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Dietary Supplementation of Dormival and Lemon Oil to Improve Growth, Immune Functions, and Health Status of Nile Tilapia, *Oreochromis niloticus*, Juveniles Cultured Under High Density

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ABSTRACT

Intensive aquaculture often exposes fish to crowding stress, impairing growth performance and overall physiological stability. This study evaluated the effects of dietary Dormival (D) and lemon oil (LO) supplementation on the growth performance, antioxidant status, and immune response of Nile tilapia (*Oreochromis niloticus*) juveniles reared under high-density conditions for 42 days. Six diets were tested, including control, 0.5% LO, 0.2%–0.4% D, and their combinations. Fish receiving D, either alone or with LO, showed significantly higher weight gain (WG), specific growth rate (SGR), and feed efficiency ($p < 0.05$) compared with the control. Moderate D inclusion (0.2%–0.4%) enhanced survival, antioxidant defense (superoxide dismutase [SOD], glutathione [GSH], total antioxidant capacity [TAC]), and immune activity (lysozyme, immunoglobulin M [IgM]). However, excessive supplementation (0.4% D + 0.5% LO) increased aspartate aminotransferase (AST) and malondialdehyde (MDA), suggesting mild metabolic stress. Proximate composition indicated higher lipid and ash with elevated D levels. Overall, 0.2% D, alone or combined with 0.5% LO, effectively mitigated crowding stress and promoted superior growth and physiological resilience in Nile tilapia.

1 | Introduction

Due to global population growth and the increased demand for animal protein in general and fish and seafood in particular [1], being a vital source of high-quality protein, rich in omega-3 fatty acids, minerals, antioxidants, and lipid-soluble vitamins [2, 3]. Furthermore, capture fisheries are unable to meet this growing demand. Therefore, it is necessary to enhance aquaculture activities and optimize the utilization of available water and land resources by developing intensive systems that maintain growth rates and overcome the stressful conditions associated with them [4, 5]. Stress in farmed fish is known to have a significant impact on both welfare and productivity; fish culture at high densities is a source of stress since it has been related to immune depression, aberrant behavior, and decreased growth, all of which raise the risk of disease [6, 7].

Additionally, *Oreochromis niloticus* is one of the most common freshwater fish species, and it can withstand a wide range of environmental conditions with rapid growth rates. However,

substantial mortalities have been recorded among tilapia farms during the summer season [8], often attributed to stress from high stocking densities [9]. Furthermore, climate changes such as less rainfall and rising temperatures have increased groundwater salinity, resulting in poor water quality, which has a severe impact on the aquaculture sector [10, 11]. The use of natural immunostimulants and antistress drugs, including prebiotics, probiotics, vitamins, minerals, and herbs, has become necessary and urgent under intensive fish farming and has been shown to enhance immunity in fish [12, 13]. The overuse of antibiotics in intensive aquaculture contributes to antimicrobial resistance and environmental contamination. Hence, a shift toward sustainable aquaculture development [14, 15].

Phytogenic feed additives, including spices, herbs, and essential oils, are natural plant-derived compounds that enhance animal performance [16, 17]. Among these, lemon essential oil is valued for its antimicrobial and antiparasitic properties and ease of incorporation into fish and shellfish diets [18, 19]. Its major constituents—D-limonene (52.85%), p-cymene (14.36%), and β-pinene (13.69%)—contributed to improved growth and health in several fish species [20–22].

Furthermore, Dormival (D) is a commercial product herbal tranquilizer that contains a combination of *Valeriana officinalis* and *Humulus lupulus*, both traditionally used to alleviate anxiety and insomnia [23, 24]. *Valeriana officinalis* exerts its sedative action by modulating neurotransmitters, enhancing GABA availability, and reducing catecholamine levels, thus alleviating stress and promoting immune modulation [25–27]. It is rich in valerenic acid, valepotriates, and volatile oils with anti-inflammatory and anxiolytic properties [28]. According to Abasali and Mohamad [29], *Valeriana officinalis* root extracts effectively inhibit swordtails' (*Xiphophorus hellerii*) stress responses. Compounds found in valerian have anticonvulsant and anxiolytic effects on zebrafish *Danio rerio* [30].

Although growth and mucosal immune parameters were not significantly affected by adding valerian extract to the diets of common carp, malondialdehyde (MDA) levels decreased, and gene expression associated with inflammation and oxidative stress changed. Notably, the group that received 0.25% valerian extract as a supplement showed the strongest effects compared with those of the other groups [31].

Similarly, *Humulus lupulus* interacts with serotonin and melatonin receptors and modulates GABA activity, contributing to its sedative effect [32]. Its bioactive compounds—humulones, lupulones, polyphenols, and essential oils—possess antimicrobial, antioxidant, and immunostimulatory activities [33], which may support fish health and enhance disease resistance under stressful aquaculture conditions. Dadras et al. [34] found that *Humulus lupulus* extract in a carp diet did not affect growth, but it could improve fat metabolism and protein deposition in whole-body composition. It can also increase the antioxidant capacity of the liver and the immune system and change the fatty acid profile of fish fillets. Regarding terrestrial animals, Hanczakowska et al. [35] reported that growing pigs given a diet supplemented with hop extract showed improvements in the fatty acid profile and decreased cholesterol levels. Wang et al. [36] revealed that adding hops to feedlot cattle may improve gut health and lower the chance of bacterial infection. It should be noted that recent studies by Yilmaz et al. [31] and Dadras et al. [34] recommended that further research with extended feeding durations and varying doses of *Valeriana officinalis* extract is required to understand its physiological effects in aquaculture better. Also, the functional concentration of hop *Humulus lupulus* extract must be selected cautiously, as its benefits vary across different doses, depending on the fish tissue. They add that our results act as a starting point for further in vivo investigations of its possible utilization, as well as the underlying antioxidant and anti-inflammatory mechanism of hop in aquaculture species. Therefore, the present study is a unique one that attempted to investigate the interaction impacts of D as a substance (including both the extract of *Humulus lupulus* and *Valeriana officinalis*) and lemon oil (LO) on growth rates, survival rate (SR), feed efficiency, liver, kidney functions, immunity, and antioxidant indices of *O. niloticus*.

2 | Materials and Methods

2.1 | Study Location and Ethical Approval

The present experiment was done at the Desert Aquaculture Research Unit (DARU), Faculty of Aquaculture and Marine Fisheries, Arish University, Arish, North Sinai Governorate, Egypt. This study was carried out in strict accordance with the recommendations of the Suez Canal University, Faculty of Agriculture Scientific Research Ethics Committee, Ismailia, Egypt, and this protocol was approved with code (21/2024). The authors confirm that all methods were performed according to the relevant guidelines and regulations and that the study is reported according to the ARRIVE guidelines.

2.2 | Experimental Fish and Culture Facility

A 42-day feeding trial was conducted to assess D and LO as feed supplements on the growth performance, SR, feed efficiency, whole-body composition, hematological parameters, and immune responses of monosex *O. niloticus*. Fish were sourced from stock ponds of the DARU greenhouse and subjected to a 1-week acclimation period, during which they were fed a basal diet containing 30% crude protein. After this period, fish were graded by weight, and a total of 180 tilapia juveniles (mean initial weight: 20.6 ± 0.17 g/fish) were randomly allocated into 18 glass aquaria (70 cm L × 30 cm W × 40 cm H; water volume, 75 L), with triplicates per treatment ($N = 30$ fish/treatment or 10 fish/aquarium [75 L], equivalent to 133 fish/m^3 [intensive stocking rate according to [37, 38]]). Fish were hand-fed twice daily (at 10:00 and 16:00) at a rate of 3% of their body weight 6 days per week. Every 14 days, fish were collectively weighed after a fasting day to readjust feed rations.

2.3 | Treatments and Preparation of Experimental Diets

Six dietary treatments were designed as follows: Group I (0.5% LO): 0.5% LO; Group II (0.2% D): 0.2% D; Group III (0.4% D): 0.4% D; Group IV (0.2% D + 0.5% LO): 0.2% D + 0.5% LO; Group V (0.4% D + 0.5% LO): 0.4% D + 0.5% LO; and the control group (C) received the basal diet without any additives.

2.3.1 | LO

Lemon essential oil was obtained from Medical Plant Marketing, Arish City, Egypt, and directly sprayed onto the 2 mm pellets (basal diet) and dried in a forced-air oven at 50°C for 60 min to ensure uniform coating and stability. The used dose of LO was according to Copatti et al. [39].

2.3.2 | D

D capsules, commercially purchased, contained 100 mg of *Valeriana officinalis* extract and 25 mg of *Humulus lupulus* extract per capsule. The tested dosages were inspired by a previous study by Yilmaz et al. [31]. A commercially formulated floating pellet diet for Nile tilapia (Skretting Egypt Co., Belbies, Sharkia, Egypt) was used as the basal control diet, containing 30% crude protein, 6% crude lipid, and an estimated gross energy of 3900 kcal/kg. The experimental diets were prepared by surface-coating the basal diet with the designated feed additives. The additive extract powder was sprayed on the pelleted basal diet (2 mm diameter) with gelatin solution (4% W/V) at 1:10 (gelatin solution:feed, v/w) and dried in the forced-air drying oven (CO 150, 220 V, 2200 W, Amb. +15°C to 250°C, Made in Korea, S/N: X01017627) at 50°C for 60 min. For the control diet, the same gelatin solution was applied to the basal diet without any additives. All experimental feeds were left to cool, then packed into plastic bags labeled with the names of the treatments, and stored in a refrigerator at 4°C.

2.4 | Water Quality Management

Throughout the trial, aquaria were continuously aerated and filled with prestored underground water. Key water quality parameters were routinely monitored to maintain optimal environmental conditions. The average water temperature was maintained at 27 ± 1°C, with salinity levels of 4 ± 0.2 ppt, dissolved oxygen concentrations of 5.6 ± 0.2 mg/L, and a pH of 7.9 ± 0.1. To maintain the water quality, uneaten feed and fecal matter were removed daily via siphoning, followed by a 35% water exchange conducted at 14:00 h. Total ammonia nitrogen was assessed biweekly using standard protocols outlined by the American Public Health Association [40], with recorded values ranging from 0.15 to 0.20 mg/L.

2.5 | Data Collection

At the end of the feeding trial, all fish were subjected to a 24-h fasting period to minimize the influence of gut content on body weight measurements. Subsequently, individual body weights and total lengths of all fish within each aquarium were accurately recorded.

2.5.1 | Fish Performance Calculations and Body Index

$$\text{Weight gain (WG, g)} = \text{final live body weight (FW, g)} - \text{initial live body weight (IW, g)},$$

$$\text{Average daily weight gain (ADG, g)} = \text{WG}/t; \text{ where } t \text{ is the experimental period (42 days)},$$

$$\text{Specific growth rate (SGR, \%)} = 100(\ln \text{FW} - \ln \text{IW})/t,$$

$$\text{Condition factor (CF, \%)} = (\text{FW}/\text{FL}^3) \times 100; \text{ where, } F_L \text{ is final total body length (cm)},$$

$$\text{Survival rate (SR, \%)} = (\text{number of fish at the end}/\text{number of fish at the beginning of trial period}) \times 100,$$

$$\text{Feed conversion ratio (FCR)} = \text{dry feed consumption (g)}/\text{weight gain (g)},$$

$$\text{Hepatosomatic index (HSI)} = 100(\text{liver weight [g]}/\text{whole body weight [g]}).$$

2.6 | Blood Sampling and Analyses

Ten fish/treatment were randomly selected and anesthetized using MS-222 (0.1 g L^{-1} tricaine methane sulfonate; Sigma–Aldrich). Approximately 3 mL of blood was drawn from the caudal vein using sterile disposable syringes. Blood was immediately divided into two portions: one transferred into tubes containing 10% ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and the other into plain tubes for serum separation. All samples were promptly transported to the Hematology and Biochemistry Laboratory of Arish University and processed within 1 h. After collecting the blood, the fish of each treatment were euthanized before they recovered from anesthesia by decapitation and then used for analyzing the body chemical composition. Whole blood (EDTA-treated) was analyzed to determine hematological parameters, including red blood cell (RBC), white blood cell (WBC), hemoglobin (Hb), and hematocrit (Hct), using a fully automatic veterinary hematological analyzer device, model BK-5000VET, brand Biobase. For serum analysis, clotted blood samples were centrifuged at 3000 rpm for 10 min using a benchtop centrifuge (PRO-RESEARCH K241R/Serial Number 209958/230V/50Hz/690W/United Kingdom).

The obtained serum was aliquoted and used for biochemical and antioxidant assessments. Levels of glucose, urea, creatinine, total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), MDA, superoxide dismutase (SOD), total antioxidant capacity (TAC), and glutathione (GSH) were determined using a UV-Vis spectrophotometer along with commercial diagnostic kits, except lysozyme which was determined by using the (LZM) ELISA Kit (Catalog Number orb1653509) according to [41]. Serum immunoglobulin was measured using immunoturbidimetric methods [42].

2.7 | The Proximate Chemical Composition

Whole-body proximate composition analyses were conducted in triplicate ($n = 3$) from each treatment group to determine moisture, crude protein, crude lipid, and ash content, following the standardized methodologies outlined by the Association of Official Analytical Chemists [43]. Moisture content was quantified by drying homogenized samples to a constant weight in a forced-air convection oven at 105°C. Crude protein was determined using the Kjeldahl method, which involved digestion in concentrated sulfuric acid at 450°C, followed by nitrogen quantification and conversion to protein using the factor N × 6.25. The lipid content was assessed gravimetrically through ether extraction using a Soxhlet apparatus. Ash content was determined by incinerating

the dried samples in a muffle furnace at 550°C for 6 h, with the residue expressed as a percentage of the dry mass.

2.8 | Statistical Analysis

Initially, the normality of the data was confirmed using the Wilk's Shapiro test. A one-way analysis of variance (ANOVA) complemented by a Waller-Duncan test was used to compare means across treatments at a significance level ($p \leq 0.05$). Means were presented as the mean (± standard error, SE; $n = 3$). Statistical analysis was performed using the SPSS [44] Statistical Package Program v.23.

3 | Results

3.1 | Growth Indicators

Table 1 showed that growth indices, including final body weight (FW), weight gain (WG), average daily gain (ADG), and specific growth rate (SGR), were significantly influenced by the dietary treatments at ($p \leq 0.05$). Fish fed diets supplemented with D at both 0.2% and 0.4%, either alone or in combination with 0.5% LO, exhibited significantly superior growth rates compared with the control and LO-only groups. The highest growth rates were recorded at 0.4% and 0.2% D, where statistical analysis did not show significant differences between them.

3.2 | Feed Conversion Ratio (FCR), SR, and Some of Morphometric Measurements

As presented in Table 2, although the statistical analysis did not appear significant among the treatments, the fish fed the diet supplemented with 0.2% D demonstrated the most efficient feed utilization, recording the lowest FCR at 1.20 ± 0.03 followed, by the 0.4% D + 0.5% LO group in comparison with the other treatments and especially the control group.

In the same trend, there were nonsignificant differences in the condition factor (CF); however, the higher values of CF were observed in fish receiving 0.4% D and the combination of 0.4% D+0.5% LO, suggesting better somatic development compared to the control group, which recorded the lowest CF. The hepatosomatic index (HSI) did not show any significant differences among treatments ($p \leq 0.05$), indicating that dietary supplementation had no adverse effect on liver development or relative liver size.

In terms of SR, fish fed 0.2% D exhibited the highest SR, followed by 0.4% D+0.5% LO, 0.2% D+0.5% LO, and 0.5% LO, whereas the control group and 0.4% D had the lowest SR ($p \leq 0.05$), respectively.

3.3 | Hematological Assay

As shown in Table 3, the hematological profiles of juvenile *Oreochromis niloticus*, including RBCs, Hb, and Hct, were not significantly affected by dietary supplementation with D and LO over a 42-day feeding period. This indicates good management of water quality and the maintenance of dissolved oxygen levels in the optimal range for fish growth. However, the WBC count was significantly higher in the group receiving 0.2% D compared with all other groups.

3.4 | Biochemical of Blood Serum

Results in Table 4 showed that glucose concentrations were significantly affected by the dietary treatments ($p \leq 0.05$). Elevated glucose levels were observed in the groups receiving 0.4% D +0.5% LO and 0.5% LO. Conversely, the lowest glucose concentrations were detected in the 0.2%, 0.4% D, and control groups. Total serum protein levels also exhibited significant differences among treatments. The 0.2% D group recorded the highest total protein concentration, suggesting enhanced protein synthesis and/or improved immune function. In contrast, the lowest value was observed in the 0.5% LO group. Kidney function indicators, including urea and creatinine values, were not significantly different among the treatments, but the 0.2% D group had the lowest values for these items. Liver function indicators, AST and ALT, were significantly influenced by the dietary treatments. The highest AST activity was recorded in the 0.4% with or without LO groups, while there was no significant difference among the other

TABLE 1 | Effect of D and LO as feed additives on growth performance of Nile tilapia juveniles that were cultured under high density for 42 days were statistically analyzed with a one-way ANOVA then complemented by a Waller-Duncan test.

Treatments	IW[1] (g)	FW[2] (g)	WG[3] (g)	ADG[4] (g)	SGR[5] (%/day)
Control	20.40 ± 0.40	33.10 ± 2.60^{b}	12.70 ± 2.20^{b}	0.32 ± 0.05^{b}	1.20 ± 0.15^{b}
0.5% LO	20.95 ± 0.25	27.35 ± 2.51^{c}	6.38 ± 2.26^{c}	0.16 ± 0.06^{c}	0.65 ± 0.21^{c}
0.2% D	21.25 ± 0.05	40.93 ± 0.03^{a}	19.68 ± 0.02^{a}	0.50 ± 0.00^{a}	1.63 ± 0.01^{ab}
0.4% D	20.20 ± 0.10	40.60 ± 0.10^{a}	20.40 ± 0.20^{a}	0.51 ± 0.01^{a}	1.74 ± 0.01^{a}
0.2% D+0.5% LO	20.26 ± 0.10	39.40 ± 0.40^{a}	19.20 ± 0.30^{a}	0.48 ± 0.01^{a}	1.66 ± 0.02^{a}
0.4% D+0.5% LO	20.60 ± 0.40	37.07 ± 0.76^{ab}	16.47 ± 0.90^{ab}	0.41 ± 0.01^{ab}	1.46 ± 0.04^{ab}
p-Value	0.07	<0.01	<0.01	<0.01	<0.01

Note: The values (mean ± SE, $n = 3$) in the same column do not share similar superscripts and are substantially different at $p \leq 0.05$.
[1]IW: initial body weight.
[2]FW: final body weight.
[3]WG: weight gain.
[4]ADG: average daily gain.
[5]SGR: specific growth rate.

TABLE 2 | Effect of D and LO as feed additives on morphometric indices of Nile tilapia juveniles that were cultured under high density for 42 days were statistically analyzed with a one-way ANOVA then complemented by a Waller-Duncan test.

Treatments	FCR[1] (%)	CF[2] (%)	HSI[3] (%)	SR[4] (%)
Control	2.85 ± 0.15	1.44 ± 0.16	2.00 ± 1.00	40.00 ± 5.70^{d}
0.5% LO	2.67 ± 0.67	1.69 ± 0.14	2.48 ± 0.33	66.60 ± 6.60^{bc}
0.2% D	1.20 ± 0.10	1.64 ± 0.02	2.49 ± 0.04	96.60 ± 3.00^{a}
0.4% D	2.80 ± 0.87	1.86 ± 0.06	2.93 ± 0.63	56.60 ± 3.00^{cd}
0.2% D+0.5% LO	2.06 ± 0.20	1.66 ± 0.11	2.41 ± 0.23	76.60 ± 9.00^{abc}
0.4% D+0.5% LO	1.30 ± 0.16	1.88 ± 0.03	2.22 ± 0.60	86.60 ± 9.00^{ab}
p-Value	0.08	0.08	0.70	<0.01

Note: The values (mean ± SE, $n = 3$) in the same column do not share similar superscripts and are substantially different at $p \leq 0.05$.
[1]FCR: feed conversion ratio.
[2]CF: condition factor.
[3]HSI: hepatosomatic index.
[4]SR: survival rate.

TABLE 3 | Effect of D and LO as feed additives on hematological parameters of Nile tilapia juveniles that were cultured under high density for 42 days were statistically analyzed with a one-way ANOVA then complemented by a Waller-Duncan test.

	Parameters			
Treatments	WBC[2] (10^3 cells/μL)	RBC[3] (10^6 cells/μL)	Hb[4] (g/dL)	Hct[5] (%)
Control	214.00 ± 60.00^{a}	1.80 ± 0.20	7.45 ± 0.65	20.41 ± 3.95
0.5% LO	169.40 ± 9.40^{b}	1.60 ± 0.10	7.30 ± 0.10	20.2 ± 0.40
0.2% D	227.50 ± 2.50^{a}	1.72 ± 0.02	7.15 ± 0.55	21.0 ± 3.00
0.4% D	171.80 ± 10.80^{b}	1.43 ± 0.37	7.00 ± 1.00	23.3 ± 3.00
0.2% D+0.5% LO	135.70 ± 4.70^{c}	1.42 ± 0.38	5.70 ± 0.30	17.2 ± 3.10
0.4% D+0.5% LO	128.50 ± 3.70^{c}	1.41 ± 0.40	6.20 ± 0.20	18.0 ± 2.80
p-Value	<0.01	0.86	0.25	0.72

Note: The values (mean ± SE, $n = 3$) in the same column do not share similar superscripts and are substantially different at $p \leq 0.05$.
[1]WBC: white blood cell.
[2]RBC: red blood cell.
[3]Hb: hemoglobin.
[4]Hct: hematocrit.

TABLE 4 | Effect of D and LO as feed additives on serum biochemical analysis of Nile tilapia juveniles that were cultured under high density for 42 days were statistically analyzed with a one-way ANOVA then complemented by a Waller-Duncan test.

	Parameters					
Treatments	Glucose (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)	Total protein (g/dL)	AST[1] (U/L)	ALT[2] (U/L)
Control	61.0 ± 1.00^{c}	24.0 ± 0.70	0.36 ± 0.04	5.75 ± 0.05^{ab}	54 ± 5.00^{c}	22 ± 0.80^{c}
0.5% LO	104.0 ± 9.00^{a}	25.0 ± 2.80	0.30 ± 0.14	4.88 ± 0.11^{b}	57 ± 3.00^{c}	52 ± 2.40^{b}
0.2% D	59.0 ± 4.20^{c}	21.0 ± 0.60	0.26 ± 0.03	9.87 ± 0.04^{a}	60 ± 0.50^{c}	25 ± 2.50^{c}
0.4% D	56.5 ± 4.90^{c}	27.5 ± 2.12	0.30 ± 0.06	5.10 ± 0.14^{ab}	106 ± 2.10^{b}	60 ± 3.20^{a}
0.2% D+0.5% LO	83.5 ± 3.50^{b}	25.5 ± 0.28	0.30 ± 0.03	5.25 ± 0.35^{ab}	66 ± 3.00^{c}	21 ± 6.10^{c}
0.4% D+0.5% LO	111.0 ± 4.24^{a}	27.0 ± 4.24	0.25 ± 0.03	5.07 ± 0.09^{ab}	142 ± 7.30^{a}	42 ± 4.10^{b}
p-Value	<0.01	0.42	0.74	<0.01	<0.01	<0.01

Note: The values (mean ± SE, $n = 3$) in the same column do not share similar superscripts and are substantially different at $p \leq 0.05$.
[1]AST: aspartate aminotransferase.
[2]ALT: alanine aminotransferase.

groups. Regarding ALT, the highest level was found in the 0.4% D group, whereas the lowest level was detected in the 0.2% D+0.5% LO group, indicating an improvement in liver function in these groups.

3.5 | Antioxidant Activities and Immunity Indices

Antioxidant and immune responses in *Oreochromis niloticus* juveniles were significantly affected by dietary supplementation with D and LO and are summarized in Figure 1a–d. MDA, a crucial marker of oxidative stress and lipid peroxidation, was considerably higher in the combination groups. (0.2% D + 0.5% LO and 0.4% D + 0.5% LO). However, the lowest MDA concentration was recorded in the group supplemented with 0.5% LO and 0.2% D. SOD activity followed a different trend; groups of 0.5% LO and 0.2% D had the highest *SOD* ($p \leq 0.05$) while the 0.4% D + 0.5% LO group had the lowest. Also, the highest total antioxidant capacity (TAC) was detected in the 0.2% D. Besides, GSH increased in the treated groups compared with the control.

Regarding immune parameters (Figure 2a), lysozyme activity—a crucial marker of innate immunity—was significantly higher in the 0.5% LO and 0.2% D groups. However, the 0.4% D + 0.5% LO group exhibited the lowest lysozyme activity. Also, immunoglobulin M (IgM) concentrations were significantly elevated in all supplemented groups except for the control and the 0.4% D + 0.5% LO group. The negative effect of interaction between 0.4% D and 0.5% LO on these indicators is indicated in (Figure 2b).

3.6 | Whole Body Composition

The effects of dietary supplementation with D and LO on the whole-body composition of *O. niloticus* juveniles after 42 days are presented in Table 5. Significant differences ($p \leq 0.05$) were observed among treatments for all measured parameters, including moisture, crude lipid, ash, and crude protein contents. Moisture, lipid, and ash contents were significantly highest in the 0.2% D+0.5% LO and 0.2% D groups, which had the lowest protein content compared with the other groups.

4 | Discussion

The integration of herbal additives into aquaculture diets has shown significant potential in enhancing the growth and health of various fish species, as evidenced by multiple studies [17, 45, 46]. In the present study, dietary supplementation with D, either individually or in combination with LO, produced clear improvements in growth performance parameters, including FW, WG, and SGR, compared to the control group ($p \leq 0.05$). The most

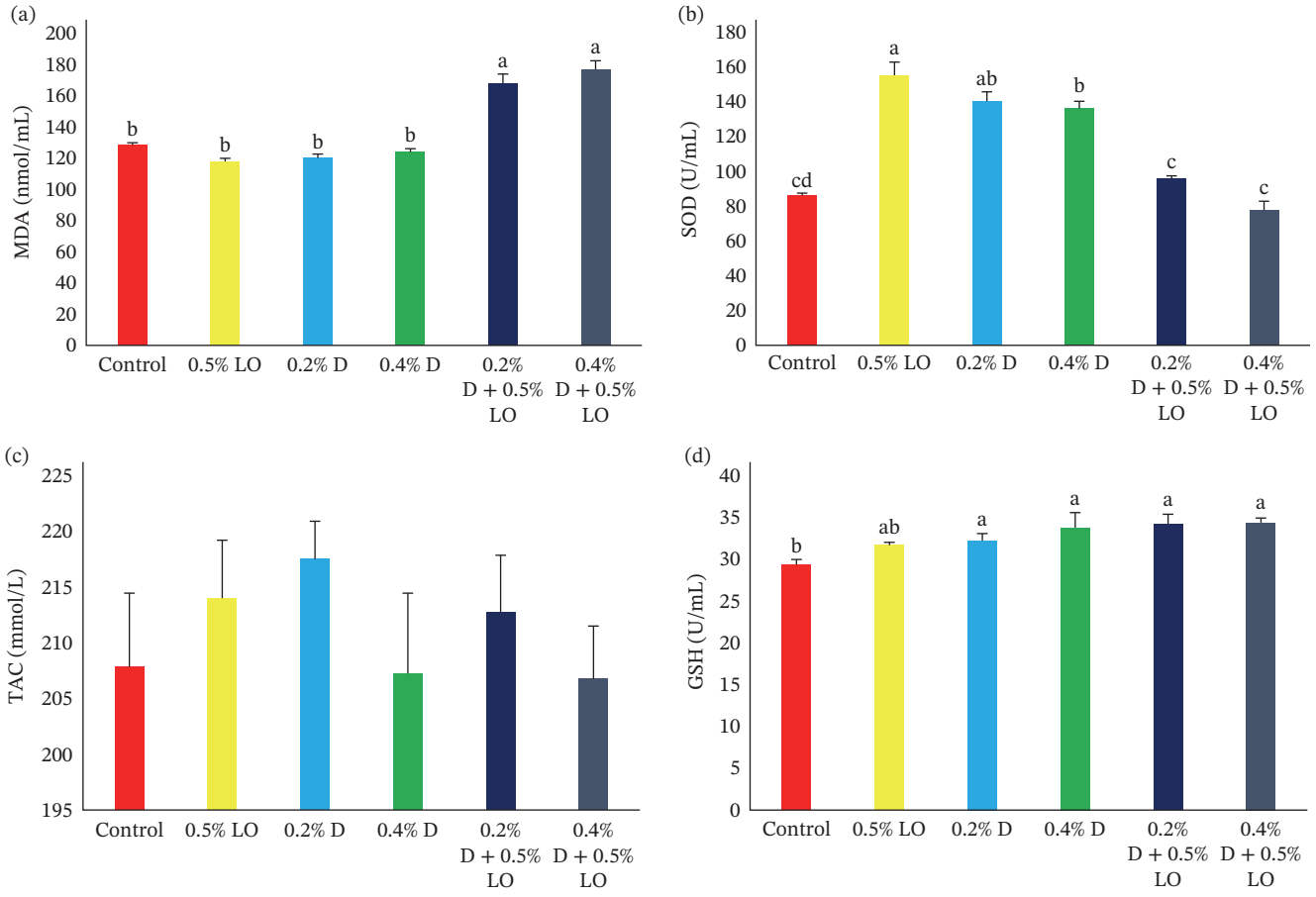

FIGURE 1 | (a–d) Effect of D and LO as feed additives on malondialdehyde (MDA, p-value < 0.01), superoxide dismutase (SOD, p-value <0.01), total antioxidant capacity (TAC, p-value = 0.67), and glutathione (GSH, p-value < 0.01) of Nile tilapia juveniles that were cultured under high density for 42 days were statistically analyzed with a one-way ANOVA then complemented by a Waller-Duncan test. Bar chart columns with error bars refer to means ± SE, and different superscript letters are significantly different ($n = 3$, $p \leq 0.05$).

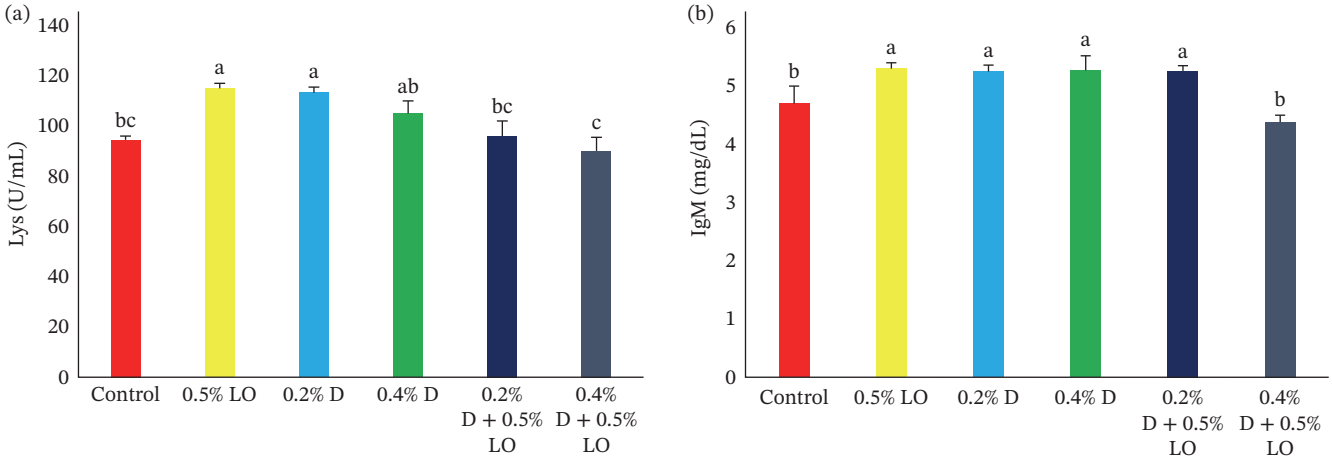

FIGURE 2 | (a and b) Effect of D and LO as feed additives on lysozyme (Lys, p-value = 0.04) and immunoglobulin M (IgM, p-value = 0.019) of Nile tilapia juveniles that were cultured under high density for 42 days were statistically analyzed with a one-way ANOVA then complemented by a Waller-Duncan test. Bar chart columns with error bars refer to means ± SE, and different superscript letters are significantly different ($n = 3$, $p \leq 0.05$).

TABLE 5 | Effect of D and LO as feed additives on whole body composition of Nile tilapia juveniles that were cultured under high density for 42 days were statistically analyzed with a one-way ANOVA then complemented by a Waller-Duncan test.

Treatments	Moisture (%)	Lipid (%)	Ash (%)	Protein (%)
Control	71.5 ± 0.36^{c}	22.4 ± 0.60^{b}	14.5 ± 0.60^{c}	63 ± 1.00^{ab}
0.5% LO	73.31 ± 0.06^{b}	18.7 ± 0.30^{c}	16.05 ± 0.50^{b}	65.05 ± 0.95^{a}
0.2% D	76.38 ± 0.07^{ab}	22.64 ± 0.20^{b}	19.685 ± 0.21^{a}	57.64 ± 1.30^{c}
0.4% D	74.5 ± 0.40^{ab}	18.4 ± 0.60^{c}	16.92 ± 0.92^{b}	64.725 ± 0.50^{a}
0.2% D+0.5% LO	76.4 ± 1.02^{a}	24.9 ± 0.10^{a}	18.14 ± 0.14^{a}	56.625 ± 0.11^{c}
0.4% D+0.5% LO	75.25 ± 0.20^{a}	22.59 ± 0.41^{b}	16.195 ± 0.60^{b}	61 ± 2.00^{bc}
p-Value	<0.01	<0.01	<0.01	<0.01

Note: The values (mean ± SE; $n = 3$) in the same column do not share similar superscripts and are substantially different at $p \leq 0.05$.

notable growth enhancement was recorded in fish fed 0.4% D and 0.2% D/0.5% LO diets, while the 0.5% LO treatment yielded the lowest performance values. These results suggest that moderate inclusion levels of D and LO effectively promoted nutrient assimilation and energy utilization, whereas excessive LO may have negatively affected the beneficial intestinal microbiota and nutrient absorption, as reported by Zeng et al. [47].

The bioactive compounds present in D—particularly valerenic acids, valepotriates, and flavonoids from *Valeriana officinalis* and prenylated flavonoids and bitter acids from *Humulus lupulus*—likely played pivotal roles in enhancing stress resilience by being associated with sedative and antioxidant activities that could reduce collective stress and metabolic costs, allowing more energy to be directed toward growth [48–49]. Using sedatives is another way to help lower the stress levels and metabolism rates of fish during transport [50].

LO contains active constituents such as limonene and β-pinene, which may stimulate digestive enzyme secretion and improve gut health at optimal doses [22]. The superior performance of fish fed 0.4% D and 0.2% D/0.5% LO is further supported by the improved FCR and CF observed in these groups, indicating more efficient feed utilization and better somatic condition. The 0.2% D group achieved the best FCR (1.2), reflecting the efficient conversion of dietary nutrients into body mass. In contrast, fish receiving 0.5% LO exhibited the poorest FCR (3.67), reinforcing the need for precise dosage optimization. These findings are in line with Nyadjeu et al. [51], who demonstrated improved FCR values at moderate phytogenic inclusion levels.

Although the 0.4% D group exhibited a relatively low SR (~55%), the surviving fish still achieved a remarkably high growth performance. This seemingly contradictory result can be attributed to reduced stocking density and intraspecific competition within the rearing units following mortality. With fewer fish per tank, the remaining individuals likely experienced improved feed availability, reduced stress levels, and better space utilization, all of which can enhance the growth efficiency. Therefore, the observed improvement in growth at this concentration may reflect a compensatory growth response associated with lower rearing density rather than a true nutritional advantage of the additive at such a high inclusion level.

Comparable trends have been documented in previous studies investigating herbal and phytogenic feed additives in aquaculture. Dada [52] reported optimal growth and nutrient utilization in Nile tilapia fed 5.0 g/kg Superliv powder. He et al. [53] found

that *Foeniculum vulgare* and *Artemisia annua* extracts significantly enhanced growth performance in largemouth bass through increased feed intake and nutrient absorption. El-Dakar et al. [46] demonstrated that chamomile and marjoram meal supplementation improved growth indices in Nile tilapia reared under biofloc systems.

Likewise, Yilmaz et al. [54] showed that PASSIF MOOD (a mixture of *Valeriana officinalis* and *Passiflora incarnata* extracts) improved appetite and nutrient utilization in rainbow trout, while Wiszniewski et al. [55] observed increased FW in sterlet (*Acipenser ruthenus*) fed bromelain-supplemented diets due to improved digestive efficiency. Moreover, Zeppenfeld et al. [56] found enhanced growth rate and feed efficiency in silver catfish fed essential oils at a 2.0 mL/kg diet, supporting the positive role of natural oils in promoting metabolic efficiency.

There were no significant differences ($p < 0.05$) in the HSI across all treatments, indicating that the addition of D and LO did not significantly affect liver size or health. The SR was significantly higher in the treatments containing D and LO compared to the control group; the highest SR was observed in the 0.2% D group. This improvement in survival may be attributed to the antioxidant and immunomodulatory bioactive compounds present in D and LO, which can enhance stress tolerance and immune defense mechanisms in fish [31, 34, 57]. These results are in agreement with Ghafarifarsani et al. [58], who reported that plant-derived bioactive components improved immune responses and survival in fish. Similarly, Radwan et al. [59] found that dietary inclusion of *Carica papaya* seed extract enhanced the SR of Nile tilapia fry, suggesting that phytogenic compounds can promote resilience and reduce stress-related mortality under culture conditions.

Hematological and biochemical indices are widely recognized as reliable indicators of fish physiological and health status, reflecting internal metabolic and immune responses to nutritional or environmental conditions [60]. In the present study, supplementation of D and LO resulted in significant effects on hematological parameters, serum biochemistry, and antioxidant-immune responses in *Oreochromis niloticus* juveniles reared under high-density conditions. The hematological profiles of juvenile *Oreochromis niloticus*, including RBCs, Hb, and Hct, were not significantly affected by dietary supplementation with D and LO. The stability of these parameters suggests that all experimental diets were nutritionally balanced and that the rearing environment was adequately managed, particularly in terms of water quality and dissolved oxygen. These findings are in agreement with previous reports where dietary inclusion of natural plant additives did not alter RBC-related indices, indicating the absence of hematological stress or anemia in fish [61].

In contrast, the WBC count showed a significant increase in the group fed 0.2% D compared to the control and other treatments. This elevation in WBCs reflects enhanced leukopoiesis and immune stimulation, which are desirable responses in aquaculture under intensive culture conditions. D is composed of *Valeriana officinalis* and *Humulus lupulus*, both of which contain bioactive compounds such as valerenic acids, flavonoids, and prenylated phenolics that possess antioxidant and immunomodulatory properties [48, 62]. These compounds may have contributed to the activation of nonspecific immune defenses by enhancing leukocyte proliferation and functional activity. These findings are consistent with Behbodi et al. [63], who highlighted the immunomodulatory effects of dietary *Curcuma longa* powder on fish species. A similar pattern of increased WBC counts was observed by Alsaiad and Al-Zayat [64] in Nile tilapia supplemented with ginger extract rich in phenolic compounds and by Effendi et al. [65] in common carp fed diets containing phytogenic feed additives.

The stimulation of WBCs under D supplementation can be further interpreted as part of the fish's adaptive response to oxidative and environmental stress induced by high stocking density. Herbal-derived antioxidants help reduce the formation of reactive oxygen species (ROS) and protect immune cells from oxidative damage, thereby maintaining efficient immune surveillance [66]. The higher WBC levels in the 0.2% D treatment may therefore indicate optimized immune readiness and improved capacity to resist opportunistic infections under intensive rearing systems.

Serum biochemical indices provide further insights into metabolic and hepatic responses (AST and ALT). The marked increase in glucose levels observed in fish fed 0.4% D + 0.5% LO and 0.5% LO diets may indicate stress-induced hyperglycemia or enhanced gluconeogenic activity, as previously reported in fish exposed to handling or stocking stress [67–68]. In contrast, lower glucose levels in the 0.2% and 0.4% D groups imply improved stress resilience and metabolic stability, consistent with valerian's sedative and adaptogenic properties [69].

The higher total protein concentration in the 0.2% D group points to improved hepatic protein synthesis and possibly enhanced humoral immunity, supported by elevated IgM and lysozyme activities in the same group. Similar findings were reported by Abdel-Tawwab et al. [70], who observed that dietary phytogenic additives such as curcumin nanoparticles increased total serum protein and immunoglobulin levels in Nile tilapia, reflecting enhanced innate and adaptive immunity. These results align with the general consensus that herbal bioactives strengthen both humoral and cellular defense mechanisms in fish [70, 71].

Liver enzyme activities (AST and ALT) were affected by the treatments, serving as reliable markers of hepatic function. The significant elevation of AST and ALT in 0.4% D and 0.4% D + 0.5% LO groups may indicate mild hepatic stress or metabolic overload associated with higher additive doses [70, 72, 73]. Elevated hepatic enzyme activity has also been linked to excessive supplementation of essential oils or plant bioactives, possibly due to increased metabolic demands or transient oxidative stress [74, 75]. Conversely, the lowest AST and ALT values in 0.2% D + 0.5% LO-treated fish suggest improved hepatocellular integrity and detoxification capacity, aligning with findings where moderate doses of phytogenic additives enhanced hepatic health and redox balance [76]. These findings are consistent with the antioxidant data, where moderate additive levels (0.2% D and 0.5% LO) enhanced SOD, GSH, and TAC and reduced MDA, reflecting strengthened antioxidant defense mechanisms [77, 78]. The elevated MDA levels observed in combined high-dose groups further confirm a potential oxidative imbalance due to excessive supplementation.

Dietary supplementation with D and LO exerted significant effects on the proximate composition of Nile tilapia juveniles reared under high density. The observed variations in moisture, lipid, protein, and ash contents reflect metabolic adjustments induced by phytogenic supplementation.

Fish fed 0.2% D + 0.5% LO and 0.2% D diets showed the highest moisture and ash contents, suggesting enhanced mineral retention and possible osmoregulatory modulation. Increased body moisture in these groups could be associated with improved hydration status and reduced metabolic dehydration under crowding stress, likely mediated by the sedative and adaptogenic effects of *Valeriana officinalis* extract in D [29, 69]. Similar increases in body moisture have been documented in fish supplemented with herbal adaptogens that improve physiological resilience under stress [70, 77].

The significantly elevated lipid content in fish receiving 0.2%D + 0.5%LO (24.9%) and 0.2%D (22.64%) diets indicates enhanced lipogenesis or efficient lipid deposition, potentially resulting from improved feed palatability and digestive enzyme stimulation by LO terpenes such as limonene and citral [21]. Phytogenic essential oils are known to stimulate bile secretion, improve lipid absorption, and modulate energy metabolism, leading to higher lipid deposition in muscle tissue [79–80]. Moreover, the mild sedative effect of D may reduce stress-related energy expenditure, redirecting available energy toward growth and lipid accumulation [81–82].

Conversely, the higher crude protein content recorded in fish fed 0.5% LO and 0.4% D diets (65.05% and 64.73%, respectively) reflects improved protein synthesis and nitrogen retention efficiency. This enhancement could be linked to the antioxidant and hepatoprotective roles of moderate doses of essential oils, which maintain hepatic integrity and protein metabolism [83]. Similar improvements in protein content were observed by Abdel-Tawwab et al. [70], who reported that dietary LO enhanced hepatic function, leading to greater amino acid utilization for protein synthesis. The lower protein percentages in high D doses (0.2% D+0.5% LO and 0.2% D groups) may reflect a shift in nutrient partitioning toward lipid deposition, as well as possible mild metabolic overload at higher sedative doses, consistent with reports of dose-dependent effects of valerian-based supplements in aquaculture [31].

Ash content followed a pattern similar to moisture, being highest in 0.2% D and 0.2% D+0.5% LO groups, suggesting improved mineral retention and bone mineralization. The phytochemical components of LO, particularly flavonoids and monoterpenes, may enhance mineral absorption and antioxidant protection, leading to greater ash accumulation [84, 85].

5 | Conclusion

Dietary supplementation with D and LO enhanced the growth, feed efficiency, and physiological resilience of *Oreochromis niloticus* under high-density culture. Moderate D levels (0.2%–0.4%) alone or combined with LO significantly improved growth indices, antioxidant status, and immune responses while supporting hepatic integrity and protein metabolism. However, higher inclusion levels (0.4% D + 0.5% LO) slightly elevated AST and MDA, suggesting mild metabolic stress. The increased lipid and ash contents observed with moderate supplementation indicate improved nutrient absorption and energy utilization. Overall, D at 0.2%, with or without LO, represents the optimal dose for promoting growth performance, antioxidant defense, and physiological stability in intensively reared Nile tilapia.

Author Contributions

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data generated or analyzed during this study are included in this article.

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