through a 3 mm die to form pellets, which were then dried at 40 °C in a drying cabinet. The final feed pellets were stored at 4 °C until feeding commenced.

Sampling and tissue collection

At the end of the experiment, fish were fasted for 24 h before sampling to reduce the effect of gut contents and were euthanized using an overdose of anesthetic (MS-222 at 300 mg L⁻¹), following ethical guidelines. Fish in all groups were weighed individually to determine the total final weights and growth performance parameters. Six fish from each tank were sampled for chemical analyses. Each sampled fish was carefully rinsed with distilled water to minimize external contamination. Fish were then re-weighed and dissected under sterile conditions, and the internal organs were removed. Liver and digestive system tissues (intestine and stomach) were aseptically excised and immediately placed in labeled, sterile containers for subsequent digestive and antioxidant enzyme activity analyses. Fillets were also obtained, with care taken to avoid cross-contamination, and were homogenized under cold conditions for biochemical and amino acid composition analyses. All tissue samples were frozen and stored at -20 °C until further analysis.

Calculations

Growth parameters such as weight gain (WG), feed conversion rate (FCR), specific growth rate (SGR), protein efficiency rate (PER), hepatosomatic index (HSI) and viscerosomatic index (VSI) were calculated using the following equations. The hepatosomatic index (HSI) and viscerosomatic index (VSI) were calculated to monitor energy mobilization and visceral development during fasting and feeding, common stress markers in fish physiology (Dernekbaşı et al. 2017; Dernekbaşı and Karayücel 2021):

- Weight gain (WG, g) = Final body weight - initial body weight
- Specific growth rate (SGR, % day⁻¹) = $[(\ln \text{ final wet weight} - \ln \text{ initial wet weight}) / \text{days}] \times 100$
- Feed conversion rate (FCR) = Total feed intake / weight gain
- Protein efficiency ratio (PER) = Wet weight gain / protein fed
- Hepatosomatic index (HSI, %) = (liver weight / body weight) × 100
- Viscerosomatic index (VSI, %) = (viscera weight / body weight) × 100

Biochemical analysis

Experimental diets and fish fillet samples were analyzed according to the standard methods of the Association of Official Analytical Chemists (AOAC 2005). Dry matter content was determined by drying the samples at 105 °C until constant weight. Ash content was measured by incinerating the samples in a muffle furnace at 550 °C for 6 h. Crude protein levels were analyzed using the Kjeldahl method, while crude lipid content was determined through extraction with petroleum ether by Soxhlet extraction.

Amino acid analysis

Amino acid analysis was performed using an Agilent Technologies 6460 Triple Quadrupole LC-MS/MS system. The analysis was conducted with a Jaseem LC-MS/MS Amino Acid Analysis Kit (Pt Berca Niaga Medica, Indonesia), which includes five separate standards, an internal standard, mobile phases, and necessary reagents. Chromatographic separation and mass detection parameters were optimized according to the kit protocol. Sample preparation involved an updated acid hydrolysis procedure to ensure accurate quantification of amino acids.

Digestive enzymes

Following the completion of the feeding experiment, digestive enzyme activities were analyzed, including amylase, lipase, and trypsin in the intestinal tissue and pepsin in the stomach. The whole digestive tract and pancreas were thoroughly homogenized using a Heidolph Silentcrusher-m (Germany) in ice-cold sterilized distilled water and centrifuged at 15,000 × g for 45 min at 4 °C. The supernatant was used as a crude enzyme source.

- Amylase activity was assessed according to the method described by Bernfeld (1955), using starch as the substrate at pH 6.9 and a temperature of 20 °C.
- Lipase activity was analyzed according to Furné et al. (2008), where p-nitrophenyl myristate served as the substrate at pH 9.0 and 30 °C. One unit of lipase activity was defined as the amount of enzyme releasing 1 pmol of p-nitrophenol per minute.
- Pepsin activity was determined using azocasein as the substrate at pH 9.5 and 25 °C, following the method of Worthington (1988).
- Trypsin activity was assessed using Na-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) as substrate, following Erlanger et al. (1961).

Specific activities of all digestive enzymes were calculated as enzyme activity per milligram of protein.

$$\text{Amylase} = \left[(\text{sample} - \text{blank})^2 \times 7.712 \right] - \left[1.082 \times (\text{sample} - \text{blank}) \right] + 0.082 = \text{Result/mg protein}$$

$$\text{Lipase} = \left[(\text{sample} - \text{blank}) \times (0.2359 + 0.0153)^2 \right] / \text{mg protein}$$

$$\text{Pepsin} = \left[(\text{sample} - \text{blank}) \times 1000 \right] / \left[10 \times \text{mg protein} \right]$$

$$\text{Absorption result} = (\text{Last result} - \text{first result}) / 10 \text{ min}$$

$$\text{Trypsin} = \left[(\text{Absorption result} \times 10^6) / 8.800 \right] / 2 = \text{Result/mg protein}$$

Antioxidant enzyme activity

At the end of both periods, fish liver samples were taken to determine the activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and lipid peroxidation (LPO). All samples were stored at -80°C until analysis.

After thawing, liver samples were washed with sterile physiological saline, blotted dry with filter paper, and approximately 1 g of tissue was homogenized in 1000 μL of homogenization buffer (0.25 M sucrose, 0.5 mM EDTA, and 10 mM Tris-HCl; pH 7.4) using a Heidolph Silentcrusher-m homogenizer. The homogenates were then centrifuged at $15,000 \times g$ for 45 min at 4°C in a refrigerated centrifuge and the resulting supernatant was used for subsequent enzyme assays.

- SOD activity was measured using a SOD Assay Kit (Sigma, Catalog No: 19160-1KT-F). SOD activity was determined using an assay method based on xanthine oxidase and a novel color reagent.
- CAT activity was determined with a Catalase Assay Kit (Cayman Chemical, Catalog No: 707002). CAT activity was determined using hydrogen peroxide as the substrate, and one unit of enzyme activity was defined as the amount that decomposed one molecule of hydrogen peroxide.
- GPx activity was assessed using the Cayman GPx Assay Kit (Catalog No: 703102). GPx activity was measured indirectly by a couple of reactions with glutathione reductase (GR).
- Lipid peroxidation (LPO) was measured using a TBARS Assay Kit (Cayman Chemical, Catalog No: 10009055). LPO was determined based on the thiobarbituric acid reaction at 95°C .

Statistical analyses

Two-way ANOVA was performed using SPSS 21 (SPSS Inc., USA) to evaluate the main effects of diet type (feed type), feeding regime (regular vs. intermittent), and their

interaction on the measured variables. When a significant main effect or interaction was detected, post-hoc comparisons were conducted using Tukey's HSD test at a 95% confidence level to identify pairwise differences among treatment groups. Post-hoc tests were applied separately for the main effects and interaction terms to clarify specific group differences. Percentage data were arcsine square root transformed prior to analysis to ensure homogeneity of variances. For data visualization, the following R packages were utilized: MASS, ggplot2, patchwork, reshape2, and factoextra. The relationship between digestive and antioxidant enzyme activities was assessed using Principal Component Analysis (PCA) performed in the Past4.03 software. Statistical significance was accepted at $p < 0.05$.

Results

Growth performance

At the end of the experiment, no statistically significant differences were observed in growth parameters among the regular feeding groups (Cfeeding, ULM5feeding, and ULM10feeding) ($P > 0.05$). Among these, the ULM5feeding group had numerically higher weight gain (WG) and specific growth rate (SGR), followed by ULM10feeding, but these differences were not significant (Table 3). In contrast, all fasting groups (Cfasting, ULM5fasting, and ULM10fasting) exhibited significantly lower final body weight (FBW), WG, and SGR compared to the feeding groups ($P < 0.05$). The lowest WG and SGR values were observed in the ULM10fasting group. Feed conversion ratio (FCR) did not differ significantly among the feeding groups ($P > 0.05$). In fasting groups, FCR values were generally higher, though only the ULM10fasting group showed a markedly elevated mean FCR (1.21), indicating reduced feed efficiency, but this was not statistically tested separately. PER values were statistically similar within feeding and fasting subgroups ($P > 0.05$), while the differences between feeding and fasting groups were significant ($P < 0.05$).

Biochemical composition

At the end of the experiment, there was no statistically significant difference in moisture content among the regular feeding groups ($P > 0.05$). The moisture content of the fasting groups was significantly higher than that of the regular feeding groups ($P < 0.05$). The highest protein contents ($21.14 \pm 0.27\%$ and $21.58 \pm 0.12\%$) were recorded in the ULM5feeding and ULM10feeding groups, and these values were significantly higher than those observed in the Cfeeding and all fasting groups ($P < 0.05$). Protein content was

Table 3 Growth, survival and other body parameters of Nile tilapia (*Oreochromis niloticus*) fed the six experimental diets for 12 weeks

Experimental groups						
Regular feeding groups				Fasting groups		
	Cfeeding	ULM5feeding	ULM10feeding	Cfasting	ULM5fasting	ULM10fasting
IW (g)	10.69 ± 0.02 ^a	10.71 ± 0.02 ^a	10.65 ± 0.02 ^a	10.69 ± 0.03 ^a	10.64 ± 0.03 ^a	10.66 ± 0.02 ^a
FW (g)	67.89 ± 4.83 ^a	70.96 ± 9.85 ^a	67.47 ± 4.58 ^a	30.17 ± 1.94 ^b	32.26 ± 0.25 ^b	26.89 ± 0.55 ^b
WG (g)	57.19 ± 4.83 ^a	60.25 ± 9.85 ^a	56.79 ± 4.60 ^a	19.48 ± 1.95 ^b	21.63 ± 0.25 ^b	16.23 ± 0.55 ^b
SGR (% day ⁻¹)	2.28 ± 0.09 ^a	2.31 ± 0.16 ^a	2.27 ± 0.08 ^a	1.28 ± 0.08 ^b	1.37 ± 0.01 ^b	1.14 ± 0.03 ^b
FCR	1.06 ± 0.05 ^b	1.05 ± 0.06 ^b	1.06 ± 0.04 ^b	1.15 ± 0.08 ^{ab}	1.05 ± 0.02 ^b	1.21 ± 0.02 ^a
PER	1.44 ± 0.12 ^a	1.51 ± 0.25 ^a	1.43 ± 0.12 ^a	0.49 ± 0.05 ^b	0.54 ± 0.01 ^b	0.41 ± 0.01 ^b
HSI (%)	2.55 ± 0.14	2.33 ± 0.28	1.74 ± 0.11	2.85 ± 0.23	1.67 ± 0.38	1.73 ± 0.17
VSI (%)	8.64 ± 0.65	8.06 ± 0.96	8.01 ± 0.52	8.15 ± 0.25	9.97 ± 0.59	10.42 ± 0.31
Survival (%)	100	100	100	100	100	100

Data are reported as mean ± standard errors of three replicates (3). Means with different superscript letter in a row are significantly different ($p < 0.05$). IBW: Initial body weight, FBW: Final body weight. WG: Weight gain, SGR: Specific growth rate, FCR: Feed conversion ratio, PER: Protein efficiency ratio, HSI: Hepatosomatic index, VSI: Viscerosomatic index

significantly lower in the fasting groups compared to the regular feeding groups ($P < 0.05$). The highest lipid content ($2.69 \pm 0.10\%$) was observed in the Cfeeding group. Lipid content in fasting groups was significantly lower than in regular feeding groups ($P < 0.05$). There was no statistically significant difference in ash content among the regular feeding groups ($P > 0.05$). However, ash content in fasting groups was significantly lower than in regular feeding groups ($P < 0.05$) (Table 4).

Amino acid composition

At the end of the experiment, in all groups fed with experimental diets, the total amounts of essential and non-essential amino acids increased significantly with the inclusion level of U. Lactuca (Fig. 1). In the regular feeding groups, ULM5 and ULM10 diets resulted in significantly higher total amino acid content compared to the control group ($P < 0.05$). Among the fasting groups, the ULM10fasting group had the highest total amino acid content (55.87 g/100 g),

which was significantly greater than that of the other fasting groups ($P < 0.05$). Although the $\Sigma\text{EAA}/\Sigma\text{NEAA}$ ratio remained relatively constant (0.21–0.25), alanine, glutamic acid, and aspartic acid levels were significantly higher in the ULM groups compared to the control ($P < 0.05$). The levels of certain essential amino acids, such as threonine and valine, were also significantly increased in the ULM10 groups under both feeding and fasting regimes ($P < 0.05$). Therefore, ULM10 diets provided the highest amino acid enrichment among the experimental treatments.

Digestive enzyme activities

At the end of the experiment, amylase, lipase, pepsin and trypsin activities of Nile tilapia fed with different feeding regimes (Regular feeding and fasting) and three different experimental diets (C, ULM5, ULM10) were determined (Fig. 2). Feeding regime and diet type had no statistically significant effect on amylase activity ($P > 0.05$). The highest lipase activities were determined in the fasting groups.

Table 4 Chemical compositions in Nile tilapia (*Oreochromis niloticus*) fillets fed the six experimental diets for 12 weeks (% wet weight)

	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
<i>Regular feeding groups</i>				
Cfeeding	77.00 ± 0.02 ^a	20.69 ± 0.16 ^{ab}	2.69 ± 0.10 ^b	2.57 ± 0.04 ^b
ULM5feeding	76.74 ± 0.03 ^a	21.14 ± 0.27 ^b	2.46 ± 0.12 ^b	2.43 ± 0.09 ^b
ULM10feeding	77.96 ± 0.07 ^a	21.58 ± 0.12 ^b	2.11 ± 0.07 ^b	2.44 ± 0.43 ^b
<i>Fasting groups</i>				
Cfasting	79.03 ± 0.19 ^b	20.26 ± 0.05 ^a	1.98 ± 0.01 ^a	1.99 ± 0.14 ^a
ULM5fasting	79.29 ± 0.08 ^b	20.26 ± 0.02 ^a	1.93 ± 0.07 ^a	1.89 ± 0.03 ^a
ULM10fasting	79.14 ± 0.14 ^b	20.45 ± 0.11 ^a	1.98 ± 0.13 ^a	1.87 ± 0.19 ^a

Data are mean ± SE. Means with different superscript letter in a row are significantly different ($p < 0.05$)

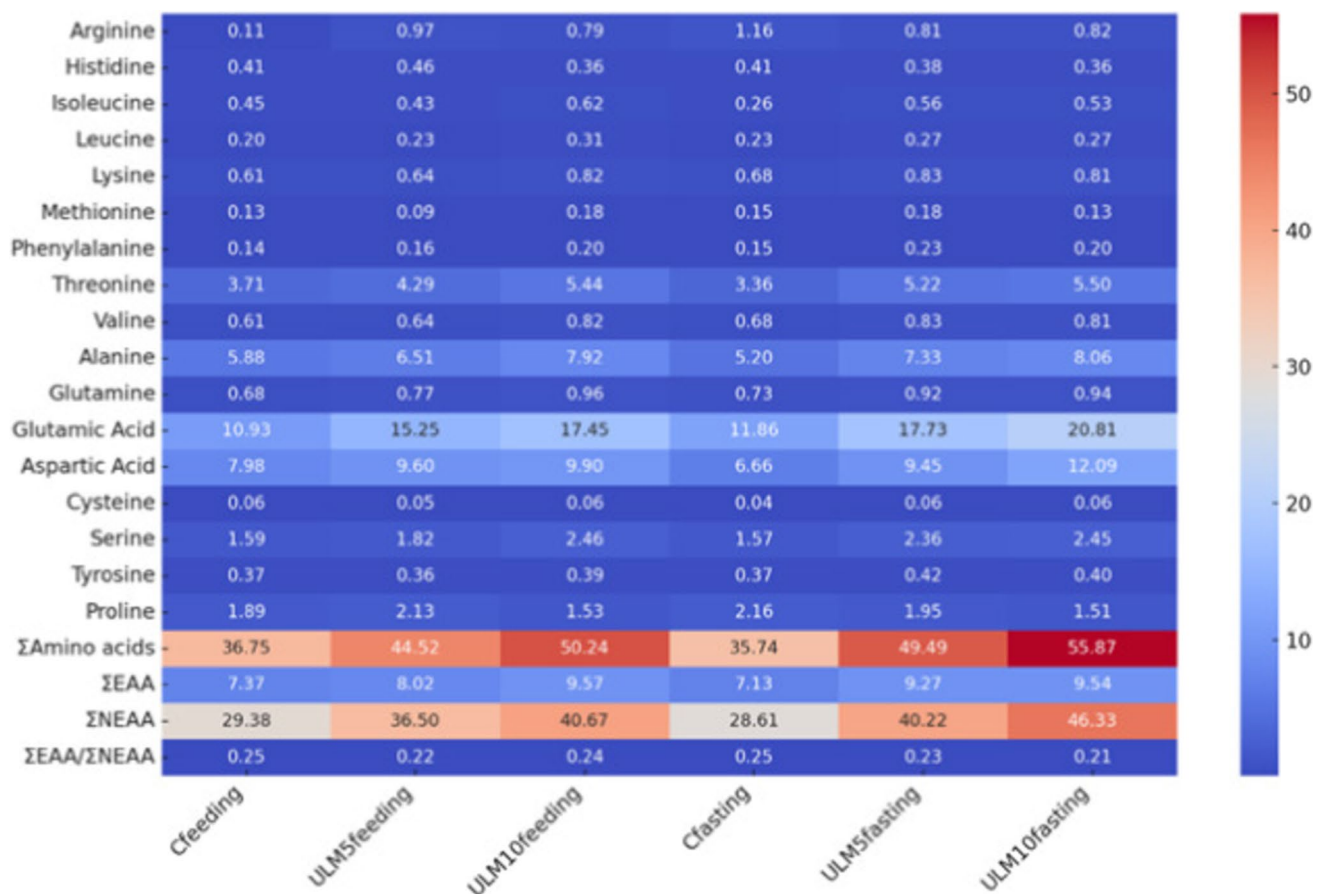


Fig. 1 Amino acid compositions in Nile tilapia fillets (*Oreochromis niloticus*) fed the six experimental diets for 12 weeks. The color scale represents values of amino acid concentrations

Especially, the ULM10fasting group exhibited the highest lipase activity ($P < 0.05$), while the Cfeeding group exhibited the lowest lipase activity ($P < 0.05$). Pepsin activity did not show any statistically significant difference between the feeding groups (Cfeeding, ULM5feeding, ULM10feeding) and the ULM5fasting group ($P > 0.05$), while the differences in the Cfasting and ULM10fasting groups were significant ($P < 0.05$). The highest trypsin activity was in the Cfeeding and ULM10feeding groups ($P > 0.05$). This was followed by ULM5fasting and ULM10fasting groups ($P > 0.05$). The lowest trypsin activity was in ULM5feeding and Cfasting groups ($P < 0.05$).

Antioxidant enzyme activities

At the end of the experiment, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and lipid peroxidation (LPO) activities of Nile tilapia fed with different feeding regimes (regular feeding and fasting) and three different experimental diets (C, ULM5, ULM10) are shown in Fig. 3. SOD activity was highest in the Cfeeding group under regular feeding conditions ($P < 0.05$). Although a decrease in

SOD activity was observed under fasting conditions in this group, the difference in SOD activity among groups was not statistically significant ($P > 0.05$). CAT activity was highest in the Cfeeding group and appeared to increase in ULM-supplemented groups under fasting conditions; however, these differences were not statistically significant ($P > 0.05$). GPx activity was significantly higher in Cfasting and ULM5fasting groups compared to their corresponding feeding groups ($P < 0.05$), while no significant difference was found between ULM10feeding and ULM10fasting groups ($P > 0.05$). LPO levels showed a decreasing trend with increasing inclusion of *U. lactuca* in the feeding groups and were higher in fasting groups; however, these differences were not statistically significant ($P > 0.05$), except for ULM10fasting, which showed the highest LPO level among all groups ($P < 0.05$).

Discussion

This study does not claim novelty in reporting general trends of enzyme activity in fed versus fasted fish. Instead, it highlights the interaction between *U. lactuca* supplementation

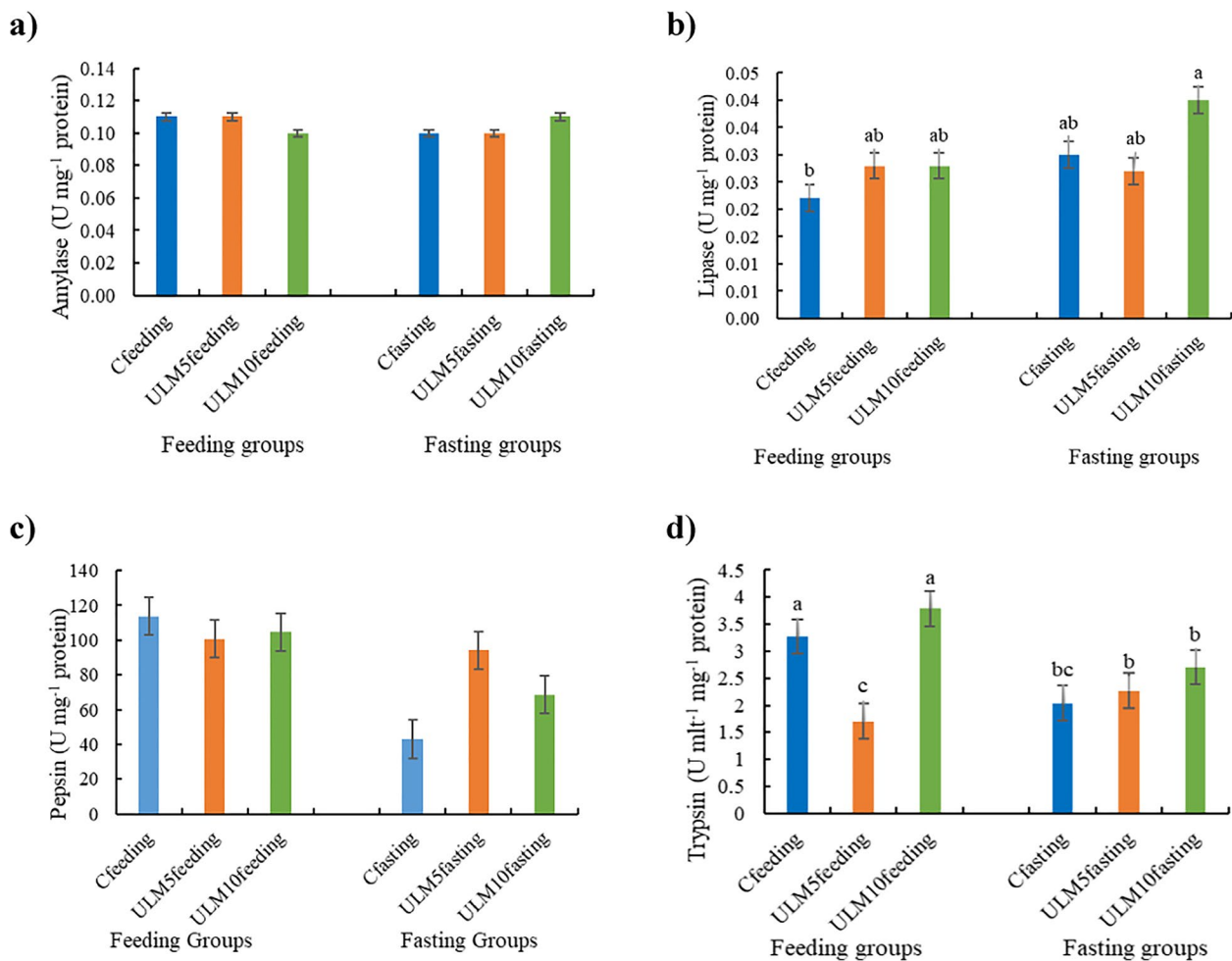


Fig. 2 Digestive enzyme activities [amylase (**a**), lipase (**b**), pepsin (**c**), trypsin (**d**)] in Nile tilapia (*Oreochromis niloticus*) fed experimental diets for 12 weeks and the relationships of these enzymes among groups. All data are presented as means $\pm$ SE (n=6). Different

letters above the bars indicate significant differences between groups ($P < 0.05$). Feeding and Fasting represent feeding regimes, C, ULM5, ULM10 represent experimental diets. Groups fed with the same diet are indicated by the same colors

and metabolic adjustments during prolonged fasting, a less-explored area. The lack of compensatory growth in starved fish is consistent with literature on energy trade-offs and the role of bioactive feed ingredients in mitigating stress-induced physiological decline. The implications for antioxidant defense and protein conservation, even during feed withdrawal, underscore the adaptive potential of Nile tilapia supported by macroalgae.

The best growth rate in both feeding and fasting groups was determined in the groups fed with 5% ULM added feed. The best FW, SGR and WG were also determined in these groups. At the end of the fasting period, the control group lost approximately twice as much weight. The ULM5fasting group showed the least weight loss. Güroy et al. (2011) reported that at the end of a 3-week fasting period in rainbow trout (*Oncorhynchus mykiss*), the control group showed approximately twice as much weight loss as the algae-fed groups. It has also been reported that weight loss was

reduced in sea bream (*Acanthopagrus schlegelii*) fed macroalgae before fasting (Nakagawa 1997). Yang et al. (2019) reported that fish receiving suboptimal feed and experiencing changes in feeding intervals may show lower growth as stored energy is mobilized to prolong survival. The results of the current study are similar to those of El-Araby et al. (2020). These studies indicated that fasting is well tolerated by Nile tilapia due to their relatively high growth rates and improved feed conversion ratio (FCR) during the refeeding period.

PER represents how efficiently an animal can utilize dietary protein for growth. A higher PER generally indicates that dietary protein is more efficiently converted into body tissue. A fish exhibits a higher PER, indicating better protein utilization and retention (Sankian et al. 2017). In the present study, PER was significantly higher in the continuously fed groups, but lower in the fasted groups. However, when looking at the chemical composition of the fish, it was

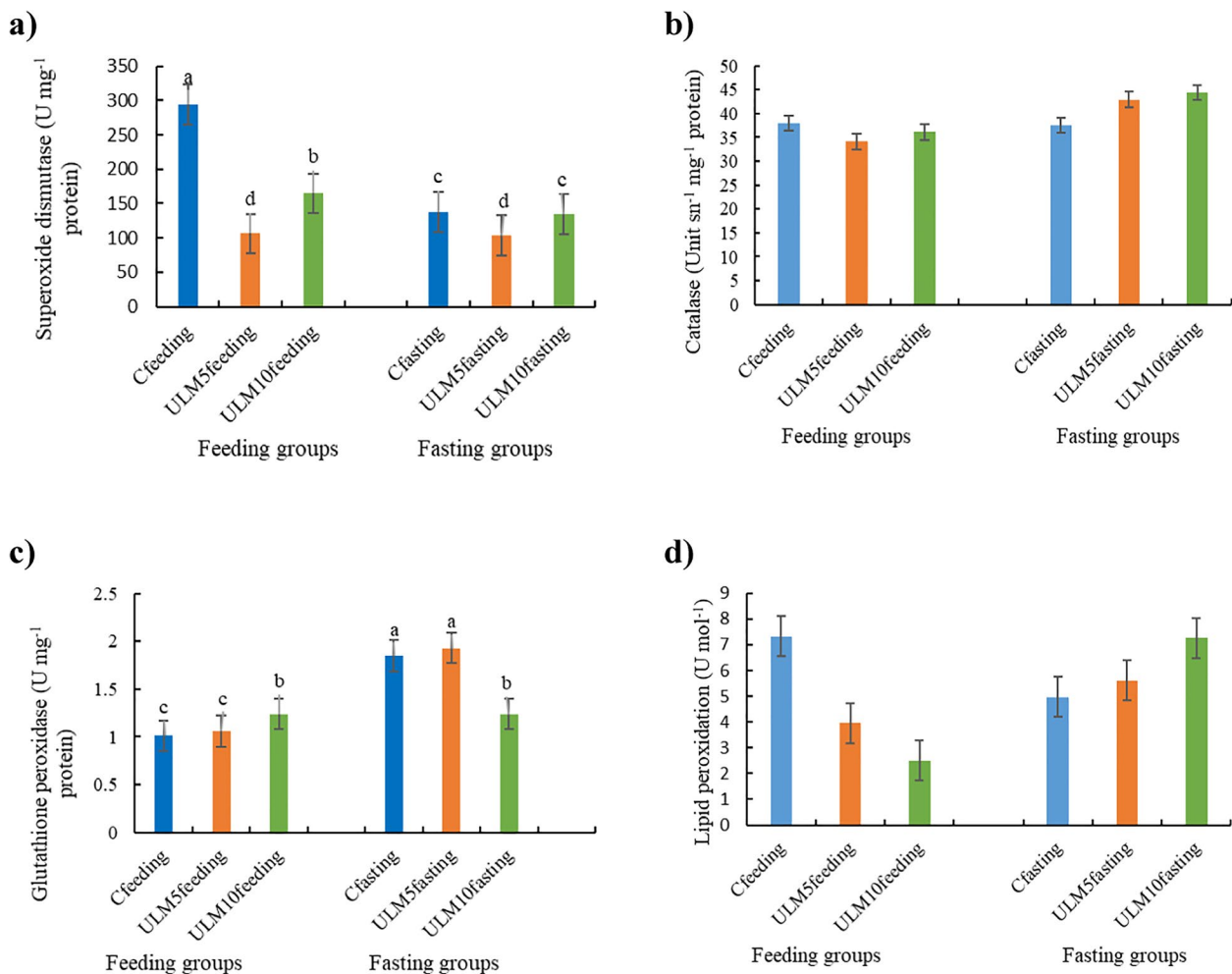


Fig. 3 Antioxidant enzyme activities [superoxide dismutase (**a**), catalase (**b**), glutathione peroxidase (**c**), and lipid peroxidation (**d**)] in Nile tilapia (*Oreochromis niloticus*) fed experimental diets for 12 weeks and the relationships of these enzymes among groups. All data are presented as means $\pm$ SE ($n=6$). Different letters above the

bars indicate significant differences between groups ($P<0.05$). Feeding and Fasting represent feeding regimes, C, ULM5, ULM10 represent experimental diets. Groups fed with the same diet are indicated by the same colors

determined that the crude protein content in the fish flesh did not fall below 20%. This suggests that the fish may tend to retain protein even though they are under fasting conditions. This study supports the hypothesis that fish fed diets with higher carbohydrate content have higher PER (Rahman et al. 2016; Schrama et al. 2018).

The use of metabolic energy reserves to provide energy to the organism during fasting periods varies among fish species (Furné et al. 2008) and depends on various factors such as the physiological state of the fish and environmental conditions. Thus, some fish utilise protein as the main energy source by maintaining the hepatic glycogen reserve through gluconeogenesis, while other fish use lipids ahead of the glycogen reserve (Alliot et al. 1974). Furthermore, not all body tissues contribute to these reserves in the same way. Some fish have a large energy reserve in the mesentery

of the digestive tract, others in the liver and others in the muscles (Furné et al. 2008). In tilapia, the liver is the organ that contains the most energy reserves. The liver plays a very important role in storing energy in the form of glycogen and lipids that can be utilised by the fish during periods of fasting or high energy demand. When we look at the HSI values in the present study, it was determined that it was quite low in the fasting groups. This indicates that tilapia fish meet their energy from the liver during the fasting period. Furné et al. (2008) reported that sturgeon showed a greater susceptibility to energy mobilisation in muscle and liver tissue during fasting than rainbow trout.

Amino acids and digestive enzymes play critical roles in fish metabolism, especially protein digestion and utilization. The production and regulation of digestive enzymes in fish can be affected by dietary factors, including the presence

and composition of amino acids. Sufficient levels of specific amino acids are required for the synthesis and activation of digestive enzymes (Hani et al. 2018). Optimal fish nutrition involves ensuring that dietary protein contains a balanced essential amino acid profile. This balance is crucial to maximize the efficiency of protein digestion, absorption and utilization, ultimately supporting the growth, health and overall metabolism of the fish (Wang et al. 2022). The presence and availability of certain amino acids in the diet can also affect the expression and activity of digestive enzymes in the gastrointestinal tract of fish. The proteins in the fish's diet must be broken down into smaller components, primarily amino acids, for absorption and use. Digestive enzymes facilitate this breakdown process in the intestine. After the proteins are broken down, the resulting amino acids are absorbed by the fish's intestinal cells and transported to various tissues for use. These amino acids serve as building blocks for synthesizing new proteins necessary for growth, tissue repair, enzyme production, and other biological functions (Shabana et al. 2019). In the present study, although the lowest total amino acid value was determined in ULM5 feed, the best growth was determined in ULM5 groups among both fasting and feeding groups. In addition, it was determined that Cfeeding and Cfasting groups fed with C feed, which had the highest total amino acid value, had the lowest total amino acid value. In the groups with the addition of *U. lactuca*, the total amino acid amounts increased in parallel with the inclusion rate of *U. lactuca*. Based on these results, it is concluded that the addition of *U. lactuca* has an effect on the amino acid profile in fish diet and the total amino acid amount increases in parallel with the addition rate of *U. lactuca*. Furthermore, glutamic acid and aspartic acid constitute a significant part (20–32%) of the total amino acids and, in fact, significant amounts of these two amino acids are responsible for the characteristic taste and “umami” aroma of seaweed (Wong and Cheung 2000; Salehi et al. 2019). In the present study, although it was observed that the amounts of glutamic acid and aspartic acid were higher in the control group feed, a significant increase was detected in the levels of these amino acids in the groups fed with ULM supplemented feeds both in feeding and fasting conditions. In this study, it was determined that the most dominant amino acids that form the characteristic aroma of algae in both feeds and fish meat were glutamic acid and aspartic acid. This finding shows that these amino acids play an important role in both feed composition and fish meat quality.

In the present study, fasting generally increased digestive enzyme activities, suggesting that the digestive system became more active to meet the energy needs. In addition, it was observed that the ULM10 diet increased lipase and trypsin activities in particular. This suggests that the content characteristics of ULM10 may play a role in promoting digestion. Changes in enzyme activities reveal that the

feeding regime and diet vary depending on the metabolic needs of the animals and their digestive system adaptations. Adequate nutrition generally stimulates the production and activity of digestive enzymes such as amylase, pepsin, lipase and trypsin. This is because the presence of food in the digestive system triggers the secretion of these enzymes to facilitate the breakdown and absorption of nutrients (Hidalgo et al. 1999). Prolonged fasting usually leads to a decrease in the activity of digestive enzymes. This decrease is a physiological response to conserve energy and resources during periods when food is scarce. Therefore, a decrease in enzyme activity may affect digestive efficiency when feeding is resumed (Zhu et al. 2014). Some species show adaptive responses to cycles of intermittent feeding and fasting. When fish or other animals resume feeding after periods of fasting, they can rapidly increase the production of digestive enzymes such as pepsin, trypsin, lipase, and amylase. This adaptive response allows rapid and efficient digestion of nutrients and maximizes nutrient uptake by the organism (Lopez-Lopez et al. 2005). Chakrabarti et al. (2006) reported that the Indian large carp (*Cirrhinus mrigala*) can temporarily increase the synthesis of digestive enzymes when refed after a period of food deprivation in order to rapidly digest and absorb nutrients. Fasting can also lead to morphological changes in the digestive system, such as a reduction in the size of the stomach and intestines. These changes may also affect the secretion and activity of digestive enzymes (Navarro and Gutiérrez 1995). Furné et al. (2012) reported that prolonged fasting in sturgeon (*Acipenser naccarii*) and trout may lead to a decrease in pepsinogen production and hence pepsin activity as part of the body's strategy to conserve energy and protein. Feeding stimulates the activity of digestive enzymes and increases the efficiency of food breakdown and absorption. Conversely, fasting leads to a decrease in digestive enzyme activity as part of the energy conservation strategy, which may affect digestive efficiency when feeding is resumed. These effects are part of the physiological adaptations that animals use to cope with changing food availability (Navarro and Gutiérrez 1995; Hidalgo et al. 1999; Zhu et al. 2014). The results of the present study provided important data to evaluate the effects of alternative feed sources (e.g. diets containing ULM) on different digestive enzymes. However, we believe that further studies would be useful to support these findings and elucidate the mechanisms. Although digestive enzyme activities were evaluated, gut microbiota composition was not assessed in this study. Future studies incorporating microbiome analysis would provide valuable insights into the microbial contributions to digestive physiology and enzyme regulation under dietary and fasting stress conditions.

In the present study, it was found that fasting generally increased oxidative stress levels (higher LPO and antioxidant enzyme activity), while these effects were less pronounced

in the regular feeding groups. The ULM10 diet provided a more effective antioxidant defense against oxidative stress by increasing CAT and GPx activities and limited lipid peroxidation by decreasing LPO levels. These findings clearly demonstrate the effects of feeding regimen and diet type on oxidative stress and antioxidant defense systems and indicate the potential benefits of alternative feed sources such as ULM10.

Fasting or lack of food intake can have various effects on antioxidant enzyme activity in organisms, including fish. Fasting can lead to a decrease in the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Muralisankar et al. (2015) believed that this decrease is a result of decreased energy availability and metabolic rate during fasting. Biomarkers such as antioxidant enzymes (SOD, CAT, GPx) and lipid peroxidation (LPO) play a critical role in controlling oxidative stress and maintaining cellular health (Cadenas and Davies 2000; Halliwell and Gutteridge 2015). SOD activity is generally increased in nutritional status. This controls reactive oxygen species (ROS) within the cell by converting superoxide anions into less harmful compounds (hydrogen peroxide). Nutrition increases metabolic activity in cells, which can increase ROS production. Increased SOD activity helps to manage this oxidative stress. In the fasting state, SOD activity is generally decreased. Decreased metabolic rate reduces ROS production, so cells no longer need high levels of SOD (Furné et al. 2008). In the present study, SOD activities decreased in fasting, even the SOD activity, which was quite high in the Cfeeding group, decreased in the fasting group.

Kiron et al. (2004) reported that CAT activity generally increases in the fed state, CAT protects cells from oxidative damage by converting hydrogen peroxide into water and oxygen, more hydrogen peroxide is produced in cells with increased nutrition and this increases CAT activity, CAT activity generally decreases in the fasting state, metabolic rate decreases and ROS production reduces the need of cells for CAT activity. However, in the current study, significant effects of both feeding regimen and diet type were observed in terms of CAT activity. CAT activity increased especially in the fasting group and it was determined that ULM10 diet supported this increase more. This may be related to the increase in cellular hydrogen peroxide levels under fasting conditions and may indicate that ULM10 provides an antioxidant capacity that can buffer this situation. Similarly, GPx activity also increases in the fed state and GPx plays a critical role in the detoxification of hydrogen peroxide and organic hydroperoxides. Feeding increases cellular activity and this may increase GPx activity. In fasting, GPx activity generally decreases. Due to the low ROS production, cells require less antioxidant enzymes such as GPx (Mourete et al. 2005). In the present study, significant

effects of feeding regimen and diet type on GPx activity were detected. Diet type (especially ULM10) increased GPx activity, indicating that nutrients contained in the ULM10 diet may support glutathione-based antioxidant defense. The increase in GPx activity in the fasting state suggests that the organism is making an adaptive response to oxidative stress and that this increase may vary depending on the diet type. Further research is needed to better understand such effects, but this finding suggests that macroalgae may have positive effects on health.

Nutritional status may also affect LPO levels. High dietary intake, especially high-fat diets, may make cell membranes more susceptible to lipid peroxidation. Antioxidant defense systems (SOD, CAT, GPx) attempt to compensate for this. LPO levels are generally reduced under fasting conditions. Decreased metabolic activity leads to less ROS production in cell membranes, which in turn reduces lipid peroxidation (Liu et al. 2015). In the present study, significant differences were observed between feeding regimen and diet type in terms of LPO levels. LPO levels were higher in the fasting group than in the regular-fed group, indicating that oxidative stress increases under fasting conditions. It was observed that the ULM10 diet provided positive effects in regulating LPO levels, suggesting that ULM10 may limit oxidative damage by reducing lipid peroxidation. Moussavou et al. (2011) investigated the antioxidant activity of *U. lactuca* essential oil and extracts collected from the coast of Gabon and found that *U. lactuca* has antioxidant properties and can potentially reduce cell damage. Therefore, it can be concluded that the addition of *U. lactuca* to the diet may have positive effects on cell health. However, these results generally need to be supported by more detailed studies.

The fasting regime applied in the present study resulted in lower growth performance in fish compared to the regularly fed groups. However, in terms of chemical composition, crude protein content in fish muscle remained above 20%, suggesting that Nile tilapia may prioritize protein retention even under fasting conditions. The observed decrease in the Hepatosomatic index (HSI) is likely due to the mobilization of liver energy reserves to meet metabolic demands during prolonged fasting, a response commonly seen in fish experiencing food deprivation. These adaptive metabolic changes underline the importance of dietary support during stress conditions such as fasting. In this context, the inclusion of *U. lactuca* in the diet appears promising; not only for its nutritional value but also for its potential to support fish health by enhancing antioxidant capacity, particularly under stressful conditions like fasting. This is further supported by the observed effects of *U. lactuca* on antioxidant enzyme activities. Moreover, the findings clearly demonstrate that regular feeding significantly enhances digestive enzyme activities, such as trypsin and amylase, whereas fasting suppresses these functions, underscoring the necessity of consistent feeding to maintain optimal digestive

efficiency. The study also emphasizes the role of antioxidant defense mechanisms in maintaining physiological balance, and suggests that integrating functional feed components like *U. lactuca* may aid in oxidative stress management. In conclusion, although the best growth was observed in the ULM5 feeding group, the inclusion of 10% *U. lactuca* enhanced antioxidant status and amino acid profiles, suggesting its utility in sustaining fish health under stressful conditions, such as intermittent fasting. These findings indicate *U. lactuca* promise as a functional feed additive in sustainable aquaculture strategies. Additionally, although this study provided sufficient time to evaluate short- and medium-term physiological responses, longer-term studies are needed to examine possible chronic effects such as persistent oxidative stress and organ-level histopathological changes.

Author contribution SD designed the work. EK and SD realised the work. DKÖ carried out the laboratory studies. KYÖ performed digestive enzymes and antioxidant analyses. IK and SD analyzed the data and interpreted the results. All authors were involved in the setup and finalisation of the experiment.

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Data Availability Data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval This experiment was carried out according to the ethical principles of animal experiments established by Sinop University Animal Experiments Control Council (HADYEK; Animal Experiments Local Ethics Committee) and the current legislation approved by the Local Animal Use Ethics Committee (Protocol 2021/04).

Competing interests The authors declare no competing interests.

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