



Nutritional modulation using *Nigella sativa* with or without feed restriction as a novel strategy to boost growth and immunity and combat edwardsiellosis in *Oreochromis niloticus*

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Abstract

Medicinal plants occupy a privileged place and are considered one of the most important natural phytogetic feed additives. Black cumin (*Nigella sativa*, NS) and its derivatives have several beneficial therapeutic properties for aquaculture farms. This study investigated the effects of NS with or without feed restriction on growth performance, haemato-biochemical profile, immune and oxidant/antioxidant status, as well as the resistance of *Oreochromis niloticus* (*O. niloticus*) to *Edwardsiella tarda* (*E. tarda*) challenge. A total of 360 fish with initial body weight (mean $\pm$ SE) 26.68 ± 0.08 g were randomly distributed into four groups with nine replicates (10 fish each) as follows: ad libitum control (Ad C), ad libitum *Nigella sativa* (Ad NS), restricted control (Res C), and restricted *Nigella sativa* (Res NS). The *O. niloticus* in the first (Ad C) and third groups (Res C) were fed with the basal control diet without NS supplementation. The second (Ad NS) and fourth (Res NS) groups were fed on diets supplemented with 3% NS. The fish in the Ad C and Ad NS groups were fed ad libitum during the experimental period, while the Res C and Res NS groups were subjected to 2 days of feed restriction weekly (third and seventh days of the week). At the end of the feeding trial (2 months), the experimental groups were combated against edwardsiellosis by intraperitoneally injection of 0.1 mL (1×10^5 CFU) *E. tarda*. The survival of the fish was evaluated for a further 7 days. The results revealed that growth metrics of fish were substantially higher in the Ad NS group, followed by the Ad C and Res NS groups. The feed conversion ratio (FCR) was substantially lower in the feed-restricted groups compared to the ad libitum groups. Biochemical analysis showed a significant decrease in hepatic enzymes as well as glucose levels in all experimental groups

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Highlights

- Fish that were fed with diets supplemented with 3% NS grew faster, and their immune-antioxidant responses were modulated.
- Feed restriction not only improved the health status and immune-antioxidant responses in *O. niloticus*, but also it reduced the mortality after challenge with *E. tarda*.
- The fish feeding programme and diet composition have a big impact on fish farming's income and health.

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compared to Ad C. Moreover, total cholesterol and LDL-c were reduced ($P < 0.05$) in both *Nigella* groups. The growth hormone and leptin were higher in all groups, particularly Res NS group compared to the Ad C group however, cortisol level significantly reduced in the Res NS group only compared to the control group. There were substantial elevations in lysozyme and IgM in all groups, particularly the Res NS group. Also, the oxidant/antioxidant status had been improved mostly in the Res NS group. Histopathological examination revealed that combining NS with feed restriction maintained normal histology in the intestine, liver, kidney, and spleen. Furthermore, the combined treatment significantly reduced mortality rates following challenges with *E. tarda*, highlighting a potential protective role. In conclusion, dietary intervention of 3% NS with or without feed restriction can enhance fish productivity, immunity, organ function, and disease resistance. Moreover, NS rectifies the detrimental impact of feed restriction on performance. Lastly, our study suggests combining NS with feed limitation as a novel approach to improve growth, immunity, and resistance of *O. niloticus* to edwardsiellosis.

Keywords Aquaculture health · *Edwardsiella tarda* · Feed restriction · *Nigella sativa* · *Oreochromis niloticus*

Introduction

One of the most pressing sustainability challenges facing humanity today is meeting the nutritional demands of a growing global population (Rossignoli et al. 2023). Recently, fish consumption has increased globally, surpassing the consumption of terrestrial animal proteins, with the majority of fish production now derived from aquaculture (Dongyu 2024). Fish is a highly nutritious food with considerable protein content, other dietary supplements, and low cholesterol. Moreover, *O. niloticus* is one of the most widely farmed and consumed fish species worldwide (Ndraha et al. 2024).

Numerous reasons have contributed to the expansion and success of aquaculture, which is mainly focused on improving growth, preventing disease, and guaranteeing the safety of food for human consumption (Falco et al. 2024). The use of phytobiotic feed additives is being employed to achieve that. NS is regarded as one of the most valuable phytobiotic additives due to its contents of alkaloids, proteins, carbohydrates, saponins, and essential oil. Thymoquinone, thymohydroquinone, dithymoquinone, thymol, and carvacrol are the main active components of NS (Ali and Blunden 2003). Supplementation of fish diets with NS can improve growth performance, reduce production cost, enhance immune responses, improve blood biochemical profiles, and protect against pathogenic bacteria (Öz et al. 2018; Abd El-Hack et al. 2021).

Feeding regimes are critical factors influencing fish health and growth (Xu et al. 2020). Feed restriction and re-feeding techniques can minimise the amount of food provided to fish. Restriction feeding regimes are widely utilised in aquaculture as strategies to promote fish health and reduce production costs (Assis et al. 2020). A dietary pattern known as intermittent fasting, which involves eating less during specific times or not eating at all during specific regular meal periods (Mattson et al. 2017), has been shown to improve physiological metabolism and growth performance (Tian et al. 2024). Moreover, fish catabolises lipids and releases fatty acids during fasting in order to adapt to the restriction in their

dietary intake that prevents accumulation in their muscles, liver, and viscera (Jeziarska et al. 1982).

Unfortunately, infectious diseases have had a major influence on the tilapia sector over the last 10 years. These diseases have resulted in high rates of morbidity and death, production losses, and trade restrictions (Wang et al. 2023). Aquaculture operations worldwide suffer significant financial losses due to the septicemic bacterial disease edwardsiellosis, which is caused by *E. tarda* (Miniero Davies et al. 2018). Antibiotics and vaccines are currently used as prophylactic methods to combat edwardsiellosis in aquaculture (Kwon et al. 2006). However, the public's health is in danger due to antibiotic residues, and the usage of antibiotics has led to harmful bacteria developing resistance to them (Banerjee and Ray 2017). Meanwhile, employing vaccinations to prevent edwardsiellosis is costly and time-consuming. Consequently, it is necessary to implement new infection control and prevention protocols. Great attention is directed to natural antibiotic alternatives such as probiotics (Ringø 2020) and medicinal plants (Tadese et al. 2022). To the best of our knowledge, no previous research has evaluated the potential of NS in preventing edwardsiellosis in fish. Additionally, the combined effects of NS supplementation and feed restriction strategies have not been explored. Thus, the purpose of this study was to examine how NS diet supplementation, both with and without feed limitation, affected *O. niloticus*'s immune/antioxidant state, stress response, and growth performance. The study also evaluated NS' possible preventive function against edwardsiellosis.

Materials and methods

Ethics statement

This study procedure was authorized by Beni-Suef University's Institutional Animal Ethics Committee in Egypt (BSU-IACUC-022–357). The NIH Guide for the Care and Use of Laboratory Animals served as the guide for all experimental fish handling practices.

Fish and rearing condition

Healthy *O. niloticus* weighing 26.68 ± 0.08 g were obtained from the Abo-Saleh fish hatchery (Beni-Suef, Egypt) and transported live to the wet laboratory of the Faculty of Veterinary Medicine at Beni-Suef University. The fish were carried in oxygenated plastic nylon bags to ensure survival during transit (FAO 2020). Upon arrival, they were acclimatised for 14 days in 500-L fiberglass tanks under controlled conditions. Throughout this period, the fish were fed a basal diet (3% of body weight) three times daily, following standard aquaculture protocols (El-Sayed 2006). The tanks were maintained with continuous aeration and 10% daily water exchange using dechlorinated tap water. After adaptation, the fish were randomly distributed into glass aquariums (82 cm length $\times$ 40 cm width $\times$ 44 cm depth, a water volume of 95 L) with ten fish per aquarium. Key water quality parameters, including temperature (26 ± 1 °C), dissolved oxygen (6.1 ± 0.77 mg/L), pH (6.4 ± 0.2), ammonia (0.01 ± 0.002 mg/L), and nitrite (0.04 ± 0.01 mg/L), were monitored twice weekly to ensure optimal conditions throughout the experimental period (Boyd and Tucker 2014).

Diet preparation and experimental design

The dietary level of NS (3%) used in this study was selected according to Andrawes et al. (2021) and Mahboub et al. (2022). NS seeds were obtained from the Agriculture Research Center at Dokki, Giza, Egypt. The seeds were milled by mechanical ball milling utilizing a planetary ball mill (Retsch PM 400, Germany). The morphological characteristics of NS seeds were studied using the field transmission scanning electron microscope (FESEM, JEOL JSM-5900, Japan). A very thin layer of gold was first applied to the samples using the direct sputtering technique.

The proximate compositions of NS and all feed ingredients were analysed according to AOAC 2005 (Table 1). Based on the nutrient contents of the ingredients, the diets were formulated to satisfy the fish's nutritional needs stated by NRC (2011). The digestible energy content of the feeds was estimated from their proximate composition according to Smith (1971). The physical and chemical composition of the control diet is displayed in Table 2.

A total number of 360 monosex 8-week-old *O. niloticus* were divided into four groups in nine replicates (10 fish/replicate) as follows: ad libitum control (Ad C), ad libitum NS (Ad NS), restricted control (Res C), and restricted NS (Res NS). The experimental period lasted for two months. The fish in the first (Ad C) and third groups (Res C) were fed on the basal diet without NS supplements (control). The second (Ad NS) and fourth (Res NS) groups were fed on diets supplemented with 3% NS. During the experimental period, *O. niloticus* fish were fed three times daily (8:00, 12:00, and 16:00 h) until satiation. The fish in the Ad C and Ad NS groups were fed ad libitum during the experimental period, while the Res C and Res NS groups were subjected to 2 days of feed restriction weekly (third and seventh days of the week).

Diets were offered in the form of pellets by the aid of gelatin and the oil ingredients, which were used as binding materials. The gelatin was added at the rate of 30 g/kg dissolved in hot water (3.00 g in 100 mL distilled water), and with the oil, well mixed with the finely ground mixture to reach a dough consistency. The dough was then extruded through a die of 3 mm diameter using a meat mincer. The extruded spaghetti-like strands were air dried, broken up, and sieved into an appropriate pellet size of 2 mm. The feeds were kept frozen in tightly closed bags at -18°C until feeding. During experimentation, different diets were kept at all times in a refrigerator at 4°C .

Growth performance

The starting body weight (IBW) and final body weight (FBW) were measured at the beginning of the trial and at the end of the 8-week period, respectively. Throughout the trial, fish weight and length were recorded weekly, while feed intake (FI) was measured daily. The condition factor is the relation between weight and length and is used as an indicator for fitness and was calculated after the following equation:

$$\text{Condition factor (K)} = \frac{\text{Weight (g)}}{\text{Length (cm)}^3} \times 100$$

Table 1 Energy value (kcal/kg) and chemical composition (%) of feed ingredients on a fresh matter basis

Ingredient	Dry matter	Digestible energy ^a	Crude protein	Methionine ^b	Lysine ^b	Ether extract	Crude fiber	Ash	Nitrogen-free extract ^c	Calcium ^d	Phosphorus ^d
Fish meal	90.24	3819.75	65.45	1.77	4.81	8.60	0.95	11.39	3.85	5.19	2.88
Soybean meal	90.37	3046.36	43.84	0.60	2.83	2.97	6.70	6.91	29.95	0.26	0.64
Corn gluten meal	90.48	3557.01	60.38	1.90	1.07	1.75	1.45	1.49	25.41	0.07	0.44
Yellow corn	87.53	2644.44	7.88	0.18	0.24	3.70	2.50	0.99	72.46	0.03	0.28
<i>Nigella sativa</i>	94.36	4292.44	20.85	0.87	1.75	30.17	15.20	4.55	23.59	0.35	0.50
Gelatin	91.67	4039.24	88.76	0.73	3.55	0.32	0.94	0.83	0.82	0.49	0.12
Soya oil	99.91	8760.00	0.00	0.00	0.00	99.92	0.00	0.00	0.00	0.00	0.00
Monocalcium phosphate	98.50	0.00	0.00	0.00	0.00	0.00	0.00	80.50	0.00	16	21

^aDigestible energy was calculated from the chemical composition of feeds, according to Smith (1971), for all ingredients except the DE of the soya oil, which was obtained from NRC of fish (2011)

^bMethionine and lysine values were obtained from NRC of fish (2011) except that of *Nigella sativa*, which was analyzed

^cNitrogen-free extract (NFE) was calculated according to the following equation: $NFE\% = 100 - (\text{moisture}\% + \text{crude protein}\% + \text{ether extract}\% + \text{crude fiber}\% + \text{ash})$ (AOAC 2005)

^dCalcium and phosphorus values were obtained from the NRC of fish (2011) except that of *Nigella sativa*, which was analyzed

Table 2 Feed ingredients and chemical composition % of the experimental diets (as fed)

Feed ingredients %	Dietary groups	
	Control	<i>Nigella sativa</i>
Fish meal	7.89	7.89
Soybean meal	37.89	36.80
Corn gluten meal	3.94	3.94
Yellow corn	42.56	41.37
<i>Nigella sativa</i>	0.00	3
<i>Gelatin</i>	3	3
Soya oil	2.50	1.80
Monocalcium phosphate	1.15	1.15
Common salt	0.30	0.30
Vitamin and mineral premix ^a	0.50	0.50
Choline chloride	0.10	0.10
Vit C	0.05	0.05
DL-methionine	0.12	0.10
Calculated chemical composition		
Digestible energy, kcal/kg	3061.44	3064.22
Crude protein	30.18	30.22
Crude fiber	3.77	4.12
Ether extract	5.86	6.08
Nitrogen-free extract	43.95	43.39
Methionine	0.66	0.66
Lysine	1.70	1.72
Calcium	0.72	0.73
Phosphorus	0.85	0.86
Sodium	0.14	0.14

^aVitamin and mineral premix (NEOFARMA), each kg contains vitamin A acetate (6,250,000 IU), vitamin D3 (cholecalciferol, 2,500,000 IU), vitamin E (α-tocopherol; 25,000 mg), vitamin K3 (menadione sodium bisulfate; 1750 mg), vitamin B1 (500 mg), vitamin B2 (2750 mg), vitamin B6 (1250 mg), vitamin B12 (10 mg), nicotinic acid (niacin; 20,000 mg), calcium pantothenate (5000 mg), folic acid (500 mg), biotin (50 mg), iron sulfate (22,000 mg), manganese oxide (31,000 mg), copper sulfate (2500 mg), zinc oxide (37,500 mg), potassium iodide (650 mg), selenium selenite (113 mg), cobaltous sulfate (50 mg), ethoxyquin (250 mg), wheat bran (carrier, 120 gm), and limestone (carrier, up to 1 kg)

From the body weight data, the specific growth rate (SGR) of fish was calculated as follows:

$$\text{SGR}(\% \text{ body weight/day}) = \frac{\ln(\text{Wt}) - \ln(\text{W0})}{t} \times 100$$

where ln (Wt) is the natural logarithm of the FBW, ln (W0) is the natural logarithm of the IBW, and (t) is the feeding period in days.

The feed conversion ratio of fish (FCR) was measured by dividing the amount of feed consumed in a certain period by the gain in weight at the same period as follows:

$$\text{Feed conversion ratio} = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

Protein efficiency ratio (PER) is defined as the weight gain per unit weight of the consumed protein. It was calculated using the following formula:

$$\text{Protein efficiency ratio} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

The fish survival rate (SR) was determined as follows:

$$\text{SR} = \frac{\text{Number of fish in each group remaining alive after the experiment period (60 days)}}{\text{Initial number of fish}} \times 100$$

Whole body composition of fish

At the start of the experiment, 18 fish were randomly selected from the initial fish population to determine initial body composition. At the end of the feeding trial, 18 fish per group were collected for the determination of the final carcass. The proximate composition of fish was analysed according to AOAC (2005) for dry matter (DM), crude protein (CP), ether extract (EE), and ash contents. The nitrogen-free extract (NFE) was calculated by difference.

Sampling

At the end of the feeding trial (8 weeks), 18 fish from each group were fasted for 24 h before blood sampling. First, the fish were anaesthetised with a 100 mg/L benzocaine solution. Two blood samples were taken from the caudal blood vessels. The first was obtained for serum separation by centrifuging the sample at $1075 \times g$ for 10 min (min). The serum was then stored at -20°C to measure the levels of biochemical and immunological parameters. The second sample was obtained on EDTA for determining the blood indices. A part of liver tissue was preserved at -20°C for homogenisation and estimation of oxidant/antioxidant indices. Also, samples from the intestine, liver, kidney, and spleen were fixed in 10% buffered neutral formalin solution for histopathological examination.

Haematological evaluation

According to Thrall et al. (2012), an automatic cell counter was used to determine the results of the haematological tests, which included red blood cell count (RBCs), hemoglobin concentration (Hb), packed cell volume (PCV%), and a leukogram (counting the total white blood cells (WBCs) and differential WBCs count). In accordance with Bain

et al. (2016), the mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) have been assessed as follows:

$$\text{MCV(fl)} = \frac{\text{PCV}}{\text{RBCs}} \times 10$$

$$\text{MCHC(\%)} = \frac{\text{Hb}}{\text{PCV}} \times 100$$

Biochemical analysis

Serum triglycerides (TG) and total cholesterol (T-CHO) were measured using the methods of Allain et al. (1974) and Fossati and Prencipe (1982), respectively. An automatic biochemistry analyzer (CHEMIX-800, Sysmex Corporation, Kobe, Japan) was used to test serum high-density lipoprotein-cholesterol (HDL-c) using a kit. Friedewald et al. (1972) used the following formula to determine serum low-density lipoprotein-cholesterol (LDL-c):

$$\text{LDL} - \text{c} \left(\frac{\text{mg}}{\text{dL}} \right) = \text{total cholesterol} - (\text{HDL} - \text{c}) - \left(\frac{\text{triglycerides}}{5} \right)$$

The techniques outlined by Henry (1964) and Doumas et al. (1971) were used to assess serum total protein (TP) and albumin, respectively. Serum globulin was calculated by subtracting the value of albumin from the TP-value.

Following the procedure described by Huang et al. (2006), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated. Glucose level was measured spectrophotometrically in serum using BioMed Diagnostic commercial kits (Cairo, Egypt).

Oxidant/antioxidant parameters

The liver tissue was homogenised in PBS (pH 7.5) after being washed three times with a cold NaCl solution (0.9%). The homogenates were then cold centrifuged at 3000 rpm for about 15 min. Lastly, a clean tube was used to collect the supernatants. Biodiagnostic commercial kits were used to measure malondialdehyde (MDA) levels in accordance with Wills' (1987) technique. The method outlined by Nishikimi et al. (1972) was used to assess the levels of superoxide dismutase (SOD). Glutathione peroxidase (Gpx) was measured according to Paglia and Valentine (1967). Antioxidant kits (SOD, MDA, and GPx) were bought from Biodiagnostic Trade Co., Dokki, Egypt.

Immune parameters

According to Ellis (1990), serum lysozyme activity (LYZ) was measured colourimetrically at 450 nm using the change in turbidity as a basis. In short, 190 µL of substrate

solution (0.2 mg of *Micrococcus lysodeikticus*/ml PSB, pH = 7.4) and 10 µL of serum sample were combined in 96 microplate tubes at lab temperature with minimal stirring. At a predetermined 5-min interval, the turbidity change is estimated. Immunoglobulin M (IgM) mg/dL was measured using a colourimetric CUSABIO kit (Wuhan, China) following the manufacturer's instructions.

ELISA estimation of hormones

Commercially available ELISA kits provided by Cusabio Technology LLC, Houston, TX, USA, were used for quantitative determination of serum growth hormone, leptin, and cortisol according to the manufacturer's instructions (catalog Nos. CSB-E12121 Fh, CSB-EL012870 FI, and CSB-E08487f, respectively).

Histopathological work up

Intestinal, liver, kidney, and spleen specimens from each group of tilapia fish were gathered and promptly preserved in a 10% buffered neutral formalin solution for a full day. They were then dehydrated in progressively increasing ethanol (70, 80, 95, 95, and 100%), cleaned in xylene, and embedded in paraffin. A microtome (Leica RM 2155, England) was used to slice paraffin sections that were 5 µm thick. Hematoxylin and eosin stains were regularly applied after the slices were made, and they were then seen under a microscope (Suvarna et al. 2018). Intestinal villi length, intestinal villi width, and absorption surface area were measured for intestinal histomorphometry. Each fish's average of these parameters was calculated using 50 well-aligned villi from each part of the intestinal segments. The villi's widths (VW) were measured at the half-height point, and their heights (VL) were measured from tip to base. A light microscope with a full HD microscopic camera and image analysis software (Leica Microsystem, Germany) was used to investigate the tissue sections. For statistical analysis, image analysis software was used to measure the parameters. The following formula was used to determine the absorption surface area (ASA) (Alaa et al. 2024) $ASA (mm^2) = villus\ height \times villus\ width$.

Challenge test

Preparation of bacterial isolate

The *E. tarda* strain used in this study was isolated from naturally infected *O. niloticus* at the Fish Diseases and Management Department, Faculty of Veterinary Medicine, Beni-Suef University, Egypt. Bacterial identification was confirmed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Germany) following standard protocols (Seng et al. 2009). For propagation, the isolate was cultured in Luria–Bertani (LB) broth (Sigma-Aldrich, USA) at 37 °C for 24 h under aerobic conditions. The bacterial suspension was centrifuged (3000 × g, 10 min), washed with phosphate-buffered saline (PBS, pH 7.4), and resuspended in fresh PBS for further experiments. The lethal dose 50 (LD50) was evaluated as described by Korní et al. (2021) with modifications. Briefly, 180 fish were allocated into six groups

(30 fish/group and 10 fish/replicate in triplicate tanks). Then bacterial concentrations (10^4 – 10^8 CFU/mL) were prepared using McFarland Standard No. 0.5 (BioMérieux, France). After that, groups 2–6 received intraperitoneal (IP) injections of 0.1 mL bacterial suspension (10^4 – 10^8 CFU/fish), while the control group (group 1) received 0.1 mL PBS. Mortality was recorded daily for 96 h, and LD50 (2×10^5 CFU/fish) was calculated using Finney's (1971) probit analysis. A sublethal challenge dose (1×10^5 CFU/fish, 50% of LD50) was selected for subsequent experiments to mimic chronic infection dynamics (Gharaei et al. 2020).

Fish group setup and infection with *E. tarda*

The remaining 54 *O. niloticus* fish per group were used. The control group (Ad C) was subdivided equally into two subgroups (27 fish each). Fish were starved for 24 h before the infection. The first subgroup from the Ad C group was IP injected with 0.1 mL PBS, regarded as control negative (C –ve). The second subgroup (control positive; C + ve) and other experimental groups were challenged IP with *E. tarda* at a dose of 0.1 mL (1×10^5 CFU). For 7 days, the clinical symptoms and mortalities were tracked every day, and SR was determined using the Kaplan–Meier model (a standard statistical tool for survival time data).

Statistical analysis

The Shapiro–Wilk test was employed to ensure that the collected data were regularly distributed. One-way analysis of variance (ANOVA) was used to look for any significant differences between treatments. Tukey multiple range tests were employed to find differences between means. The statistical analysis was performed using SPSS Version 17 for Windows (SPSS Inc., Chicago, IL, USA). The data was expressed using the means $\pm$ standard error of the mean (SEM).

Results

The characteristic morphological feature of milled NS is illustrated in Fig. 1. The milled NS appears as spherical agglomerated particles with micro-scale size (2.25 ± 1.07 μ m).

Growth metrics

In comparison to the Res C, the Ad NS group had significantly higher fish FBW, BWG, SGR, PER, CF, and FI ($P < 0.05$), followed by the Ad C and Res NS groups. In comparison to the ad libitum groups, the feed-restricted groups had a significantly reduced FCR ($P < 0.05$). Nevertheless, Table 3 shows that there was no difference ($P > 0.05$) in the FBL between the experimental groups. All experimental groups showed 100% SR at the end of the feeding trial (Table 3).

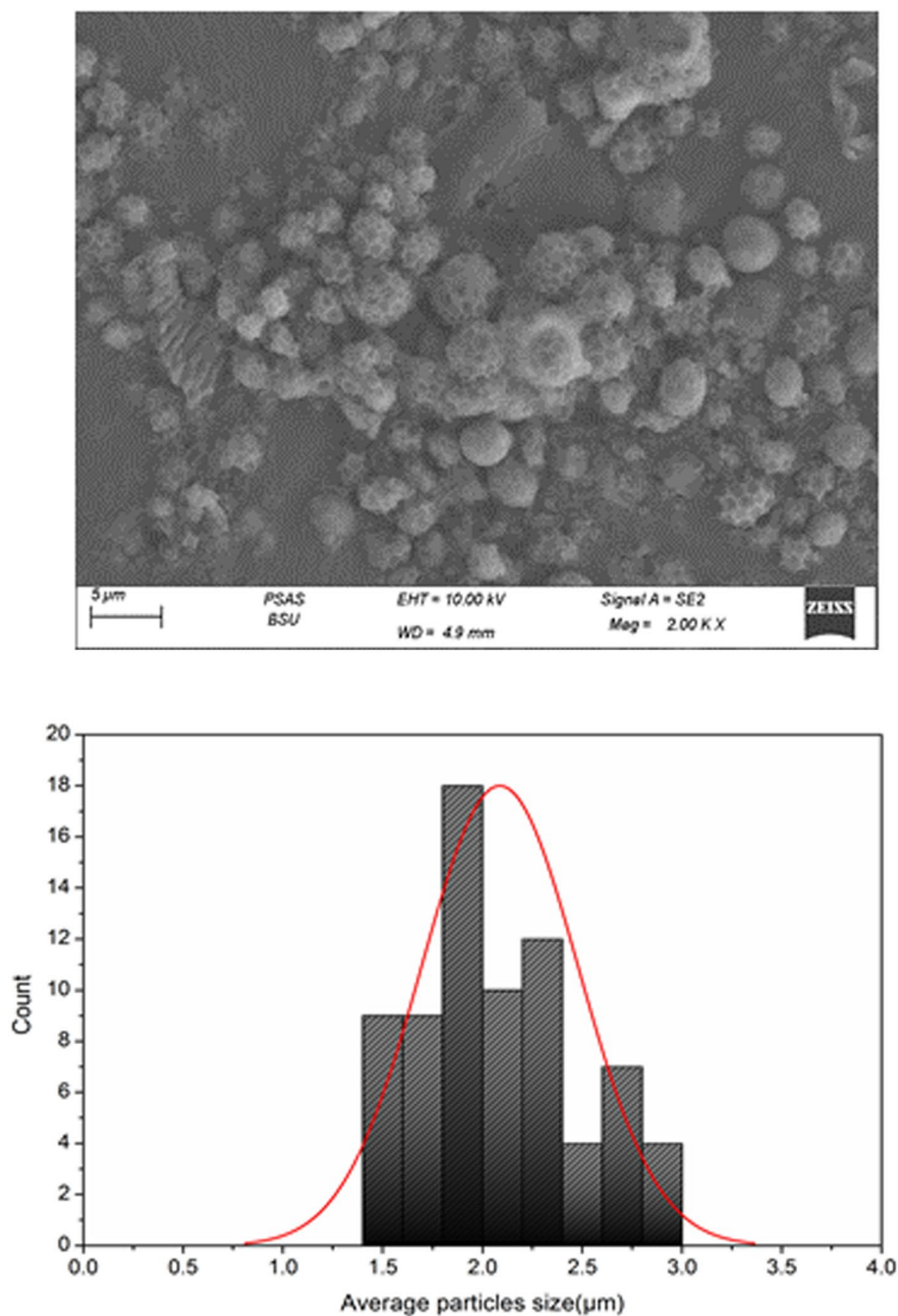


Fig. 1 SEM images and particles size distribution of NS

Table 3 Growth performance of *O. niloticus* fish fed experimental diets with and without feed restriction

Parameters	Experimental groups			Res NS	P-value
	Ad C	Ad NS	Res C		
IBW (g)	26.57 ± 0.10 ^a	26.61 ± 0.08 ^a	26.80 ± 0.07 ^a	26.73 ± 0.08 ^a	0.20
FBW (g)	57.18 ± 1.53 ^b	64.95 ± 2.37 ^a	49.91 ± 0.76 ^c	54.45 ± 0.89 ^{bc}	< 0.001
BWG (g)	30.61 ± 1.57 ^b	38.34 ± 2.36 ^a	23.11 ± 0.80 ^c	27.72 ± 0.96 ^{bc}	< 0.001
SGR (%)	1.37 ± 0.05 ^c	1.59 ± 0.07 ^{ab}	1.55 ± 0.04 ^b	1.77 ± 0.05 ^a	< 0.001
FI (g)	51.40 ± 2.24 ^b	65.78 ± 4.19 ^a	37.33 ± 1.29 ^c	46.02 ± 2.18 ^{bc}	< 0.001
FCR	1.68 ± 0.02 ^a	1.72 ± 0.03 ^a	1.62 ± 0.01 ^b	1.66 ± 0.03 ^{ab}	0.03
PER	1.97 ± 0.03 ^b	1.93 ± 0.02 ^b	2.05 ± 0.02 ^a	2.00 ± 0.03 ^a	0.02
TL (cm)	13.19 ± 0.19 ^a	13.57 ± 0.14 ^a	13.07 ± 0.10 ^a	13.30 ± 0.20 ^a	0.20
CF (g/cm ³)	2.50 ± 0.08 ^a	2.61 ± 0.11 ^a	2.24 ± 0.05 ^b	2.32 ± 0.08 ^{ab}	0.02
SR (%)	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a	0.84
Body composition on DMB (%)					
DM	28.51 ± 0.75 ^a	28.89 ± 90 ^a	26.98 ± 0.27 ^a	27.81 ± 0.18 ^a	0.21
CP	55.95 ± 1.80 ^a	54.82 ± 0.97 ^a	56.36 ± 0.31 ^a	56.26 ± 0.63 ^a	0.74
EE	17.81 ± 0.59 ^a	17.89 ± 0.46 ^a	15.33 ± 0.67 ^b	13.63 ± 0.32 ^b	0.001
Ash	17.01 ± 0.81 ^b	21.04 ± 0.50 ^a	17.90 ± 0.84 ^b	21.34 ± 0.44 ^a	0.004
NFE	9.23 ± 1.48 ^a	6.25 ± 1.14 ^a	10.41 ± 0.49 ^a	8.77 ± 0.13 ^a	0.08

Values in the same row that did not share the same superscripts are significantly different ($n = 18$ /group; $P < 0.05$; one-way ANOVA). The body composition of *O. niloticus* at the beginning of the experiment on DMB was 22.45% DM, 54.34% CP, 14.46% EE, 17.84% ash, and 13.36% NFE

IBW initial body weight, FBW final body weight, BWG body weight gain, SGR specific growth rate, FI feed intake, FCR feed conversion ratio, PER protein efficiency ratio, TL total body length, CF condition factor, SR% survival rate, DM dry matter, CP crude protein, EE ether extract, NFE nitrogen-free extract, DMB dry matter basis, Ad C fish group fed a basal diet on ad libitum basis, Ad NS fish group fed with experimental diet containing 3% *Nigella sativa* on ad libitum basis, Res C fish group fed basal control diet and subjected to 2 days of feed restriction weekly, Res NS fish group fed with experimental diet containing 3% *Nigella sativa* and subjected to 2 days of feed restriction weekly

Table 4 Haematology of *O. niloticus* fed experimental diets with or without feed restriction

Parameters	Experimental groups				
	Ad C	Ad NS	Res C	Res NS	P-value
RBCs ($\times 10^6/\mu\text{L}$)	3.84 ± 0.06^a	3.87 ± 0.05^a	3.76 ± 0.06^a	3.76 ± 0.04^a	0.41
Hb (g/dL)	9.10 ± 0.11^a	9.03 ± 0.09^a	8.73 ± 0.09^a	8.80 ± 0.06^a	0.06
PCV (%)	33.30 ± 0.25^a	33.79 ± 0.46^a	32.70 ± 0.52^a	33.18 ± 0.32^a	0.36
MCV (fL)	86.83 ± 0.85^a	87.39 ± 1.13^a	87.07 ± 1.52^a	88.25 ± 0.43^a	0.79
MCHC (%)	27.34 ± 0.54^a	26.75 ± 0.51^a	26.72 ± 0.56^a	26.52 ± 0.16^a	0.67
WBCS ($\times 10^3/\mu\text{L}$)	3.84 ± 0.04^a	4.02 ± 0.05^a	4.12 ± 0.06^a	4.20 ± 0.18^a	0.13
Neutrophils ($\times 10^3/\mu\text{L}$)	0.36 ± 0.02^a	0.51 ± 0.08^a	0.54 ± 0.05^a	0.57 ± 0.13^a	0.36
Lymphocytes ($\times 10^3/\mu\text{L}$)	3.24 ± 0.05^a	3.24 ± 0.14^a	3.30 ± 0.08^a	3.34 ± 0.08^a	0.85
Monocytes ($\times 10^3/\mu\text{L}$)	0.24 ± 0.003^a	0.27 ± 0.04^a	0.29 ± 0.02^a	0.29 ± 0.03^a	0.66

Values in the same row that did not share the same superscripts are significantly different ($n = 18/\text{group}$; $P < 0.05$; one-way ANOVA)

Ad C fish group fed a basal diet on ad libitum basis, *Ad NS* fish group fed with experimental diet containing 3% *Nigella sativa* on ad libitum basis, *Res C* fish group fed basal control diet and subjected to 2 days of feed restriction weekly, *Res NS* fish group fed with experimental diet containing 3% *Nigella sativa* and subjected to 2 days of feed restriction weekly

Chemical composition of fish body

Body composition of the fish at the beginning of the experiment on a dry matter basis (DMB) was 22.45% DM, 54.34% CP, 14.46% EE, 17.84% ash, and 13.36% NFE. The entire fish body composition at the end of the feeding trial on DMB was affected by the dietary supplementation of NS and the feeding regime. The ash content was

Table 5 Biochemical parameters of *O. niloticus* fed experimental diets with or without feed restriction

Parameters	Experimental groups				
	Ad C	Ad NS	Res C	Res NS	P-value
ALT (U/L)	25.03 ± 0.36^a	20.67 ± 1.11^b	21.04 ± 0.38^b	18.42 ± 0.60^b	< 0.001
AST (U/L)	37.27 ± 0.61^a	29.48 ± 2.18^b	30.07 ± 0.39^b	26.14 ± 0.48^b	< 0.001
TP (g/dL)	5.09 ± 0.06^a	5.05 ± 0.05^a	4.69 ± 0.33^a	4.62 ± 0.14^a	0.12
Albumin (g/dL)	3.25 ± 0.05^a	3.23 ± 0.04^a	2.88 ± 0.26^a	2.85 ± 0.04^a	0.13
Globulins (g/dL)	1.84 ± 0.19^a	1.83 ± 0.06^a	1.81 ± 0.07^a	1.77 ± 0.18^a	0.22
A/G ratio	1.77 ± 0.08^a	1.76 ± 0.08^a	1.59 ± 0.08^a	1.61 ± 0.17^a	0.15
Glucose (mg/dL)	70.03 ± 2.29^a	67.95 ± 3.67^a	48.27 ± 1.58^b	43.04 ± 1.62^b	< 0.001
Total cholesterol (mg/dL)	101.45 ± 2.70^a	91.13 ± 0.71^{bc}	96.63 ± 2.52^{ab}	87.17 ± 1.33^c	< 0.005
Triglycerides (mg/dL)	84.46 ± 2.91^a	85.61 ± 2.62^a	81.22 ± 2.27^a	76.37 ± 2.37^a	0.12
HDL-c (mg/dL)	50.73 ± 1.63^a	52.45 ± 2.55^a	50.31 ± 0.59^a	50.37 ± 1.37^a	0.79
LDL-c (mg/dL)	33.83 ± 3.66^a	21.55 ± 1.99^b	30.06 ± 2.63^{ab}	21.54 ± 0.27^b	< 0.02

Values in the same row that do not share the same superscripts are significantly different ($n = 18/\text{group}$; $P < 0.05$; one-way ANOVA)

Ad C fish group fed a basal diet on ad libitum basis, *Ad NS* fish group fed with experimental diet containing 3% *Nigella sativa* on ad libitum basis, *Res C* fish group fed basal control diet and subjected to 2 days of feed restriction weekly, *Res NS* fish group fed with experimental diet containing 3% *Nigella sativa* and subjected to 2 days of feed restriction weekly

substantially higher ($P < 0.05$) in the NS-supplemented groups with and without feed restriction compared to the control groups (Ad C and Res C). The EE was substantially lower ($P < 0.05$) in the feed-restricted groups (Res C and Res NS) compared to the ad libitum groups. However, there was no difference ($P > 0.05$) in the values of DM, CP, and NFE among all experimental groups (Table 3).

Haematological parameters

At the end of the treatment period, no significant differences were observed in erythrocyte or leucocyte indices between the different groups ($P > 0.05$), as shown in Table 4.

Biochemical analysis

As presented in Table 5, the findings of ALT and AST activities indicated a substantial reduction ($P \leq 0.001$) in all groups when compared to the control (Ad C), with no significant differences observed among these groups (Ad NS, Res C, and Res NS). Nonetheless, no notable alterations were observed in the serum protein profile (total protein, albumin, globulins, and A/G ratio) ($P > 0.05$) among the control and various experimental groups. However, non-significant reductions have been noticed in total protein and albumin of both fasted groups.

In contrast to the control group and NS-treated group (Ad NS), there were notable reductions ($P < 0.001$) in glucose levels in the Res C and Res NS groups. The inclusion of NS in *O. niloticus* diets significantly impacted cholesterol ($P < 0.005$) and LDL-c ($P < 0.02$), resulting in substantial reductions in Res NS followed by Ad NS compared to the control and restricted group (Res C). However, triglycerides and HDL-c demonstrated no notable changes.

Table 6 Hormonal, immunological, and oxidant/antioxidant parameters of *O. niloticus* fed experimental diets with or without feed restriction

Parameters	Experimental groups				
	Ad C	Ad NS	Res C	Res NS	P-value
Cortisol (ng/ml)	0.08 ± 0.003 ^a	0.07 ± 0.003 ^{ab}	0.06 ± 0.002 ^{ab}	0.05 ± 0.003 ^b	< 0.001
GH (pg/ml)	488.57 ± 10.57 ^d	643.86 ± 22.86 ^c	822.99 ± 6.73 ^b	995.36 ± 2.93 ^a	< 0.001
Leptin (pg/ml)	65.18 ± 0.56 ^c	108.01 ± 4.85 ^b	118.51 ± 2.66 ^b	136.13 ± 2.85 ^a	< 0.001
Lysozyme (ng/ml)	5.63 ± 0.35 ^d	8.34 ± 0.29 ^c	10.34 ± 0.41 ^b	12.49 ± 0.16 ^a	< 0.001
IgM (ug/ml)	1.44 ± 0.09 ^c	2.22 ± 0.07 ^b	2.65 ± 0.11 ^b	3.36 ± 0.15 ^a	< 0.001
MDA (nmol/g)	0.99 ± 0.02 ^a	0.93 ± 0.02 ^a	0.83 ± 0.02 ^b	0.68 ± 0.03 ^c	< 0.001
GPx (U/g)	1.71 ± 0.03 ^b	1.81 ± 0.03 ^{ab}	1.84 ± 0.04 ^{ab}	1.96 ± 0.03 ^a	< 0.01
SOD (U/g)	1.71 ± 0.02 ^b	1.81 ± 0.04 ^b	1.93 ± 0.07 ^{ab}	2.09 ± 0.09 ^a	< 0.01

Values in the same row that do not share the same superscripts are significantly different ($n = 18/\text{group}$; $P < 0.05$; one-way ANOVA)

Ad C fish group fed a basal diet on ad libitum basis, Ad NS fish group fed with experimental diet containing 3% *Nigella sativa* on ad libitum basis, Res C fish group fed basal control diet and subjected to 2 days of feed restriction weekly, Res NS fish group fed with experimental diet containing 3% *Nigella sativa* and subjected to 2 days of feed restriction weekly

Immune and oxidant/antioxidant parameters

According to the results of Table 6, the Res NS group had significantly higher levels of lysozyme and IgM ($P < 0.001$) than the control group, followed by Res C and Ad NS, respectively. Comparing the MDA results to the Ad C and Ad NS groups, there was a substantial decrease ($P < 0.001$) in Res NS, followed by the Res C group. In the Res NS group, GPx activity data showed no significant changes when compared to the Ad NS and Res C groups; however, there was a substantial ($P < 0.01$) increase when compared to the control. Comparing the Res NS group to the control and Ad NS groups, SOD activity increased significantly ($P < 0.01$), but there were no discernible changes when compared to the Res C group.

Hormonal analysis

The cortisol level in the Res NS group decreased significantly ($P < 0.001$) compared to the control group, according to the results in Table 6; however, there were no substantial changes between the Ad NS and Res C and Res NS groups. Growth hormone levels in the Res NS group were significantly higher ($P < 0.001$) than in the control group, followed by Res C and Ad NS, respectively. When compared to the control group, leptin levels were considerably ($P < 0.001$) higher in all groups; however, this elevation was much higher in the Res NS group than in the Res C or Ad NS groups.

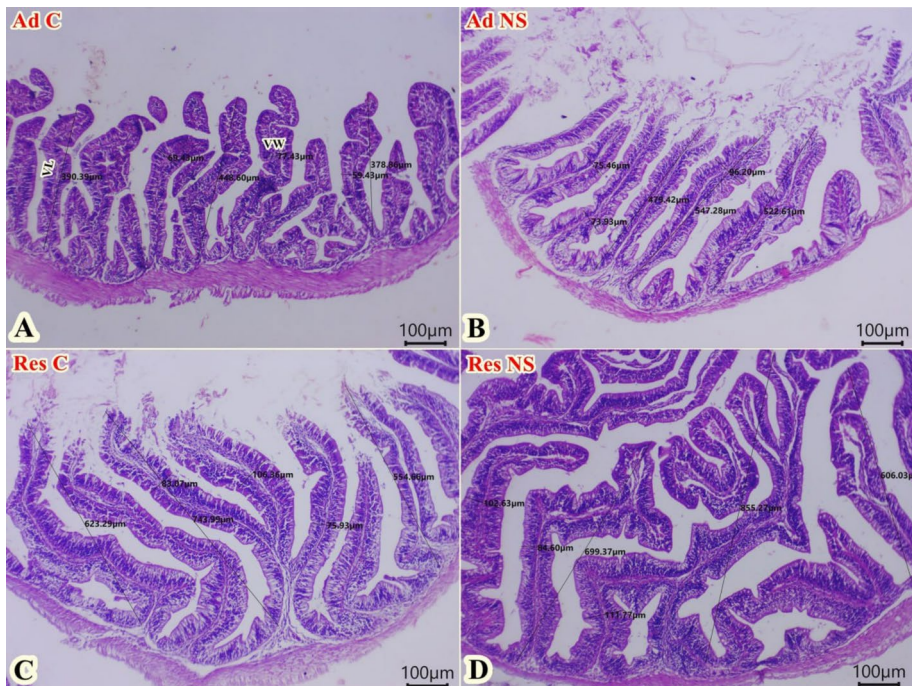


Fig. 2 Photomicrograph of H&E-stained sections from the intestine (scale bar, 100 μm) showing the least intestinal measurements particularly the villous length (VL) and villus width (VW) at Ad C group (A) and a gradual increase in intestinal parameters at Ad NS (B), Res C (C), Res NS (D) groups, respectively. The highest level of villous length (VL) and villus width (VW) is shown in group 4 (Res NS group)

Table 7 Intestinal morphometry of *O. niloticus* fed experimental diets with or without feed restriction

Parameters	Experimental groups				
	Ad C	Ad NS	Res C	Res NS	P-value
Villus length (μm)	405.33 ± 21.61^c	640.00 ± 55.22^{ab}	514.67 ± 18.83^{bc}	720.00 ± 72.64^a	0.01
Villus width (μm)	68.33 ± 5.21^a	88.86 ± 9.29^a	81.33 ± 7.35^a	99.00 ± 7.94^a	0.11
Absorption surface area, ASA, (mm^2)	0.03 ± 0.00^b	0.06 ± 0.01^{ab}	0.04 ± 0.01^{ab}	0.07 ± 0.01^a	0.04

Values in the same row that do not share the same superscripts are significantly different ($n = 18/\text{group}$; $P < 0.05$; one-way ANOVA)

Ad C fish group fed a basal diet on ad libitum basis, *Ad NS* fish group fed with experimental diet containing 3% *Nigella sativa* on ad libitum basis, *Res C* fish group fed basal control diet and subjected to 2 days of feed restriction weekly, *Res NS* fish group fed with experimental diet containing 3% *Nigella sativa* and subjected to 2 days of feed restriction weekly

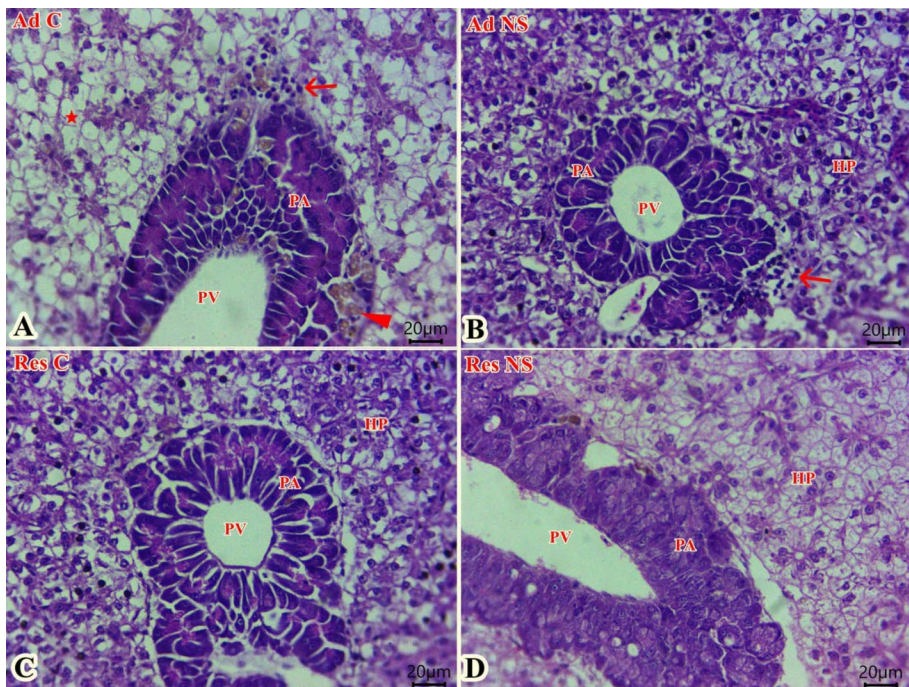


Fig. 3 Photomicrograph of H&E-stained sections from the liver (scale bar, 20 μm) showing severe degrees of hydropic degenerative changes within large numbers of hepatic cells (star) beside prominent melanomacrophage centers (arrowhead) and peripancreatic inflammatory cell aggregates (arrow) at Ad C group (A); more pronounced vacuolated hepatocytes and peripancreatic inflammatory cell infiltrates (arrow) at Ad NS group (B); and normal histology of vacuolated hepatic cells and exocrine pancreatic cells around branches of the portal vein in groups Res C (C) and Res NS (D). Hepatic cells (H), pancreatic acini (PA), portal vein (PV)

Histopathological findings

All groups' intestinal sections showed normal villi, lamina propria, submucosa, and muscle layer histomorphology. The Res NS group had the highest levels of villus length (VL) and villus width (VW). In contrast, the Res C, Ad NS, and Ad C groups showed a progressive decrease in intestinal parameters (Fig. 2). The absorption surface area (ASA), villus length (VL), and villus width (VW) histomorphometrics for the various treated groups were shown in Table 7. In relation to the liver (Fig. 3), the Res NS and Res C groups displayed polyhedral hepatic cells with narrow hepatic sinusoids and normal histology. The majority of the liver cells were vacuolated. Around the portal vein's branches, acinar types of exocrine pancreatic cells were observed. The pancreatic cells had round basal nuclei and were columnar epithelium. These cells feature basophilic staining at the basal ends and eosinophilic granules at the apical ends. However, the Ad NS group showed more noticeable infiltrates of peripancreatic inflammatory cells and vacuolated hepatocytes. The Ad C group showed clusters of peripancreatic inflammatory cells and a significant degree of hydropic degenerative alterations in a large number of hepatic cells in addition to substantial melanomacrophage centers.

Both the Res NS and Res C groups showed normal morphological structures of renal tubules, glomerular corpuscles, and other stromal tissues, as shown in Fig. 4. However,

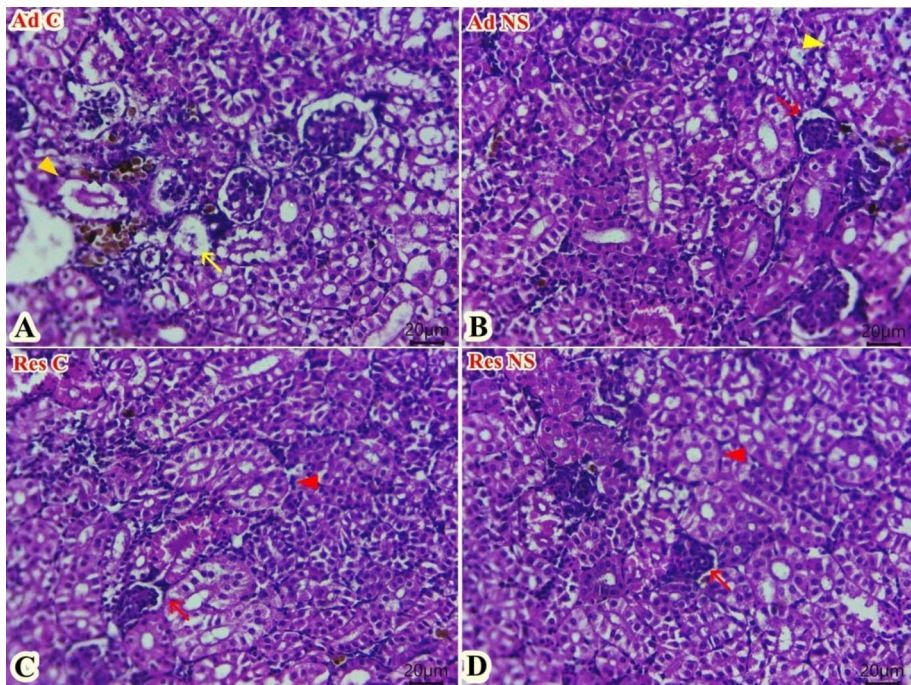


Fig. 4 Photomicrograph of H&E-stained sections from the kidney (scale bar, 20 µm) showing atrophic glomerular tufts (yellow arrow), moderate number of necrotic renal tubules (yellow arrowhead), and prominent melanomacrophage centers at Ad C group (A); apparent normal glomeruli (red arrow) and most renal tubules with few renal tubular necrosis (yellow arrowhead) in Ad NS group (B); and normal morphological structures of glomerular corpuscles (red arrows), renal tubules (red arrowheads), and other stromal tissues in both Res C (C) and Res NS (D) groups

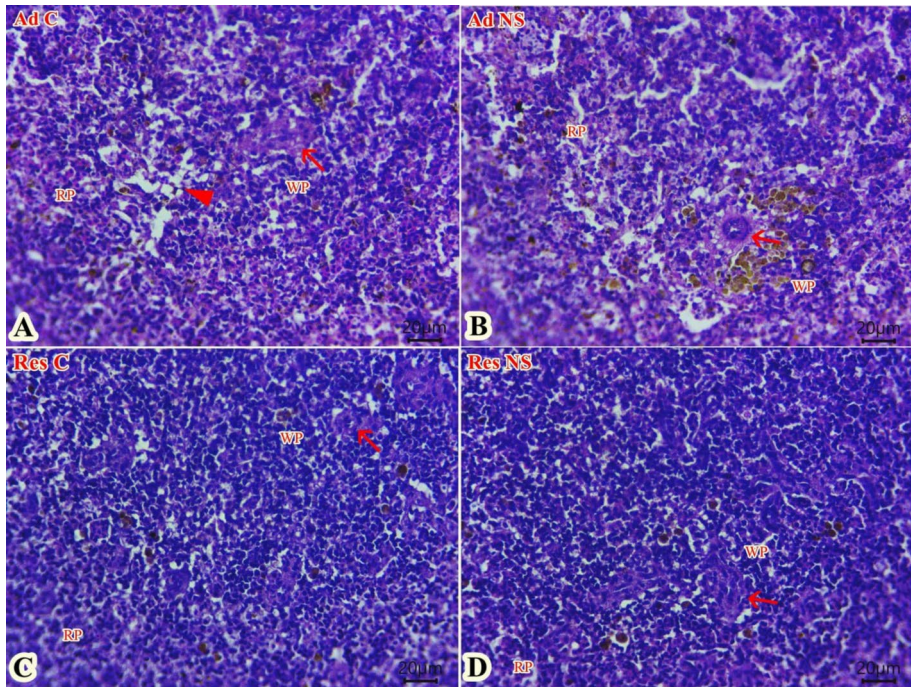


Fig. 5 Photomicrograph of H&E-stained sections from the spleen (scale bar, 20 μ m) showing mild depletion of white pulps (arrowhead) with numbers of lymphocytic elements around ellipsoid arterioles and aggregations of different haematopoietic series within dilated sinusoids at Ad C group (A); lymphocytic series at white pulp beside foci of melanomacrophage centers (MMCs) and dilated sinusoids at Ad NS group (B); and normal cytoarchitectures of lymphoid elements around ellipsoid arterioles beside normal red pulps in both Res C (C) and Res NS (D) groups. White pulp (WP), red pulp (RP), ellipsoid arterioles are indicated by arrows

the majority of the renal tissues in the Ad NS group appeared normal, with very little renal tubular necrosis. Additionally, the Ad C group had noticeable melanomacrophage centers, moderate numbers of necrotic renal tubules, and atrophic glomerular tufts. Lastly, the spleens of both the Res NS and Res C groups displayed typical cytoarchitectures of lymphoid components surrounding ellipsoid arterioles that alternated with red pulps. In contrast, the Ad NS group found lymphocytic series at the white pulp next to dilated sinusoids and melanomacrophage center (MMC) foci. Additionally, the Ad C group showed aggregations of various haematopoietic series within dilated sinusoids and a moderate depletion of white pulps with numbers of lymphocytic components around ellipsoid arterioles (Fig. 5).

Challenge test results

The C+ ve group had a greater mortality rate (35%) during the *E. tarda* challenge than the control group (0%), as seen in Fig. 6. The mortality rate for the (Res NS) group was lower than that of the C+ ve group (5.55%), followed by that of the (Res C) group (11.11%) and the Ad NS group (14.81%). After 3 days, fish showed signs of skin darkening and minor body haemorrhages. Hepatomegaly and splenomegaly with swollen internal organs were

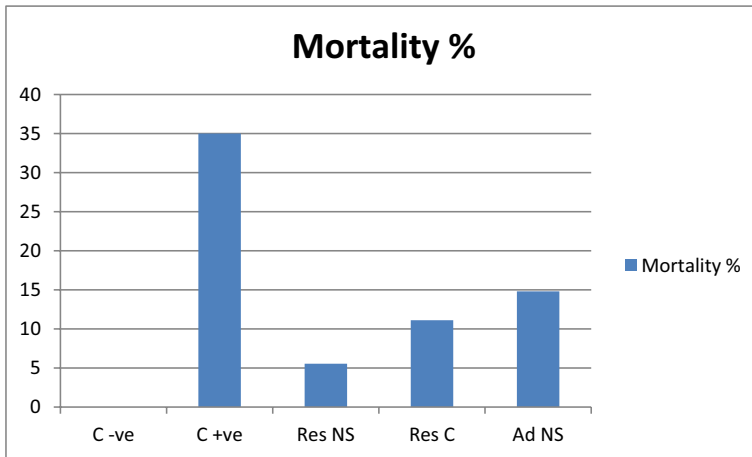


Fig. 6 Mortality % of *O. niloticus* in all experimental groups during the *E. tarda* challenge for 7 days. C– ve, the fish group fed a basal diet ad-libitum and not challenged; C+ ve, the fish group fed a basal diet ad-libitum and challenged with *E. tarda*; Res NS, fish group fed experimental diet contained 3% NS and subjected to 2 days of feed restriction and challenged with *E. tarda*; Res C, fish group fed a basal control diet and subjected to 2 day of feed restriction and challenged with *E. tarda*; Ad NS, fish group fed experimental diet contained 3% NS on ad-libitum basis and challenged with *E. tarda*, log-rank = 0.005 (Mantel–Cox)

among the post-mortem abnormalities. While the C– ve group showed normal clinical indications and no postmortem modifications, the C+ ve group had more severe postmortem lesions and clinical indicators than the other experimental groups.

Discussion

In this study some attempts were made to improve the productive performance, immunity, and health status of *O. niloticus* using natural phytogetic feed additives, *Nigella sativa*, in addition to getting some benefit from these plants in the feed restriction programme. Using phytogetic feed additives as growth promoters and immune-stimulants in fish is more useful than antibacterial drugs that cause adverse side effects for fish, the environment, and consumers (Dorucu et al. 2009). Moreover, feed restriction is one of the feeding strategies that improve immunity, feed conversion, and economic return and reduce fat deposition in carcasses (Bolívar et al. 2006; Chowdhury et al. 2021). Because of the feed restriction, feed intake and growth were expected to be reduced with an end weight less than normal in spite of an improvement in feed conversion and carcass quality (Afram et al. 2021). So, this study tested the effect of NS in correcting the negative effect of restriction on *O. niloticus* performance.

In the current study, *O. niloticus* fed diets enriched with NS for 8 weeks showed improved growth parameters due to its active components, thymoquinone, dithymoquinone, thymol, nigellidine, and nigellidine (Abou-Zeid 1998, Ahmed et al. 2013 and Sani et al. 2025). Alkaloids, saponins, minerals (copper, iron, phosphorous, and zinc), and vitamins (folic acid, pyridoxine, riboflavin, vitamin E, and thiamin) are also reported to be present in NS (Al-Jassir 1992). The combined effects of these elements may have improved tilapia growth

performance. Our findings are corroborated by Al-Dubake et al. (2012), Abouelezz et al. (2020), and Latif et al. (2020), who reported that by stimulating digestion through their aromatic substances or essential oils, a diet containing 1–3% black seed improved growth rate and gut morphology when compared to a control. Additionally, black seeds contain a wealth of antibacterial, antioxidant, and essential amino acids (methionine, threonine, lysine, arginine, glutamic acid, leucine, tyrosine, and proline) that improve intestinal health, digestion, and absorption, all of which improve growth performance (Latif et al. 2020). The control group (Res C) clearly experienced the effects of the feed limitation, whereas the NS group (Res NS) did not. The Res C group's weight gain fell as a result of restriction because it reduced feed intake and the amount of nutrients available (Riche et al. 2004). Because of the growth promoter and NS appetizer effects, the Res NS group's performance was unaffected by restriction (Zaki et al. 2012; Latif et al. 2020). Every group's body length did not differ from the control group. These findings are consistent with those of Bektaş et al. (2019) and Hussein et al. (2020), who demonstrated that the length differences between the groups administered dietary NS were negligible. This study's findings on fish body composition are consistent with those of Soliman et al. (2000), Hassan et al. (2009), and Abozaid et al. (2018). According to these authors, fish fed diets supplemented with NS showed a considerable rise in body ash but no significant change in body DM, CP, or NFE. Additionally, EE was much lower in feed-restricted groups than in ad libitum groups.

Estimating the haematological index often produces important indicators of the species' physiological reactions and nutritional state (Hassaan et al. 2018). This study illustrated that neither fasting for 2 non-consecutive days per week nor the addition of 3% NS to the ration of the *O. niloticus* has a significant effect on the whole haematological picture. This result is in accordance with Pourmand et al. (2025), who found that treatment of *Oncorhynchus mykiss* with NS had no significant effect on haematological indices. Moreover, Najafi et al. (2015) found that refeeding after 16 days of food deprivation had no discernible impact on the haematological and immunological indicators of *M. sharpeyi* fingerlings. Our results can be discussed as the period of food restriction was too short to elicit measurable alterations in haematological markers.

Our results revealed a significant decrease in liver enzymes in NS-treated groups; this could be due to the hepatoprotective activities of NS resulting from the thymoquinone antioxidant effect (El-Tahir and Bakeet 2006). Moreover, the results are compatible with those of Yousefi et al. (2021), who reported that *Cyprinus carpio* fed diets enriched with NS at different concentrations exhibited lower ALT and AST activities. Also, in our study, intermittent fasting led to a reduction of hepatic enzymes, but this result is not compatible with the previous studies (Bjornvad et al. 2004; Mehjebin et al. 2023), which found that short-term fasting elevated liver enzymes. The decreased activity of the investigated liver enzymes was most likely a result of improving the liver functions by intermittent fasting (Ranjbar et al. 2025) otherwise, the body's decreased ability to regenerate proteins due to the lack of amino acids from the gastrointestinal tract and the low level of gluconeogenesis from amino acids during the studied fasting period (Tlak et al. 2008).

Regarding serum protein profile results, including total protein, albumin, globulins, and A/G ratio, no significant changes between all experimental groups were observed. This may be attributed to the short fasting period (2 days/week) and no difference in protein percentage in diets of different groups (Kaki et al. 2018). Serum glucose levels in both restricted groups were lower than in ad libitum groups due to the negative effect of restriction on hepatic gluconeogenesis (Wang et al. 2019). Furthermore, NS supplemented groups showed lower serum glucose levels than both control groups due to NS hypoglycemic effects (Fararh et al. 2004; Heshmati et al. 2015; Niroomand et al. 2023). The active

components of NS, such as thymoquinone, dithymoquinone, linoleic acid, and oleic acid, can improve the insulin production from pancreatic β -cells (Badar et al. 2017) and increase insulin sensitivity in peripheral tissue (Benhaddou-Andaloussi et al. 2011).

The addition of NS in the diet together with feed constriction (Res NS) led to decreasing total serum cholesterol and LDL-c levels when compared to control, while feed constriction alone had no effect. This can be discussed as NS contains a lot of unsaturated fatty acids, which may promote the oxidation and excretion of cholesterol into the colon (Khodary et al. 1996 and Mustafa et al. 2024).

Fish welfare is impacted by cortisol which is a component of the primary (neuroendocrine) stress response that alters behaviour and affects brain and physiological functions (Ellis et al. 2012). According to our findings, the cortisol level in the Res NS group was significantly lower than in the control group (Ad C). The synergistic effect of fasting and NS enrichment may be the cause of this outcome. The effect of fasting is consistent with the study of Small and Peterson (2005) in channel catfish, while the effect of NS supplementation is consistent with Mohan et al. (2023). The beneficial outcomes of NS can be mainly related to its volatile oil content, mainly the thymoquinone (Kannan et al. 2022), which has anti-anxiety properties by regulating levels of GABA and nitric oxide in the brain or plasma (Gilhotra and Dhingra 2011).

Growth hormone (GH) was elevated in restricted fish and/or received diets containing NS when compared to the control group because GH production would be triggered by increased pituitary sensitivity to GHRH (Small and Peterson 2005). The increases in plasma GH match the results of a previous study in which plasma levels were increased after 3, 5, and 7 days of fasting in the tilapia (Riley et al. 2008). Also, another previous study by Company et al. (1999) reported an increase in plasma GH in Chinook salmon after fasting for 4 days. It is unclear how fasting raises circulating GH concentrations; it could be partly caused by increased pituitary sensitivity to GHRH, possibly as a result of decreased inhibitory somatostatin, or by increased release of GHRH and synthesis of GHRH receptors.

The hormone leptin was first discovered by Kurokawa et al. (2005) in fish and has since been identified in numerous fish species. In mammals, it is a key factor in the long-term regulation of hunger and energy homeostasis (Wynne et al. 2005). According to Peelman et al. (2006), leptin is a 16-kDa pleiotropic peptide hormone that is included in a number of biological activities, including energy balance, obesity, bone formation, wound healing, reproduction, and immunity. Our results indicated that there is a highly significant increase in leptin in the Res NS group, followed by Res C and Ad NS, when compared to the control (Ad C). This result is not in accordance with the results of a previous study by Al-Asoom et al. (2014), who studied the cardiac hypertrophy-associated biomarkers induced by NS supplementation in the diet of exercise-trained rats and found no significant elevation in leptin in the NS-treated group when compared to the control group. While our result is in line with Trombley et al. (2012) who found significant increase in *lepa1* expression and leptin plasma level in feed restricted juvenile Atlantic salmon. One of the physiological processes of leptin in mammals is to mobilize and deplete stored lipids, particularly in the liver (Cohen and Friedman 2004).

The immune system comprises various components, and the lysozyme activity is an important indicator of innate immunity in fish (Dhanarso et al. 2021). In fish, lysozyme has been shown to operate as an opsonin, stimulating phagocytes and the complement system (Grinde 1989). The humoral adaptive immune reaction is delivered by B cells, which yield high-affinity immunoglobulins (Ig) specific to their target antigen (Velázquez et al. 2018). IgM is the primary Ig found in serum and the systemic reaction, as well as all immune tissues of tilapia (Zhang et al. 2010). IgM is commonly used as a measure of systemic antibody responses (Mai et al. 2021).

According to our data, the Res NS group exhibited a substantial increase in lysozyme and IgM, followed by Res C and Ad NS, respectively, as compared to the Ad C group. The

beneficial effect of NS on immunity is thanks to its thymoquinone (Ahmad et al. 2021) and antioxidant compounds (Hannan et al. 2021). Our results are consistent with Awad et al. (2013) and Ibrahim et al. (2021) who reported that dietary supplementation of NS oil and seeds had elevated the lysozyme and IgM in fish. Moreover, Liao et al. (2021) and Wang et al. (2019) reported that lysozyme elevated after starvation of fish because feed restriction enhances lysosome activity primarily by promoting autophagy.

Lipid peroxidation is a biomarker to assess damage caused by oxidative stress in animal tissues (Faheem and Lone 2018). Antioxidant characteristics, including CAT, GPx, GST, and GSH, are crucial for an organism's defense because they keep reactive oxygen species (ROS) at the lowest possible level within cells (Lesser 2006). In contrast to the other two experimental groups (Ad C and Ad NS), MDA results in our investigation indicated a considerable reduction in Res NS, followed by Res C. As compared to the control group, the Res NS group showed a significant increase in SOD and GPx activity because thymoquinone in NS can boost the expression of antioxidant enzymes like GPx and SOD while suppressing the expression of iNOS, which is involved in oxidative stress states (Al-Okbi et al. 2013). Furthermore, thymoquinone can lower NADH, which would lower the NADH-NAD⁺ changes and prevent hepatocyte lipogenesis (Khalife and Lupidi 2007). Our results agree with Latif et al. (2020), who added 30 g/kg NS in the Nile tilapia diet and recorded an increase in the expression levels of SOD and GPx and a decrease in the level of lipid peroxidation. Moreover, Wang et al. (2019) stated that serum SOD and CAT activities increased in Nile tilapia in the first 3 days of fasting because the high levels of lipid and fatty acid oxidation during early starvation result in the production of excessive ROS and trigger antioxidant enzymes (Barim-Öz Ö 2017).

The inclusion of NS in fish feed has demonstrated significant effects on the histomorphology of key organs such as the liver, intestine, kidney, and spleen. This is primarily attributed to the antioxidant, anti-inflammatory, immunomodulatory, and hepatoprotective properties of thymoquinone (Ciesielska and Wojciechowicz 2023).

The intestine plays a crucial role in nutrient absorption and immunity. NS supplementation has been shown to improve gut morphology, increasing villi height and intestinal fold density, enhancing nutrient absorption (Hartono et al. 2021). In this study, the longest and widest villi were seen in the Res NS and Res C groups, then Ad NS, and finally Ad C, suggesting the synergy between NS (anti-inflammatory) and feed restriction (reduced metabolic burden) improving nutrient absorption (Hartono et al. 2021). Furthermore, in the current study Res NS and Res C groups were also proved to have the strongest hepatoprotection, which can be discussed as the combined antioxidant effects of NS and feed restriction (Alagawany et al. 2020). In addition, fasting reduced lipid accumulation, so it improved liver enzyme profiles (ALT, AST ↓), as we also recorded in our study, but lacked the anti-inflammatory benefits of NS. Therefore, the combination of NS with feed restriction provides optimal liver protection by enhancing detoxification while preventing over-nutrition-related stress.

The kidney is essential for osmoregulation and waste excretion. NS supplementation contributes to renal health by reducing tubular degeneration due to the antioxidant properties that mitigate oxidative damage in renal tissues (Mahboub et al. 2022). Effects on the kidney were in line with effects on the intestine and liver, where Res NS and Res C groups showed normal morphological structures of glomerular corpuscles, renal tubules, and other stromal tissues in contrast to atrophic glomerular tufts, moderate numbers of necrotic renal tubules, and prominent melanomacrophage centers that were noticed in the Ad C group. This can be caused by the restriction of food that reduces the nitrogenous wastes, while NS counters oxidative stress (Mahboub et al. 2022).

The spleen is a major immune organ in fish. NS enhances splenic function by boosting immune responses; increased lymphocyte proliferation and macrophage activity enhance disease resistance (Hussein et al. 2020). It follows logically that Res NS and Res C groups showed the normal cytoarchitectures of lymphoid elements around ellipsoid arterioles that alternated with red pulps in the spleen. Conversely, mild depletion of white pulps with numbers of lymphocytic elements around ellipsoid arterioles and aggregations of different haematopoietic series within dilated sinusoids were seen in the Ad C group. These findings imply that NS stimulated immunity, while feed restriction reduced metabolic immunosuppression (Hussein et al. 2020).

All these promising findings were finally confirmed by the challenge experiment, which revealed the lowest mortality and milder pathological signs in restricted fish groups than Ad NS group compared with the Ad C group (Fig. 1). This protective effect of NS is attributed to its antimicrobial, antioxidant, and immunomodulatory properties. Interestingly, feed restriction alone (Res C) provided a higher level of protection over the Ad NS group; it can be attributed to the induction of autophagy or enhanced leukocyte function during nutrient stress (Qin et al. 2016). However, its combination with NS resulted in a significantly enhanced protective effect, suggesting that nutritional immune-stimulants might work best in optimized metabolic states.

Conclusion

The present study has depicted that using NS at a level of 3% in *O. niloticus* diets can enhance the growth performance, haemato-biochemical parameters, and organ-gut health status of fish. The combined implementation of feed restriction and NS supplementation yields superior results in aquaculture production. NS can counteract the negative effect of restriction on fish performance. Moreover, supplementation of NS to the fish diet with feed restriction stimulated the immunity and enhanced the resistance of *O. niloticus* to *E. tarda* challenge. NS can be used as a natural antimicrobial growth promoter and help in solving the global problem of antimicrobial resistant bacterial strains responsible for human and animal diseases.

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Declarations

Ethics approval This experimental protocol was approved by Beni-Suef University's Institutional Animal Ethics Committee in Egypt (BSU-IACUC-022–357).

Consent to participate Every author contributed to this work.

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