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Effects of Dietary Supplementation with *Bacillus subtilis natto* on Growth, Digestive Enzyme Activity, Immune Response, and Intestinal Microorganisms of Red Sea Bream, *Pagrus major*

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Abstract: This study investigated the influence of dietary supplementation with *Bacillus subtilis natto* (BSN) on various physiological parameters in red sea bream (*Pagrus major*). Key areas of focus included growth performance, enzymatic activity related to digestion, blood biochemical markers, immune function, and intestinal microorganisms. Fish were fed diets containing the following five levels of BSN: 0 (BN0), 1×10^7 (BN1), 1×10^8 (BN2), 1×10^9 (BN3), and 1×10^{10} (BN4) CFU kg^{-1} for 56 days. Fish in the BN3 and BN4 groups exhibited significantly enhanced growth performance compared to the BN0 group ($p < 0.05$). Additionally, the activities of amylase and protease were markedly higher in the BN3 and BN4 groups ($p < 0.05$), while lipase activity was significantly elevated in fish fed the BN3 diet ($p < 0.05$). Plasma total protein levels also showed a significant increase in the BN3 group ($p < 0.05$). Hematocrit values were significantly improved in the BN2, BN3, and BN4 groups ($p < 0.05$). The intestinal microbiota of the BN4 group revealed a significant increase in the populations of *B. subtilis* and *Lactobacillus*, along with a notable decrease in *Escherichia coli*, compared to all other groups ($p < 0.05$). The observed improvements in specific growth rate and the upregulation of growth-related gene expression further highlight the potential of BSN to enhance the growth performance of red sea bream. In conclusion, dietary supplementation with BSN at 1×10^9 and 1×10^{10} CFU kg^{-1} shows promise in improving the growth, health, and immune response of red sea bream.

Keywords: *Bacillus subtilis natto*; red sea bream; intestinal microorganisms; immune response

Key Contribution: This study highlights the potential of *Bacillus subtilis natto* (BSN) as a dietary probiotic for enhancing the growth performance, digestive enzyme activity, immune response, and intestinal microbiota balance in red sea bream (*Pagrus major*), providing evidence for its use as a sustainable and functional feed additive in aquaculture.

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1. Introduction

Aquaculture plays a pivotal role in ensuring sustainable development and global food security. According to the Food and Agriculture Organization of the United Nations (FAO), the growth rate of the global consumption of fish for food has consistently outpaced the world population growth rate over the past 60 years. Global fish consumption grew by an

average of 3.0% per year between 1961 and 2021, outpacing the annual population growth rate of 1.6% over the same period. Per capita fish consumption is expected to reach 21.3 kg by 2032, with aquaculture expected to account for 60% of the human supply of fish for food, up from 57% in 2022 [1]. However, the intensification of aquaculture systems has led to a higher incidence of diseases caused by pathogenic bacteria, viruses, fungi, and parasites, significantly impacting productivity and economic outcomes. Conventional treatments like antibiotics pose risks, such as promoting antibiotic-resistant bacterial strains and environmental degradation. Thus, there is an urgent need for sustainable alternatives like probiotics, which have been increasingly studied for their role in promoting growth and disease resistance in aquaculture [2,3].

Various probiotics, both Gram-negative and Gram-positive, such as *Lactobacillus* (*Lb.*), *Bacillus*, *Enterococcus*, *Lactococcus*, and *Pseudomonas* species, have been investigated for their positive effects in aquaculture [4,5]. Microorganisms isolated from the digestive systems of aquatic animals, seafood sources, and various commercial probiotic formulations have shown potential in enhancing growth performance, feed conversion, immune system activity, and resilience to stress in species such as turbot (*Scophthalmus maximus*), red sea bream, Nile tilapia (*Oreochromis niloticus*), and South American white shrimp (*Litopenaeus vannamei*) [6–8]. Adherence of probiotics to the intestinal mucosa has been identified as a key mechanism, preventing pathogenic colonization and promoting immune modulation and intestinal barrier function [9]. Among the various probiotics, *Bacillus subtilis natto* (BSN), a subspecies of *Bacillus subtilis* (*B. subtilis*) originally isolated from the traditional Japanese fermented food natto, has garnered attention due to its diverse benefits. BSN inhibits pathogenic bacteria by producing antimicrobial compounds like organic acids and bacteriocins, while also regulating intestinal microbiota to enhance nutrient absorption [10]. Additionally, BSN can decompose complex macromolecules in feed, enriching it with bioavailable nutrients such as amino acids, oligosaccharides, and organic acids. Its metabolic byproducts include digestive enzymes and vitamins, which promote intestinal mucosal cell proliferation and improve overall gut health [10,11].

The red sea bream (*Pagrus major*), a highly valued marine fish in East Asia, is renowned for its attractive appearance, delicate flesh, and nutritional richness. This species is extensively cultured in Japan, China, and other coastal regions due to its high market demand. Despite the growing body of research on the immunological benefits of BSN in terrestrial animals, its effects on the growth of red sea bream remain underexplored [2,4]. Given the scarcity of research on BSN within marine aquaculture, particularly regarding its effects on the growth and health of red sea bream, this study seeks to assess the influence of BSN supplementation at different concentrations on growth metrics, digestive enzyme activities, immune responses, and gut microbiota composition. This research aims to offer new insights that could contribute to more sustainable and ecologically friendly farming practices.

2. Materials and Methods

2.1. Fish and Experimental Setup

The feeding trials took place at Kinkowan Station, Faculty of Fisheries, Kagoshima University, Japan. Red sea bream juveniles (*Pagrus major*) were acclimated to laboratory conditions for a period of two weeks in a 500 L holding tank. After acclimatization, the fish were transferred to 100 L polycarbonate tanks with 80 L of water in a flow-through seawater system. The system maintained a constant water flow rate of 1.5 L/min to ensure optimal rearing conditions, with each tank featuring individual water inlets, outlets, and aeration to maintain optimal water quality. The tanks were exposed to natural light/dark cycles, and seawater, drawn from a deep-sea basin in Kagoshima Bay, was gravel-filtered prior to entering the system.

Following the acclimation period, juvenile fish were homogenized and then randomly distributed into 15 tanks, with 20 fish (mean initial weight = 11.23 $\pm$ 0.11 g) assigned to each tank. The feeding trial lasted for 56 days, and the fish were fed to apparent satiation

twice daily at 8:30 and 16:00. Every dietary treatment group had three replicates. Daily feed intake was carefully tracked by collecting uneaten feed one hour after feeding, which was subsequently dried and weighed. Fish were batch-weighed every two weeks to monitor growth and overall health. Water temperature was kept stable at 21.8 ± 1.9 °C, with a pH of 8.1 ± 0.7 and salinity at 3.35 ± 0.05%, ensuring that water quality was consistently monitored to provide ideal conditions for the fish.

2.2. Experimental Diet

The experimental diet formulations are presented in Table 1. Five experimental diets were designed to evaluate the effects of BSN (5×10^9 CFU/mL), which was cultivated in a previous experiment. The BSN strain np1 was sourced from the Yuzo Takahashi Laboratory, Co. Ltd. (Kaminoyama, Japan). The strain was added to the basal diets at different concentrations, resulting in the following experimental groups: BN0 (control, 0 CFU/kg), BN1 (1×10^7 CFU/kg), BN2 (1×10^8 CFU/kg), BN3 (1×10^9 CFU/kg), and BN4 (1×10^{10} CFU/kg). After diet elaboration, the actual number of cultivable BSN cells was determined using a standard plate counting method [12]. BSN counts and chemical compositions of the experimental diets are shown in Table 2.

Table 1. Formulation of the experimental diets.

Ingredient	Test Diet (g/kg)
Brown fish meal [1]	600.0
Wheat flour	100.0
Soybean lecithin [2]	30.0
Pollack liver oil [3]	45.0
HUFA (DHA + EPA)	5.0
Vitamin premix [4]	30.0
Mineral premix [5]	30.0
Stay-C [6]	3.0
Activated gluten [7]	50.0
α-Cellulose [8]	78.0
Amino acid premix [9]	9.0
CMC [10]	10.0
BSN Prep [11]	10.0
Total	1000

[1] Nippon suisan Co. Ltd., Tokyo, Japan. [2] Kanto Chemical Co., Inc. Tokyo, Japan. [3] Riken Vitamin, Tokyo, Japan. [4] Vitamin premix (mg/kg diet): β-carotene (0.10), vitamin D3 (0.01), menadione sodium bisulfite (K3) (0.05), DL-α-tocopheryl acetate (E) (0.38), thiamine nitrate (B1) (0.06), riboflavin (B2) (0.19), pyridoxine HCl (B6) (0.05), cyanocobalamin (B12) (0.0001), biotin (0.01), inositol (3.85), niacin (nicotinic acid) (0.77), calcium pantothenate (0.27), folic acid (0.01), choline chloride (7.87), para-aminobenzoic acid (0.38), and cellulose (1.92). [5] Mineral premix (mg/kg diet): $MgSO_4$ (5.07), Na_2HPO_4 (3.23), K_2HPO_4 (8.87), iron citrate (1.1), calcium lactate (12.09), $Al(OH)_3$ (0.01), $ZnSO_4$ (0.13), $MnSO_4$ (0.03), $Ca(IO_3)_2$ (0.01), $CoSO_4$ (0.04). [6] Stay-C 35: L-ascorbyl-2-phosphate-Na/Ca complex. [7] A-Glu SS, Glico Nutrition Company Ltd. Osaka, Japan. [8] Nippon Paper Chemicals, Tokyo, Japan. [9] Amino acid premix (alanine, betaine, and glycine). [10] Carboxymethyl Cellulose. [11] BSN was diluted to different levels with culture medium. BSN Prep (CFU/mL): BN0 (0); BN1 (1×10^6); BN2 (1×10^7); BN3 (1×10^8); BN4 (1×10^9).

Table 2. BSN counts after diet elaboration and chemical composition (% dry matter basis).

Ingredient	Test Diet				
	BN0	BN1	BN2	BN3	BN4
BSN counts (CFU/Kg)	2.33×10^3	9.71×10^6	9.68×10^7	9.64×10^8	9.34×10^9
Crude Protein (g/kg)	50.16	50.19	50.20	50.28	50.49
Crude Lipid (g/kg)	13.91	13.87	13.95	13.98	14.01
Ash (g/kg)	10.10	10.08	10.11	10.05	9.97
Gross energy (kJ/g) *	21.77	21.77	21.78	21.81	21.84

* Energy content was calculated using combustion values of 23.6 kJ/g for protein, 39.5 kJ/g for lipid, and 17.2 kJ/g for carbohydrate. Carbohydrate content was calculated as 100 minus the sum of protein, lipid, ash, and moisture contents.

To achieve a uniform distribution, BSN was emulsified with Pollack liver oil and soybean lecithin using a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan) before blending with the other dry ingredients. The diets were prepared by first mixing all dry components for 15 min using a food mixer. Water, accounting for 35–40% of the dry matter, was added to the blend, and the mixture was further processed for another 15 min. The feed was then extruded through a pellet mill with 2.1 mm diameter dies, air-dried at room temperature, and stored at −20 °C until required for the feeding trials.

To maintain the viability of BSN, fresh diets were prepared every two weeks. The actual BSN counts in the feed were verified using a selective medium. Approximately 0.1 g of feed was homogenised in 1 mL phosphate-buffered saline (PBS, pH 7.4). Serial dilutions were prepared, and the samples were plated on selective medium before incubation at 37 °C for 2–3 days. The selective medium consisted of 1% polypeptone, 0.5% dry yeast, 2% agar, and 1% NaCl [13].

2.3. Sample Collection

At the beginning of the study, an initial sample set of 15 fish was collected for whole-body composition analysis and stored at −20 °C. Upon completion of the feeding experiment, samples were taken after a 24 h fasting period. The fish were individually weighed and measured in each tank to assess growth metrics. From each tank, three fish were randomly chosen, rinsed with distilled water, and frozen at −20 °C for later whole-body analysis. In addition, blood samples were collected from three randomly selected fish from each tank replicate. Caudal vein puncture was performed using heparinized syringes for some analyses, whereas non-heparinized syringes were used for serum-based assessments. Heparinized whole blood was used to measure the hematocrit and hemoglobin levels. After the blood was left to rest for about 30–45 min, plasma and serum were separated by centrifugation at 3000× *g* for 15 min at 4 °C and stored at −80 °C for future biochemical analyses.

For digestive enzyme assessments, the digestive tract was removed, segmented, rinsed with distilled water, pooled, and stored at −80 °C. Additionally, the livers and intestines were excised from the sampled fish, weighed, and used to calculate the hepatosomatic index (HSI) and viscerosomatic index (VSI). Skin mucus was collected by rinsing the fish with distilled water and extracting mucus from the skin before storage at −80 °C for subsequent analysis [14].

2.4. Digestive Enzyme Analysis

Preparation of Crude Enzyme Solution: For each treatment, nine starved (24 h fasting) red sea bream were sampled (three fish per tank) and subsequently dissected. After thawing, a segment of the intestine weighing 0.5 g was collected from each fish and placed into 10 mL centrifuge tubes. Sterile saline was added in a 1:9 ratio (sample:saline), resulting in a total volume of 4.5 mL. The mixture was homogenized for 3–5 min using an automatic homogenizer and maintained in an ice bath to preserve enzyme activity. Following homogenization, the samples were centrifuged at 4 °C for 30 min at 5000 rpm. The resulting supernatant was carefully collected, aliquoted, and stored in a refrigerator at 4 °C for subsequent analysis, ensuring that the corresponding enzymatic assays were performed within 24 h. Protein content in the tissue homogenate was quantified using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Enzymatic Activity Assays: 1. Non-specific protease activity was assessed using the method described by Cupp-Enard [15]. In this assay, protease activity was measured based on the enzyme's ability to cleave peptide bonds, utilizing casein as the substrate; 2. Amylase activity was determined following the protocols outlined in the Worthington Enzyme Manual [16]. The absorbance at 540 nm was measured to determine the maltose concentration, which was compared against a standard curve for quantification; 3. Specific enzyme activity (U) = μmol of maltose produced per mg of enzyme in the reaction mixture

over 3 min; 4. Lipase activity was determined following the procedure outlined by Mustafa et al. [17]. Each enzymatic assay was performed in triplicate, and the standard deviation of the mean was calculated to ensure the statistical reliability of the results.

2.5. Biochemical and Blood Analysis

To assess the nutritional composition of the experimental diets and the whole bodies of *Pagrus major*, samples were analyzed in triplicate for moisture, crude protein, total lipid, and ash content using standardized protocols as per AOAC guidelines [18]. Hematocrit values were measured using the microhematocrit technique. Plasma biochemical parameters, including glucose, blood urea nitrogen (BUN), total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglycerides, total cholesterol, and total protein, were quantified using commercial reagent kits (Arkray Inc., Kyoto, Japan). These analyses were performed using an automated biochemical analyzer (SPOTCHEM™ EZ model SP-4430, Arkray Inc., Kyoto, Japan), adhering to the manufacturer's recommendations to ensure precision and consistency in the results.

2.6. Immunological Parameter Evaluation

The nitro blue tetrazolium (NBT) reduction assay was performed based on a modified version of the method outlined by Anderson and Siwicki [19]. In brief, 0.1 mL of whole blood was transferred into each well of a microtiter plate followed by the addition of 0.2% NBT solution (Sigma, Saint Louis, MO, USA). After a 30 min incubation at room temperature, 0.05 mL of the blood–NBT mixture was transferred into glass tubes containing 1 mL of N,N-dimethylformamide (Sigma, USA) and centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant was measured at 540 nm with a spectrophotometer using blank N,N-dimethylformamide.

Serum and mucus bactericidal activities were evaluated according to the method described by Yamamoto and Iida [20]. The samples were diluted in Tris buffer (pH 7.5) and mixed with an *Escherichia coli* (*E. coli*) K12 (DH5α) bacterial suspension (0.001 g/mL). After 24 h incubation at 25 °C on a microtube rotator (MTR-103, AS ONE, Osaka, Japan), the samples were plated on TSA plates for another 24 h at the same temperature. Colony-forming units (CFU) were quantified, and bactericidal activity was expressed as a percentage reduction in CFU, calculated using the following formula:

$$\text{Bactericidal activity (\%)} = (\text{CFU of the blank group} - \text{CFU of each group})/\text{CFU of blank group} \times 100$$

Lysozyme activity in serum and lysozyme activity in mucus was quantified using a turbidimetric assay adapted from Ellis [21]. A 10 µL sample was added to a microplate well containing 190 µL of *Micrococcus lysodeikticus* (Sigma, USA) cell suspension (0.2 mg/mL) in PBS (pH 7.4). Absorbance was monitored at 450 nm with an Immuno Mini NJ-2300 microplate reader (System Instruments, Tokyo, Japan) at 1 and 5 min after gentle shaking at room temperature. One unit of lysozyme activity was defined as a decrease in absorbance of 0.001 per min.

2.7. Growth-Related Genes Analysis (IGF-1 and IGF-2)

Juvenile fish were euthanized and skeletal muscle samples were immediately excised for RNA extraction. Samples were preserved in RNAlater® solution (Invitrogen, Thermo Fisher Scientific, Yokohama, Japan) and stored at −80 °C to maintain RNA integrity. Total RNA was extracted from 30 mg of muscle tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted RNA was purified by centrifuging the tissue homogenates and mixing the supernatant with 70% ethanol for further purification.

cDNA was synthesized using a PrimeScript™ RT Master Mix Kit (Takara Bio Inc., Shiga, Japan) for reverse transcription. The primers used for the analysis of growth-related genes are shown in Table 3. Real-time quantitative PCR (qPCR) was performed using the

SYBR™ Select Master Mix (Thermo Fisher Scientific, Waltham, MA). *β-actin* served as the internal reference gene for data normalization. qPCR amplification was conducted using a CFD-3120 Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc., Hercules, CA, USA) under the following thermal cycling conditions: 95 °C for 2 min for initial denaturation followed by 40 cycles at 95 °C for 15 s and 65 °C for 30 s for annealing and extension. Each sample was analyzed in triplicate to ensure the reliability of the gene expression data.

Table 3. Primers for the analysis of growth-related genes.

Name	Primer Sequence: 5′-3′	Accession Number
IGF-1 (F) *IGF-1* (R)	TAAACCCACACCGAGTGACA GCGATGAAGAAAAGCTACGG	AB050670.1
IGF-2 (F) *IGF-2* (R)	CGGCAAACTAGTGATGAGCA CAGTGTCAAGGGGGAAGTGT	AB360966.1
β-actin (F) * *β-actin* (R)	TCTGTCTGGATCGGAGGTC AAGCATTTGCGGTGGACG	JN226150.1

* where *β-actin* was the housekeeping gene.

2.8. Intestinal Bacterial Analysis

Quantification of Intestinal Bacterial Populations Using Real-time PCR [22]. DNA concentrations were then assessed via spectrophotometry and working solutions were prepared at a concentration of 15 ng/μL. SYBR Green-based absolute quantitative real-time PCR (qPCR) was used to quantify the populations of *B. subtilis*, *Lb.*, *E. coli*, and total bacteria in the intestinal samples.

DNA standards were generated by amplifying the bacterial DNA of the 16S rRNA gene region using species-specific primers (Table 4) for *B. subtilis*, *Lb.*, *E. coli*, and total bacteria via PCR. The PCR products were purified, inserted into the pMD19-T vector (Takara Bio Inc., Shiga, Japan), and transformed into *E. coli* DH5α competent cells. Plasmid DNA was extracted from selected positive clones using a TIANprep Mini Plasmid Kit (Tiangen, Beijing, China) and served as a DNA standard for qPCR. Standards were serially diluted (30 ng/μL) to generate calibration curves [23,24]. The copy number for each standard was calculated using the molecular weight and DNA concentration.

Table 4. Primers for total bacteria, *B. subtilis*, *Lb.*, and *E. coli*.

Items	Primer	Primer Sequence (5′-3′)	Amplicon Size (bp)
Total bacteria	Forward Reverse	CGGCAACGAGCGCAACCC CCATTGTAGCACGTGTGTAGCC	130 (125–146)
B. subtilis	Forward Reverse	TCTGCTCGTGAACGGTGCT TTTCGCCTTATTTACTTGG	319
Lb.	Forward Reverse	TGGAAACAGRTGCTAATACCG GTCCATTGTGGAAGATTCCC	222
E. coli	Forward Reverse	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	96

The qPCR reactions were conducted using a Bio-Rad iQ5 PCR System (Bio-Rad Laboratories Inc., Hercules, CA, USA) with a 20 μL reaction mix containing SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), 0.3 μM primers, and 30 ng of template DNA. The thermal cycling conditions included an initial denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. Triplicates were performed for each sample, and the mean Ct values were used to determine bacterial counts based on the calibration curves. Bacterial counts were expressed in colony-forming units (CFU) per gram of intestinal content.

2.9. Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) to assess differences between treatment groups, with significance set at $p < 0.05$. Where significant differences were observed, the Tukey–Kramer post-hoc test was applied to determine pairwise group differences. Results are presented as mean ± standard deviation (SD), and all analyses were performed using SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Growth Performance, Survival, and Feed Utilization

Red sea bream fed diets supplemented with BSN at BN3 and BN4 levels exhibited significantly higher final body weight (FBW) and specific growth rate (SGR) than those fed the non-supplemented control diet (BN0) ($p < 0.05$). Additionally, the weight gain rate (WGR) was markedly elevated in the BN4 group compared to the BN0 and BN1 groups ($p < 0.05$), indicating that growth performance was enhanced only when BSN reached a certain dosage.

While no significant improvements were observed in feed intake (FI), feed conversion efficiency (FCE), or protein efficiency ratio (PER) between the BSN-supplemented and control groups ($p > 0.05$), there was an evident upward trend in these metrics, suggesting potential benefits from BSN inclusion.

The survival rates remained consistently high across all groups, ranging from 93.33% to 96.67%, with no statistically significant differences between them ($p > 0.05$), indicating that BSN supplementation did not negatively affect fish survival (Table 5).

Table 5. Growth performance and nutrient utilization in red sea bream fed the test diets for 56 days.

Parameters	Test Groups				
	BN0	**BN1**	**BN2**	**BN3**	**BN4**
IBW [1]	11.23 ± 0.10	11.23 ± 0.11	11.25 ± 0.17	11.23 ± 0.15	11.24 ± 0.13
FBW [2]	33.75 ± 0.40 [a]	33.77 ± 0.55 [a]	35.35 ± 0.48 [ab]	36.41 ± 0.67 [b]	36.28 ± 0.60 [b]
WGR [3]	200.66 ± 4.12 [a]	200.71 ± 5.77 [a]	214.22 ± 4.56 [ab]	224.22 ± 6.58 [ab]	222.78 ± 5.43 [b]
SGR [4]	1.96 ± 0.03 [a]	1.97 ± 0.04 [ab]	2.04 ± 0.04 [ab]	2.10 ± 0.04 [b]	2.09 ± 0.03 [b]
FI [5]	46.48 ± 1.08	46.48 ± 0.88	45.36 ±1.24	47.04 ± 2.04	48.72 ± 2.31
FCE [6]	48.45 ± 2.14	48.49 ± 2.55	53.13 ± 2.87	53.53 ± 3.12	51.40 ± 2.98
PER [7]	96.59 ± 4.12	96.62 ± 4.52	105.84 ± 4.10	106.46 ± 4.87	101.79 ± 5.21
SR [8]	95.00 ± 5.00	96.67 ± 5.77	95.00 ± 5.00	96.67 ± 2.89	93.33 ± 5.77

Values are means ± SD of triplicate groups. Within a row, means with the same letters are not significantly different ($p > 0.05$), taking $p < 0.05$ (lowercase letters) as significant. [1] IBW: initial body weight (g). [2] FBW: final body weight (g). [3] WGR: weight gain rate (%) = (FBW − IBW) × 100/IBW. [4] SGR: specific growth rate (% day^{-1}) = {Ln (FBW) − Ln (IBW)/duration} × 100. [5] FI: feed intake (g) = $\sum_{i=1}^{56}$ day i feed intake (g fish^{-1} days^{-1}) = $\sum_{i=1}^{56}$ day i (dry diet given − dry remaining diet recovered)/No. of fish. [6] FCE: feed conversion efficiency = wet weight gain (g)/FI (g) × 100. [7] PER: protein efficiency ratio = WG (g)/dry protein intake (g) × 100, [8] SR: survival rate (%) = 100 × (final no. of fish/initial no. of fish).

3.2. Digestive Enzyme Activities

Protease and amylase activities showed significant increases in fish fed diets BN3 and BN4, with BN4 exhibiting the highest levels compared to the control (BN0) group ($p < 0.05$; Figure 1A,B). However, significant enhancement of lipase activity was observed only in the BN3 group relative to BN0 ($p < 0.05$; Figure 1C), with no notable differences among the remaining groups ($p > 0.05$). Within the BSN-supplemented groups, BN4 displayed significantly higher protease activity than BN1 ($p < 0.05$; Figure 1A).

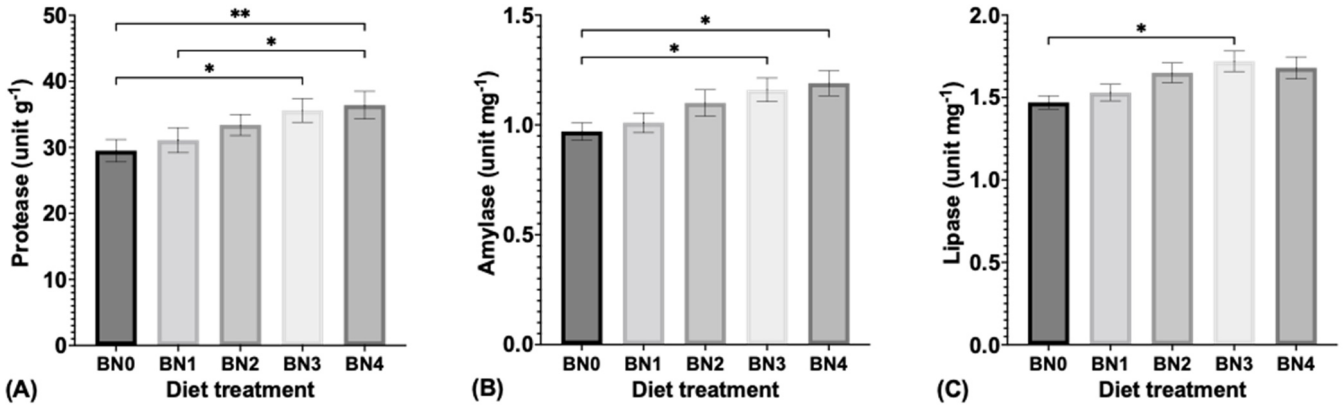

Figure 1. Specific activities of (**A**) protease, (**B**) amylase, and (**C**) lipase enzymes in juvenile red sea bream fed test diets for 56 days. Data represent means ± SD. The level of significance between the means was calculated using the Tukey–Kramer test, taking * as significant($p < 0.05$), and ** as highly significant($p < 0.01$).

3.3. Blood Chemistry

Most of the blood chemical parameters of red sea bream were unaffected by the dietary treatments, with the exceptions of hematocrit, total bilirubin (T-Bill), and plasma total protein (Table 6). Fish in the BN2, BN3, and BN4 groups showed significantly elevated hematocrit levels compared to the control ($p < 0.05$). Additionally, the T-Bill levels in the control group were significantly lower than those in the experimental diet group ($p < 0.05$). Plasma total protein was notably higher in the BN3 group than in the BN0 group ($p < 0.05$), whereas no significant differences were detected between the other groups ($p > 0.05$).

Table 6. Blood parameters of juvenile red sea bream fed test diets for 56 days.

Parameters	Test Groups				
	BN0	**BN1**	**BN2**	**BN3**	**BN4**
Hematocrit (%)	32.3 ± 1.5 ^{a}	37.0 ± 3.6 ^{ab}	38.0 ± 1.6 ^{b}	40.5 ± 1.3 ^{b}	39.7 ± 1.5 ^{b}
Hemoglobin (mg/dL)	12.63 ± 2.42	11.87 ± 1.23	12.56 ± 1.04	13.66 ± 3.12	14.65 ± 2.44
T-Cho (mg/dL) ^{1}	169.3 ± 6.9	166.3 ± 9.8	171.5 ± 10.2	172.7 ± 12.1	182.0 ± 10.4
BUN (mg/dL) ^{2}	6.3 ± 0.3	6.2 ± 0.6	6.7 ± 0.9	7.0 ± 1.3	6.3 ± 0.8
T-Bill (mg/dL) ^{3}	0.31 ± 0.03 ^{a}	0.56 ± 0.06 ^{b}	0.74 ± 0.15 ^{b}	0.88 ± 0.24 ^{b}	0.78 ± 0.19 ^{b}
GOT (IU/L) ^{4}	68.0 ± 12.0	80.5 ± 16.5	73.0 ± 10.0	84.0 ± 5.51	96.0 ± 21.07
GPT(IU/L) ^{5}	58.3 ± 17.1	56.5 ± 6.5	64.3 ± 13.2	45.7 ± 19.9	68.7 ± 13.3
TG (mg/dL) ^{6}	185.0 ± 45.5	180.7 ± 25.1	207.0 ± 18.9	182.37 ± 31.7	156.0 ± 26.5
T-Pro (g/dL) ^{7}	3.17 ± 0.23 ^{a}	3.67 ± 0.35 ^{ab}	3.76 ± 0.36 ^{ab}	4.22 ± 0.26 ^{b}	4.10 ± 0.26 ^{ab}
GLU (mg/dL) ^{8}	53.5 ± 5.5	52.0 ± 4.3	55.0 ± 9.1	57.3 ± 7.9	57.7 ± 10.2

Values are means ± SD of triplicate groups. Within a row, means with the same letters are not significantly different ($p > 0.05$), taking $p < 0.05$ (lowercase letters) as significant. ^{1} T-Cho: total cholesterol. ^{2} BUN: blood urea nitrogen. ^{3} T-Bill: Total bilirubin. ^{4} GOT: glutamyl oxaloacetic transaminase. ^{5} GPT: glutamic pyruvate transaminase. ^{6} TG: triglyceride. ^{7} T-Pro: total protein. ^{8} GLU: glucose.

3.4. Immune Responses

NBT activity was significantly elevated in fish fed the BN2, BN3, and BN4 diets relative to the control ($p < 0.05$). Among the BSN-supplemented groups, NBT levels were significantly higher in BN3 and BN4 than in BN1 ($p < 0.05$). Serum bactericidal activity was notably higher in BN3 than in both the control and BN1 groups ($p < 0.05$), whereas mucus bactericidal activity was significantly enhanced only in the BN3 group compared to the control ($p < 0.05$). Fish in the BN3 and BN4 groups exhibited significantly higher serum lysozyme activity than the control ($p < 0.05$), although mucus lysozyme activity did not differ significantly between the groups (Table 7).

Table 7. Immune indexes of experimental red sea bream.

Parameters	Test Groups				
	BN0	BN1	BN2	BN3	BN4
NBT (OD at 540 nm)	0.61 ± 0.02^{aA}	0.67 ± 0.04^{abAB}	0.78 ± 0.04^{bcAB}	0.82 ± 0.03^{cB}	0.81 ± 0.03^{cB}
Serum bactericidal activity (%)	68.30 ± 2.30^{a}	67.97 ± 3.50^{a}	76.88 ± 6.50^{ab}	85.23 ± 4.20^{b}	81.00 ± 5.26^{ab}
Mucus bactericidal activity (%)	62.30 ± 2.40^{a}	64.97 ± 2.90^{ab}	66.88 ± 2.65^{ab}	75.23 ± 3.10^{b}	76.40 ± 5.60^{ab}
Serum lysozyme activity (%)	14.20 ± 2.40^{a}	20.50 ± 2.90^{ab}	21.80 ± 2.65^{ab}	25.30 ± 3.10^{b}	26.20 ± 3.40^{b}
Mucus lysozyme activity (%)	23.20 ± 2.50	22.80 ± 2.90	26.88 ± 3.65	25.23 ± 3.10	26.40 ± 5.60

Values are means ± SD of triplicate groups. Within a row, means with the same letters are not significantly different ($p > 0.05$), taking $p < 0.05$ (lowercase letters) as significant and $p < 0.01$ (uppercase letters) as highly significant. Experimental conditions: upon completion of the 56 days feeding experiment, after a 24 h fasting period, blood samples were collected from three randomly selected fish from each tank.

3.5. Intestinal Microbiota

Intestinal microbiota analysis revealed significant increases in *B. subtilis* and *Lb.* populations in the BN3 and BN4 groups, respectively, compared to the control ($p < 0.05$). Additionally, the *E. coli* count was significantly reduced in the BN4 group compared to that in all other groups ($p < 0.05$). Notably, *B. subtilis* levels were higher in BN3 and BN4 than in BN1. However, no significant differences were observed in the total bacterial population across the dietary treatments (Table 8).

Table 8. Number of *B. subtilis*, *Lb.*, *E. coli*, and total bacteria in red sea bream intestines (lg (copies)/g content).

Parameters	Test Groups				
	BN0	BN1	BN2	BN3	BN4
B. subtilis	3.87 ± 0.45^{aA}	3.98 ± 0.42^{aAB}	5.50 ± 0.62^{abAB}	5.69 ± 0.71^{bAB}	6.21 ± 0.87^{bB}
Lb.	4.38 ± 0.54^{a}	4.53 ± 0.34^{ab}	5.00 ± 0.66^{ab}	6.01 ± 0.69^{b}	5.90 ± 0.51^{b}
E. coli	3.26 ± 0.20^{b}	3.11 ± 0.18^{b}	2.79 ± 0.17^{b}	2.59 ± 0.11^{b}	2.02 ± 0.08^{a}
Total bacteria	6.23 ± 0.25	6.43 ± 0.24	6.87 ± 0.31	7.02 ± 0.43	7.10 ± 0.50

Values are means ± SD of triplicate groups. Within a row, means with the same letters are not significantly different ($p > 0.05$), taking $p < 0.05$ (lowercase letters) as significant and $p < 0.01$ (uppercase letters) as highly significant.

3.6. Relative Gene Expression of Growth Factors

Expression of the skeletal muscle genes *IGF-1* and *IGF-2* is shown in Figure 2. Fish fed the BN2, BN3, and BN4 diets exhibited significantly higher IGF-1 mRNA levels than the control group ($p < 0.05$). Similarly, *IGF-2* mRNA expression was significantly elevated in the BN2, BN3, and BN4 groups relative to that in the control ($p < 0.05$).

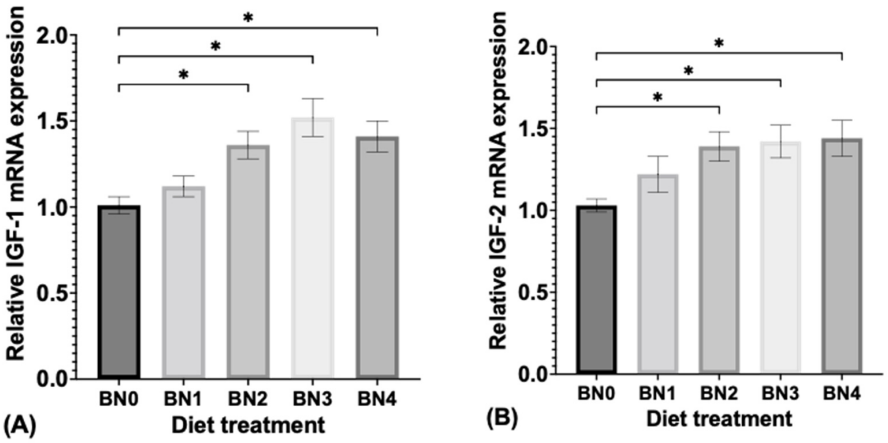

Figure 2. qPCR analysis of the relative expression of growth-related genes *IGF-1* (**A**) and *IGF-2* (**B**) in the skeletal muscle of red sea bream. Data represent means ± SD. The level of significance between the means was calculated using the Tukey–Kramer test, taking * as significant ($p < 0.05$).

4. Discussion

The results of this study revealed significant improvements in growth performance, digestive enzyme activities, immune responses, and intestinal microbiota composition in red sea bream fed diets supplemented with *Bacillus subtilis natto* (BSN). These findings align with and extend prior research on probiotics in aquaculture, particularly the use of *Bacillus* spp., which are known to promote fish health and performance by improving digestion, immune responses, and intestinal homeostasis [25–28].

4.1. Growth Performance and Feed Utilization

The significant increase in final body weight (FBW), weight gain rate (WGR), and specific growth rate (SGR) observed in fish fed BSN-supplemented diets, particularly at the BN3 and BN4 levels, suggests that BSN plays a vital role in promoting growth. This enhancement can be attributed to the probiotic's ability to improve nutrient assimilation and feed efficiency, supported by its positive effect on digestive enzyme activity, as indicated by the significant increase in protease and amylase activities [10,29,30]. Previous studies in tilapia (*Oreochromis niloticus*) and grouper (*Epinephelus coioides*) have reported similar improvements in growth performance with *Bacillus* spp. supplementation [27,31,32]. The presence of beneficial *Bacillus* strains in the gut enhances nutrient breakdown by producing extracellular enzymes, such as amylases and proteases, which aid in the digestion of carbohydrates and proteins, ultimately improving nutrient utilization [33,34].

Interestingly, while feed intake (FI) and feed conversion efficiency (FCE) were not [31,35,36] significantly different between the groups, an increasing trend was observed in BSN-supplemented diets. This suggests that BSN may enhance nutrient absorption efficiency without necessarily increasing feed intake, which is consistent with previous findings in other species, such as *Labeo rohita* [25,26] and catfish (*Clarias gariepinus*) [37]. *Bacillus* spp. have also been shown to produce essential micronutrients, such as vitamins and growth factors, which may promote better feed utilization [38–40].

Furthermore, the exogenous enzymatic activity of probiotics not only improves digestibility but also reduces the environmental impact of aquaculture by minimizing the waste load from undigested feed [35,36,41]. This could be critical for managing waste in intensive aquaculture systems. Future research could explore the role of BSN in enhancing the digestibility of alternative protein sources, such as plant-based ingredients, to reduce fish-meal dependency.

4.2. Digestive Enzyme Activity

BSN supplementation significantly increased protease and amylase activities in the gut—a result corroborated by studies in common carp and tilapia [42,43]. *Bacillus* probiotics enhance gut enzyme activity by promoting beneficial microbial colonization, which aids in nutrient breakdown and absorption [44,45]. This increased enzymatic activity is essential for optimizing the utilization of complex carbohydrates and proteins, which are often present in plant-based aquafeeds. Other species, such as Nile tilapia (*Oreochromis niloticus*) and Indian major carp (*Labeo rohita*), also show enhanced digestive enzyme activity when supplemented with probiotics [46,47]. Therefore, BSN's beneficial effects on digestive enzyme activity could have wide applicability across various commercially important aquaculture species.

4.3. Blood Biochemistry and Immune Response

BSN supplementation significantly increased hematocrit (Hct) and total plasma protein levels, indicating improved oxygen transport capacity and enhanced immune function. These findings are consistent with earlier studies showing that probiotics can boost immune parameters in aquaculture species [44,46,48]. Increased Hct reflects improved oxygen delivery, whereas elevated plasma protein levels are associated with enhanced immune responses [44,47]. Moreover, enhanced respiratory burst and lysozyme and serum bactericidal activities were observed, indicating a stronger non-specific immune response. *B.*

subtilis can stimulate macrophage activity and increase lysozyme levels, thus strengthening the host's resistance to pathogens, as observed in various probiotic studies [47,49]. Other fish species, such as grass carp (*Ctenopharyngodon idella*), have shown similar enhancements in non-specific immune responses following probiotic supplementation [49].

4.4. Intestinal Microbiota

BSN altered the intestinal microbiota composition of red sea bream, increasing beneficial bacteria, such as lactic acid bacteria and *Bacillus* species, while reducing harmful bacteria, such as *E. coli*. The balance of the gut microbiota is crucial for fish health, as beneficial bacteria produce short-chain fatty acids (SCFAs), maintain gut immune homeostasis, and inhibit pathogen growth [36,50]. The ability of probiotics to modulate the gut microbiota can improve feed utilization efficiency and decrease disease incidence, which is essential for sustainable aquaculture production [51–53]. Recent microbiome studies further suggest that probiotics can modulate gene expression in the host gut, thereby affecting nutrient absorption and metabolism [54–56]. Future research should explore the molecular mechanisms by which BSN regulates the intestinal microbiota to identify potential pathways for enhancing gut health and nutrient absorption.

4.5. Growth-Related Gene Expression

This study demonstrated that BSN supplementation upregulated the expression of growth-related genes (*IGF-1* and *IGF-2*) in the livers of red sea bream. This finding supports the hypothesis that BSN promotes growth through the IGF signaling pathway, which is crucial for muscle development and growth regulation [57]. In species such as golden pompano [58] and rainbow trout (*Oncorhynchus mykiss*) [59], IGF expression has been shown to correlate directly with growth rate. Further studies are needed to investigate how BSN modulates the IGF pathway at different developmental stages in fish.

5. Conclusions

This study demonstrated that dietary supplementation with BSN at concentrations of 1×10^9 and 1×10^{10} CFU/kg of feed positively influences growth, digestive enzyme activity, hematology, and intestinal microbial balance in red sea bream. These findings suggest that BSN supplementation not only supports efficient nutrient absorption and utilization but also strengthens both humoral and mucosal immune responses, making it a promising probiotic intervention in aquaculture. By modulating the gut microbiota and enhancing enzyme activity, BSN contributes to optimized digestive efficiency and improved overall health, promoting sustainable fish production. Future research should prioritize large-scale, multi-system trials to confirm BSN's efficacy across diverse aquaculture environments, further validating its role as a sustainable and effective feed additive for red sea bream and potentially other species.

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Institutional Review Board Statement: Rules of animal experimentation at Kagoshima University do not extend to fish. Ethical review and approval were waived for this study due to the absence of animal- or human-related studies. However, the protocol in the present study took due consideration for animal care (e.g., number of fish, fish handling, weighing of fish, and blood sampling).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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