Growth, feed efficiency, liver biomarkers, antioxidants, hematological indices, immune gene expression, and immunomodulatory effect of *Mesosphaerum suaveolens* leaf extract in grass carp (*Ctenopharyngodon idellus*) against *Aeromonas hydrophila*

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Abstract

Background The study was to examine the effects of *Mesosphaerum suaveolens* (MS) leaf extract on growth performance, whole body composition, immunological responses, hematological parameters, digestive enzymes, and antioxidant status in grass carp (*Ctenopharyngodon idellus*).

Methods A total of 525 grass carp, with an average body weight of 110.0 ± 1.0 g, were utilized in a 70-day feeding experiment. Fish were randomly allocated to one of five groups. Each group had a triplicate tank with 35 fish per tank. Dietary treatment composed of a basal diet was given to the control group (MS0); MS1, MS2, MS3, and MS4 were substituted with 0, 1, 2, 3, and 4 g/kg of the basal diet, respectively. Fish, relative percent survival (RPS) was measured 14 days after *Aeromonas hydrophila* ($1x10^7$ cells/mL) was given to the fish at the end of the feeding trial.

Results Significant enhancements were shown in growth performance indicators and the body's crude lipid and moisture of the grass carp fed increasing levels of MS compared with the control. Serum levels of total triglycerides, total cholesterol, total protein, glucose, globulin, albumin, oxalate transaminase, glutamic pyruvate transaminase, and serum content of red blood cells, hemoglobin, hemocrit, and white blood cells significantly increased, and mean cell hemoglobin and mean cell hemoglobin concentrations significantly decreased in the grass carp fed the MS-supplemented diets. The liver catalase enzyme, total antioxidant capacity, superoxide dismutase, and glutathione peroxidase activities were increased, and liver malondialdehyde was lower in the in the MS groups compared to the control. Dietary MS supplementation improved the respiratory burst, myeloperoxidase, lysozyme, alternative complement, ceruloplasmin, antiprotease, haemagglutination, bacterial agglutination, and total immunoglobulin levels compared with the control. After being exposed to *A. hydrophila* for seventy days, the grass carp that was given MS extracts showed a better rate of survival than the control group at the end of the experiment; the highest survival rates were seen in the MS3 and MS4. The transcriptional examination of these gene expressions showed that the spleen and head kidney of grass carp 14-day infection had significantly elevated expression levels of major beta 2 microglobulin (β2M), Toll-like receptor 22 (TLR 22), tumor necrosis factor-alpha (TNFα), Lysozyme-C, and Lysozyme-G.

Conclusions Overall, the present research's results suggest that MS extract's growth promotion, immunological responses, hematological parameters, digestive enzymes, and antioxidant status make it an acceptable option for use as a feed additive in grass carp farms.

Background

Over the past two decades, China and India have dominated global aquaculture production [1]. Nevertheless, with the rapid emergence of the aquatic sector, various diseases and water pollution have improved and confined the viable emergence of aquaculture [2–4]. Antibiotics are frequently used to combat diseases in aquaculture, and their overuse has led to the formation of resistant strains and a

considerable amount of antibiotic residue in the environment and palatable tissues of aquatic organisms, which is harmful to human health [5–7].

Recently, the vision of antibiotic-free agriculture has been sketchily appreciated and applied worldwide [2]. The utilization of herbal plants as “green medicine” has received increasingly attention, and they are also widely utilized as feed additives in fish [7, 8]. Various herbal plants include many health-enhancing products like tannins, flavonoids, terpenoids, alkaloids, steroids, saponins, and polysaccharides [9]. Several studies have indicated that certain herbs could be used to cure fish ailments [10, 11] and that different herbal extracts may enhance growth performance [7, 12]. Because *M. suaveolens* (MS) is extensively dispersed throughout India, China, South Korea, Europe, and America, the traditionally utilized extract from the plant is attractive [9]. This species roots, leaves, stems, and seeds are frequently used in regional and traditional medicine to treat a variety of ailments, including gastrointestinal and respiratory disorders, dysentery, indigestion, boils, cramps, wounds, tumors, headaches, colds, fevers, body pain, leukorrhea, skin conditions, cancer, renal problems, and inflammatory conditions [13]. Some biological activities of MS have been reported, including antioxidant, anti-diabetic, anti-bacterial, immune function, anti-fungal, anti-diabetic, wound healing, anti-malarial, cytotoxic, and anti-inflammatory properties, as well as being utilized as an antidote for snake bites [14]. Some biological activities of MS have been reported, including antioxidant, anti-diabetic, anti-bacterial, immune function, anti-fungal, anti-diabetic, wound healing, anti-malarial, cytotoxic, and anti-inflammatory properties, as well as being utilized as an antidote for snake bites [14]. Therefore, MS, a major constituent of triterpenoids, carbohydrates, flavonoids, and saponins, may well affect the production of fish. MS-induced pharmacological activities that appear to have an impact on humans or different experimental animals have been extensively documented. Still, the effects of MS extract as a feed supplement on grass carp have not been thoroughly investigated. We hypothesized that extracts from MS chosen based on different physiological effects would contain several active ingredients that work well together to maximize productive performance. Thus, we tried to assess the effectiveness of MS as a natural growth promoter in the diets of grass carp in this study. The assessment's components included monitoring physiological changes in growth performance, nutrient utilization, whole-body composition, immunological responses, hematological parameters, digestive enzymes, and antioxidant status.

Material methods

Experimental Samples

M. suaveolens (MS) is derived from a climbing plant widely distributed in the north-east of India (Fig. 1). To avoid photolysis and heat degradation, the fresh leaves were gathered, shade dried for one week at room temperature (25–31°C), and then ground into a powder. By using typical extraction techniques, crude extracts were produced [15, 16]. An electrical stainless-steel blender was used to chop dried leaves into small pieces and then grind them into a coarse powder (Kumar et al. 2014). Using 500 ml of methanol and 48 hours at 45–80°C, the powdered material (100 g) was extracted using the Soxhlet technique [17]. In order to form the crude extract, it was kept at 4°C [18] for further studies.

Experimental feed and setup

The grass carp we used in our research were tamed for two weeks to acclimate to the test conditions. They were purchased from a local fish farm in Chumoukedima, Nagaland. During the prescribed experiment, 375 grass carp weighing approximately 110.0 ± 1.0 g were carefully chosen and arbitrarily allocated to five treatments. 25 fish are stocked in 1 m x 3 m x 1 m aquariums in three duplicates for each treatment. Sattanathan et al. [19] were followed in creating the baseline diet as a control diet, which is displayed in Table 1. A diet plan consisting of feeding one of the five meals to fish in duplicate tanks twice a day (08:30–09:30 and 16:30–17:30) at a ratio of 4% of their body mass was applied at random to the fish until they showed signs of satiation. A basal diet was given to the control group (MS0); MS1, MS2, MS3, and MS4 were substituted with 0, 1, 2, 3, and 4 g/kg of the basal diet, respectively. The dietary treatments continued for ten weeks, during which time uneaten food and excrement were removed from each tank. One-third of the entire volume of water was replaced daily in order to clean the excrement in the tanks. The water temperature was kept between 13 and 18°C during the experiment, dissolved oxygen was 6.0 and 8.0 mg/L; ammonia nitrates were ≤ 0.021 mg/L; and pH was 7.4 and 7.6.

Table 1
Composition (g /kg) of the four experimental diets supplemented at different levels of M. suaveolens

Ingredients (g/kg)	MS0	MS1	MS2	MS3	MS4
Rice bran	170	169	168	167	166
Soybean meal	530	530	530	530	530
Fish meal	50	50	50	50	50
Wheat flour	20	20	20	20	20
Corn flour	150	150	150	150	150
Vegetable oil	50	50	50	50	50
Iodized salt	5	5	5	5	5
Mineral mix*	25	25	25	25	25
MS extract (g/kg)	0	1	2	3	4
Chemical composition					
Crude protein	38.24	38.25	38.26	38.27	38.62
Total lipid	28.5	28.47	28.61	28.97	28.75
Ether extract	12.21	12.25	12.85	12.56	12.47
Moisture (%)	7.25	7.28	7.65	7.95	7.65
Ash (%)	13.54	13.84	13.74	13.47	13.95

*Composition of the mixture per kg dry weight (Suplevite M ontained from Sarbahi chemicals, Wadi, Baroda-390007, India); Vit A (700,000 IU), Vit D3 (70,000 IU), Vit E (250 mg), Nicotinamide (1000 mg), Co (150 mg), Cu (1200 mg), Io (325 mg), Fe (1500 mg), Mn (1500 mg), K (100 mg), Se (10 mg), Na (5.9 mg), S (0.72%), Zn (9600 mg), Ca (25.5%), P (12.75%).

After 70 days of rearing, all fish in each tank were weighed, and the final weight (FW), net growth (NG), weight gain rate (WGR), daily growth rate (DGR), specific growth rate (SGR), feed conversion rate (FCR), feed efficiency (FE), condition factor (CF), hepato-somatic index (HSI), viscero-somatic index (VSI), and survival rate (SR) were calculated according to Ahmadi et al. [20].

Whole body composition analysis

The analytical procedures for the estimated compositions of fish fillets were based on previously reported methods [21]. Whole-body nutrient compositions like crude protein, moisture, and ash were examined using conventional techniques (AOAC, International Association of Official Analytical Chemists) [22]. Crude lipid levels were estimated according to the Soxhlet extraction method [23].

Liver markers and Haematological parameters

Six fish were selected and gathered via the caudal vein using sterile 1 mL syringes. Fishes were randomly sampled and euthanized using tricaine methanesulfonate (MS-222) at a concentration of 20 mg/L for sample collection. The serum was gathered and kept at -20°C for the following steps: standing overnight at 4°C and centrifuging at 1000 × g for 10 minutes at 4°C. The glucose, globulin, albumin, total triglycerides (TG), total cholesterol (TC), total protein (TP), glutamic oxalate transaminase (GOT), and glutamic pyruvate transaminase (GPT) were as determined by an automatic biochemical analyzer (Model 7020, Hitachi, Tokyo, Japan). The hematological parameters like hemoglobin (HB), hemocrit (HCT), white blood cell (WBC), mean cell volume (MCV), mean cell hemoglobin (MCH), red blood cell (RBC), and mean cell hemoglobin concentration (MCHC) were analyzed using the method [24].

Immunological parameters

The different immune characteristics, both specific and non-specific, were observed by [26–28]. The method of Tukmechi et al. [29] was used to measure the lysozyme activity in serum. In accordance with Villamil et al. [30], serum bactericidal activity against *Aeromonas hydrophila* (MTCC 1739) was assessed. In accordance with Rao and Chakrabarti [31], the levels of serum total antiprotease and a1-antiprotease were determined. Ceruloplasmin activity in serum was determined as p-phenylene diamine oxidase activity using the previously reported method, with a few minor modifications [32]. One unit of ceruloplasmin was defined as the quantity of oxidase that catalyzed a 0.001 min^{-1} drop in absorbance at 550 nm. The overall level of myeloperoxidase activity was calculated using the procedure outlined in [33]. The OD was measured at 450 nm using a microplate reader. In accordance with Stasiack and Bauman [34], the respiratory burst content was assessed. Yuvan et al. [35] reportedly offered a modified version of Kohshahi et al. [36] alternate complement activity calculation method. The hemagglutination assay was conducted in accordance with Dinh et al.'s [37] protocol. The bacterial hemagglutination assay was used to evaluate the antigen-specific antibody response [38]. The technique developed by Khalil et al. [38] was used to calculate the total immunoglobulin concentration in plasma.

Antioxidant status

To decrease their enzyme activity during the dissection step, the sedated fish were positioned close to the ice. Following the removal of their viscera, cold distilled water was used to wash the liver tissue. The liver samples were homogenized near ice in a 0.2 M NaCl solution. After that, the suspension was centrifuged for 30 minutes at 5000 rpm and 4°C. The supernatant was then separated and put to use in antioxidant experiments. The antioxidant enzymes such as catalase enzyme (CAT), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were analyzed using the method [39].

Digestive enzymes activity

The midgut of the fish (n = 3) was separated, and the enzyme extract was prepared by washing it with distilled water. This was done in order to assess the impact of different experimental treatments on the activity of digestive enzymes (amylase, cellulase, and lipase). For 2.0 minutes, tissues were homogenized at a 5:1 ratio using buffer. After that, it was centrifuged for 20 minutes at 10,000 rpm and

4°C. In order to assess soluble protein and enzyme activity, the supernatant was kept at -20°C [40]. Using this technique, the digestive enzymes lipase, cellulase, and amylase were examined [41].

Challenge test

A. hydrophila (MTCC-1739) was shown to have a seven-day lethal dosage (LD_{50}) of 10^7 cells/mL (Sattanathan et al., 2020). Following a 70-day feeding trial, 12 fish from each treatment group received an intraperitoneal injection of 100 µL of PBS containing one million live *A. hydrophila* cells per milliliter. For 14 days, the challenged fish were maintained under obseration and given a basal diet. Up to 14 days after the challenge, the survival rate in each group was noted. Relative percentage survival (RPS) was used to calculate the survival rate [42].

Immune-Related Gene Expression Analysis

Tissue collection

After 14 days of immunization, microbiological tests were performed on the spleens and head kidneys of three dead fish (n = 3), and the results showed the presence of bacteria. Following the pathogenicity period, fish were examined using current methods to determine the expression of immune genes.

Gene expression Analysis

The protocols for kidney and spleen RNA extraction, reverse transcription, and qPCR were carried out in accordance with the guidelines set forth by Sattanathan et al. [42]. RNA was extracted using RNAiso Plus (Takara Bio, Kusatsu, Japan), treated with DNase I (Beyotime), and then quantified and purified using agarose gel electrophoresis and spectrophotometry (A260/280 ratio), in that order. Reverse transcription was carried out using the Takara Bio PrimeScriptTM RT Reagent Kit. The qPCR reaction kit (Invitrogen, Bangalore, India) contained 1 µL of complementary DNA, 0.2 µL ROX, 5 µL SYBR Premix Ex Taq, 0.4 µL primers, and 3.4 µL nuclease-free water. The reaction was conducted in a QuanStudio 5Real-Time System (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Table 2 lists the oligonucleotide primers that were utilized. To compute the changes in target gene expression in relation to β-actin levels, the 2 − ΔΔCT technique was employed.

Table 2
Primers that are used for real-time quantitative PCR

Target Gene	Primer Sequence (5'-3')	Optimum Annealing Temperature (◦C)	Size of PCR Amplification (bp)	Reference
NKEF- B	F- ACTGTGACCATCGAGTTC R-TGGGCAAGTAATTGCTG	49	285	Chen et al. [86]
Lysozyme–C	F- CTGTGATGTTGTCCTATCTTC R-GTAACTTCCCCAGGTATCC	52.7	321	Huttenhuis et al. [87]
Lysozyme G	F-CTTATGCAGGTTGACAAACG R-GGCAACAACATCACTGGAGTAATC	53.5	249	Mohanty and Sahoo [88]
TNF α	F-CCAGGCTTTCACTTCAGG R-GCCATAGGAATCGGAGTAG	51.6	102	Gonzalez et al. [89]
TLR22	F-TCACCCCATTTCGAGGAATGTC R-GAAGGCGTCGTACTGGCTAACAT	56.0	520	Saurabh et al Chen. [90]
β2M	F-TCCAGTCCCAAGATTCAGGTG R-TGGTGAGGTGAAACTGCCAG	59.7	175	Mohanty and Sahoo [88]
β-actin	F-GACTTCGAGCAGGAGATGG R-CAAGAAGGATGGCTGGAACA	55.3	138	Mohanty and Sahoo [88]

Statistical Analysis

The findings were displayed as means ± standard errors of the mean, or SEM. Every data set was analyzed using one-way ANOVA. Duncan multiple range rest was used to compare the group means further when there were significant differences ($P < 0.05$). With SPSS 21.0 (SPSS, IL, USA), all statistical analyses were carried out.

Results

Growth performance and body chemical composition

During the experiment period, grass-carp-fed diets supplemented with MS had increased FW, NG, WGR, DGR, SGR, FCR, HSI, and SR compared with the control. The CF and FCR of the MS3 and MS4 groups decreased ($P < .05$) compared to other groups. However, no effects were shown in the FE and VSI among all groups (Table 3). Growth, specific growth rate, daily growth rate, and feed conversion rate were

analyzed using polynomial regression while grass carp were fed increasing amounts of dietary MS extracts for 10 weeks. G, SGR, and DGR determined the dietary MS extract needed to be 2.99 g/kg, and FCR determined the dietary MS extract needed to be 2.79% (Fig. 2). The dietary inclusion of MS can improve the body's crude lipids and moisture in grass carp. Therefore, no differences were observed in the crude protein and moisture among all groups (Table 4).

Table 3
Growth performance of grass carp fed different levels of M. suaveolens

Parameters	MS0	MS1	MS2	MS3	MS4
IW (g)	110.46 ± 0.03	110.33 ± 0.05	110.45 ± 0.09	110.37 ± 0.05	110.38 ± 0.03
FW (g)	121.13 ± 0.30^{d}	125.67 ± 0.12^{c}	129.67 ± 0.11^{b}	131.09 ± 0.32^{a}	129.28 ± 0.33^{b}
NG (g)	10.66 ± 0.29^{d}	15.34 ± 0.18^{c}	19.21 ± 0.09^{b}	20.71 ± 0.32^{a}	18.99 ± 0.30^{b}
WGR (%)	9.65 ± 0.29 ^{d}	13.90 ± 0.17^{c}	17.39 ± 0.09 ^{b}	18.76 ± 0.29 ^{a}	17.12 ± 0.27 ^{b}
DGR (g/day)	0.15 ± 0.004 ^{d}	0.21 ± 0.002^{c}	0.27 ± 0.001^{b}	0.29 ± 0.004^{a}	0.26 ± 0.004^{b}
SGR (%/day)	0.13 ± 0.003^{d}	0.18 ± 0.002^{c}	0.22 ± 0.001^{b}	0.24 ± 0.003^{a}	0.22 ± 0.003^{b}
FCR	2.40 ± 0.06^{a}	2.25 ± 0.03^{ab}	2.20 ± 0.02^{b}	2.18 ± 0.03^{b}	2.21 ± 0.003^{b}
FE (%)	43.42 ± 1.21	44.45 ± 0.65	45.46 ± 0.52	45.69 ± 0.70	45.09 ± 0.62
CF (K)	1.43 ± 0.01^{b}	1.45 ± 0.001^{b}	1.54 ± 0.001^{a}	1.14 ± 0.006^{c}	1.12 ± 0.01^{c}
HSI (%)	1.24 ± 0.02^{b}	1.34 ± 0.03^{a}	1.32 ± 0.08^{a}	1.38 ± 0.01^{a}	1.38 ± 0.01^{a}
VSI (%)	2.63 ± 0.01	2.60 ± 0.01	2.59 ± 0.02	2.67 ± 0.07	2.64 ± 0.06
SR (%)	96.6 ± 1.92^{b}	97.7 ± 1.11^{ab}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

Values are means ± SD of three replicates. Mean values with different letters in the same row are significantly different ($P < 0.05$). IW: Initial weight; FW: Final weight; NG: net growth; WGR: Weight gain rate; DGR: Daily growth rate; SGR: Specific growth rate; FCR: Feed conversion rate; FE: Feed efficiency; CF: Condition factor; HIS: Hepato-somatic index; VSI: Visero-somatic index; SR: Survival rate.

Table 4
The carcass compositions (percentage of wet weight) of grass carp fed different levels of M. suaveolens

	MS0	MS1	MS2	MS3	MS4
Crude lipid (%)	23.83 ± 0.24[b]	24.72 ± 0.03[a]	24.72 ± 0.08[a]	24.77 ± 0.32[a]	24.55 ± 0.16[a]
Crude protein (%)	52.54 ± 0.05	52.63 ± 0.19	52.78 ± 0.07	52.94 ± 0.22	52.88 ± 0.33
Ash (%)	10.47 ± 0.05	10.43 ± 0.05	10.49 ± 0.02	10.50 ± 0.08	10.54 ± 0.09
Moisture (%)	72.40 ± 0.03[b]	72.47 ± 0.08[b]	72.54 ± 0.01[b]	72.78 ± 0.03[a]	72.79 ± 0.09[a]
Values are means ± SD of three replicates. Mean values with different letters in the same row are significantly different (P < 0.05)					

Liver marker and Haematological parameters

As shown in Table 5, compared with the additional control diet group, the MS diet group could increase GPT, GOT, TG, TC, TP, glucose, albumin, and globulin in the serum of grass carp (P < .05). The GOT, GPT, TG, TC, TP, glucose, albumin, and globulin reached a high in the MS3 and MS4 groups. Additionally, the increasing levels of MS supplements improve the HB, RBC, WBC, HCT, and MCV levels and decrease the MCH and MCHC levels. The RBC level was higher and the MCH level was lower in the in the MS3 group (Table 6).

Table 5
The biochemical blood parameters of grass carp fed different levels of M. suaveolens

	MS0	MS1	MS2	MS3	MS4
GPT (U/mL)	30.27 ± 0.03^{d}	31.86 ± 0.33^{c}	33.01 ± 0.34^{c}	34.94 ± 0.40^{b}	37.48 ± 0.63^{a}
GOT (U/mL)	35.64 ± 0.11^{e}	36.59 ± 0.13^{d}	38.47 ± 0.06^{c}	40.47 ± 0.08^{a}	46.78 ± 0.33^{a}
TG (mg/dL)	0.42 ± 0.003^{d}	0.45 ± 0.008^{cd}	0.46 ± 0.006^{bc}	0.48 ± 0.003^{ab}	0.49 ± 0.012^{a}
TC (mg/dL)	10.27 ± 0.04^{d}	11.20 ± 0.38^{c}	12.60 ± 0.08^{b}	13.55 ± 0.11^{a}	14.18 ± 0.33^{a}
Glucose (mg/dL)	3.60 ± 0.03^{c}	3.81 ± 0.04^{bc}	3.84 ± 0.14^{b}	4.15 ± 0.08^{a}	4.27 ± 0.03^{a}
TP (mg/dL)	3.54 ± 0.15^{c}	4.28 ± 0.20^{b}	4.23 ± 0.001^{b}	5.48 ± 0.13^{a}	5.34 ± 0.001^{a}
Albumin (mg/dL)	12.50 ± 0.08^{c}	13.17 ± 0.33^{bc}	13.64 ± 0.004^{b}	14.53 ± 0.15^{a}	14.77 ± 0.32^{a}
Globulin (mf/dL)	1.48 ± 0.03^{c}	1.49 ± 0.09^{c}	1.71 ± 0.003^{b}	1.90 ± 0.035^{a}	1.84 ± 0.032^{ab}

Values are means ± SD of three replicates. Mean values with different letters in the same row are significantly different ($P < 0.05$).

Table 6
The haematological parameters of grass carp fed different levels of M. suaveolens

	MS0	MS1	MS2	MS3	MS4
HB (g/dL)	9.40 ± 0.05^{d}	9.53 ± 0.01^{b}	9.61 ± 0.02^{bc}	9.73 ± 0.01^{a}	9.70 ± 0.03^{ab}
RBC (10^6/mm^3)	35.58 ± 0.11^{e}	36.59 ± 0.13^{d}	38.47 ± 0.06^{c}	46.787 ± 0.33^{a}	40047 ± 0.08^{b}
WBC (10^3/mm^3)	40.33 ± 0.12^{c}	41.88 ± 0.02^{b}	42.17 ± 0.06^{b}	43.09 ± 0.07^{a}	42.68 ± 0.26^{a}
HCT (%)	17.18 ± 0.76^{c}	18.02 ± 0.34^{c}	19.76 ± 0.28^{b}	21.77 ± 0.07^{a}	21.34 ± 0.44^{a}
MCV (fl)	4.82 ± 0.20^{c}	4.92 ± 0.08^{bc}	5.13 ± 0.28^{ab}	5.37 ± 0.02^{a}	4.56 ± 0.08^{bc}
MCH (pg)	2.64 ± 0.02^{a}	2.60 ± 0.01^{a}	2.49 ± 0.001^{b}	2.07 ± 0.02^{d}	2.40 ± 0.01^{c}
MCHC (g/dL)	54.96 ± 2.83^{a}	52.92 ± 0.95^{ab}	48.65 ± 0.76^{bc}	44.72 ± 72^{c}	45.51 ± 1.03^{c}

Values are means ± SD of three replicates. Mean values with different letters in the same row are significantly different ($P < 0.05$). HB: haemoglobin; RBC: red blood cell; WBC: white blood cell; HCT: haematocrit; MCV: mean cell volume; MCH: mean cell haemoglobin; MCHC: mean cell hemoglobin concentration.

Antioxidant activity

As depicted in Table 7, the liver CAT, SOD, GSH-Px, and T-AOC activities were increased in the MS groups compared with the control ($P < 0.05$). However, the liver MDA levels were decreased by the MS treatments compared to the control ($P < 0.05$).

Table 7
The antioxidant status of grass carp fed different levels of M. suaveolens

	MS0	MS1	MS2	MS3	MS4
MDA (U/mL)	12.2 ± 0.24^{a}	10.40 ± 0.35^{b}	9.94 ± 0.40^{b}	8.95 ± 0.30^{c}	10.37 ± 0.30^{b}
SOD (U/mL)	148.34 ± 0.68^{c}	148.37 ± 0.34^{bc}	149.50 ± 0.08^{ab}	150.34 ± 0.02^{a}	149.32 ± 0.57^{ab}
CAT (U/mL)	39.01 ± 0.31^{c}	39.44 ± 0.15^{bc}	40.04 ± 0.30^{ab}	40.81 ± 0.32^{a}	40.87 ± 0.23^{a}
GSH-PX (U/mL)	84.80 ± 1.12^{c}	97.24 ± 3.40^{b}	110.92 ± 0.48^{a}	112.91 ± 1.17^{a}	115.84 ± 0.28^{a}
T-AOC (U/mL)	4.77 ± 0.25^{c}	6.05 ± 0.05^{b}	6.44 ± 0.26^{b}	7.21 ± 0.22^{a}	6.27 ± 0.03^{b}

Values are means ± SD of three replicates. Mean values with different letters in the same row are significantly different ($P < 0.05$). CAT: catalase enzyme; SOD: superoxide dismutase; T-AOC: total antioxidant capacity; GSH-Px: glutathione peroxidase; MDA: malondialdehyde.

Immunological parameters

As shown in Figs. 3 and 4, the activities of nitroblue tetrazolium, myeloperoxidase, lysozyme, alternative complement, ceruloplasmin, antiprotease, haemagglutination, bacterial agglutination, and total immunoglobulin improved with MS inclusion and acquired the greatest values in the MS3 group ($P < 0.05$).

Digestive enzymes activity

As displayed in Fig. 5, for midgut digestive enzyme activity, compared to the control diet, the increasing level of the MS diet group improved the activities of amylase and cellulose and decreased the activities of lipase in the midgut of grass carp ($P < 0.05$).

Challenge test

During the trial period, there was no mortality. Following an A. hydrophila infection, grass carp given 2 and 4 g/kg MS-enriched diets had high RPS (88 and 86%). On the other hand, 63% of the control fish that were fed the baseline diet perished (Fig. 6).

Gene expression studies

The spleen immune-gene expression of TNFα, β2M, TLR 22, Lysozyme-C, and Lysozyme-G improved with MS inclusion and reached the highest values in the MS3 group ($P < 0.05$), as Fig. 7 illustrates. Consequently, no variation in NKEF-B was noted across any group. Similar patterns were observed in the head kidney gene expression of TNFα, TLR 22, β2M, Lysozyme-G, NKEF-B, and TLR 22 with MS diets ($P < 0.05$). Consequently, there were no variations in Lysozyme-C across any group (Fig. 8).

Discussion

Aquafeeds containing plant or herbal extracts have improved aquatic species' growth, immunological response, and feed consumption [4, 43]. It has been demonstrated that adding powdered or extracted medicinal plants to fish diets enhances appetite, growth, immunity, and stress tolerance [44, 45]. In the current study, the inclusion of grass carp diets has beneficial effects on feed utilization and the growth of the fish. Although there may be a few differences based on the plant species from which the active substance is extracted and the species of fish that are being cultivated, the favorable results are generally consistent with the trend of the use of phytogenics as reported in the literature [46]. Mocanu et al. [47] reported a reduced FCR of grass carp dietary phytogenics supplementation. Since feed accounts for more than half of the overall cost of grass carp production, the FCR is a critical economic measurement. Feed manufacturers often optimize their feed formulas to minimize FCR; however, there may be variances based on aquaculture type, water quality, temperature, and other factors [48]. In the current study, MS supplementation lowers the FCR, whereas other research has shown that the use of phytogenics decreases the FCR [49]. There was an increase in crude lipid and moisture contents of the body composition among the MS group compared with the control. This is comparable to a previously published study that found that the nutritional profile of whole fish is improved by the inclusion of phytogenic feed additives [7]. The largemouth bass increased crude lipid content, which could be partially responsible for the higher HSI.

Blood is widely utilized as a promising indicator of pathological and physiological changes in toxicological research and environmental monitoring [50]. The hematological parameters are commonly studied to assess toxic effects triggered by aquatic pollutants. Fish in aquatic environments are often subjected to a wide range of physical and chemical stresses, which could weaken their immune systems and increase their susceptibility to infections by pathogenic microorganisms [51, 52]. The hematocrit value is an important tool in aquaculture to determine the health status of fish [53]. In the current study, dietary MSE increased the levels of HB, RBC, WBC, HCT, and MCV and reduced the levels of MCH and MCHC in grass carp in all the MS extract treatments compared with the control. Parallel studies have also been recorded in other fish species, including Huso huso [54] and common carp, *C. carpio* [55]. Burducea et al. [46] reported an increase in the levels of HB, RBC, and MCV and a reduction in the levels of MCH in common carp-fed phytogenic feed additives. It is believed that fish with higher serum albumin, globulin, and total protein levels have stronger innate immune responses [53]. Transaminases, including GPT and GOT, are essential for proteins and amino acids; while oxicants cause tissue damage or dysfunction, they can release these enzymes into the plasma [56]. Globulin and albumin are the major plasma proteins in fish. The current study showed an improvement in the levels of TG, TC, TP, GOT, GPT, glucose, albumin, and globulin in grass carp in all the MSE groups compared to the control. This is consistent with earlier research employing quercetin [57], garlic [58], *Avena sativa* [53], and herbal seed [52], all of which elevated serum TG, TC, TP, glucose, albumin, and globulin levels in various fish species. Many studies on herbal and medicinal plants [4, 53] have shown that including herbal seeds in diets has erythropoietic and immune-boosting properties. According to reports, these effects improve cellular

resistance to infections, oxidative stress adaptation, oxygen transport capability, and eventually lower death rates [59].

Overall, the results of the feeding experiment showed that as the content of MS extracts increased, it also increased the activities of the antioxidant enzymes. Although the role of the role of antioxidant enzymes in grass carp growth has not been fully understood, the assessment of antioxidant enzyme activities is frequently done to evaluate an organism's ability to defend against oxidative stress [60]. The present study's results are consistent with earlier research findings that demonstrated enhanced antioxidative mechanisms in fish with the supplementation of plant extract [61]. SOD and AOC are vital antioxidant enzymes that can be found in almost every organism [60, 62]. Improvements in superoxide anion and hydrogen peroxide, along with increases in the activities of CAT, GSH-Px, SOD, and AOC, may be indications of enhanced NADPH-oxidase activity and mass production of reactive oxygen species [63, 64]. Reactive oxygen species are a defense mechanism against microbial infections, but high concentrations can harm an organism's immune system by affecting essential proteins, unsaturated fatty acids, and nucleic acids [60]. Lipid peroxidation produces MDA, which is a byproduct that puts cells under toxic stress and is used as a biomarker for measuring an organism's amount of oxidative stress. Consequently, antioxidant activity is typically associated with a living system defense mechanism. The improved levels of SOD, T-AOC, CAT, and GSH-Px activities indicated that the grass carp may have benefited from the MS extract during inclusion. Our results are in agreement with Hoseinifar et al. [65], who reported an improvement in the activity of SOD, GSH-Px, and AOC and a decrease the activity of MDA in the liver of common carp through dietary supplementation with phytogenic feed additives. Salomón et al. [66] reported that medical plant extract can increase SOD and GSH-Px and decrease MDA in the gut gut of juvenile gilthead seabream. However, Das et al. [67] established that dietary inclusion of coriander oil has beneficial effects on the antioxidant activity of Nile tilapia. Regulation of these selective antioxidant enzymes by MS was detected by the nuclear factor E2-related factor 2/Kelch-like ECH-related protein 1 signaling pathway, which plays an important role in protecting the body from oxidative damage [68].

Fish have a more developed innate immune system than mammals, which is their primary line of defense against a wide range of infections [12]. The humoral immune components respiratory burst, myeloperoxidase, lysozyme, alternative complement, ceruloplasmin, antiprotease, haemagglutination, bacterial agglutination, and total immunoglobulin are closely linked to the adaptive and innate immune responses in fish and are tested to determine the health state of fish, its capacity to stimulate phagocytes and the complement system, as well as its activity towards both Gram-negative and Gram-positive bacteria [12, 40, 42]. Mohseni et al. [40] reported increased myeloperoxidase, lysozyme, and alternative complements due to Japanese eel-fed *Eucommia ulmoides* Oliver bark extracts supplemented. The dietary supplementation of phytogenics can increase lysozyme activity in turbot [69]. In the present study, the dietary supplementation of MS extracts could improve the activities of nitroblue tetrazolium, myeloperoxidase, lysozyme, alternative complement, ceruloplasmin, antiprotease, haemagglutination, bacterial agglutination, and total immunoglobulin in the serum of grass carp. The major organ of fish that synthesizes complement protein is the liver [40]. However, the enhanced liver

health, boosted by increased serum glucose, albumin, and globulin activities, might be responsible for the improvement in the alternative complement activity of grass carp fed MS-supplemented diets. Myeloperoxidase, nitroblue tetrazolium activity, ceruloplasmin,, and antiprotease could activate neutrophils and activate macrophages [70]. Due to its opsonic properties, ability to activate phagocytes and complement systems, and ability to fight Gram-negative and Gram-positive bacteria, an essential component of the innate immune system is lysozyme [71]. Because of this, dietary supplements containing MS may offer a cost-effective and sustainable means of enhancing the innate immunity of grasscar and minimizing their need for chemotherapy while protecting them against various infections.

Nutrients are digested by digestive enzymes like cellulose, amylase, and lipase [40], and it has been reported that food handling can affect enzyme activity [72]. Since the activity of digestive enzymes impacts the effectiveness of nutrient absorption, their characterization offers significant insight into fish's capacity to hydrolyze fat, protein, and carbohydrates in diets. Digestive enzymes are important for fish utilization [73, 74]. A lack of MS in animals has been shown to cause digestive issues in various ways, even though the mechanisms by which MS impacts fish digestive action are unknown. In a study by Almeida-Bezerra et al. [9], the vagus nerve innervates the established enteric neural system of the gastrointestinal tract, which can function autonomously and promote digestive secretions. Thus, deficiencies in MS may impact these areas' metabolic processes, potentially leading to adverse consequences for the gastrointestinal system [68]. Additionally, cellulose conversion and protein digestibility are decreased while a stomach's pH increases [75, 76]. Accordingly, the decrease of lipase and increase of amylase and cellulose in this study could be related to a possible improvement in gastric pH. Additionally, it improves the amount of enzymes secreted into the midgut [7], which is responsible for the increase of midgut amylase and cellulose and the reduction of midgut lipase activity in MS fed the control diet. Improvement in digestive enzymes activated by dietary herbal extract inclusion has been reported in grass carp [77, 78], tilapia [79], and white leg shrimp [60]. It has high growth efficiency in fish. To address the association between the growth performances of grass carp fed varied doses of dietary MS extract and the digestive enzymes, more research is required.

In the current study, grass carp exhibited increased resistance to A. hydrophila in a dose-dependent manner in dietary MS extract, even after intraperitoneal injection of 1×107 cells/mL of the pathogen. Compared to 36% in the control diet, the survival rate increased to 47%, 63%, 88%, and 86% in MS1, MS2, MS3, and MS4. Das et al. [67] showed that dietary phytogenic supplementation improved the survival rate of Nile tilapia against A. hydrophila. MS mainly causes damage to bacterial membranes, which leads to the loss of membrane potential, respiratory activity, and efflux activity, and finally results in cell death [9]. Besides, MS extracts also increase the immunological state by controlling the innate immune indicators, including lysozyme and immunoglobulin expression, in grasscarp. Previously, Sattanathan et al. [80] documented that *C. aerea* in *L. rohita* exhibits anti-fungal and anti-bactericidal effects against fish infections. However, dietary supplementation with phytogenics could show an increased survival percentage after confronting *A. hydrophila* [81]. Therefore, an increased survival percentage after conflict with *A. hydrophila* has been shown in rainbow trout supplemented with Coriandrum sativum extract [82].

The immunomodulation mechanism in grass carp infected with *A. hydrophila* is still unclear. In response to immunization with *A. hydrophila*, we examined the expression levels of immune-related genes (NKEF-B, Lysozyme-C, Lysozyme-G, TNFα, TLR 22, β2M) in the grass carp head kidney and spleen in the present study. Because the head kidney is the leading main lymphoid tissue for the B-lymphocyte line and the spleen is a prominent peripheral lymphoid organ, these organs are appropriate for investigating the expression of such genes in vivo [42, 83]. Major antibacterial enzymes called lysozymes are crucial to fish's innate immunity. The study of fish lysozymes helps manage pathogen-induced fish illnesses. TLR22 and β2M, exclusive to antigen-presenting cells and activating CD4 + T cells [84], are helpful markers for comprehending the processes of epitope recognition and tracking the migration of immune-related cells. Following immunization, there was a significant rise in TCRα expression levels [85]. In the grass carp, the mRNA levels of expression NKEF-B, Lysozyme-C, Lysozyme-G, TNFα, TLR 22, and β2M improved after 14 days of A. hydrophila challenge supplemented with MS extract. After A. hydrophila infection, Lysozyme, TNFα, and β2M are thought to exacerbate the inflammatory response in grass carp. The explanation of species-specific immune reactions in *A. hydrophila* could be found in the expression patterns of immune-related genes.

Conclusion

In conclusion, dietary MS supplementation enhanced growth performance, whole body composition, immunological responses, hematological parameters, digestive ezymes, and antioxidant status in grass carp. As fed to fish at 2 and 4 g/mg, it improves the growth performance, whole body composition, immunological responses, hematological parameters, digestive ezymes, and antioxidant status of grass carp. However, the consistency and quality of MS in fish feed may be impacted by the ingredient, diet production method, methods of extraction, and components. Grass carp were strongly pathogenicized by *A. hydrophila*, while grass carp seemed to have increased gene expression following *A. hydrophila* infection. Our findings offer important insights into the immunological response mechanism of A. hydrophila-infected grass carp. Consequently, there is increasing interest in various encapsulation techniques to enhance the stability of MS in fish feed and shield them from interactions with the host, food, and environment. But MS can be beneficial in reducing illness and assisting in the avoidance of antibiotic use, which can cause microorganisms to become resistant to drugs. Still, further research is needed to understand how these immune-related genes function.

Abbreviations

CAT
catalase enzyme
CF
Condition factor
DGR
Daily growth rate
FE

Feed efficiency
FCR
Feed conversion rate
FW
final weight
GSH-Px
glutathione peroxidase
GOT
glutamic oxalate transamisnase
GPT
glutamic pyruvate transaminase
HB
haemoglobin
HCT
haematocrit
HSI
Hepato-somatic index
MDA
malondialdehyde
MCH
mean cell haemoglobin
MCHC
mean cell hemoglobin concentration
MCV
mean cell volume
MS
Mesosphaerum suaveolens
NG
net growth
RBC
red blood cell
SGR
Specific growth rate
SR
Survival rate
SOD
superoxide dismutase
TG
total triglycerides
TC

total cholesterol
TP
total protein
T-AOC
total antioxidant capacity
VSI
Visero-somatic index
WBC
white blood cell
WGR
Weight gain rate.

Declarations

Ethics approval and consent participate

The St. Joseph University, Nagaland, India (SJU/ZOO/12/2023) Ethics Committee on Animal Experimentation accepted the protocols for in-vivo studies.

Authors' contributions

Govindhrajan Sattanathan: conceptualization, writing - original draft, methodology, data curation, review & editing, software analysis. Muniyappan Madesh : writing - original draft, methodology, software, review & editing, software analysis, Hairui Yu: conceptualization, investigation, supervision, project administration, funding acquisition, review & editing. Swaminathan Padmapriya: resources, methodology, data curation, review & editing, software analysis. Demin Cai:methodology, data curation, review & editing, Venkatalakshmi: methodology, data curation, review & editing, software analysis, Rajesh Ramasamy: investigation, and formal analysis.

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Consent for publication

Not applicable.

Competing interests

The authers declare that they have no competing interests.

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Figures

Figure 1

Morphology of *Mesosphaerum suaveolens*

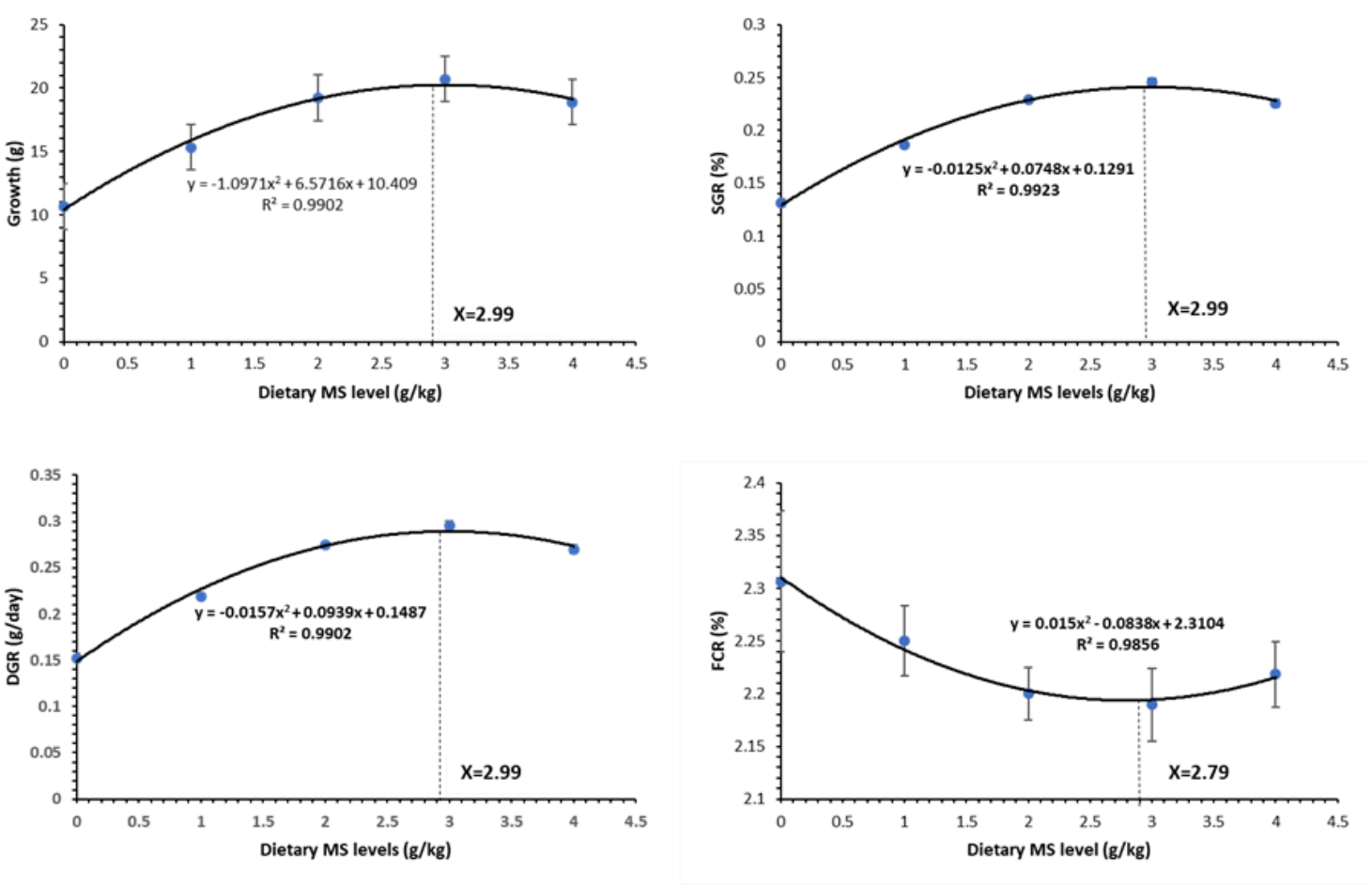

Figure 2

Growth, Specific growth rate, daily growth rate and Feed conversion rate were analyzed using polynomial regression while grass carp were fed increasing amounts of dietary MS extracts for 10 weeks. G, SGR and, DGR determined the dietary MS extract needed to be 2.99 g/kg and FCR determined the dietary MS extract needed to be 2.79%.

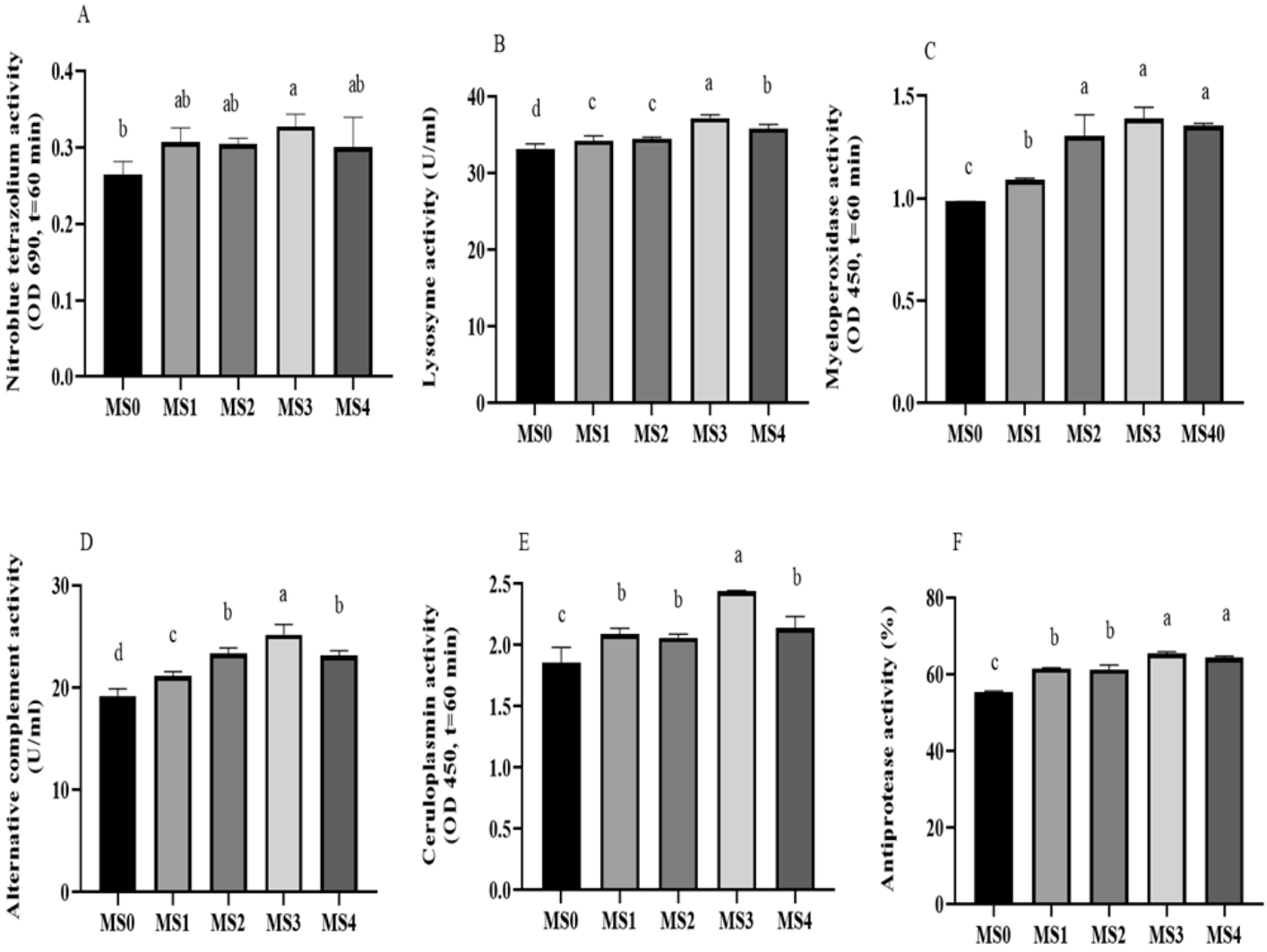

Figure 3

Effects of *M. suaveolens* leaf extract (MS) on immunological parameters grass carp. (A) Nitroblue tetrazolium. (B) Lysozyme. (C) Myeloperoxidase. (D) Alternative complement. (E) Ceruloplasmin. (F) Antiprotease. Data are presented as means ± SD.

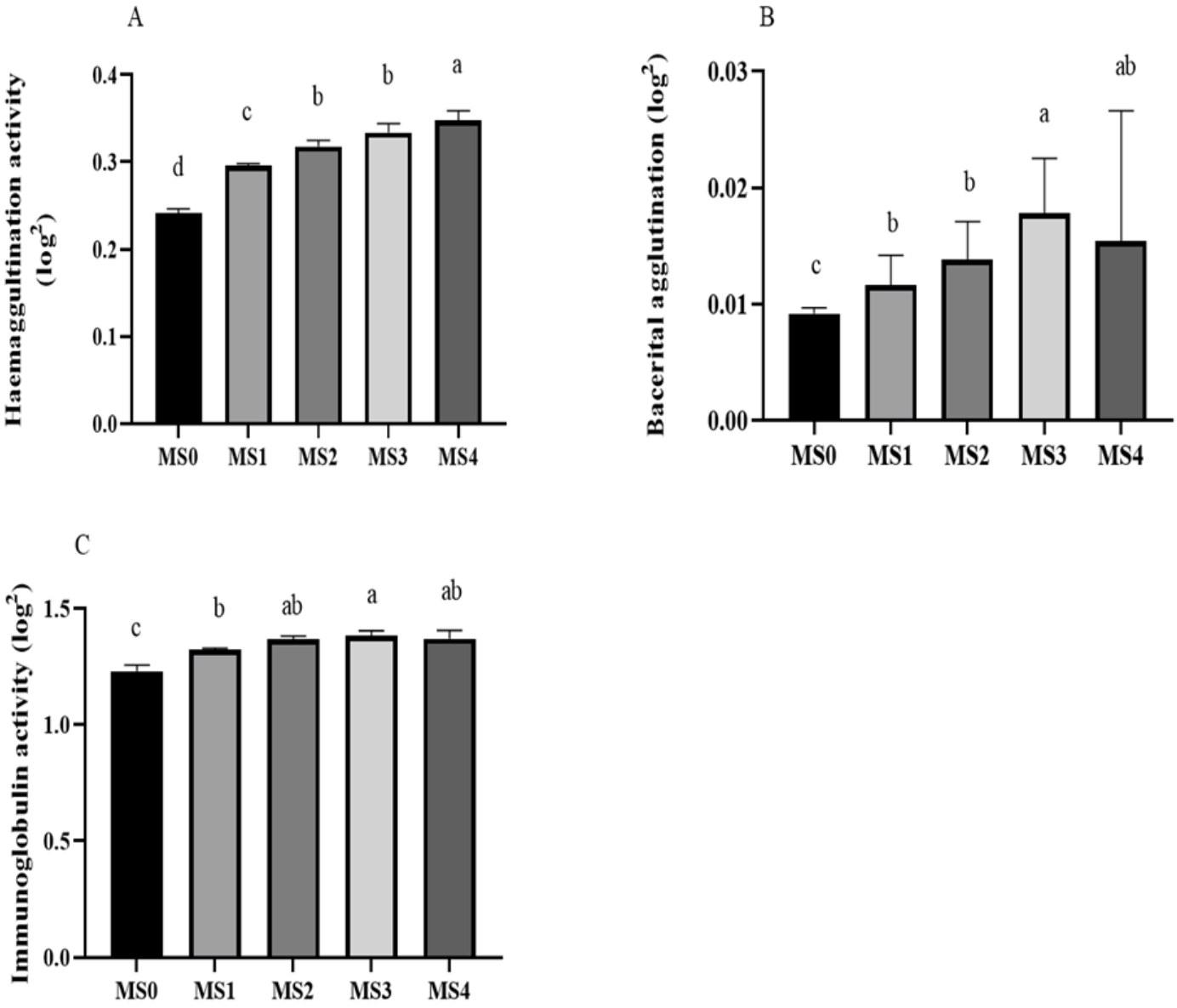

Figure 4

Effects of *M. suaveolens* leaf extract (MS) on immunological parameters grass carp. (A) Haemagglutination. (B) Bacterial agglutination. (C) Total immunoglobulin. Data are presented as means ± SD.

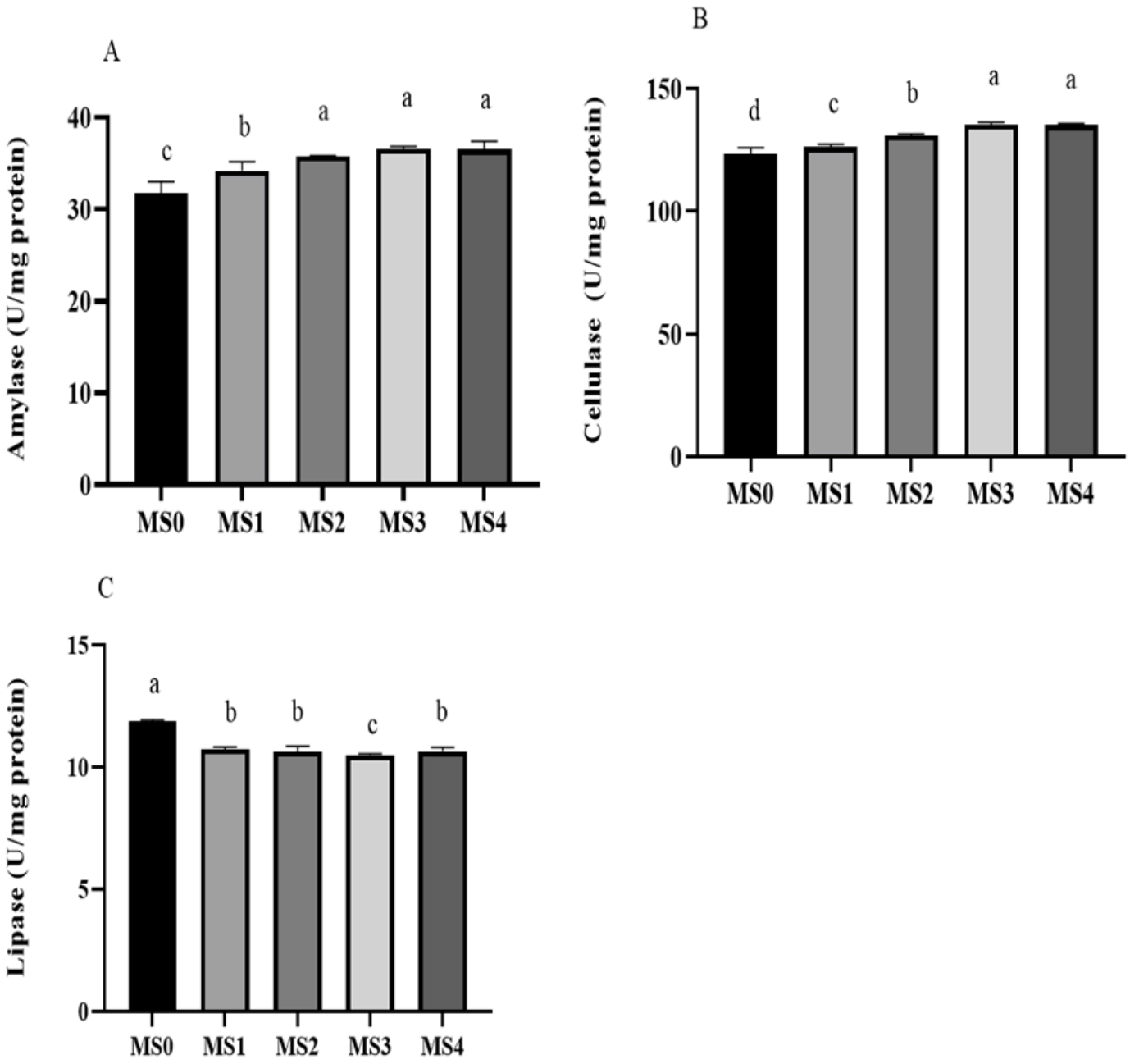

Figure 5

Effects of *M. suaveolens* leaf extract (MS) on Antioxidant activity grass carp. (A) Amylase. (B) Cellulase. (C) Lipase. Data are presented as means ± SD.

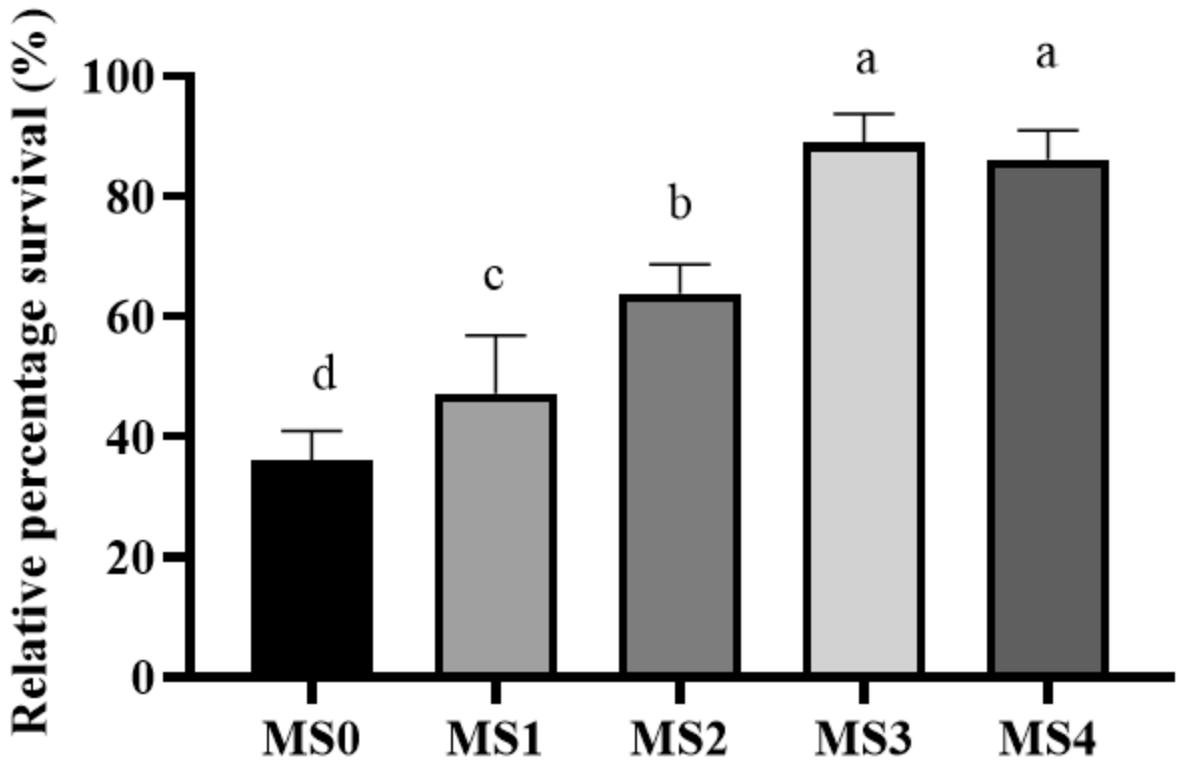

Figure 6

Relative percent survival rate (%) of grass carp post feeding with five experimental feeds with increasing level of *M. suaveolens* leaf extract such as 0, 1, 2, 3, 4 g/kg in control and treatment MS0, MS1, MS2, MS3 and MS4 groups respectively after intraperitoneal injection of 1×10^{7} cfu/mL of *Aeromonas hydrophila* recorded up to a period of 14 days.

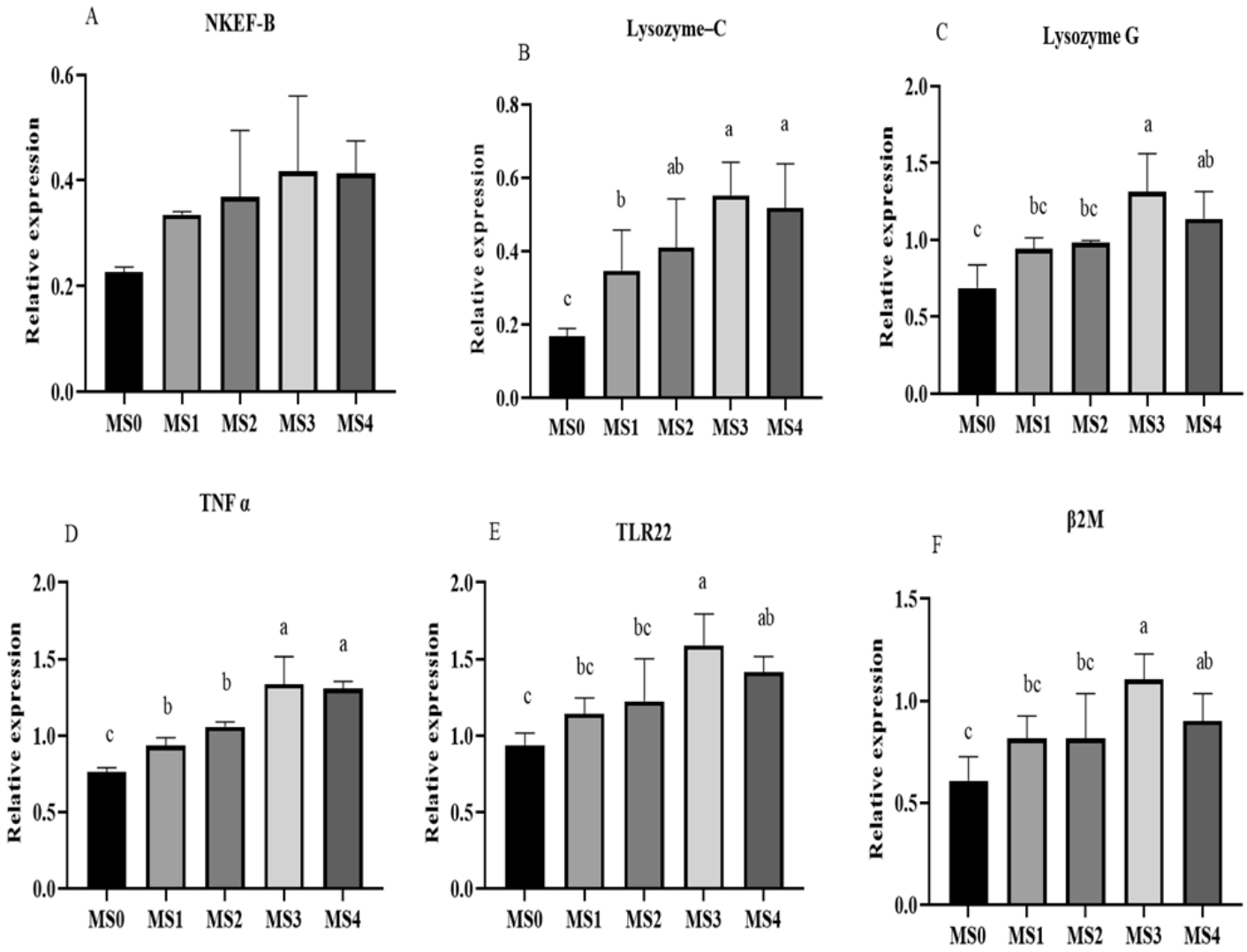

Figure 7

Relative mRNA expression levels (fold-change) for grass carp, immune-relevant gene. (A) NKEF-B. (B) Lysozyme-C. (C) Lysozyme-G. (D) TNFα, (E) TLR 22, (F) β2M in spleen tissue at after *A. hydrophila* injection 14 days. mRNA expression levels are normalized to the reference gene, β-actin. Data are presented as means ± SD.

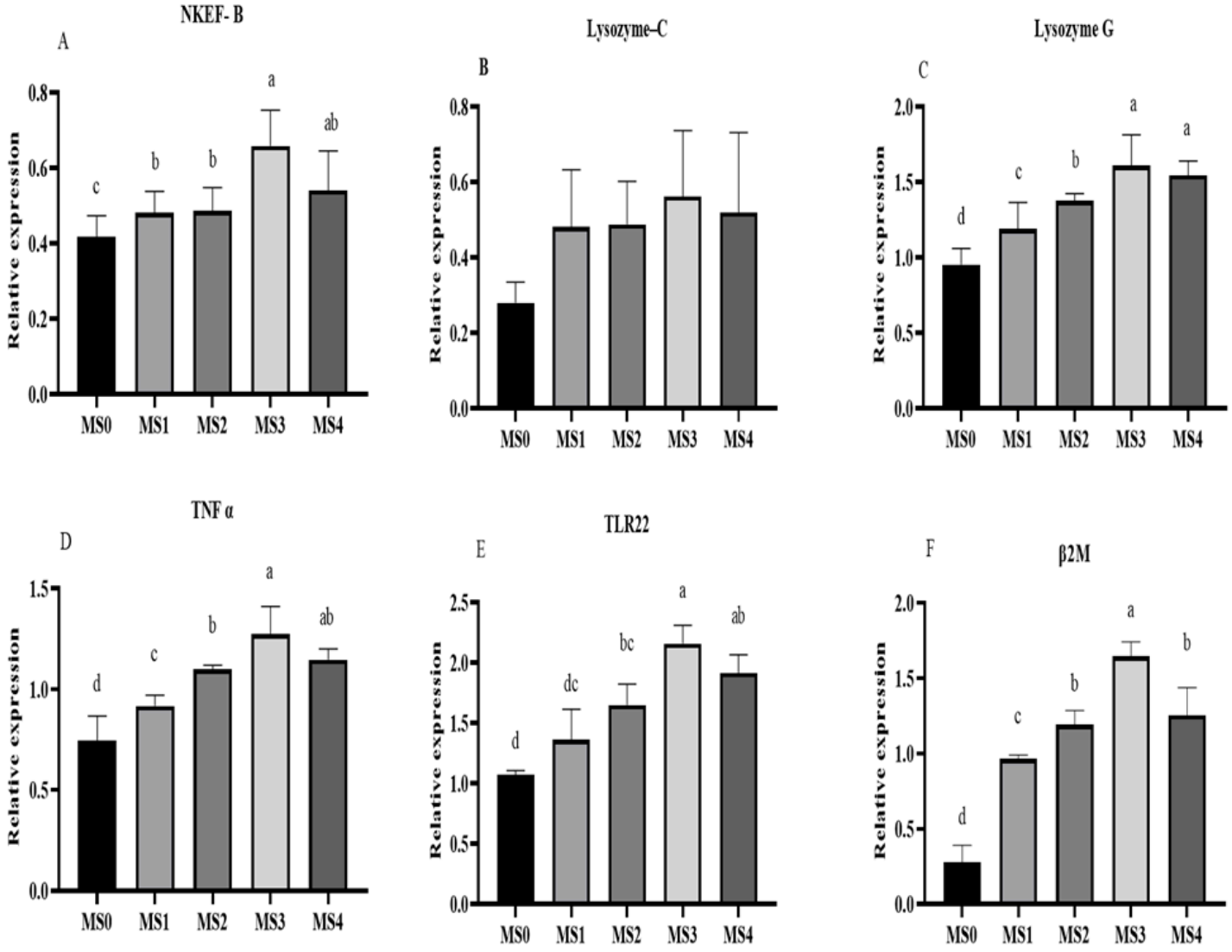

Figure 8

Relative mRNA expression levels (fold-change) for grass carp, immune-relevant gene. (A) NKEF-B. (B) Lysozyme-C. (C) Lysozyme-G. (D) TNFα, (E) TLR 22, (F) β2M in Head kidneytissue at after *A. hydrophila* injection 14 days. mRNA expression levels are normalized to the reference gene, β-actin. Data are presented as means ± SD.