Effects of probiotic *Bacillus subtilis* and *B. licheniformis* on water quality, growth, physiology, gene expression, and disease resistance in *Sparus aurata* fingerlings

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This study evaluated the effects of a probiotic blend of *Bacillus subtilis and
Bacillus licheniformis*, administered as a water additive, on the growth
performance, feed efficiency, body composition, blood biochemistry,
histology, gene expression, and resistance to *Vibrio parahaemolyticus* infection
in *Sparus aurata* fingerlings. A total of 240 healthy fingerlings (6.10 ± 0.06 g) were
distributed into 12 tanks (3 tanks per group), with 20 fish per tank. Over a period of
10 weeks, the fish were exposed to four treatments with increasing probiotic
concentrations (0, 0.01, 0.02, and 0.03 g/m^3), designated as Control, BSL1, BSL2,
and BSL3, respectively. The water additives of BSL significantly increased the
dissolved oxygen (mg/L) in a dose-dependent manner, while the values of TAN
were significantly reduced by increasing the levels of BSL in the water. The NH_3
levels were the lowest in BSL2 and BSL3 compared to other groups; however,
BSL1 was lower than the control group. The BSL3 group exhibited higher growth
performance (final body weight, BWG, survival rate) compared to the other
groups ($P < 0.05$). Adding BSL significantly improved the crude protein and ash
content in S. aurata, while it significantly reduced the lipid content ($P<0.05$). BSL
also significantly improved blood hematology parameters (PCV, RBCs, and Hb)
and immune responses (phagocytic activity, phagocytic index, lysozyme activity,
IgM, total Ig, and WBCs) in a dose-dependent manner ($P<0.05$) compared to the
control group. Blood biochemical parameters (Total protein, albumin, globulin,
and glucose), digestive enzymes (amylase and lipase) and antioxidant status (TAC,
SOD, CAT) were significantly increased in a dose-dependent manner with
increasing levels of probiotic in the water ($P<0.05$). Liver enzymes and MDA

were significantly decreased by BSL-water addition (P<0.05). BSL enhanced the intestinal structure integrity of *Sparus aurata*. The addition of *B. subtilis* and *B. licheniformis* probiotics significantly improved the growth factors (IGF-1, IGF-2, and GHR) and immune-related genes (TNF-α, IL-1β, and IL-10) compared to the control group (P<0.05) in a dose-dependent manner. Importantly, probiotic-treated fish exhibited increased resistance to *V. parahaemolyticus* infection. These findings suggest that water addition of *Bacillus subtilis* and *Bacillus licheniformis* probiotics at a concentration of 0.2-0.3g/m3 improved the growth and overall health of Sparus aurata by regulating the immune responses and antioxidant status.

Introduction

The ultimate goal of aquaculture is to achieve high production while maintaining maximum profitability. However, the expansion and intensification of aquaculture operations have generated concerns about physiological state and potential systematic difficulties with disease outbreaks on farms (Chauhan and Singh, 2019; Darafsh et al., 2020). The gilthead seabream (*Sparus aurata*) is one of the most extensively farmed marine fish, and it is popular with consumers (Torrecillas et al., 2024). Decades of research and development have developed aquaculture techniques for gilthead seabream, resulting in efficient and dependable output. While the gilthead seabream is well-known for its adaptability, growth, nutritional value, and flavor, modern aquaculture procedures have included improvements to improve growth and reduce bacterial infections (Tzortzatos et al., 2024).

Aquaculture has encountered significant challenges due to the widespread occurrence of infectious diseases during fish farming operations. To mitigate bacterial infections, there has been a substantial reliance on antibiotics (Yu et al., 2023). *Vibrio parahaemolyticus* is commonly found in temperate and tropical coastal waters, is a major pathogen responsible for considerable economic losses in aquaculture production (Millard et al., 2021; Eissa et al., 2025a). Numerous studies have documented antibiotic resistance in *Vibrio* species, particularly resistance to drugs such as ampicillin, ceftriaxone, and imipenem, which have been isolated from farmed shrimp (Costa et al., 2015). The use of antibiotics, however, poses risks not only to aquatic animals but also to human health, primarily due to the accumulation of antibiotic residues in seafood products (Li et al., 2017; Abd El-Aziz et al., 2024; Eissa et al., 2024b; Mathew et al., 2025). In response, regulatory restrictions on antibiotic use have intensified global research efforts aimed at identifying alternative strategies, particularly the use of functional feed additives. These alternatives include antimicrobial peptides, plant-derived extracts, probiotics, prebiotics, and synbiotics, all of which are being explored for their potential to enhance fish health without the drawbacks of antibiotics.

Among these alternatives, probiotics have gained considerable attention as a sustainable method for controlling infectious diseases in aquaculture (Eissa et al., 2023; Hendam et al., 2023). Their application offers several advantages, including enhancing water quality, promoting digestion, immune function and improving fish growth, Probiotics used in aquaculture water function by altering the microbial ecology, inhibiting infections, and increasing fish health via several processes: competitive pathogen exclusion, antimicrobial compound production, immunological system stimulation, water quality improvement and biofilm formation (Wuertz et al., 2021). By stimulating digestive enzyme activity and supporting a balanced intestinal microbiota, probiotics also contribute to improved feed conversion efficiency in aquatic species, making them a valuable tool for advancing sustainable aquaculture practices. This results in increased nutrient use efficiency, and enhanced fish reproduction (Abuljadayel et al., 2023). Probiotic supplementation also improves appetite and organism digestion (Banerjee and Ray, 2017). *Bacillus licheniformis* and *B. subtilis* and bacteria are crucial probiotic additions that help aquatic animals develop and operate normally by delivering vitamins, minerals, and digestive enzymes (Monier et al., 2023). These parameters improve feed consumption, nutritional absorption, and growth performance (Eissa et al., 2024a). *Bacillus* species offer a range of beneficial effects in aquaculture, notably enhancing feed utilization, producing and releasing exogenous enzymes, and promoting the growth of beneficial gut microbiota that support intestinal physiological functions (Dighiesh et al., 2024). As a result, fish fed diets supplemented with various *Bacillus* strains have demonstrated notable improvements in growth performance indicators (Soltani et al., 2019; Redhwan et al., 2024). In addition, modulation of the intestinal microbial community—by reducing harmful bacteria and increasing beneficial populations—can strengthen both innate and adaptive immune responses while maintaining intestinal integrity in the host (Hoseinifar et al., 2019). Therefore, this study aims to investigate the impacts of *B. subtilis* and *B. licheniformis* probiotics

Material and methods

Diet and experimental design

This research was conducted at a private fish farm in Ismailia Province, Egypt. Two hundred forty Sea bream (initial average body weight 6.10 ± 0.06 g) were randomly assigned to four experimental groups (three replicates per treatment). The fish were housed in 12 fiberglass tanks (1 m³) with 20 fish per tank. The control group was without supplementation. The three treatment groups received supplementation with Bacillus species probiotics, which are commercially sold as SANOLIFERPRO-W (a mixture of *Bacillus subtilis* and *B. licheniformis* (BSL) at 5×10^5 CFU/g; INVE Aquaculture, Belgium) at levels of 0.01, 0.02, and 0.03 g/m³ water, respectively. Fish were fed on a basal diet consisted of a commercial fish feed sourced from Aller Aqua (ALLER MARINE 42/15 EX) (https://www.aller-aqua.com). The given feed was divided equally into three portions and offered to fish three times a day (8.00, 12.00, and 16.00 h). The chemical composition of this feed was: Crude protein (%); 42, Crude fat (%); 15, NFE (%); 24.4, Ash (%); 7.4, Fibre (%); 3.2, P (%); 1.1, Gross energy (MJ); 20.6 and Digestible energy (MJ); 14.8. Tanks were equipped with compressed air through air stones using air pumps. A daily water change rate of 25% for the control group and 5% for the treatment groups was used throughout the ten-week experiment.

Physico-chemical analyses of water

Water quality was monitored throughout the 10-week experiment. A SensoDirect150 MultiMeter was utilized to evaluate salinity, dissolved oxygen, temperature, and pH. Nitrogenous compounds (NH_3 and TAN) were analyzed with a DREL 2000 spectrophotometer (HACH) following (APHA. American Public Health Association, 1998) guidelines.

Fish performance and feed utilization

Fish growth performance and feed utilization were evaluated using standard equations established by (Cho and Kaushik, 1990), and further referenced by (Eissa et al., 2024a). Key parameters assessed included average weight gain (AWG), average daily gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (%). These metrics are essential for accurately assessing growth efficiency, nutrient utilization, and the overall health and performance of the fish.

Body composition and blood analysis of experimental fish

The proximate composition of the experimental fish crude protein, dry matter, ash, and crude lipid was determined following (AOAC, 2000) protocols. At trial end, three fish per replicate (n = 9 per treatment) were frozen at -18°C for analysis. Dry matter was assessed by drying at 105°C, ash by incineration at 550°C, crude lipid via Soxhlet extraction, and crude protein using the Kjeldahl method (N × 6.25). Dry weight was determined after dehydration at 55°C.

For blood analysis, samples (n = 6 per treatment) were collected after anesthetizing fish with clove oil (5 mL/L). Blood drawn from caudal vessels (2 mL) was divided into two portions: one with anticoagulant (0.1 mL sodium citrate) for hematological assessments (Red blood cells (RBCs), hematocrit, hemoglobin, and phagocytic activity), and another without anticoagulant for serum analysis. The serum was centrifuged at 2000 × g for 10 min. and saved at -20°C for biochemical assessments, including lysozyme activity and immune responses.

Biochemical parameters were analyzed using standard protocols: Red blood cells (RBCs) and Packed Cell Volume (PCV) were determined with a Neubauer hemocytometer, hemoglobin concentration via the cyanomethemoglobin method, and serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities using commercial kits. Total protein, albumin, and globulin levels were measured following established methods.

Hematological assessments included Total immunoglobulin (Ig) and Immunoglobulin M (IgM) levels using assay kits. Serum lysozyme activity was evaluated as per (Ellis, 1990), while phagocytic activity and index were calculated following (Kawahara et al., 1991). Antioxidant activity was measured using diagnostic kits: Superoxide dismutase (SOD) activity via (Nishikimi et al., 1972), catalase (CAT) by (Koroliuk et al., 1988), Malondialdehyde (MDA) following (Buege and Aust, 1978), and Total Antioxidant Capacity (TAC) using (Galaktionova et al., 1998).

Digestive enzyme activities

Amylase and lipase were assayed in serum using Mindray BS-230 kits and a Tecan Infinite 200 PRO spectrophotometer. Amylase activity (405 nm) was determined kinetically based on 2-chloro-4-nitrophenol formation, with results expressed as U/mg protein (Eissa et al., 2025b). Lipase activity (580 nm) was measured using a kinetic assay (García-Meilán et al., 2023), with a buffer containing Tris, taurodeoxycholate, deoxycholate, tartrate, DGGR, $CaCl_2$, mannitol, and colipase (pH 8.3). Lipase activity was also expressed as U/mg protein.

Histological analysis

Anterior intestinal samples from *S. aurata* (n = 3 per treatment) were carefully collected, tissues were trimmed into small pieces (~1 cm³) before fixation to ensure proper penetration and immediately fixed in 10% buffered neutral formalin for 24 hours. Following fixation, the tissues were dehydrated via a series of ethanol (70%, 80%, 95%, and 100%) (half hour for each conc.), cleared in xylene I, and xylene II (1hour and half for each solu.), and embedded in paraffin wax I, and II (1hour and half for each wax.). Sections of 5 μm thickness were cut using a Leica RM 2155 microtome (Leica, England). These sections were stained routinely with hematoxylin and eosin, then examined under a light microscope according to standard histological procedures (Suvarna et al., 2018).

For histomorphometric analysis, key measurements included villus width (VW), villus height (VL), and absorptive surface area (ASA). Fifty well-oriented villi were selected from each intestinal section, and the values were averaged per fish. Villus height (VL) was measured from the tip to the base, while VW was measured at the midpoint of the villus. The ASA was calculated using the formula: ASA (mm²) = VL × VW, as described by (Mohammady et al., 2021). All measurements were done using a high-resolution light microscope with an HD camera (Leica Microsystems, Germany) and image J analysis software version 1.x.

Relative gene expression analysis

Total RNA was extracted from fish liver tissues (n = 3 per treatment) using a commercial kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. DNase treatment was applied during RNA extraction to eliminate any residual genomic DNA contamination. RNA purity and concentration were measured with a NanoDrop Lite spectrophotometer and all samples showed acceptable A260/280 ratios within the 1.8-2.0 range. For cDNA

synthesis, 1 μg of RNA was reverse-transcribed using the SuperScript™ III system (Invitrogen, USA) with Oligo-dT primers and stored at -20°C. Gene expression of *IGF-1*, *IGF-2*, *TNF-α*, *GHR*, *IL-10*, and *IL-1β* was quantified by qPCR (SensiFast SYBR Lo-Rox kit, Bioline, UK) under thermal cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 85°C for 5 min. qPCR was performed using (Applied Biosystems QuantStudio series, QuantStudio 7 Pro) instrument and melting curve was included to confirm the specificity of the amplified products. Expression levels were normalized to *EF-1 α* using the $2^{-\Delta\Delta CT}$ method (Table 1).

Challenge assay against *Vibrio parahaemolyticus*

Twenty fish from each treatment group, totaling 80 fish, were placed in 50-liter tanks. Both treated and control groups were exposed to a virulent strain of *V. parahaemolyticus* (obtained from the Sakha Animal Production Research Station, Egypt) at a concentration of 10^7 CFU/mL. The bacterial challenge was conducted via immersion in water containing the prepared suspension for 24 hours at 28°C, following the method described by (Balcázar et al., 2007).

Statistical analysis

The Kolmogorov-Smirnov and Levene's tests were used to check the normal distribution of the data and the homogeneity of variances. All data were analyzed with IBM SPSS Statistics (version 25.0) and are reported as the mean ± SE. To compare means across groups, a one-way analysis of variance (ANOVA) was employed. Before ANOVA, the assumption of equal variances was verified using Levene's test. If this assumption was met, *post hoc* analysis was completed using the LSD test to determine specific group

TABLE 1   Primer sequences for the selected genes used in the qPCR analysis.

Gene	Primer Sequence 5´ - 3´	bp	Accession no/ref.
IGF-1	F: GGGCGAGCCCAGAGA R: GCCGTAGCCAGGTTTACTGAAATAA	98	XM_030440256.1
IGF-2	F: GTCGGCCACCTCTCTACAG R: TGCTTCCTTGAGACTTCCTGTTTT	66	XM_030425968.1
GHR	F: ACCTGTCAGCCACCACATGA R: TCGTGCAGATCTGGGTCGTA	99	XM_030417994.1
TNF-α	F: TTCCGACTGGTGGACAATAAG R: GAGATCCTGTGGCTGAGAGG	143	XM_030392876.1
IL-1β	F: AGCGCAGTAGAAGAGCGAAC R: CACTCGGACTAAGTGCCTCTG	117	XM_030416076.1
IL-10	F: CTCACATGCAGTCCATCCAG R: TGTGATGTCAAACGGTTGCT	98	XM_030420872.1
EF-1 α	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	263	XM_030411990.1

Insulin growth factor -1(IGF-1), and 2 (IGF-2), Growth honrone recpetor (GHR), Tumor Necrosis Factor-alpha (TNF-α), interluekin-1β (IL-1β), interluekin-10 (IL-10), Eukaryotic translation elongation factor 1 alpha 1 (EF-1 α).

differences. The threshold for statistical significance was set at p < 0.05. The heatmap was visualized using GraphPad Prism 8.

Results

Water quality

The impacts of various BSL (0, 0.01, 0.02, and 0.03 g/m³, designated Control, BSL1, BSL2, and BSL3, respectively) on water quality are explained in Table 2. The water additives of *B. subtilis* and *B. licheniformis* significantly increased the dissolved oxygen (mg/L) in a dose-dependent way. In contrast, the values of TAN were significantly reduced by increasing the levels of BSL in the water. The NH_3 levels were the lowest in BSL2 and BSL3 compared to other groups; however, BSL1 was lower than the control group. For pH, BSL2 and BSL3 significantly reduced the water pH values compared to the remaining groups. The salinity and temperature of the water were not affected by the administration of *B. subtilis* and *B. licheniformis* (Table 2).

Growth performance and feed efficiency

Supplementation of water treatments with *B. subtilis* and *B. licheniformis* showed significant effects on feed efficiency and growth performance in *Sparus aurata* (Table 3). The BSL3 group exhibited higher final body weight, weight gain, specific growth rate (SGR), and average daily gain (ADG) compared to the other groups (P < 0.05). The feed conversion ratio (FCR) was higher in the control group, with the lowest values observed in the BSL3 group (P < 0.05). Feed intake decreased in the BSL3 group but increased in the other experimental groups (P > 0.05). The survival rate was improved by BSL administration. Overall, supplementing *S. aurata* water with BSL significantly enhanced growth indices and feed efficiency, particularly at a dose of 0.03 g/m³ water.

Approximate body composition analysis

The dry matter was not affected by the water administration of *B. licheniformis* and *B. subtilis* probiotics (Table 4). Adding *B.*

TABLE 2 Impacts of *B. subtilis* and *B. licheniformis* water additives on water quality parameters in *S. aurata* over 10 weeks.

Parameters	Control	BSL1	BSL2	BSL3
Salinity (g/L)	31.85 ± 0.51	31.37 ± 0.27	31.35 ± 0.34	31.33 ± 0.28
Temperature °C	27.10 ± 0.06	27.17 ± 0.03	27.10 ± 0.01	27.20 ± 0.01
Dissolved oxygen (mg/L)	7.33 ± 0.02[d]	7.44 ± 0.02[c]	7.52 ± 0.01[b]	7.86 ± 0.02[a]
pH	8.18 ± 0.01[a]	8.17 ± 0.01[a]	8.14 ± 0.01[b]	8.13 ± 0.01[b]
TAN (mg/L)	1.20 ± 0.01[a]	0.72 ± 0.01[b]	0.54 ± 0.01[c]	0.45 ± 0.01[d]
NH_3 (mg/L)	0.12 ± 0.01[a]	0.07 ± 0.01[b]	0.05 ± 0.01[c]	0.04 ± 0.01[c]

Fish were exposed to four treatments with increasing probiotic concentrations (0, 0.01, 0.02, and 0.03 g/m³), designated as Control, BSL1, BSL2, and BSL3, respectively. [a–d]Different letters in each row indicate significant differences between the groups (*P* < 0.05). Data presented as mean ± SE.

TABLE 3 Impact of *B. licheniformis* and *B. subtilis* administered as water additives on the growth indices and feed utilization of *S. aurata* during a 10-week period.

Parameters	Control	BSL1	BSL2	BSL3
Initial fish weight(g)	6.07 ± 0.03	6.10 ± 0.06	6.10 ± 0.06	6.07 ± 0.03
Final fish weight (g)	31.40 ± 0.51[c]	35.33 ± 0.52[b]	36.27 ± 0.55[b]	38.13 ± 0.20[a]
Weight gain	25.33 ± 0.49[c]	29.23 ± 0.47[b]	30.17 ± 0.50[b]	32.07 ± 0.18[a]
SGR (%/fish/day)	2.35 ± 0.02[d]	2.51 ± 0.01[c]	2.55 ± 0.01[b]	2.63 ± 0.01[a]
Feed intake (g)	38.22 ± 0.21[a]	38.43 ± 0.36[a]	38.43 ± 0.36[a]	38.22 ± 0.21[a]
FCR (g feed/g gain)	1.51 ± 0.02[a]	1.31 ± 0.01[b]	1.27 ± 0.01[b]	1.19 ± 0.00[c]
ADG (g)	0.36 ± 0.01[c]	0.42 ± 0.01[b]	0.43 ± 0.01[b]	0.46 ± 0.00[a]
Initial Fish number (n)	20.00 ± 0.01	20.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00
Fish final number (n)	18.33 ± 0.33[a]	19.33 ± 0.67[a]	19.00 ± 0.01[a]	19.67 ± 0.33[a]
Fish biomass (per 1m³)	575.57 ± 11.70[c]	682.87 ± 22.40[b]	689.07 ± 10.54[b]	750.07 ± 16.10[a]
Survival rate (%)	91.67 ± 1.67[a]	96.67 ± 3.33[a]	95.00 ± 0.01[a]	98.33 ± 1.67[a]

FCR, Feed conversion ratio; SGR, specific growth rate; ADG, average daily gain. Fish were exposed to four treatments with increasing probiotic concentrations (0, 0.01, 0.02, and 0.03 g/m³), designated as Control, BSL1, BSL2, and BSL3, respectively. [a–d]Different letters in each row indicate significant differences between the groups (*P* < 0.05). Data presented as mean ± SE.

licheniformis and *B. subtilis* probiotics to water significantly improved the crude protein content in *S. aurata*, with maximum values observed in the BSL3 group. Ash content in all BSL groups was greater compared to the control group (P < 0.05). Lipid content significantly decreased with increasing levels of probiotics in the water (P < 0.05). BSL3 had the lowest lipid content and the highest ash content compared to other groups (P < 0.05).

Blood hematology and biochemical parameters

The water addition of combined probiotic *B. licheniformis* and *B. subtilis* significantly improved the PCV, RBCs, and Hb in a dose-dependent way (P<0.05) compared to the control group (Table 5). BSL3 exhibited the highest values of PCV, RBCs, and Hb compared to other groups. The values of MCV, MCH, and MCHC were higher in BSL2 and BSL3 groups compared to other groups (P<0.05). BSL1 had greater MCV and MCH than those of the control group (P<0.05). Total protein, albumin, globulin, and glucose were significantly increased in a dose-dependent manner by increasing the levels of probiotic in the water (P<0.05). BSL3 had the highest values of total protein, albumin, and globulin compared to other BSL and control groups (P<0.05). BSL2 and BSL3 showed similar levels of glucose, urea, and creatinine (P>0.05). The addition of probiotics significantly reduced the levels of AST and ALT compared to the control group, with the lowest values shown in BSL3 followed by BSL2 (P<0.05). Urea levels were decreased by the addition of *B. licheniformis* and *B. subtilis* (P < 0.05).

TABLE 4   Impact of various water additive *B.licheniformis* and *B. subtilis* probiotic on approximate body composition analysis of *S. aurata* for 10 weeks.

Parameters	Control	BSL1	BSL2	BSL3
Dry matter (%)	30.52 ± 0.37	31.10 ± 0.06	31.37 ± 0.45	31.30 ± 0.09
Crude protein (%)	51.92 ± 0.12[c]	52.58 ± 0.34[b]	52.98 ± 0.11[ab]	53.24 ± 0.03[a]
Lipids (%)	28.62 ± 0.04[a]	28.38 ± 0.04[b]	28.23 ± 0.02[c]	28.10 ± 0.02[d]
Ash (%)	15.91 ± 0.03[d]	16.46 ± 0.03[c]	16.87 ± 0.03[b]	17.08 ± 0.01[a]

Fish were exposed to four treatments with increasing probiotic concentrations (0, 0.01, 0.02, and 0.03 g/m³), designated as Control, BSL1, BSL2, and BSL3, respectively. [a–d]Different letters in each row indicate significant differences between the groups (*P* < 0.05). Data presented as mean ± SE.

TABLE 5   Impact of *B. licheniformis* and *B. subtilis* water additives on the blood hematology and biochemical parameters of *S. aurata*.

Parameters	Control	BSL1	BSL2	BSL3
Blood hematology				
PCV (%)	28.81 ± 0.25[d]	30.91 ± 0.13[c]	34.06 ± 0.10[b]	35.92 ± 0.08[a]
RBCs (10⁶/μL)	2.34 ± 0.01[d]	2.39 ± 0.00[c]	2.58 ± 0.02[b]	2.72 ± 0.01[a]
Hb (g/dL)	8.87 ± 0.03[d]	9.66 ± 0.21[c]	11.05 ± 0.05[b]	11.75 ± 0.03[a]
MCV (fL)	122.93 ± 0.91[c]	129.51 ± 0.70[b]	132.03 ± 0.81[a]	132.07 ± 0.46[a]
MCH (pg/cell)	37.87 ± 0.16[c]	40.47 ± 0.86[b]	42.83 ± 0.25[a]	43.20 ± 0.13[a]
MCHC (g/dL)	30.81 ± 0.18[b]	31.25 ± 0.67[b]	32.44 ± 0.04[a]	32.71 ± 0.12[a]
Blood biochemical parameters				
Total protein (g/dL)	2.95 ± 0.07[d]	3.75 ± 0.01[c]	4.39 ± 0.04[b]	5.32 ± 0.07[a]
Albumin (g/dL)	1.58 ± 0.05[d]	2.14 ± 0.02[c]	2.50 ± 0.04[b]	2.97 ± 0.06[a]
Globulin (g/dL)	1.37 ± 0.02[d]	1.61 ± 0.02[c]	1.89 ± 0.03[b]	2.35 ± 0.01[a]
Glucose (mmol/L)	13.08 ± 0.04[c]	14.01 ± 0.14[b]	14.57 ± 0.05[a]	14.46 ± 0.02[a]
AST (U/L)	20.98 ± 0.07[a]	20.21 ± 0.04[b]	19.36 ± 0.31[c]	17.78 ± 0.19[d]
ALT (U/L)	31.43 ± 0.22[a]	29.91 ± 0.11[b]	29.15 ± 0.16[c]	28.27 ± 0.12[d]
Uric acid (mg/dL)	3.28 ± 0.04[a]	2.35 ± 0.05[b]	2.18 ± 0.03[c]	1.73 ± 0.05[d]
Urea (mg/dL)	102.18 ± 0.17[a]	101.97 ± 0.23[a]	100.55 ± 0.06[b]	100.08 ± 0.18[b]
Creatinine (mg/dL)	0.49 ± 0.01[a]	0.43 ± 0.01[b]	0.40 ± 0.00[c]	0.38 ± 0.01[c]

Fish were exposed to four treatments with increasing probiotic concentrations (0, 0.01, 0.02, and 0.03 g/m³), designated as Control, BSL1, BSL2, and BSL3, respectively. [a–d]Different letters in each row indicate significant differences between the groups (*P* < 0.05). Data presented as mean ± SE.

Immunoglogical parameters

The results in Table 6 indicate that the water additives Bacillus subtilis and B. licheniformis significantly increase the phagocytic activity, phagocytic index, lysozyme (LYZ) activity, IgM, total Ig, and white blood cells (WBCs) compared to the control group. This increase in the investigated immunological parameters of S. aurata was independent (P<0.05). The highest levels for the highest values of immunological parameters of S. aurata were shown with the addition of 0.3 g/m³.

Antioxidant biomarkers parameters

Significant reductions in MDA levels were shown in all water additive B. subtilis and B. licheniformis groups (Table 7), with the lowest values shown in BSL2 and BSL3 groups (P < 0.05). In contrast, adding B. subtilis and B. licheniformis at various levels significantly improved the antioxidant status, such as SOD, CAT, and TAC, of S. aurata. The BSL3 group had the greatest values of SOD, CAT, and TAC, followed by BSL2 and BSL1 with statistical differences among all groups (P < 0.05).

Digestive enzyme activities

Supplementation with combined probiotics at different levels of B. subtilis and B. licheniformis (0.1, 0.2, and 0.3 g/m3) significantly improved the amylase (Figure 1A) and lipase (Figure 1B) activity in Sparus aurata. The high levels of probiotics exhibited the greatest

values of lipase and amylase, followed by the BSL2 groups with significant differences (P<0.05).

Histological study

All groups of fish intestines in the "control to BSL3" category exhibited normal histological structures (Figure 2). The intestinal villi were lined with simple columnar epithelium, and both the submucosal and muscular layers appeared intact and well-organized, indicating no signs of pathological alterations across the examined groups. Moreover, a gradual improvement in villous length and width was demonstrated from the control group to the BSL3 group, respectively (Figure 2A–D). Figure 3 illustrates the histo-morphometric analysis of villus length (Figure 3A), width (Figure 3B), and absorption surface area (Figure 3C) among different groups. Despite improvements in villus length, width, and absorption surface area in all BSL groups with increasing levels, there were no statistically significant differences among all groups (P > 0.05).

Gene expression

Effect of various water additives B. subtilis and B. licheniformis probiotics on gene expression of Sparus aurata for 10 weeks are shon in Figures 4A–F. These genes include mRNA expressions of growth factors IGF-1 (Figure 4A), IGF-2 (Figure 4B), and GHR (Figure 4C), as well as immune-related genes such as TNF-α (Figure 4D), IL-1β (Figure 4E), and IL-10 (Figure 4F). The

TABLE 6 Effect of various water additive *Bacillus subtilis* and *B. licheniformis* probiotic on immunoglogical parameters of *S. aurata* for 10 weeks.

Parameters	Control	BSL1	BSL2	BSL3
Phagocytic activity (%)	21.47 ± 0.12[d]	23.83 ± 0.19[c]	25.53 ± 0.41[b]	28.27 ± 0.26[a]
Phagocytic index (%)	2.04 ± 0.02[d]	2.15 ± 0.01[c]	2.54 ± 0.02[b]	3.00 ± 0.04[a]
LYZ activity (Unit/mL)	0.11 ± 0.01[d]	0.15 ± 0.01[c]	0.23 ± 0.01[b]	0.27 ± 0.01[a]
IgM (ng/mL)	3.24 ± 0.05[d]	4.29 ± 0.03[c]	4.61 ± 0.04[b]	4.94 ± 0.05[a]
Total Ig (mg/mL)	1.12 ± 0.01[d]	1.27 ± 0.01[c]	1.40 ± 0.01[b]	1.70 ± 0.01[a]
WBCs (mm³)	22.48 ± 0.65[d]	24.73 ± 0.17[c]	26.61 ± 0.41[b]	28.61 ± 0.19[a]

Fish were exposed to four treatments with increasing probiotic concentrations (0, 0.01, 0.02, and 0.03 g/m³), designated as Control, BSL1, BSL2, and BSL3, respectively. [a-d]Different letters in each row indicate significant differences between the groups (*P* < 0.05). Data presented as mean ± SE.

TABLE 7 Effect of various water additive *B. subtilis* and *B. licheniformis* probiotic on antioxidant biomarkers parameters of *S. aurata* for 10 weeks.

Parameters	Control	BSL1	BSL2	BSL3
MDA (nmol/mL)	23.80 ± 0.38[a]	21.00 ± 0.15[b]	19.77 ± 0.15[c]	19.00 ± 0.25[c]
SOD (U/mg protein)	2.30 ± 0.12[d]	3.20 ± 0.06[c]	3.90 ± 0.10[b]	4.57 ± 0.03[a]
CAT (U/mg protein)	1.98 ± 0.07[d]	2.40 ± 0.04[c]	2.98 ± 0.08[b]	3.25 ± 0.01[a]
TAC (mM/L)	0.40 ± 0.02[d]	0.51 ± 0.02[c]	0.61 ± 0.03[b]	0.67 ± 0.01[a]

Fish were exposed to four treatments with increasing probiotic concentrations (0, 0.01, 0.02, and 0.03 g/m³), designated as Control, BSL1, BSL2, and BSL3, respectively. [a-d]Different letters in each row indicate significant differences between the groups (*P* < 0.05). Data presented as mean ± SE.
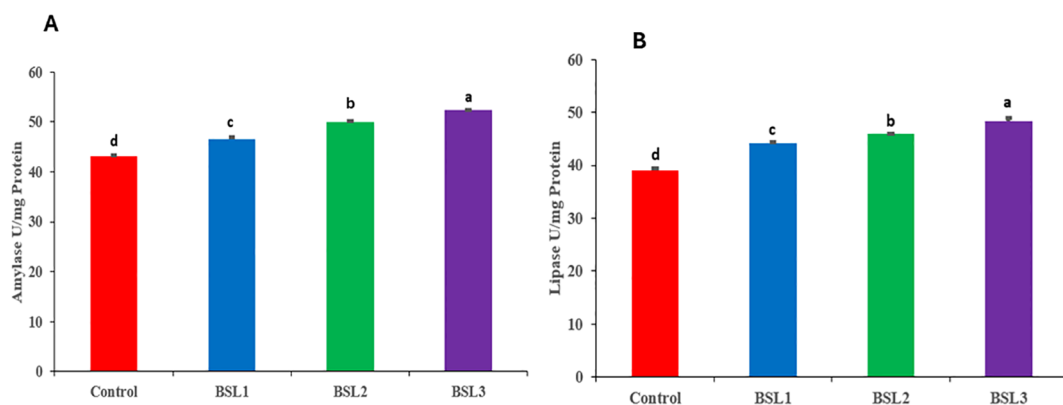
FIGURE 1

Effect of various water additives *Bacillus subtilis* and *Bacillus licheniformis* probiotics on digestive enzyme activities such as amylase **(A)** and lipase **(B)** of *Sparus aurata* for 10 weeks. Superscripts represent significant (P < 0.05) differences among treatments.

addition of *B. subtilis* and *B. licheniformis* probiotics significantly improved the growth factors IGF-1 (Figure 4A), IGF-2 (Figure 4B), and GHR compared to the control group (P<0.05) in a dose-dependent manner. It was shown that increasing the levels of *B. subtilis* and *B. licheniformis* significantly increased the levels of

growth genes (P<0.05) with statistical differences among all groups. The same trend was shown for immune-related genes such as TNF-α (Figure 4D), IL-1β (Figure 4E), and IL-10 (Figure 4F), indicating the growth beneficial effects of probiotics and immune modulatory effects.
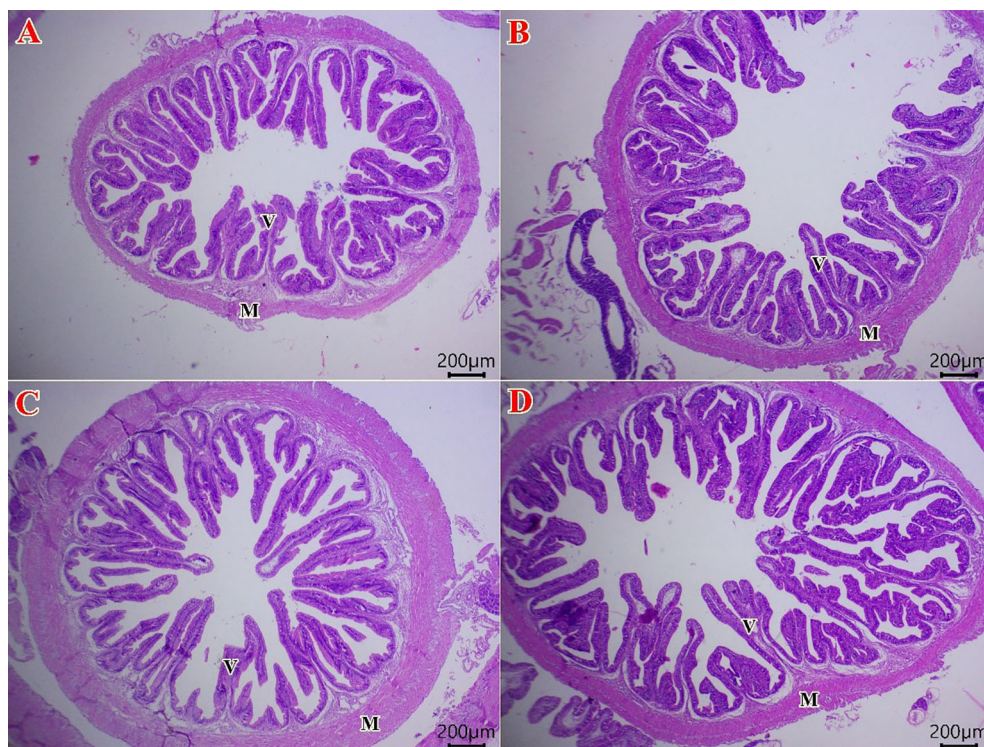

FIGURE 2

Photomicrograph of H&E stained sections from the intestine of *Sparus aurata* fish (Scale bar 200 µm). The fish intestine in control group **(A)** shows the normal histology of a simple columnar epithelial lining intestinal villi (V), submucosa, and muscular layer (M) in all groups from the control group to BSL3 group. There is a gradual improvement in villous length and width in fish treated with 0.1 **(B)**, 0.2 **(C)**, and 0.3 **(D)** g/m³ of *B. subtilis* and *B. licheniformis* probiotic.
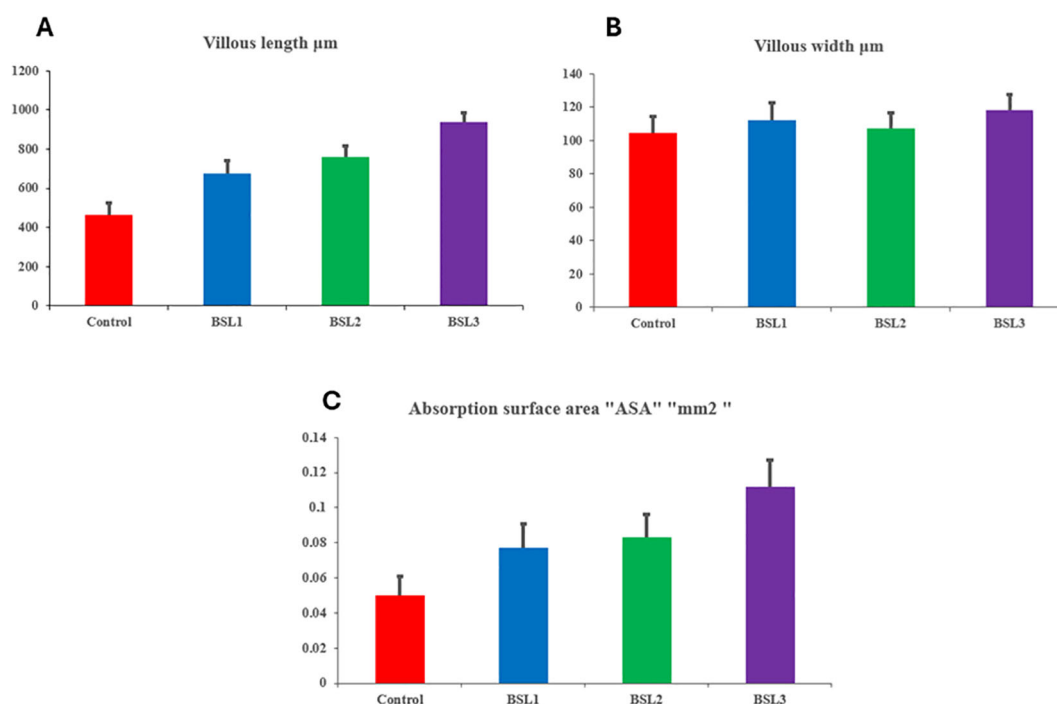
FIGURE 3
The histo-morphometric analysis of villus length **(A)**, width **(B)**, and absorption surface area **(C)** in of *Sparus aurata* fish treated with 0.1, 0.2, and 0.3 g/m^3 of *B. subtilis* and *B. licheniformis* probiotic.

Challenge assay against *Vibrio parahaemolyticus*

In the post-challenge period, fish reared in water with BSL showed improved immune status, resulting in reduced mortality rates in the treated groups (Figure 5). BSL3 exhibited significantly lower mortality (25%), followed by BSL2 (30%), BSL1 (40%), and Control (60%).

Correlation analysis

This heatmap illustrates the normalized values of key biochemical parameters (e.g., total protein, albumin, AST, ALT, urea, creatinine) and immunological parameters (e.g., phagocytic activity, lysozyme, immunoglobulins, WBCs) across different probiotic treatments (Control, BSL1, BSL2, BSL3) (Figure 6). The color gradients indicate relative increases or decreases compared to other groups. The BSL3 group exhibits the most favorable biochemical and immunological profile, suggesting improved health status with probiotic supplementation.

Discussion

In response to the ban on antibiotics, the use of natural molecules like phytochemicals or probiotics has emerged as a sustainable and environmentally friendly strategy to promote growth performance, enhance immune function, modulate blood health, and improve disease resistance. However, the combined effects of these natural molecules on sea bream blood health, immunity, and growth indices have not been thoroughly investigated.

Water quality is the most important factor affecting fish development and production. The combination of physical and biological components influences water quality (Khademzade et al., 2020). Probiotics play a significant role in improving water quality by reducing the levels of organic contaminants, keeping the water clean, and creating an optimal environment for fish in the pond. Both *B. subtilis* and *B. licheniformis* have been found to effectively enhance water quality while maintaining it within acceptable levels for fish farming. Maintaining optimal water quality parameters is essential for promoting growth and reducing disease incidence in fish farming. The high alkalinity and buffering properties of saline water in this study resulted in minimal pH fluctuations, making it an excellent environment for aquaculture (Boyd and Tucker, 1998).

According to the study by (Zhang et al., 2011), the addition of *B. licheniformis* as a denitrifying bacterium to rearing water effectively reduced toxic compounds such as (TAN and NH$_3$), while also promoting the breakdown of residual feed proteins and starches. Moreover, *Bacillus* species play a key role in the biodegradation of nitrogenous waste through mineralization processes, thereby contributing to improved water quality (Eissa et al., 2024a). Ensuring good water quality is essential for the survival of aquatic species, especially Broadstock fish, as ammonia and nitrite nitrogen are key indicators in aquaculture. Elevated levels of these compounds can harm cultured animals, making efficient water management vital for production. Introducing beneficial
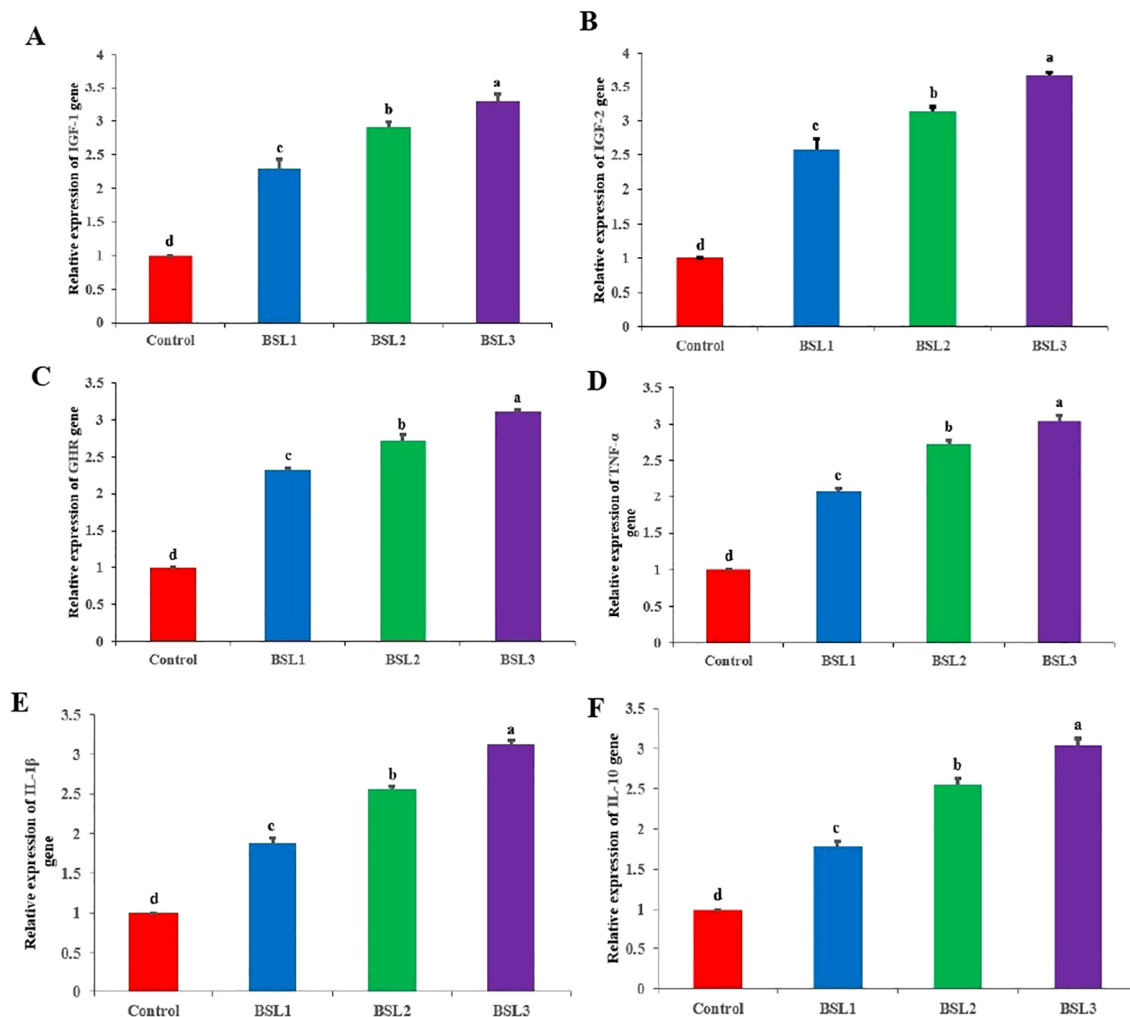
FIGURE 4

Effect of various water additives *Bacillus subtilis* and *Bacillus licheniformis* probiotics on gene expression of *Sparus aurata* for 10 weeks. These genes include mRNA expressions of growth factors IGF-1 **(A)**, IGF-2 **(B)**, and GHR **(C)**, as well as immune-related genes such as TNF-α **(D)**, IL-1β **(E)**, and IL-10 **(F)**. Superscripts represent significant (P < 0.05) differences among treatments.
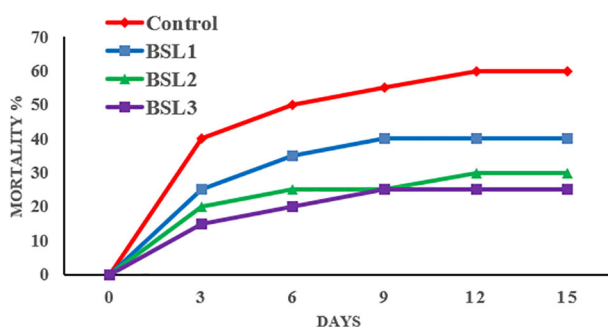

FIGURE 5

Effect of various water additive *B. licheniformis* and *B. subtilis* probiotic on resistance of *S. aurata* against *Vibrio parahaemolyticus* for 15 days.

microbial communities helps recycle organic waste and maintain cleaner water (Qiu et al., 2023). Studies have shown that *B. subtilis* (10^9 CFU/mL) supplementation significantly lowers total nitrogen and ammonia levels.

Here, adding a *Bacillus* probiotic mixture to water significantly enhanced seabream growth, likely due to the growth-promoting properties of these bacteria. Similarly, *B. licheniformis* has been shown to improve weight gain and specific growth rate in prawns (Chen et al., 2020) and *Litopenaeus vannamei* (Cao et al., 2022). In line with these findings, dietary inclusion of *B. subtilis* and *B. licheniformis* blend improved growth and feed efficiency in Kutum fry (Azarin et al., 2015) and Nile tilapia (Abarike et al., 2018). Moreover, studies by (Monier et al., 2023; Eissa et al., 2024a) informed notable improvements in the growth parameters of whiteleg shrimp and red tilapia, respectively, following treatment with these *Bacillus* strains.
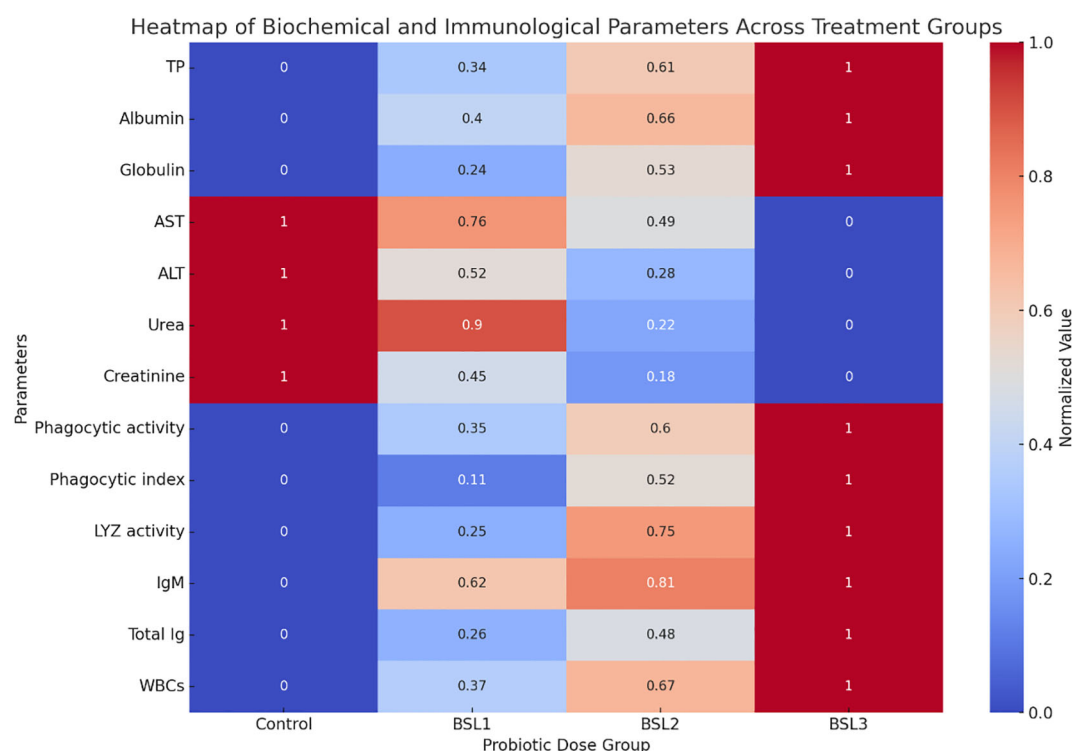
FIGURE 6
Heatmap of biochemical and immunological profiles in sparus aurata across probiotic dose groups. This heatmap illustrates the normalized values of key biochemical parameters (e.g., total protein, albumin, AST, ALT, urea, creatinine) and immunological parameters (e.g., phagocytic activity, lysozyme, immunoglobulins, WBCs) across different probiotic treatments (Control, BSL1, BSL2, BSL3). The color gradients indicate relative increases or decreases compared to other groups. The BSL3 group exhibits the most favorable biochemical and immunological profile, suggesting improved health status with probiotic supplementation.

Haemato-biochemical parameters are widely recognized as valuable indicators of fish health (Hamada et al., 2025). In the present study, the inclusion of BSL in the rearing water improved the hematological profile of *Sparus aurata*. Treated groups showed significant increases in hemoglobin (HB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and packed cell volume (PCV), reflecting enhanced blood oxygen-carrying capacity (Yaqub et al., 2021). Moreover, water supplementation with *B. subtilis* led to notable improvements in serum levels of albumin, total protein, and globulin (Ghiasi et al., 2018; Hassaan et al., 2018).

Changes in blood serum composition—particularly under probiotic treatment—are indicative of physiological status and organ function, notably the kidneys, liver, and circulatory system. Hepatic enzymes such as AST and ALT are regularly used as biomarkers for liver health. In this trial, groups receiving *B. licheniformis* and *B. subtilis* exhibited substantially reduced liver enzyme levels, suggesting improved hepatic condition. These results are consistent with previous findings in Nile tilapia (Redhwan et al., 2024).

Additionally, probiotic supplementation led to significant reductions in serum urea, creatinine, and uric acid levels, implying better renal function. This contrasts with the study by (Zhao et al., 2022), which reported no significant changes in these

parameters among probiotic-treated groups. The graded inclusion of *B. subtilis* and *B. licheniformis* also enhanced antioxidant enzyme activities, (SOD and CAT), in agreement with results by (Eissa et al., 2023).

There is a well-established link between diet composition and the activity of digestive enzymes in aquatic species. In this study, the application of *Bacillus* strains in water significantly elevated digestive enzyme activity compared to the control. Notably, *B. licheniformis* was found to enhance nutrient digestion by stimulating enzymes such as amylase and cellulase. Similarly the probiotic *Bacillus* strains increased digestive enzyme activities in *Litopenaeus vannamei*, with elevated amylase and lipase levels likely contributing to improved growth performance (Monier et al., 2023). These findings suggest that *B. licheniformis* enhances nutrient absorption and utilization (Yaqub et al., 2021).

Histological examination in this study further confirmed the effective impacts of *B. subtilis* and *B. licheniformis* on intestinal tissue architecture in *S. aurata*. These outcomes are consistent with prior research showing that probiotics and prebiotics can improve the microscopic structure of digestive organs in fish (Ngamkala et al., 2020; Ruiz et al., 2020).

Tumor necrosis factor alpha (TNFα) was initially identified in fish as a single gene expressed in activated leukocytes of the Japanese flounder. It is now recognized as a crucial cytokine

involved in antibacterial defense and inflammatory responses (Abd El-Aziz et al., 2024). In fish, the *GH* and *IGF-I* genes play vital roles in regulating growth and cellular functions through various signaling pathways (Wang et al., 2020). The *GHR* facilitates the activity of *GH* by binding peptide hormones and mediating signaling through the JAK-STAT pathway, thereby regulating growth (de Vos et al., 1992; Dehkhoda et al., 2018). Interleukin-1 beta (*IL-1β*) functions to attract leukocytes in fish and controls their migration via activation of G protein-coupled receptors and chemokine gradients.

In this study, both pro-inflammatory cytokines (*TNF-α* and *IL-1β*) and the anti-inflammatory cytokine *IL-10* were selected to assess their involvement in cytokine signaling pathways under stimulation. The administration of *B. subtilis* and *B. licheniformis* across different treatments led to upregulation of *IL-1β*, *TNF-α*, *IL-10*, *IGF-1*, and *GH*, indicating a shift in the immune response toward enhanced protection. Furthermore, the expression levels of *IGF-1* and *GH* genes were significantly higher in fish cultured in water treated with *B. subtilis* and *B. licheniformis* compared to the control group, aligning with improvements observed in growth performance and serum biochemical parameters.

These results corroborate previous studies by (El-Kady et al., 2022; Abd El-Aziz et al., 2024), who reported that probiotic supplementation notably increased *IGF-1* and *GH* gene expression in Nile tilapia. Similarly, probiotics added to the rearing water of Yellow Perch significantly elevated the expression of *GH* and *IGF-I* genes relative to controls (Wang et al., 2020). Additionally, probiotic treatment in Yellow Perch enhanced the expression of *IL-1β* and *IL-10* genes compared to untreated groups (Dighiesh et al., 2024).

Research on fish immunology has primarily concentrated on developing preventive strategies to enhance disease resistance and improve fish survival following pathogen exposure (Magnadottir, 2010). In this study, all fish fed diets supplemented with the probiotic *B. subtilis* (BS) demonstrated significantly enhanced protection against *V. parahaemolyticus* infection compared to the control group, with the BSL3 dosage offering the greatest level of protection. This marked improvement in tilapia's resistance supports the idea that *Bacillus* spp. probiotics can effectively stimulate and prolong the immune response against pathogenic challenges. Many previous natural compounds have been used for optimizing the overall health and well-being in animals (Saleh et al., 2015; Bakeer, 2021; Bakeer et al., 2022) and aquatic fish (Magnadottir, 2010; Darafsh et al., 2020). Comparable increases in disease resistance have been reported in Nile tilapia fed *Bacillus* spp. mixtures (Abarike et al., 2018; Redhwan et al., 2024), as well as in Persian sturgeon (Darafsh et al., 2020), and in whiteleg shrimp and red tilapia reared in water treated with *Bacillus* spp. blends (Monier et al., 2023; Eissa et al., 2024a). The overall enhancement in biochemical and immunological parameters across probiotic-treated groups is visually summarized in Figure 5, highlighting the dose-dependent health benefits, particularly in the BSL3 group.

Conclusion

This study demonstrates that supplementing *Sparus aurata* rearing water with a probiotic blend of *Bacillus subtilis* and *B. licheniformis*, particularly at a concentration of 0.03 g/m³, significantly improves water quality, growth performance, feed efficiency, and survival rates. The probiotics enhance fish physiological health by optimizing body composition, hematological and biochemical parameters, and antioxidant defenses, while boosting immune responses and digestive enzyme activities. Improved intestinal morphology and upregulated expression of growth and immune-related genes further support these benefits. Importantly, probiotic-treated fish show greater resistance to *V. parahaemolyticus* infection. Overall, this probiotic mixture offers a promising, eco-friendly approach to enhancing the health, welfare, and productivity of sea bream in aquaculture systems.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

All animal care and experimental procedures were approved by the Zagazig University, Egypt (Approval No. ZU-IACUC/1/F/84/2025). Moreover, all experimental procedures and animal handling were followed according to both institutional guidelines and the ARRIVE guidelines for the ethical treatment of animals.

Author contributions

SN: Writing – review & editing, Visualization, Validation. OA: Validation, Writing – review & editing, Visualization. RE: Validation, Writing – review & editing, Visualization. FM: Visualization, Writing – review & editing, Validation. EE: Investigation, Visualization, Writing – review & editing, Validation. RO: Visualization, Validation, Writing – review & editing. ME: Resources, Validation, Formal analysis, Writing – review & editing, Visualization, Methodology, Writing – original draft, Data curation, Investigation, Conceptualization, Software. EE: Writing – original draft, Formal analysis, Resources, Visualization, Project administration, Supervision, Methodology, Writing – review & editing, Conceptualization, Data curation,

Software, Validation, Investigation. NA: Writing – review & editing, Validation, Visualization, Investigation.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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