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EFFECTS OF DIETARY ROSEMARY (*ROSMARINUS OFFICINALIS*) LEAF POWDER AND *BACILLUS SUBTILIS* ON THE GROWTH PERFORMANCE, DIGESTIVE ENZYMES, ANTIOXIDANT AND IMMUNE-RELATED GENE EXPRESSION, AND RESISTANCE TO *AEROMONAS HYDROPHILA* IN COMMON CARP, *CYPRINUS CARPIO*

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Abstract

Due to increasing demand about the application of natural feed additives in aquaculture, the efficacy of separate and the combination of rosemary (*Rosmarinus officinalis*) and *Bacillus subtilis* on the growth performance, blood indices, innate immunity, antioxidant defense, digestive enzymes activity, the expression levels of genes with immunity modulation, as well as resistance against *Aeromonas hydrophila*, in common carp, *Cyprinus carpio* is reported. Fish (44.63±0.04 g) were divided into four treatments including the T0 (control treatment) fed with basal diet, T1 fed with rosemary at the rate of 3%, T2 fed with 1.5×10^7 CFU g^{-1} *B. subtilis*, and T3 fed with a combination of rosemary at the rate of 3% and 1.5×10^7 CFU g^{-1} *B. subtilis.* After 56 days enriched feed with *B. subtilis* and combination of rosemary and *B. subtilis* caused significant improvement in weight gain (WG %), feed conversion ratio (FCR), hematological and biochemical indices, the innate immune responses including total Ig level, lysozyme and ACH50 activities as well as the chymotrypsin, pepsin, protease, and amylase activities correlated to the control group (P<0.05). Additionally, the serum CAT and GPX activities were significantly enhanced in the T3 group (P<0.05). The mRNA expression of *CAT* and *LYS* genes significantly increased in T2 and T3 contrary to T0 (P<0.05). Fish challenged with *A. hydrophila* had variable survival and the best survival was in the T3. In summary, the combination of the rosemary and *B. subtilis* enhanced immune parameters and the ability of fish to cope with *A. hydrophila* as a fish pathogen. These findings indicate the potential of these substances as a promising feed additive.

Key words: *Rosmarinus officinalis*, *Bacillus subtilis*, common carp, feed additives, immune response, *Aeromonas hydrophila*

The utilization of antibiotics has become a common trend for the treatment and control of bacterial fish diseases and to enhance growth performance. Nevertheless, the unconscious use of antibiotics causes the emergence of antibiotic-resistant strains, antibiotic residues in aquatic products and consequently elevated food safety concerns and various problems in the aquatic environment (Larsson and Flach, 2022; Mello et al., 2022; Wang et al., 2023). In this circumstance the enhancement of immunity via immune stimulants is crucial to optimize resistance against infectious diseases and reduce the reliance on antibiotics (Dawood and Koshio, 2018). Researchers have made efforts to find a cost-effective, natural, and environmentally safe substitution for replacement of the antibiotics in aquaculture such as probiotics (Dawood and Koshio, 2016; Van Doan et al., 2019) and herbal plants (Vallejos-Vidal et al., 2016; Hoseini et al., 2019; Ahmadifar et al., 2021). Medical plants are an alternative approach to disease control, to mitigate the adverse effects of other ways (Vijayaram et al., 2024). The existence of numerous bioactive compounds has been extensively proven in relation to the enhancement of fish growth, stimulation of appetite, modulation of immunity, as well as their antimicrobial and anti-stress properties (Vallejos-Vidal et al., 2016; Hoseini et al., 2019; Awad and Awaad, 2017; Ahmadifar et al., 2019 b; Ghafarifarsani et al., 2021, 2022).

Rosemary (*Rosmarinus officinalis*) is known for its medicinal properties (Gao et al., 2014; Zoral et al., 2017). Numerous evidence demonstrated that this plant possesses various beneficial effects, such as anti-inflammatory, anticancer, and antioxidant activities (Gao et al., 2014; Takayama et al., 2016; Zoral et al., 2017).

So far a multitude of research studies have demonstrated that probiotics as a variety of beneficial microorganisms have an important place among these alternative feed supplements and their effectiveness has been proven in many fish and shrimp species today (Yilmaz et al., 2022; Abdel-Latif et al., 2022; Ahmadifar et al., 2020). The bacterial species classified within the genera *Bacillus*, *Lactobacillus*, and *Bifidobacterium* hold significant relevance in the realm of probiotic feed additives due to their capacity to colonize the intestinal microbial milieu, in addition to their proficiency in synthesizing vitamins, bacteriocins, organic acids, growth-enhancing metabolites, and digestive enzyme excretions. Probiot-

ics as natural immune stimulators and growth promoters have been extensively confirmed in aquaculture. They can regulate immune-related genes, increase pathogen resistance, and improve non-specific immunity (Dawood and Koshio, 2016). *Bacillus* species are widely employed owing notable capacity to withstand various conditions and remarkable functional attributes (Dawood et al., 2019).

Common carp (*Cyprinus carpio*) is a major culture species all over the world with high economic benefits (FAO, 2018). It is known that adding the combination of herbs and probiotics to fish feeds has shown synergistic effects on immunity status in common carp (Abdel-Latif et al., 2023). In previous studies rosemary (Yousefi et al., 2019; Gao et al., 2014; Zoral et al., 2017) and *Bacillus subtilis* (Lee et al., 2017; Won, 2020) the individual incorporation of rosemary and *B. subtilis* into aquaculture feeds has been demonstrated to yield beneficial outcomes concerning piscine health and developmental progress; however, there exists a paucity of research investigating the implications of their simultaneous inclusion in aquatic diets on overall fish well-being and growth metrics. The efficacy of separate and the co-addition of rosemary and *B. subtilis* on the growth, hematological and biochemical parameters, innate immunity status, antioxidant defense, digestive enzymes activity, resistance against *Aeromonas hydrophila*, and expression levels of genes with immunity modulation in common carp is studied.

Material and methods

Provision of rosemary (*Rosmarinus officinalis*) and probiotic *Bacillus subtilis*

Initially, rosemary leaves were gathered and transported to the laboratory and were oven-dried at a temperature of 45°C for 72 hours. Following this, the dried leaves were ground using a mill, and passed through the mesh, then stored in a zip pack at 4°C (Yousefi et al., 2019).

To add the probiotic, *B. subtilis* (PTCC 1204) bacteria provided from the Iranian Research Organization for Science and Technology (IROST), were cultured in MRS medium (Basingstoke, UK) at 30°C for 24 h. After incubation, the bacterial cells were centrifuged (20 minutes at 2000 rpm), the supernatant was discarded and the sediment bacteria were mixed with 9% sodium chloride, and this was added to the diet (Kumar et al., 2008). The ground plates were dried and stored at 4°C. To mix, first, 7.4 grams of each probiotic substance was dissolved in 200 cc of distilled water and 5.51 grams of gelatin in 200 cc of fully dissolved water was added to the rest of the distilled water and the prepared solution was slowly added to the diet until it took the form of a paste. It was dried in a closed space and away from direct sunlight, and finally, the feed was placed at –20°C.

Experimental diet

Four enriched diets were provided by adding rosemary (*Rosmarinus officinalis*) leaf powder and *B. subtilis* to the basal diet (Table 1) including control group (T0), rosemary at the rate of 3% (Yousefi et al., 2019) (T1), 1.5×10^7 CFU g^{-1} *B. subtilis* (Kumar et al., 2008) (T2), and combination of 3% rosemary and 1.5×10^7 CFU g^{-1} *B. subtilis* (T3). The components were mixed by a grinder and transformed into pellets, subsequently subjected to air-drying and preserved in bags at a temperature of 4°C until they were used. The approximate composition of the diets was verified in accordance with the established protocols (AOAC, 1998).

Table 1. Ingredients and composition of common carp experimental diets (Hoseinifar et al., 2016)

Ingredient	%	Composition	%
Fish meal	40	Dry matter	89.5
Wheat flour	21	Crude protein	38.22
Soybean meal	13.5	Crude lipid	13.24
Wheat gluten	5.5	Ash	3.45
Soybean oil	6	Fiber	11.2
Fish oil	6		
Mineral premix[a]	3		
Vitamin premix[a]	2		
Binder[b]	2		
Anti fungal[c]	0.5		
Antioxidant[d]	0.5		

[a]Vitamin mixture (g/100 g vitamin mix except vitamins A and D_3): vitamin A, 180 000 IU; vitamin D_3, 60 000 IU; vitamin E, 4; vitamin C, 6; K_3, 0.3; thiamine, 0.8, riboflavin, 0.6; niacin, 2; pyridoxine, 0.4; pantothenic acid, 3.5; biotin, 0.5; folic acid, 0.3; cyanocobalamin, 0.8; inositol, 2; Mineral mixture (g/100 g mineral mix): Fe, 2.5; Mg, 3; K, 3.5; Mn, 1.7; Co, 0.05; Cu, 0.5; Se, 0.25; I, 0.1.

[b]Amet binder™, Mehr Taban-e-Yazd, Iran.

[c]ToxiBan antifungal (Vet-A-Mix, Shenan-doah, IA).

[d]Butylated hydroxytoluene (BHT) (Merck, Germany).

Fish and experimental design

One hundred and eighty healthy juvenile common carp (44.63±0.04 g) were acquired from a local farm situated in Golestan (Iran), taken to the laboratory (Technical and Vocational Center, Gorgan) and placed in tanks with a capacity of 300 liters each. Throughout 14 days of acclimatization, they were provided with the basal diet and were randomly assigned to 12 fiberglass tanks (150 liter). Fifteen pieces of fish were allocated in each tank and supplied with continuous aeration. Over the course of 56 days, the fish of four treatments were fed (3% body weight) daily. In order to regulate the feeding rate, the biomass of the fish was measured and recorded every two weeks. The tanks underwent daily siphoning and were replenished with half a volume of fresh water. The measurement of water physicochemical factors (dissolved oxygen, temperature, and pH levels) in the experimental period was conducted utilizing a portable apparatus (WTW, Munich, Germany). These parameters were meticulously maintained at values of 7.13±0.21 mg/l, 23.9±0.3°C, and 7.4±0.7, respectively.

Growth performance

The growth performance was measured at the end of the 56 days. Fish were anesthetized by clove oil (100 mg/l, Sigma Aldrich, Germany) and weighed. The following factors were determined by the following formula (Yousefi et al., 2019; Chekani et al., 2021; Abiri et al., 2022).

Survival rate (%) = [(final number of fish) ÷ (initial number of fish)] × 100.
Weight gain (WG, g) = W2 – W1 / 56 (days)
Specific growth rate (SGR) (%/day) = [(Ln Final weight - Ln initial weight) ÷ time] × 100.
Feed conversion ratio (FCR) = Feed intake ÷ Weight gain

Blood sampling

Six fish were chosen in each tank and put under anesthesia with clove oil (100 milligram per liter) to gather blood (2 ml) via the puncture of caudal vein with non-heparinized syringes (Hajek et al., 2006). The collected blood was transferred into heparinized (5 mg/ml blood, Sigma Aldrich, Germany) and non-heparinized tubes. The blood samples were placed in non-heparinized tubes for a duration of 2 hours. Following this, the sera were separated through centrifugation at a force of 1600 g for 10 minutes (Labofuge, Heraeus Sepatech, Germany). Subsequently, the separated sera were stored at a temperature of –20°C. On the other hand, the heparinized blood was promptly utilized for the purpose of conducting hematological and biochemical analyses.

Biochemical and hematological analysis

The red blood cells (RBC) and white blood cells (WBC) were enumerated utilizing a Neubauer hemocytometer. Hematocrit (Hct) was assessed through the utilization of the standard microhematocrit technique. Hemoglobin levels (Hb) were quantified utilizing the cyanomethemoglobin spectrophotometry procedure outlined by Blaxhall and Daisley (1973). The blood indicators, inclusive of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured (Wintrobe, 2008). Differential leukocyte counts (monocytes, lymphocytes, neutrophils, and eosinophils) were carried out by the preparation of the blood smears (Sepperumal and Saminathan, 2013).

Blood total protein, albumin, triglyceride, cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were evaluated applying commercial kits (Pars Azmoon Company, Tehran, Iran) and a biochemical autoanalyzer (Eurolyser, Belgium). The globulin amount was determined by diminishing the albumin level from the total protein (Binaii et al., 2014).

Immunological assays

Total immunoglobulin (total Ig) levels of serum were calculated subsequent to polyethylene glycol precipitation, employing the methodology outlined by Siwicki (1993). To assess the activity of serum lysozyme, a turbidimetric assay was implemented as claimed by Ellis et al. (1990) employing *Micrococcus luteus* (Sigma, USA) as a target in 0.05 M phosphate buffer (pH = 6.2). Serum alternative complement (ACH50) activity was ascertained using the approach outlined by Yano et al. (1992). This method involved using rabbit red blood cells (RRBC) as the designated target.

Antioxidant assays

Glutathione peroxidase (GPX) activity was examined through the utilization of a protocol that involves the oxidation of NADPH (Greenwald, 2018). The measurement of superoxide dismutase (SOD) was conducted by applying SOD Kit (Sigma-Aldrich, 19160) as claimed by the manufacturer's directions (Shahkar et al., 2015). The measurement of catalase (CAT) activity was achieved by the spectrophotometric determination of H_2O_2 (Goth, 1991). Malondialdehyde (MDA) activity was assessed through the Buege and Aust (1988) technique.

Digestive enzymes activity

Lowry method was used to measure protein concentration for the samples (Lowry et al., 1951). Elisa kits (Eastbiopharma Company, China) were used to measure trypsin and chymotrypsin activity. The amylase activity was detected by the former method (Bernfeld, 1955). Briefly, the samples (50 µl) were blended with starch solution (250 µl) and incubated (30 minutes at 37°C). Dinitro salicylic acid 1% (0.5 ml) was incorporated into the mixture and boiled. The mixture was cooled and finally, absorbance was reported at 540 nm. To assay the activity of lipase, fish homogenate (20 µL) was mixed with 60 µL p-nitrophenyl butyrate (50 mM) and 1 mL buffer (Tris-HCl 50 Mm and ethanol 4%). Absorbance was constantly reported at 405 nm for 5 min (Bulow and Mosbach, 1987). The Sheikhzadeh et al. (2012) method was used to measure protease activity. First, fish homogenate was mixed with reaction buffer (ammonium bicarbonate 100 mM and azocasein 0.7%). After that trichloroacetic acid (4.6%) was added to the mixture and centrifuged (10 min at 5000 g). Finally, the superficial materials were removed and blended with NaOH (0.5 M) and absorbance was reported at 450 nm (Worthington, 1991).

RNA extraction, cDNA, and RT-PCR

For analysis of the expression of antioxidant (*CAT* and *SOD*) and immune-related genes (*LYS* and *TNFα*), samples of intestine and liver (100 mg) were employed to separate the total RNA. This was accomplished by utilizing a kit (Gene All Kit, Gene All Biotechnology, Seoul, Korea). Subsequently, the purity and quantity of obtained RNA were measured spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, USA) at 260/280 nm. The first-strand complementary DNA (cDNA) was performed by reverse transcription using the protocols of a commercial kit (transcription kit, GeneAll Biotechnol-

ogy Co., Ltd., Korea), and the protocol prescribed by the manufacturer was strictly followed throughout this process. Designing the primer sets was carried out based on the sequences obtained from the GenBank database for common carp (Table 2). The quantitative real-time PCR assay was performed to determine the relative expression of the selected genes utilizing the SYBR green method. The normalization of the expression levels of the selected genes was achieved through the use of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as the housekeeping gene. The analysis of the relative mRNA levels was conducted according to the methodology described by Livak and Schmittgen (2001).

Challenge study

A. hydrophila (PTCC 1890), provided by the IROST was cultured in tryptic soy broth (TBS) medium at 37°C for 24 hours, and then adjusted to a concentration of 1×10^8 CFU/mL, as previously described by Soltanian and Fereidouni (2016). During 8 weeks five fish were selected from each group and subjected to intraperitoneal injection of the bacteria (0.1 mL) and 15 fish from the T0 were injected with 0.1 mL of PBS as a negative control. During the 14-day post-challenge period cumulative mortality was calculated. Dead fish carcass specimens were subjected to cultivation on tryptic soy agar to authenticate the existence of *A. hydrophila* (Sadeghi et al., 2021). The measurement of the RPS was performed by Amend prescribed methodology (1981):

RPS (Relative percentage of survival) = 100 – (Test mortality/Control mortality) ×100

Statistical analysis

The Kolmogorov–Smirnov test was used to evaluate the data normality distributions. Polynomial regression was utilized to analyze the growth performance parameters. Effects of dietary rosemary and *B. subtilis* were tested by one-way ANOVA and Duncan's tests. All data were analyzed by SPSS 21.0 (SPSS Inc., USA) and expressed as mean ± standard deviation (SD). Kaplan–Meier survival analysis was performed to determine the mean survival time of fish injected with *A. hydrophila*, and differences were assessed using the log-rank (Mantel-Cox) test for pairwise comparisons (Kaplan and Meier, 1958).

Results

Growth performance

Table 3 represents the results of the growth performance. The final body weight and weight gain (WG) of carps that were fed with *B. subtilis* (T2) and a combination of rosemary and *B. subtilis* (T3) were significantly higher than the control group (T0) ($P<0.05$); however, there was no notable difference between carps fed with only rosemary (T1) and T0 ($P>0.05$). The FCR of T2 and T3 was significantly lower than the T0 ($P<0.05$). The SGR and survival rate exhibited no remarkable difference in various treatments ($P>0.05$).

Hematology and biochemistry

The outcome of common carp hematological assays after the challenge period is shown in Table 4. The count of RBC rose markedly in carp that were fed with *B. subtilis* (T2) and a combination of rosemary and *B. subtilis* (T3) in contrast to the control group (T0). The count of WBC and the hematocrit were elevated significantly in the T3 group contrary to the T0 group ($P<0.05$). Fish in T1 group displayed non-significant difference with the T2 group ($P>0.05$). The hemoglobin amount boosted markedly in fish fed T1, T2, and T3 diets in comparison with the control group ($P<0.05$). MCV boosted significantly in the T3 group; conversely, the supplemented diets did not affect MCH and MCHC. The counts of monocyte, lymphocyte, neutrophil, and eosinophil in common carps revealed no significant difference among different treatments ($P>0.05$). The results of the biochemical analysis have been presented in Table 5. The total serum protein levels of carps subjected to T1, T2, and T3 treatments showed higher values contrary to the control group ($P<0.05$), and the highest total protein was recorded in the T3 group. The blood creatinine and albumin were not affected by supplemented diets while the blood triglyceride decreased considerably ($P<0.05$) in the supplemented groups in contrast to the control group. The levels of cholesterol and glucose in carps in the T2 and the T3 groups were lower significantly ($P<0.05$) in comparison with T0.

Table 2. The list of primers for the expression of selected genes in common carp

Gene	Primer	Length (pb)	Tm (°C)	NCBI Reference
Catalase	F: 5' TGGTGGATAATAACAGTTGGG 3' R: 5' ACACGATACAACACTGCTGC 3'	21 20	55.26 58.85	XM_019068441.1
Superoxide dismutase	F: 5' TGTGGGGTTCTGCCTCTTG 3' R: 5' TGGGAACATAGTGAGGGAGA 3'	19 20	59.54 57.06	XM_019081090.1
Lysozyme	F: 5' CCACTCTCCATCTGAACTCC 3' R: 5' ACATCAGACACAAGACAGCAA 3'	20 21	57.02 57.80	XM_019104788.1
Tumor necrosis factor-α	F: 5' AGGAGGAAGTCAGGCGTCT 3' R: 5' AGCGTGAAGCAGACAGCAG 3'	19 19	57.89 57.89	XM_019080541.1

Table 3. Growth performance and feed utilization of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0)

T	IW (g)	FW (g)	WG (g)	SGR (%/day)	FCR	Survival rate (%)
T0	43.72±2.58 a	79.94±3.35 c	36.21±3.59 c	1.00±0.10 a	2.00±0.19 a	100
T1	44.91±2.42 a	82.46±7.25 bc	37.54±6.28 bc	1.00±0.12 a	1.96±0.33 ab	100
T2	44.47±3.04 a	90.21±5.49 ab	45.73±6.20 ab	1.17±0.14 a	1.59±0.20 cb	100
T3	44.50±2.93 a	91.78±3.94 a	47.27±5.96 a	1.20±0.15 a	1.54±0.20 c	100

IW: initial weight; FW: final weight; WG: weight gain; SGR: specific growth rate; FCR: food conversion ratio; SR: survival rate. Values in row assigned with different letter denote significant difference (P<0.05). Data are presented as mean ± SD.

Table 4. Hematological indices of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0)

T	RBC (10^6/ml)	WBC (10^3/ml)	Htc (%)	Hb (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Monocytes (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)
T0	1.25± 0.05 c	3.25± 0.12 b	38.8± 0.63 b	7.4± 0.36 b	309.37± 28.5 b	59.57± 4.1 a	19.30± 0.96 a	2.5± 1.04 a	13.66± 1.63 a	79.33± 5.92 a	1.5± 0.54 a
T1	1.31± 0.04 bc	3.46± 0.13 ab	41.8± 2.31 b	8.2± 0.21 a	317.64± 11.9 ab	62.42± 1.4 a	19.67± 0.78 a	3.66± 1.21 a	14.50± 1.51 a	81.00± 2.60 a	1.5± 0.54 a
T2	1.37± 0.02 ab	3.18± 0.12 b	43.8± 4.95 b	8.2± 0.23 a	319.73± 33.0 ab	60.49± 2.5 a	19.12± 2.40 a	4.00± 0.89 a	14.16± 3.12 a	79.33± 5.35 a	1.6± 0.51 a
T3	1.40± 0.06 a	3.65± 0.16 a	49.1± 1.94 a	8.7± 0.45 a	350.55± 1.4 a	62.24± 2.1 a	17.77± 0.85 a	4.00± 1.41 a	13.5± 1.87 a	80.50± 4.03 a	1.5± 0.54 a

RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

Values in row assigned with different letter denote significant difference (P<0.05). Data are presented as mean ± SD.

Table 5. Biochemical indices of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0)

T	Creatinine (mg/dl)	Albumin (mg/dl)	Triglyceride (mg/dl)	Cholestrol (mg/dl)	Glucose (mg/dl)	Total protein (mg/dl)
T0	0.40±0.014 a	8.42±0.37 a	141.83±10.83 a	122.67±4.41 a	145.0±6.87 a	20.37±1.51 c
T1	0.42±0.036 a	8.46±0.26 a	105.17±4.665 b	114.17±3.65 ab	135.3±5.64 ab	23.01±1.00 bc
T2	0.44±0.028 a	8.29±0.33 a	101.33±4.844 b	108.0±8.46 bc	122.6±8.47 bc	23.61±1.77 b
T3	0.42±0.028 a	8.44±0.39 a	96.16±13.40 b	103.1±6.79 c	110.1±13.3 c	29.83±2.11 a

Values in row assigned with different letter denote significant difference (P<0.05). Data are presented as mean ± SD.

Figure 1 depicts the outcomes of the ALP, ALT, and AST enzymes. Determination of serum ALP revealed non-significant difference between the various dietary treatments and the control group (P< 0.05), whereas the ALT decreased significantly in the T3 group. Additionally, AST showed notably lower levels in the T2 and the T3 groups.

Immune response

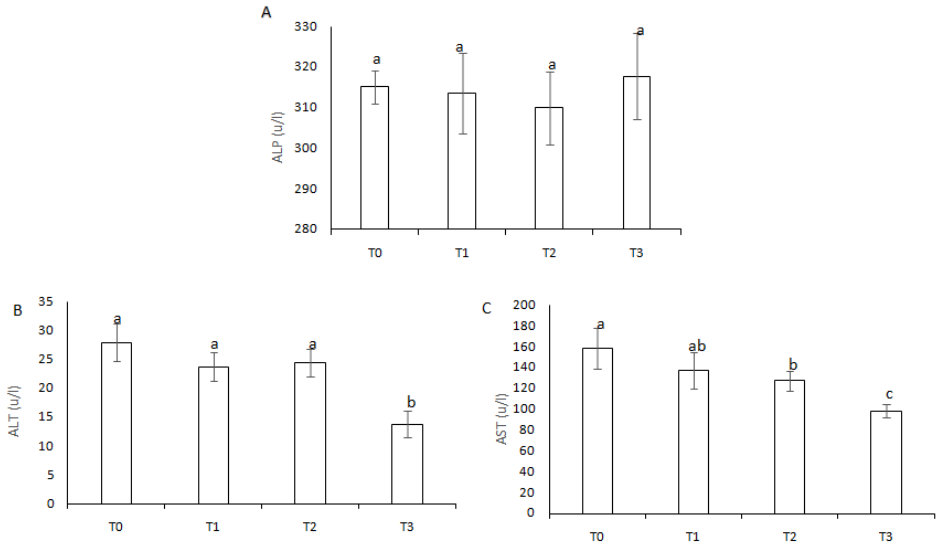
Serum immune parameters in common carp after 56 days are presented in Figure 2. Total Ig level, lysozyme activity, and ACH50 activity were significantly enhanced in the T3 group, in comparison with the control group (P<0.05). The lysozyme activity of fish in T1, T2, and T3 showed significantly higher values than the control group (P<0.05). Whereas the ACH50 values were observed more only in the T3 group while no marked difference was found in T1 and T2 as correlated to the control group.

Antioxidant enzymes

Serum antioxidant enzymes activity in common carp after 56 days is given in Figure 3. The CAT, GPX, and MDA activity was higher significantly in T2 and T3 in comparison with T0 (P>0.05). SOD was significantly decreased in the supplemented treatments as related to the control group (P>0.05).

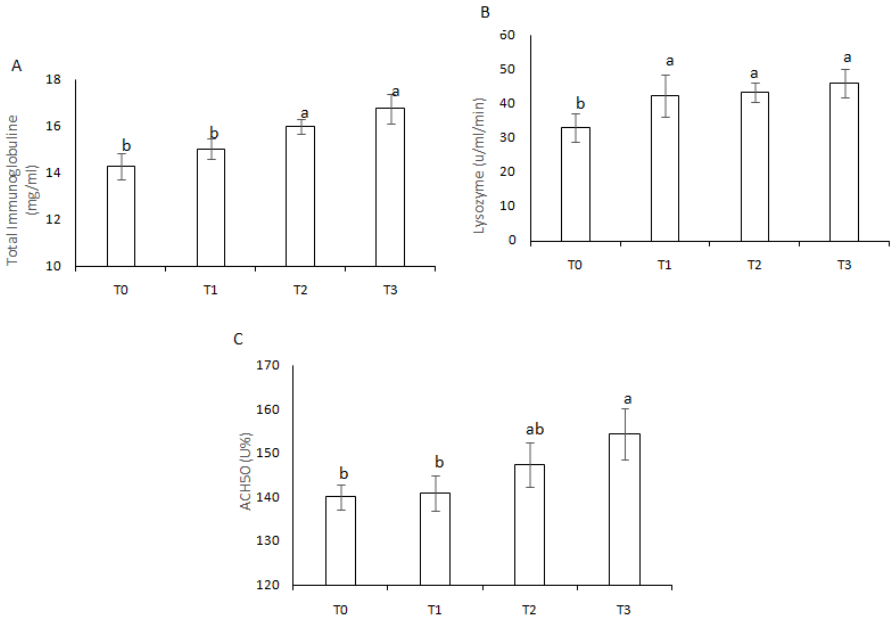
Digestive enzyme activity

Table 6 exhibits the results of digestive enzyme activity. Utilizing enriched diets led to a significant enhancement in the chymotrypsin, pepsin, protease, and amylase activities in fish fed in the T3 group contrary to the control group (P<0.05). Conversely, trypsin and lipase activities had no notable difference among the groups (P>0.05).



(A): Serum ALP, (B): serum ALT, (C): serum AST.

Figure 1. Liver enzymes level of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0). Data are expressed as the mean ± SD



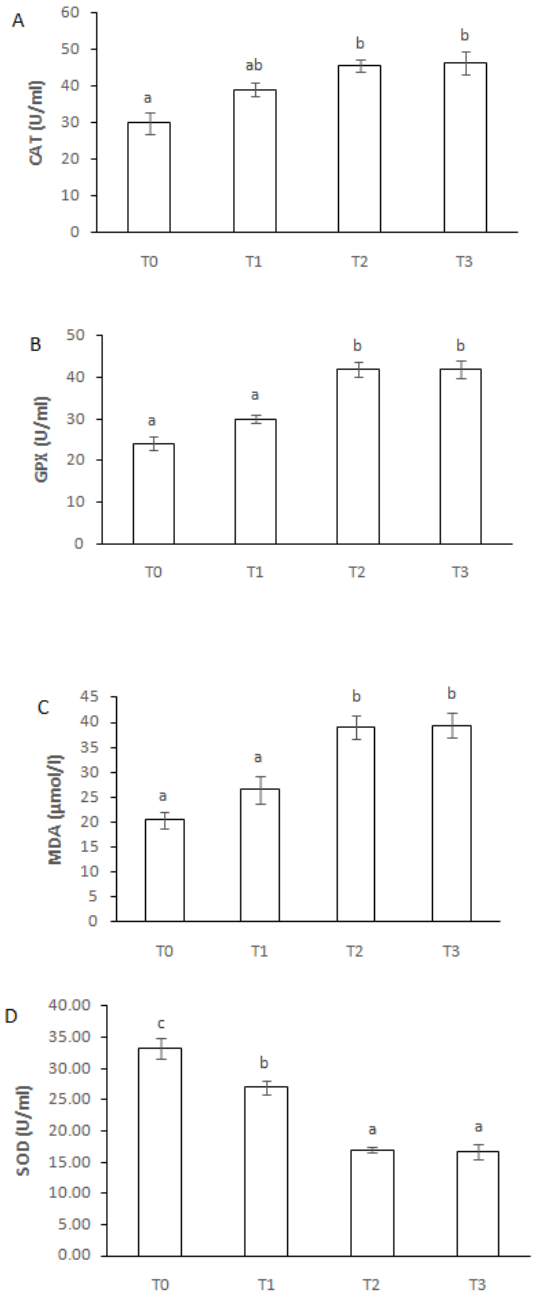
(A): serum total immunoglobulin, (B): serum lysozyme, (C): serum ACH50.

Figure 2. Serum immune parameters of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0). Data are expressed as the mean ± SD.

Table 6. Digestive enzyme activities of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0)

T	Trypsin (μ/mg protein)	Chymotrypsin (μ/mg protein)	Pepsin (μ/mg protein)	Protease (μ/mg protein)	Amylase (μ/mg protein)	Lipase (μ/mg protein)
T0	0.112±0.001 a	0.018±0.001 c	0.055±0.004 b	5.05±0.31 b	7.22±0.08 b	1.82±0.100 a
T1	0.111±0.006 a	0.019±0.001 bc	0.059±0.006 ab	5.17±0.34 b	7.33±0.13 b	1.82±0.100 a
T2	0.111±0.001 a	0.022±0.001 b	0.063±0.005 ab	5.30±0.30 b	7.46±0.22 b	1.79±0.106 a
T3	0.114±0.005 a	0.027±0.002 a	0.066±0.007 a	5.93±0.47 a	8.40±0.30 a	1.96±0.184 a

Values in row assigned with different letter denote significant difference (P<0.05). Data are presented as mean ± SD.

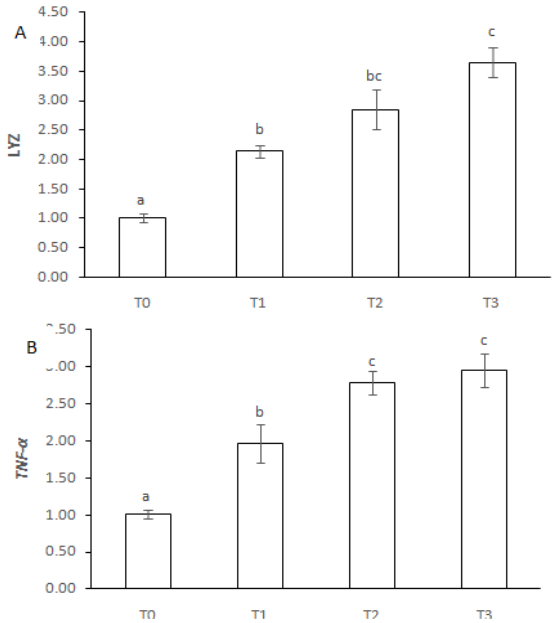


(A): CAT, (B): GPX, (C): MDA, (D): SOD.

Figure 3. Antioxidant enzymes of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0). Data are expressed as the mean ± SD

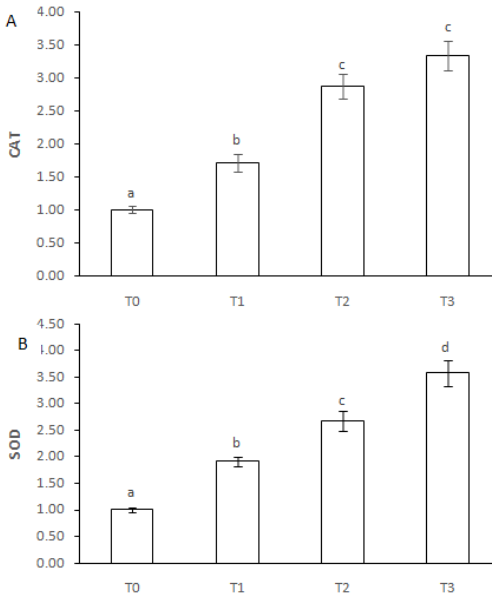
Gene expression

The effects of dietary supplementation on expression of immune-related and antioxidative-related genes are presented in Figures 4 and 5, respectively. The *LYS* gene expression had a remarkable difference in T3 ($P>0.05$). The expression of pro-inflammatory cytokines *TNF-α* was higher significantly in T1, T2 and T3 in comparison with T0 ($P>0.05$). Relative expression detected in the *CAT* gene, was remarkably upregulated in the T2 and T3 contrary to T0 ($P<0.05$). The expression of the *SOD* gene had insignificant difference in various groups ($P>0.05$).



(A): *LYS*, (B): *TNF-α*.

Figure 4. The transcription of immune-related genes of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0). Data are expressed as the mean ± SD



(A): *CAT*, (B): *SOD*.

Figure 5. The transcription of antioxidative-related genes of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0). Data are expressed as the mean ± SD

The challenge against *A. hydrophila* infection

Fish challenged with *A. hydrophila* had variable survival rate and common carp fed the combination of rosemary and *B. subtilis* had the best survival rate contrary to the control group ($P<0.05$).The mortality rate was lower in fish of all groups compared to the control group, and this difference was significant ($P<0.05$) and the highest was recorded in the control group (Table 7 and Figure 6).

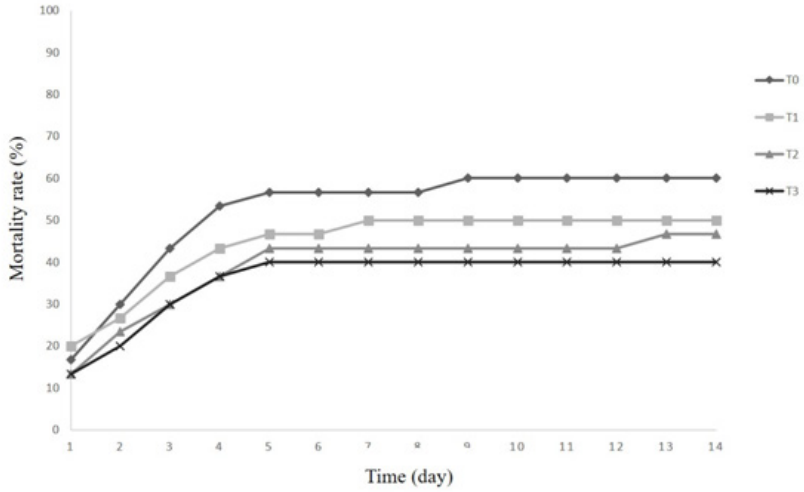


Figure 6. Survival (%) of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0), 14 days post challenge with *Aeromonas hydrophila*

Table 7. Survival rate (%) of common carp fed with dietary rosemary (T1) and *B. subtilis* (T2) separately and in combination (T3) for 56 days in comparison with control group (T0)

Treatment	Mortality rate (%)	Survival rate (%)	RPS
T0	60.00 a	40.00	–
T1	50.00 b	50.00	16.67
T2	43.33 ab	56.67	27.78
T3	40.00 b	60.00	33.33

Values in row assigned with different letter denote significant difference (P<0.05).

Discussion

Medicinal plants and probiotics have undergone extensive research for their potential efficacy in stimulating growth and immunity in fish. This investigation aimed to identify the effects of separate and the co-addition of rosemary (*Rosmarinus officinalis)* and *B. subtilis* on the growth performance, hematological parameters, innate immunity, antioxidant defense, digestive enzymes activity, immune-related gene expression, and resistance against *A. hydrophila* in common carp.

The findings showed that the mixture of the rosemary and *B. subtilis* (T3) significantly improved the growth performance as well as the activities of chymotrypsin, pepsin, protease, and amylase in comparison to the T0 (P<0.05). Consequently, the heightened presence of digestive enzymes in the T3 group may contribute to the improved utilization of nutrients and the enhanced performance of the common carp. It is widely recognized that *Bacillus* species elevates the production of cofactors, vitamins, and enzymatic activity (Han et al., 2015). Beneficial bacteria possess the capability to release exoenzymes such as protease and lipase, thereby assisting in the process of digestion Additionally, these microorganisms aid in the decomposition of indigestible compounds, produce antibacterial materials and vitamins, and exert a substantial impact on both growth and survival rates (Ringø et al., 2020). Furthermore rosemary is a medicinal herb that is rich in growth promoters and antioxidants (Zoral et al., 2017).

Consequently, the co-addition of rosemary and *B. subtilis* could have boosted the growth through augmenting the feed digestibility and absorption. Previous studies have revealed that rosemary (Gao et al., 2014; Zoral et al., 2017) and *B. subtilis* (Lee et al., 2017; Won, 2020) supplementations significantly enhanced digestive enzyme activities and growth performance in fish species. Nevertheless, according to our knowledge so far, there are no reports regarding the usage of the co-addition of rosemary and *B. subtilis* as a growth promoter supplement in common carp feed. To assess the impact of additives, it is customary to examine not only the growth performance and activity of digestive enzymes but also the immune responses, serum indices (Yılmaz et al., 2022; Sarhadi et al., 2020).

The results obtained from the hematological experiment revealed a notable raising in the WBC count in fish fed with *B. subtilis* and both rosemary and *B. subtilis*-supplemented diets. In the same way *Bacillus*-enriched diet elevated WBCs in Nile tilapia (Hassaan et al., 2018). In this investigation, it is noteworthy to mention that the inclusion of a supplemented diet did not result in any

significant alterations to the number of monocytes, lymphocytes, neutrophils, and eosinophils in common carp which can be related to the absence of infection. However, a significant boosting in hematological values, such as RBC counts, hematocrit ratios, and hemoglobin levels, was observed in carp that were fed with *B. subtilis* (T2) and a combination of rosemary and *B. subtilis* (T3).

As it was mentioned previously biochemical parameters analysis can be considered as a fundamental biomarker for evaluating the health status of fish (Yilmaz and Ergün, 2018). Serum and/or tissue enzyme activities are accepted as important indicators of tissue damage, for example ALT, AST, and ALP are closely related with liver status, whereby elevation of these enzymes is indicative of liver injury (Ueno and Komatsu, 2017). In this investigation, serum ALP showed no significant difference, however, it was observed that both ALT and AST levels decreased markedly in fish fed with both rosemary and *B. subtilis* (T3), which showed that the examined additives have no harmful effect on the liver function.

Total protein of carps in dietary treatments showed higher values contrary to the T0, and the highest amount was seen in the T3 group. It was proven that the total protein is the representative of a majority of the serum immunity (Binaii et al., 2014). Likewise, the consumption of *Bacillus* in the diet has been documented to enhance the concentrations of total protein of Nile tilapia (Hassaan et al., 2018).

Dietary administration of a combination of the rosemary and *B. subtilis* markedly improved the levels of total Ig, lysozyme, and ACH50 activities compared to T0 ($P<0.05$). Increases in these factors are usually assumed to be connected to heavier immune responses in fish (Yilmaz and Ergün, 2018). In the current investigation, the incorporation of probiotics into the diet was observed to enhance the expression of genes associated with immune function. These results substantiate the immunomodulatory properties attributed to probiotic supplementation in *Cyprinus carpio*. The outcomes align with the findings of several previous studies, which suggest that dietary enhancement with probiotic supplements may bolster the innate immune response (Dawood et al., 2019; Abdel-Latif et al., 2023) and expression of immune-related genes (Won et al., 2020; Krishnaveni et al., 2021) in fish.

The findings of our investigation exhibited an elevation in the lysozyme activity in fish that were provided with supplemented diets. The lysozyme activity functions by assaulting the peptidoglycans present in the cell wall of bacteria, thereby impeding semi-adverse effects on the host (Esteban, 2012). Correspondingly, an increase in the lysozyme activity was observed when the common carp consumed rosemary (Yousefi et al., 2019). The administration of probiotics, prebiotics, and immune stimulators to various fish species is well known to augment their immune responses (Adel et al., 2016; Dawood et al., 2020 a; Srichaiyo et al., 2020). Additionally, the results of the study conducted on common carp revealed that when rosemary and *B. subtilis* were given for eight weeks, the mortality rate subsequent to *A. hydrophila* infection decreased. In the same way, Yousefi et al. (2019) documented that the mortality rate of fish challenged with *A. hydrophila* was diminished when they were provided with dietary rosemary. The enhanced immune responses exhibited against pathogens are often attributed to the effectiveness of probiotics and herbal additives in fish or shellfish culture.

In accordance with Yousefi et al. (2019) in the present investigation, serum CAT was markedly increased in the T2 and T3 as well as GPX and dietary administration of rosemary led to a decrease in SOD activity. The enzymatic antioxidant protectors, namely catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX), are integral to the processes of detoxification, while malondialdehyde (MDA) is commonly employed as a biomarker for assessing lipid peroxidation (Oberley and Oberley, 1988). The results of the present investigation can be correlated with the antioxidant properties exhibited by probiotics. Prior literature has demonstrated that probiotics are capable of synthesizing various metabolites including glutathione, exopolysaccharides, folate, and butyrate, which have been shown to augment the antioxidant potential in aquatic species (Hoseinifar et al., 2020).

It was found that the expression of *CAT* was significantly enhanced in the T2 and T3, and *LYS* gene expression had a remarkable difference in T3 contrary to T0 ($P<0.05$). The expression of the *SOD* gene had insignificant difference in various groups ($P>0.05$). Immunity is a crucial physiological mechanism of fish to protect against infections and provide balance and homeostasis, which is bisected into innate (non-specific) and the acquired (specific) immune system immunity. The innate immune system is a fundamental defense mechanism of fish that is divided into physical barriers, cellular and humoral components. The humoral parameters encompass a range of factors such as growth inhibitors, diverse lytic enzymes, and components of the complement pathways, agglutinins, precipitins, natural antibodies, cytokines, chemokines, and antibacterial peptides (Magnadóttir, 2006). The inflammatory response made by cytokines is a substantial important part of the cellular immune response in fish. Inflammatory and pro-inflammatory cytokines, including TNF-α, are secreted from immune cells in disease and stress conditions to regulate the immune response and inflammation in the body.

The expression of pro-inflammatory cytokines *TNF-α* was higher significantly in supplemented groups in comparison with T0 ($P>0.05$). Similarly, dietary green tea powder increased the expression of *TNF-α* of rainbow trout (*Oncorhynchus mykiss*) (Nootash et al., 2013). Also, it has been reported that Nile tilapia (*Oreochromis niloticus*) fed with blackberry syrup revealed upregulated *TNF-α* (Yilmaz, 2019). *TNF-α* is responsible for the regulation of the immune response by cell proliferation, differentiation and apoptosis (Dawood et al., 2020 b).

Based on Choi et al.'s study (2018) on tilapia, *TNF-α* expression is increased after exposure to micro plastics, which is expected to regulate apoptosis and immune response. The increased expression of inflammatory genes in disease and stress conditions play a role in the regulation of apoptosis and immune response (Hollerova et al., 2023; Choi et al., 2018). In the study of Mousavi et al. (2021), following the consumption of the grape seed extract (GSE) the expression of *C3* and *Lys* genes was upregulated in the three studied mucosal surfaces in rainbow trout fed with GSE.

The disease challenges are typically employed to assess the potential of utilizing medicinal herbs (Ahmadifar et al., 2019 a, c). The infection caused by *A. hydrophila* poses a significant threat to the aquaculture industry as it leads to an elevation in the mortality rate (Zhou et al., 2019). It was indicated that the combination of rosemary and *B. subtilis* significantly enhanced the resistance of common carp after being experimentally infected with *A. hydrophila*. The findings showed a significant decrease in cumulative fish mortalities after an observation period of 14 days. They were endorsed by enhancement of immune responses, antioxidant parameters, and expression of immune and antioxidant-related genes in fish that were fed with probiotic and herbal supplemented diet. The benefits of probiotics, herbal additives, and their combination to enhance the fish diseases resistance might be due to their activating immunity by releasing cytokines from macrophages and lymphocytes (Lin et al., 2019; Krishnaveni et al., 2021), stimulation of humoral immune functions (Lee et al., 2017), and secretion of antimicrobial metabolites that serve as a protective shield in intestine against pathogenic bacteria. However, a better understanding of the probiotic ingredients for enhancing the immunity and resistance to disease in fish might require further studies.

In the study of Sheikhzadeh et al. (2011), following the consumption of green tea (*Camellia sinensis*) extract, the resistance of common carp against *Yersinia ruckeri* increased, which indicates the boosting of the immune system of fish. The results of this research are consistent with the above results. Furthermore Soltani et al. (2010) studied the effect of *Zataria multiflora* essential oil on the immune response of common carp against *A. hydrophila*, which showed the increased immunity in fish.

Conclusion

To conclude, this study indicated that the administration of a combination of the rosemary and *B. subtilis* for 8 weeks in common carp could effectively promote both growth and immunity and can be applied as a promising feed additive to enhance the resistance of common carp in possible outbreaks. It is recommended that future studies investigate the effect of this combination on histopathology and other genes.

Availability of data and materials

All obtained data are available upon request from the corresponding author.

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Conflicts of interest

All authors state that they have declared no conflicts of interest**.**

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