

Effects of Dietary *Bacillus subtilis* Supplementation on Growth Performance, Liver Antioxidant Indices, Intestinal Microbiota Structure, and Disease Resistance in Juvenile Blunt Snout Bream (*Megalobrama amblycephala*) (Postprint)

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Abstract

This experiment was conducted to investigate the effects of dietary *Bacillus subtilis* supplementation on growth performance, hepatic antioxidant indices, intestinal microbiota structure, and disease resistance of juvenile blunt snout bream (*Megalobrama amblycephala*). A total of 360 juvenile fish with an initial body weight of (1.81 ± 0.01) g were randomly allocated into four groups (three replicates per group, 30 fish per replicate): group T1, 2×10^8 CFU/g *B. subtilis* (group T2), and 2×10^9 CFU/g *B. subtilis* (group T3). The feeding trial lasted for 8 weeks. The results showed that: 1) Compared with the control group, the weight gain rate and specific growth rate of juvenile blunt snout bream in groups T1 and T2 were significantly increased ($P < 0.05$), and the feed conversion ratio in group T1 was significantly decreased ($P < 0.05$). 2) Compared with the control group, the activities of hepatic antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) in group T1 were significantly increased ($P < 0.05$), while the hepatic malondialdehyde content in this group was significantly decreased ($P < 0.05$). 3) No significant differences were observed in whole-body moisture, crude protein, crude lipid, and crude ash contents among all groups ($P > 0.05$). 4) Denaturing gradient gel electrophoresis (DGGE) profiles revealed that the intestinal microbiota structure of juvenile blunt snout bream fed diets with different *B. subtilis* supplementation levels was altered. 5) Challenge with *Aeromonas hydrophila* showed that no significant difference in cumulative mortality was observed among groups after 96 h ($P > 0.05$), but the cumulative mortality in groups T1 and T2 was lower than that in the control and T3 groups. In conclusion, dietary supplementation of 2×10^7 CFU/g *B. subtilis* could improve growth performance, antioxidant capacity, and alter intestinal microbiota

structure in juvenile blunt snout bream, but had no significant effects on body composition and disease resistance.

Full Text

Effects of Dietary *Bacillus subtilis* on Growth Performance, Liver Antioxidant Indices, Intestinal Microflora Structure and Disease Resistance of Juvenile Blunt Snout Bream (*Megalobrama amblycephala*)

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Abstract: This experiment was conducted to investigate the effects of dietary *Bacillus subtilis* on growth performance, liver antioxidant indices, intestinal microflora structure, and disease resistance of juvenile blunt snout bream (*Megalobrama amblycephala*). A total of 360 juvenile blunt snout bream with an initial body weight of (1.81 ± 0.01) g were randomly divided into four groups (three replicates per group, 30 fish per replicate): T1 group, 2×10^8 CFU/g *B. subtilis* (T2 group), and 2×10^9 CFU/g *B. subtilis* (T3 group) for eight weeks. The results showed that: (1) The weight gain rate (WGR) and specific growth rate (SGR) of fish in T1 and T2 groups were significantly higher than those in the control group ($P < 0.05$), and the feed conversion ratio (FCR) of T1 group was significantly lower than that of the control group ($P < 0.05$). (2) Compared with the control group, the activities of liver antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) in T1 group were significantly increased ($P < 0.05$), while the hepatic malondialdehyde (MDA) content in this group was significantly decreased ($P < 0.05$). (3) No significant differences were observed in moisture, crude protein, crude lipid, and ash contents of whole body among all groups ($P > 0.05$). (4) Denaturing gradient gel electrophoresis (DGGE) profiles revealed that the intestinal microflora structure of juvenile blunt snout bream was altered when fed diets supplemented with different levels of *B. subtilis*. (5) Following challenge with *Aeromonas hydrophila*, no significant differences in cumulative mortality were observed among groups after 96 h ($P > 0.05$), but the cumulative mortality in T1 and T2 groups was lower than that in the control and T3 groups. It is concluded that supplementation of 2×10^7 CFU/g *B. subtilis* in the diet can improve growth performance and antioxidant capacity, and alter intestinal microflora structure in juvenile blunt snout bream, but has no significant effect on body composition and disease resistance.

Keywords: blunt snout bream (*Megalobrama amblycephala*); *Bacillus subtilis*; growth; antioxidant ability; intestinal microflora structure

With the rapid development of high-density intensive aquaculture, the aquaculture water environment has deteriorated, leading to disease outbreaks and mass mortality in cultured animals. Given the drawbacks of traditional antimicrobial agents, such as residues in animal tissues and induction of bacterial resistance, researchers worldwide have focused on developing alternatives to antibiotics. Consequently, probiotics have attracted widespread attention as a novel feed additive in the post-antibiotic era. Probiotics applied in cultured fish are defined as microbial cells that, when added to feed or applied to aquaculture water, can improve the water environment or intestinal microbial balance, thereby conferring beneficial effects on the host, including immune stimulation, improved gastrointestinal morphology, enhanced growth and feed utilization, and better flesh quality. Compared with other probiotics, *Bacillus subtilis* offers advantages such as good stability, strong resistance, high temperature and acid-alkali tolerance, and abundant enzyme production. Previous studies have shown that dietary supplementation with *B. subtilis* not only promotes growth and improves digestive enzyme activity and non-specific immunity in aquatic animals, but also enhances their intestinal microflora structure.

Blunt snout bream (*Megalobrama amblycephala*), belonging to Osteichthyes, Cypriniformes, Cyprinidae, Abramidinae, and *Megalobrama*, is one of the main cultured fish species in China due to its delicious meat, fast growth, and high economic value. With the expansion and promotion of intensive aquaculture models, diseases and major epidemics have frequently occurred in blunt snout bream due to high-density culture, increased feeding frequency, and water pollution. As nutrients are among the most important and easily regulated factors affecting disease resistance in aquatic animals, research on the application of *B. subtilis* in juvenile blunt snout bream diets is rarely reported. This study aims to investigate the effects of dietary *B. subtilis* supplementation on growth performance, liver antioxidant indices, intestinal microflora structure, and disease resistance of juvenile blunt snout bream, providing a scientific basis for the application of *B. subtilis* in blunt snout bream aquaculture.

1.1 *Bacillus subtilis* Preparation

The *B. subtilis* preparation used in this experiment was the B115 strain, provided by the Fish Disease Laboratory of Zhejiang Institute of Freshwater Fisheries. According to the experimental design, the preparation was cultured at 30°C with shaking at 180 r/min to produce bacterial suspensions containing different concentrations of *B. subtilis*. The bacterial count in the preparation was determined using the plate count method before use.

1.2 Experimental Diets

A basal diet was formulated using fish meal, soybean meal, rapeseed meal, cottonseed meal, and wheat flour as main ingredients, with its composition and

nutrient levels shown in Table 1 . Three experimental diets were prepared by replacing the blank bacterial liquid in the basal diet with bacterial suspensions containing different concentrations of *B. subtilis*. The basal diet contained 0 CFU/g *B. subtilis* (T0 group, as control), while the three experimental diets contained 2×10^7 (T1 group), 2×10^8 (T2 group), and 2×10^9 (T3 group) CFU/g *B. subtilis*, respectively. Feed ingredients were ground and passed through a 60-mesh sieve, mixed according to the proportions in Table 1. Minor components were incorporated using the stepwise dilution method. After adding the *B. subtilis* suspension, the mixture was thoroughly blended again and processed into 2.0 mm sinking pellets using an SLP-45 pellet mill (developed by Fishery Machinery and Instrument Research Institute, Chinese Academy of Fishery Sciences). The pellets were dried at 50°C and stored at 4°C until use.

1.3 Feeding Management

Juvenile blunt snout bream for the experiment were obtained from the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Fish were reared in circular tanks (820 mm diameter, 700 mm height) with a temperature-controlled recirculating water system. A total of 360 healthy juvenile blunt snout bream with an initial average weight of (1.81 ± 0.01) g were randomly divided into four groups with three replicates each (30 fish per replicate). During a two-week acclimation period prior to the experiment, fish were fed the control diet without *B. subtilis* until they exhibited active feeding behavior. Subsequently, fish in each group were randomly fed experimental diets containing different levels of *B. subtilis*. Fish were hand-fed to satiation four times daily at 08:00-08:30, 11:00-11:30, 14:00-14:30, and 17:00-17:30. The daily feeding rate was 2-4% of body weight and adjusted appropriately based on feeding and growth conditions. The experiment lasted for eight weeks, after which fish in each group were weighed and sampled. During the culture period, water temperature was maintained at 26-30°C, pH at 7.0-8.0, dissolved oxygen concentration above 5 mg/L, ammonia nitrogen below 0.4 mg/L, and nitrite nitrogen below 0.06 mg/L.

1.4 Sample Collection and Analysis

1.4.1 Sample Collection and Preparation At the end of the experiment, fish were fasted for 24 h before counting survival numbers and measuring body weight and length. Three fish were randomly selected from each group, immediately dissected to remove the liver, which was weighed and stored in centrifuge tubes at -80°C for determination of malondialdehyde (MDA) content and superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities. The intestine was also isolated, placed in sterile centrifuge tubes, and stored at -80°C for intestinal microflora structure analysis. Another three fish from each group were sampled for whole-body proximate composition analysis.

1.4.2 Determination of Liver Antioxidant Indices The activities of SOD, CAT, and GSH-Px, as well as MDA content in the liver, were determined using

assay kits from Nanjing Jiancheng Bioengineering Institute according to the manufacturer' s instructions. Liver protein content was determined using the Coomassie brilliant blue method.

1.4.3 Determination of Whole-Body Proximate Composition Moisture content was determined by drying samples to constant weight in an oven at 105°C under normal pressure. Crude protein content was measured using the Kjeldahl method (GB/T 6432-1994). Crude lipid content was determined using the Soxhlet extraction method (GB/T 6432-1994). Ash content was measured using the 560°C incineration method (GB/T 6438-1992).

1.4.4 Determination of Intestinal Microflora Structure Intestinal microflora structure was analyzed by denaturing gradient gel electrophoresis (DGGE) followed by cloning and sequencing. Bacterial genomic DNA was amplified using universal bacterial primers 518R and 341F with GC-clamp, targeting the V3 variable region of bacterial 16S rDNA. Amplified products were detected by 1% agarose gel electrophoresis. PCR products were recovered and purified from the 1% agarose gel using a gel extraction purification kit (KAPA 2G Robust PCR Kit, Dalian Bao Biological Engineering Co., Ltd.) according to the manufacturer' s instructions. PCR amplicons were separated by DGGE using a 35-55% denaturing gradient and 8% polyacrylamide gel (chemical denaturant: 100% urea 7 mol/L and 40% acrylamide). Electrophoresis was performed at 150 V and 60°C in 1×TAE buffer for 5 h. After electrophoresis, gels were silver-stained and visualized using a gel imaging system.

1.4.5 Determination of Growth Performance Growth performance indices were calculated using the following formulas: - Weight gain rate (WGR, %) = $100 \times (\text{final average weight} - \text{initial average weight}) / \text{initial average weight}$ - Specific growth rate (SGR, %/d) = $100 \times (\ln \text{final average weight} - \ln \text{initial average weight}) / \text{culture days}$ - Feed conversion ratio (FCR) = $\text{dry feed intake} / (\text{final body weight} - \text{initial body weight})$ - Survival rate (SR, %) = $100 \times \text{final fish number} / \text{initial fish number}$

1.5 Challenge Test

After sampling, the remaining blunt snout bream in each group continued to be fed their respective diets for one week before the *Aeromonas hydrophila* challenge test. Feeding was stopped during the challenge period. A preliminary test with different concentration gradients of *A. hydrophila* was conducted to determine the median lethal dose (LD₅₀). Based on the preliminary results, fish were intraperitoneally injected with 0.5 mL of physiological saline containing *A. hydrophila* at a concentration of 5×10^7 CFU/mL (equivalent to 50 g body weight). Mortality was recorded 96 h post-injection.

1.6 Data Analysis

Experimental data were analyzed using one-way ANOVA in SPSS 16.0 statistical software package. If significant differences were detected, Tukey' s multiple comparison test was performed. The significance level was set at $P < 0.05$. All results are expressed as mean \pm standard error (mean \pm SE).

2.1 Growth Performance

As shown in Table 2 , the final body weight, weight gain rate, and specific growth rate of fish in T1 and T2 groups were significantly higher than those in the control group without *B. subtilis* supplementation ($P < 0.05$), and the feed conversion ratio of T1 group was significantly lower than that of the control group ($P < 0.05$). However, T3 group with the highest *B. subtilis* supplementation level showed lower weight gain rate and specific growth rate compared with T1, T2, and control groups, indicating that excessive dietary *B. subtilis* had an inhibitory effect on the growth of juvenile blunt snout bream.

2.2 Body Composition

Table 3 shows that no significant differences were observed in moisture, crude protein, crude lipid, and ash contents of whole body among fish fed diets with different *B. subtilis* supplementation levels and the control group ($P > 0.05$).

2.3 Liver Antioxidant Indices

As shown in Table 4 , the activities of SOD, CAT, and GSH-Px in T1 group were significantly higher than those in the control group ($P < 0.05$), while the MDA content in T1 group was significantly lower than that in the control group ($P < 0.05$), indicating that appropriate dietary *B. subtilis* supplementation could enhance antioxidant enzyme activities and reduce MDA accumulation in the liver of juvenile blunt snout bream. No significant differences in SOD, CAT, GSH-Px activities, or MDA content were observed between T2, T3 groups and the control group ($P > 0.05$).

2.4 Intestinal Microflora Structure

As shown in Figure 1 [Figure 1: see original paper], the similarity range of DGGE profiles and cluster analysis was 38-86%. The similarity between the control group and the three treatment groups (T1, T2, and T3) was relatively low, indicating that the intestinal microflora structure of blunt snout bream fed diets containing *B. subtilis* was significantly altered.

2.5 Challenge Test

Table 5 shows that the cumulative mortality of all groups exceeded 50% at 96 h after intraperitoneal injection with *A. hydrophila*. The cumulative mortality

rates for the control, T1, T2, and T3 groups were 61.7%, 51.7%, 53.3%, and 58.3%, respectively. Although T1 and T2 groups showed lower cumulative mortality than the control and T3 groups, no significant differences were observed among groups ($P>0.05$).

3.1 Effects of Dietary *Bacillus subtilis* on Growth Performance of Juvenile Blunt Snout Bream

Previous studies have shown that *B. subtilis* significantly affects the composition of intestinal microflora in fish, and probiotics play an important role in establishing a healthy and stable intestinal microflora system in larvae. Research has demonstrated that various *B. subtilis* strains added to larval fish diets have achieved good results, promoting larval growth. The present study found that appropriate dietary *B. subtilis* supplementation improved the weight gain rate and specific growth rate while reducing the feed conversion ratio in juvenile blunt snout bream, indicating a growth-promoting effect. These results are consistent with findings in Nile tilapia (*Oreochromis niloticus*), Pacific white shrimp (*Litopenaeus vannamei*), and broiler chickens (*Gallus gallus*). However, excessive *B. subtilis* supplementation showed an inhibitory effect on growth, which aligns with the dosage recommendations for probiotics by Gao et al. This may be attributed to two factors: first, excessive enzyme production by bacilli may inhibit endogenous enzyme activity, reducing nutrient breakdown; second, excessive *B. subtilis* supplementation may disrupt the microbial balance in fish, consequently affecting growth performance.

3.2 Effects of Dietary *Bacillus subtilis* on Liver Antioxidant Indices of Juvenile Blunt Snout Bream

SOD plays a crucial role in the antioxidant enzyme system of blunt snout bream and other aquatic animals. Excess superoxide anion radicals in fish are eliminated by antioxidant enzymes including SOD and CAT, primarily through SOD-mediated dismutation of radicals into hydrogen peroxide (H_2O_2), which is then decomposed into harmless water (H_2O) by CAT and GSH-Px, thereby preventing radical accumulation and toxicity. The present study demonstrated that dietary supplementation with 2×10^7 CFU/g *B. subtilis* significantly promoted antioxidant enzyme activities while reducing lipid peroxidation product MDA content in juvenile blunt snout bream.

3.3 Effects of Dietary *Bacillus subtilis* on Intestinal Microflora Structure of Juvenile Blunt Snout Bream

This experiment revealed that different *B. subtilis* supplementation levels affected the intestinal microflora structure of juvenile blunt snout bream. Bands in DGGE profiles represent potential dominant bacterial groups or operational taxonomic units (OTUs). The mean number of bands in the control group was

higher than in the three treatment groups, indicating that *B. subtilis* supplementation reduced the diversity of intestinal microflora structure. Consequently, cluster analysis showed low similarity between the control group and the three treatment groups (T1, T2, and T3), confirming that the intestinal microflora structure of blunt snout bream fed *B. subtilis*-supplemented diets was significantly altered.

3.4 Effects of Dietary *Bacillus subtilis* on Disease Resistance of Juvenile Blunt Snout Bream

Aeromonas hydrophila is one of the main pathogenic bacteria causing disease in blunt snout bream and other freshwater fish. In this study, fish were intraperitoneally injected with a certain dose of *A. hydrophila*, and after 96 h, T1 and T2 groups showed lower cumulative mortality than the control group, while T3 group exhibited the highest mortality. These results indicate that appropriate *B. subtilis* supplementation can improve disease resistance in juvenile blunt snout bream, consistent with findings in striped bass and Nile tilapia. Based on the observed enhancement of antioxidant capacity and improvement of intestinal microflora structure by appropriate *B. subtilis* supplementation, we hypothesize that dietary probiotics can enhance the digestive capacity of indigenous beneficial bacteria, strengthen the antioxidant system, and consequently improve immunity and disease resistance in fish.

Appropriate dietary *Bacillus subtilis* supplementation can improve growth performance, antioxidant capacity, and alter intestinal microflora structure in juvenile blunt snout bream.

The optimal dietary supplementation level of *B. subtilis* for juvenile blunt snout bream is 2×10^7 CFU/g.

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