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Effects of Probiotics on the Water Quality, Growth Performance, Immunity, Digestion, and Intestinal Flora of Giant Freshwater Prawn (*Macrobrachium rosenbergii*) in the Biofloc Culture System

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Abstract: In order to explore the effects of probiotics on the water quality, growth performance, nonspecific immunity, digestion, and intestinal flora of *Macrobrachium rosenbergii* in the biofloc culture system, three groups (six replicates in each group) were set up and divided into no bacteria (control group, Con), *Bacillus subtilis* (BS), and effective microorganisms (EM) in the current experiment. After dissolution of the water, the carbon source (glucose) was evenly sprayed in the aquaculture tank to construct a biofloc with a C/N of 15. A total of 1260 giant freshwater prawn (*Macrobrachium rosenbergii*) with an initial body weight of (2.09 ± 0.03) g were randomly assigned to 18 tanks (70 per tank). BS group and EM group significantly reduced total nitrogen concentration. Both BS and EM groups significantly increased final mean body weight, weight gain, and specific growth rate, but significantly decreased feed conversion rate of *Macrobrachium rosenbergii*. BS group significantly increased plasma superoxide dismutase, lysozyme, and acid phosphatase contents, but significantly decreased plasma malonaldehyde content. EM group significantly increased serum acid phosphatase content and intestinal trypsin activity but significantly decreased the Chao and ACE index of species richness. BS group and EM group significantly decreased the abundance of Chloroflexi and Verrucomicrobiota. BS group significantly increased the abundance of *Bacillus*. Overall, adding probiotics affected water quality, *Macrobrachium rosenbergii* performance, and microbial community. The results showed that *Bacillus subtilis* is a good biofloc probiotic additive.

Keywords: *Bacillus subtilis*; effective microorganisms; water quality; growth performance; immunity; intestinal flora; *Macrobrachium rosenbergii*

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1. Introduction

With the continuous expansion of aquaculture industry in China, feed inputs are high while the utilization rate of these inputs is low for aquaculture animals, leading to the continuous accumulation of ammonia nitrogen in water [1]. Ammonia nitrogen is the most harmful pollution factor to aquatic animals under the current inland intensive breeding mode. When the ammonia nitrogen in water accumulates to a certain extent, it tends to exert adverse effects on the growth, feeding, energy metabolism, osmotic regulation, and other life activities of the breeding objects [2]. In severe cases, it will cause large-scale death of aquatic animals and massive economic losses. So, we urgently need new sustainable technologies to solve the problem.

Biofloc technology (BFT) is a new water quality control technology developed in recent years. By adding organic carbon to the body of water and maintaining a certain

carbon–nitrogen ratio, nitrogen-containing waste in the body of water is transformed into microbial protein to purify the quality of the water [3], provide bait [4,5], and improve the immunity of aquaculture animals [6,7]. Biofloc is composed of heterotrophic bacteria, algae, protozoa, phytoplankton, rotifers, annelids, nematodes, copepods, and unfed food [8]. The composition of microorganisms in biofloc plays an important role in the performance of functions, and beneficial microorganisms play a role in protecting animal health [9,10]. At the same time, the presence of harmful microorganisms also causes concern [11]. By adjusting the species and abundance of beneficial microorganisms and letting them dominate the flora, the abundance of harmful microorganisms can be reduced [12]. In this way, the function of biological flocs can be strengthened, which is conducive to the popularization and application of BFT in aquaculture production. At present, the ways of affecting the flora in biofloc include carbon source [13], C/N [14], Ph [15], and the adding of exogenous probiotics [16].

Probiotics is a kind of live bacteria preparation that can improve animal immunity by using various beneficial microorganisms and special processes [17]. Studies have shown that probiotics can provide beneficial bacteria to aquaculture animals, make the intestinal microenvironment more stable [16], and help aquaculture animals inhibit the growth of pathogenic bacteria in the host [18], which is beneficial for the prevention and treatment of diseases. In addition, beneficial bacteria can also interact with the mucosal immune system to form a biological barrier [19,20], which can help the body resist invasion by pathogenic bacteria. Studies have found that the adding of probiotics to aquaculture water can reduce harmful substances in the water environment and decompose large particulate organic matter, thus purifying water quality [21]. At present, numerous studies have shown that adding probiotics to BFT can consume a large amount of oxygen in biofloc, thereby preventing the growth of aerobic pathogens [22]. Furthermore, the addition of probiotics to biofloc can balance the living environment of microorganisms, algae, and cultured animals in water [23]. Effective microorganisms are a kind of compound microbial agent which is cultivated by *photosynthetic bacteria*, *yeast*, *lactic acid bacteria*, and *actinomycetes* [24]. *Bacillus subtilis* has the advantages of being free from residues, which can rapidly spread in the intestinal tract of animals and inhibit the reproduction of harmful bacteria in the intestinal tract [25,26]. Several studies have shown that *Bacillus subtilis* can improve immunity [27], increase feed conversion rate [28], improve the breeding environment [29,30], and inhibit oxidative stress and other functions [31]. Santos et al. [32] showed that adding *Bacillus subtilis* to the BFT system can effectively improve water quality and improve the growth performance of farming tambaqui (*Colossoma macropomum*). Effective microorganisms can absorb or degrade organic or toxic substances in aquaculture water [33], promote the growth of aquatic animals [34], promote the proliferation of immune cells and immune organs [35], improve nonspecific immunity and specific immunity [36], and contribute to the colonization of beneficial bacteria [37].

The giant freshwater prawn (*Macrobrachium rosenbergii*) is popular in China due to its rapid growth, short breeding cycle, strong resistance to disease, and high economic benefits. The intensive breeding mode limits the development of the *M. rosenbergii* industry. With the deterioration of water quality, the invasion of pathogenic bacteria has a great impact on the health of *M. rosenbergii*. At present, there are few studies on the effect of adding beneficial bacteria to biofloc on *M. rosenbergii*. In this experiment, different probiotics were introduced into the biofloc system to explore the effects of microecologically cultured biofloc on the water quality, growth performance, nonspecific immunity, digestion, and intestinal flora of *M. rosenbergii*.

2. Materials and Methods

2.1. Probiotics

Glucose was purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. *Bacillus subtilis* and Effective Microorganisms (EM) were purchased from Guangdong Haida Group Co., Ltd., Guangzhou China.

2.2. Experimental Design and Diet

The experiment was carried out in the greenhouse of the College of Life Sciences of Huzhou University for 60 days. In the experiment, three groups were established, respectively. They were no bacteria (control group), *Bacillus subtilis* (BS group), and effective microorganisms (EM group). A total of 1260 *M. rosenbergii* (2.09 ± 0.03 g) was randomly assigned to 18 tanks (2 m × 1 m × 1 m) with 70 per replicate. The bacteria were added every 6 days and the dose of *Bacillus subtilis* and effective microorganisms was 10^9 CFU/mL. The carbon source was added by a C:N of 15 to construct biofloc according to Avnimelech [38]. The carbon source was dissolved in water and evenly sprinkled in the breeding tank. The feed was fed at 7:30 and 17:30 every day according to 2% of the body weight of *M. rosenbergii* (Table 1). The experiment was conducted with 24 h aerating, water temperature 26 ± 1 °C, pH 7.6 ± 0.1, and dissolved oxygen 6.5 ± 1 mg/L.

Table 1. Ingredients and chemical composition of the experimental diets (%).

Ingredients	Composition of Diets (%)
White fish meal	32.00
Gluten flour	8.00
Soybean meal	16.00
Corn starch	24.98
Corn protein	8.00
Soybean oil	2.00
Soybean lecithin	2.00
Fish oil	0.50
Calcium dihydrogen phosphate	2.00
Choline chloride	0.50
Arginine	0.37
Cholesterol	0.50
Sodium carboxymethylcellulose	2.00
Vitamin premix [1]	0.50
Mineral premix [2]	0.50
Magnesium sulfate	0.15
Proximate composition (% dry matter)	
Crude protein	39.17
Crude lipid	6.84
Ash	10.00

[1] Vitamin premix: provided by Guangzhou Xintun Fishery Technology Co., Ltd., Guangzhou, China. [2] Mineral premix: The amount of mineral elements added per kilogram of feed: $MnSO_4 \cdot H_2O$ 35.80 mg, $CoCl_2$ 29.94 mg, $FeSO_4 \cdot 7H_2O$ 9.54 mg, $CuSO_4 \cdot 5H_2O$ 30.23 mg, $ZnSO_4 \cdot H_2O$ 15.76 mg, Na_2SeO_3 0.89 mg, KI 1.06 mg.

2.3. Water Quality Monitoring and Measurement

During the breeding experiment, the portable multi-parameter water quality detector (YSI Pro Plus) was used to measure the water temperature, pH, and dissolved oxygen at the same location every day. When the temperature and pH reading were stable, the probe of the water quality detector was put into the water for 30 s and recorded. The determination of dissolved oxygen value required the probe of the water quality detector to swing left and right in the water, and was recorded after the reading rose to a stable high value. Water samples were taken every six days. The water samples were filtered through a 0.45 μm microporous membrane using a vacuum pump. The filtrate was used for the determination of total nitrogen (alkaline potassium persulfate digestion ultraviolet spectrophotometry), ammonia nitrogen (salicylic acid spectrophotometry), and nitrite nitrogen (N-(1-naphthyl)-ethylenediamine spectrophotometry) in water.

2.4. Growth Performance

At the end of the experiment, the weight of *M. rosenbergii* was weighed in each pond and the number of remaining survival was recorded. Survival rate, weight gain, feed

conversion ratio, and specific growth rate of *M. rosenbergii* were calculated. The relevant formulas are as follows:

$$\text{Weight gain (WG, \%)} = (W_f - W_i)/W_i \times 100;$$

$$\text{Feed conversion ratio (FCR)} = \text{Feed consumed (g)}/(W_f - W_i);$$

$$\text{Survival rate (\%)} = (N_f/N_i) \times 100;$$

$$\text{SGR (Specific growth rate, \%/day)} = ((\ln W_f - \ln W_i)/\text{days}) \times 100;$$

where W_f and W_i are the initial and final body weight, while N_f and N_i are the final and initial numbers of *M. rosenbergii*.

2.5. Sample Collection and Analysis

Three *M. rosenbergii* in the molting interval were randomly selected from each replicate, and the hemolymph was extracted from the ventral surface between the carapace and the first abdominal segment of the shrimp by oblique insertion with a disinfected No.5 needle and a 1 mL sterile syringe. Samples were centrifuged at 4 °C for 10 min and stored in a refrigerator at −80 °C for determination of superoxide dismutase (SOD), malondialdehyde (MDA), acid phosphatase (ACP), and lysozyme (LZM).

Three *M. rosenbergii* were randomly selected from each replicate and placed on an ice tray. The intestine and hepatopancreas were separated and the homogenate was homogenized in an ice bath with a high-speed tissue homogenizer in a mass volume ratio of 1:9 with normal saline. The homogenate was centrifuged at 4000 rpm at 4 °C for 10 min and the supernatant was a crude enzyme solution. The supernatant was divided into 1.5 mL centrifuge tubes and stored in a −80 °C refrigerator to determine amylase, trypsin, and lipase activities.

2.6. Microbiota Analysis

At the end of the experiment, three *M. rosenbergii* were randomly selected from each replicate, and intestinal contents were collected and placed in a sterile 1 mL centrifuge tube, and sent to Shanghai Meiji Biomedical Technology Co., Ltd. using 16S rRNA high-throughput sequencing, microbial community structure, and bioinformatic analysis.

2.7. Statistical Analysis

All the experimental data were analyzed by one-way ANOVA using the software SPSS 25.0. Duncan's multiple comparisons test was used to determine the treatment groups' differences. Results were considered statistically significant at the level of $p < 0.05$.

For high-throughput sequencing data, the Kruskal–Wallis rank sum test was used for multiple comparisons to test the significant difference between groups, and $p < 0.05$ was the significant difference.

3. Results

3.1. Water Quality Parameter

As shown in Figure 1, the total nitrogen concentration of each group showed an upward trend during the experiment, and the total nitrogen concentration of the BS group and the EM group was significantly lower than that of the Con group ($p < 0.05$). The concentration of ammonia nitrogen in each group first increased, then decreased, and then stabilized. The concentration of ammonia nitrogen reached its maximum on the 18th day. The concentration of ammonia nitrogen in the BS and EM groups was significantly lower than that in the Con group on the 48th, 54th, and 60th days ($p < 0.05$). The concentration of nitrite nitrogen in each group first increased, then decreased, and then tended to remain stable. Nitrite nitrogen concentration reached its maximum on the 24th day. There were no significant differences in the concentration of nitrite nitrogen between the groups ($p > 0.05$).

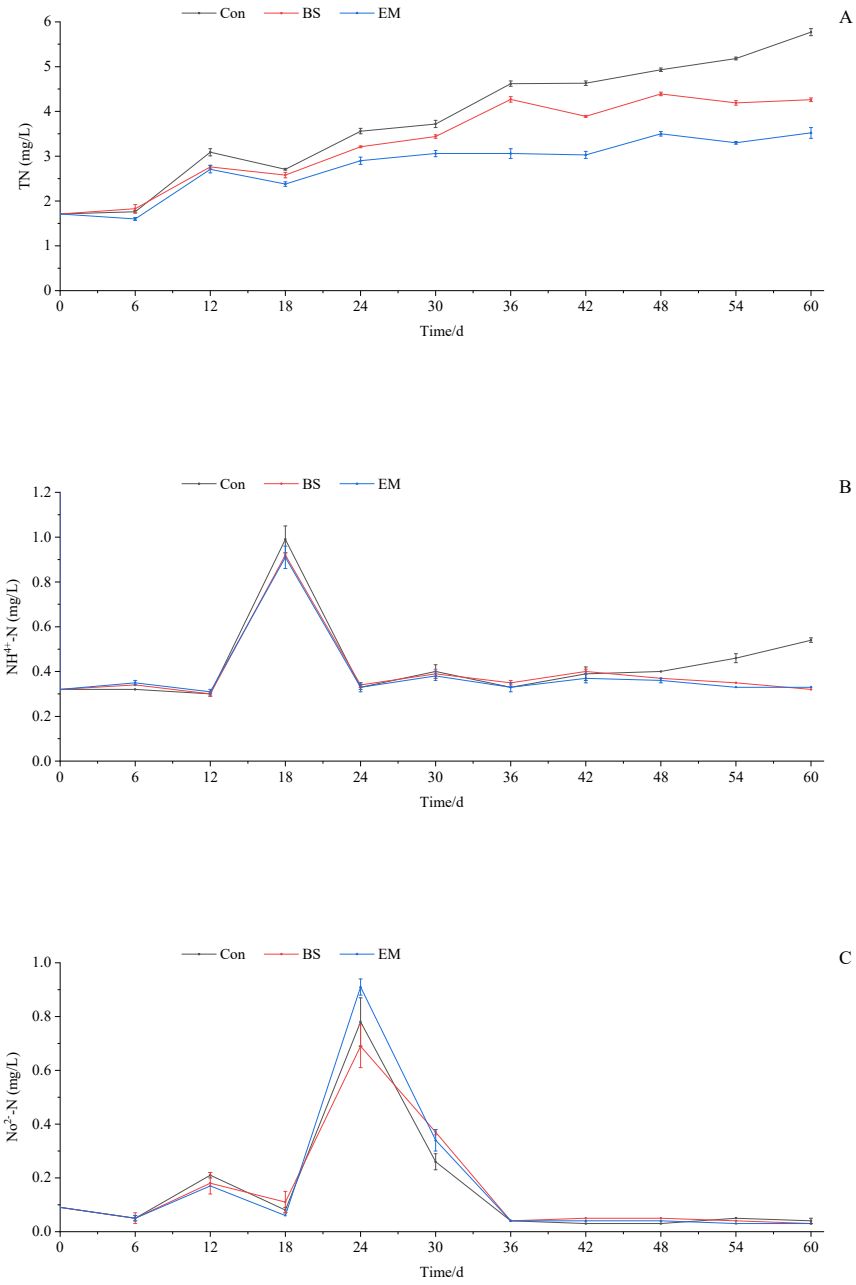

Figure 1. Effect of probiotics on water quality parameters. (**A**) TN, total nitrogen; (**B**) NH^{4+}-N, ammonia nitrogen; (**C**) NO^{2-}-N, nitrite nitrogen; Error bars are presented as the mean ± standard error (n = 3).

3.2. Growth Performance

The results showed that compared to the control group, the BS and EM groups significantly increased FBW, WG, and SGR, but significantly decreased FCR ($p < 0.05$). There were no significant differences in SR between the groups ($p > 0.05$) (Table 2).

Table 2. Effects of probiotics on growth performance of *M. rosenbergii*.

Groups	IBW(g)	FBW (g)	SR (%)	SGR (%/Day)	WG (%)	FCR
Con	2.09 ± 0.02	9.69 ± 0.26 ^{b}	63.57 ± 2.01	2.55 ± 0.06 ^{b}	364.37 ± 15.74 ^{b}	1.45 ± 0.03 ^{a}
BS	2.09 ± 0.03	11.35 ± 0.21 ^{a}	66.43 ± 1.32	2.82 ± 0.03 ^{a}	443.10 ± 8.33 ^{a}	1.35 ± 0.01 ^{b}
EM	2.09 ± 0.05	10.66 ± 0.29 ^{a}	66.19 ± 3.01	2.71 ± 0.03 ^{a}	409.09 ± 9.39 ^{a}	1.39 ± 0.01 ^{b}

Note: IBW: Initial body weight; FBW: Final body weight; WG: Weight gain; SR: Survival rate; FCR: Feed conversion rate; SGR: Specific growth rate; Values are presented as means ± standard error (n = 6). Mean values in the same row with different superscripts are significantly different ($p < 0.05$).

3.3. Plasma Immune Indices

Compared with the control group, the BS group significantly increased the plasma SOD content. The BS group significantly decreased the plasma MDA content ($p < 0.05$); the EM group significantly increased the plasma MDA content ($p < 0.05$). The BS group and the EM group significantly increased ACP content. The BS group significantly increased plasma LZM content ($p > 0.05$) (Table 3).

Table 3. Effects of probiotics on plasma immunity of *M. rosenbergii*.

Groups	SOD (U/mL)	MDA (nmol/mL)	ACP (U/100 mL)	LZM (μg/mL)
Con	72.16 ± 2.24 ^{b}	37.29 ± 0.26 ^{b}	6.23 ± 0.08 ^{b}	2.84 ± 0.05 ^{b}
BS	95.38 ± 1.70 ^{a}	33.11 ± 0.40 ^{c}	15.24 ± 0.30 ^{a}	3.52 ± 0.09 ^{a}
EM	68.33 ± 2.28 ^{b}	49.81 ± 0.28 ^{a}	15.01 ± 0.59 ^{a}	2.70 ± 0.17 ^{b}

Note: SOD: Superoxide dismutase; MDA: Malonaldehyde; ACP: Acid phosphatase; LZM: Lysozyme; Values are presented as means ± standard error (n = 6). Mean values in the same row with different superscripts are significantly different ($p < 0.05$).

3.4. Intestinal Digestive Enzyme Activity

Compared to the control group, the trypsin activity of the BS group increased significantly ($p < 0.05$). There were no significant differences in amylase and lipase activities among all groups ($p > 0.05$) (Table 4).

Table 4. Effects of probiotics on digestive enzyme activity of *M. rosenbergii*.

Groups	Amylase (U/mgprot)	Trypsin (U/mgprot)	Lipase (U/mgprot)
Con	0.81 ± 0.12	283.41 ± 32.85 ^{b}	11.23 ± 0.99
BS	0.84 ± 0.14	615.66 ± 48.71 ^{a}	14.00 ± 0.94
EM	0.85 ± 0.14	285.08 ± 11.42 ^{b}	11.88 ± 0.65

Note: Values are presented as means ± standard error (n = 6). Mean values in the same row with different superscripts are significantly different ($p < 0.05$).

3.5. Analysis of Intestinal Microbial Diversity

Compared to the control group, the EM group significantly decreased the species richness Chao and ACE index ($p < 0.05$). There were no significant differences in Shannon diversity index between groups ($p > 0.05$) (Table 5).

Table 5. Effects of probiotics on intestinal microbial diversity of *M. rosenbergii*.

Groups	Chao	Ace	Shannon
Con	656.65 ± 35.45 ^{a}	648.90 ± 35.50 ^{a}	1.91 ± 0.10
BS	572.64 ± 20.86 ^{ab}	610.32 ± 16.37 ^{ab}	2.13 ± 0.15
EM	450.74 ± 40.94 ^{b}	502.62 ± 28.74 ^{b}	1.66 ± 0.13

Note: Values are presented as means ± standard error (n = 4). Mean values in the same row with different superscripts are significantly different ($p < 0.05$).

3.6. Analysis of Predominant Intestinal Flora

The dominant bacteria at the biofloc level were analyzed. The results are shown in Figure 2. The top four dominant bacteria in biofloc abundance were Firmicutes, Proteobacteria, Actinobacteriota, and Chloroflexi. The abundance of Chloroflexi and Verrucomicrobiota decreased significantly in the BS group and the EM group ($p < 0.05$) (Figure 3).

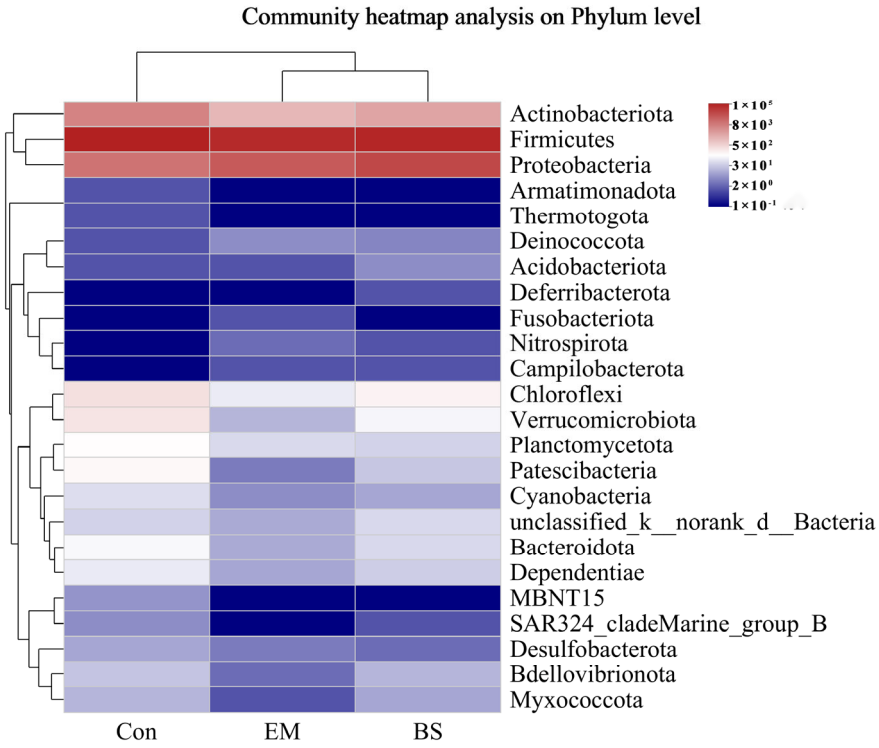

Figure 2. Analysis of dominant flora at phylum level in the intestine of *M. rosenbergii*.

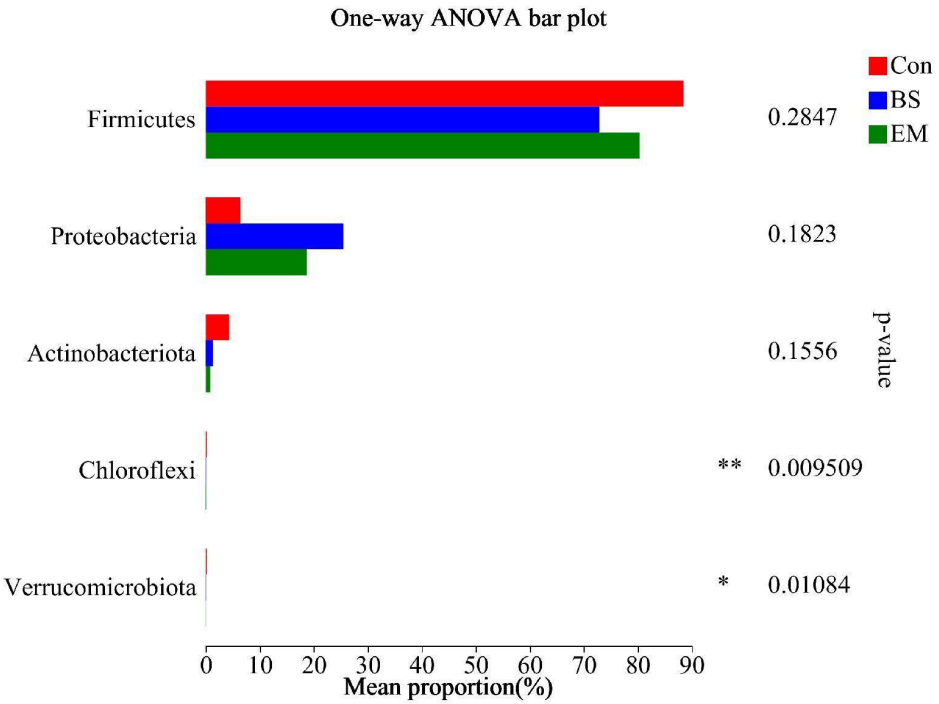

Figure 3. Analysis of significant differences in the dominant flora of the intestinal tract of *M. rosenbergii* at the phylum level. Significance ($p < 0.05$) indicated by *. Significance ($p < 0.01$) indicated by **.

The dominant flora of the genus biofloc level was analyzed and the results are shown in Figure 4. The three main dominant bacteria in the Con group were *Lactococcus* (87.31%), *Aeromonas* (2.98%) and *Nocardioides* (1.50%). The BS group were *Lactococcus* (69.40%), *Aeromonas* (21.26%), and *Bacillus* (2.69%). The EM group were *Lactococcus* (80.66%), *unclassified_f_Enterobacteriaceae* (13.19%), and *Aeromonas* (2.86%). Compared to the control group, BS group significantly increased the abundance of *Bacillus* ($p < 0.05$) (Figure 5).

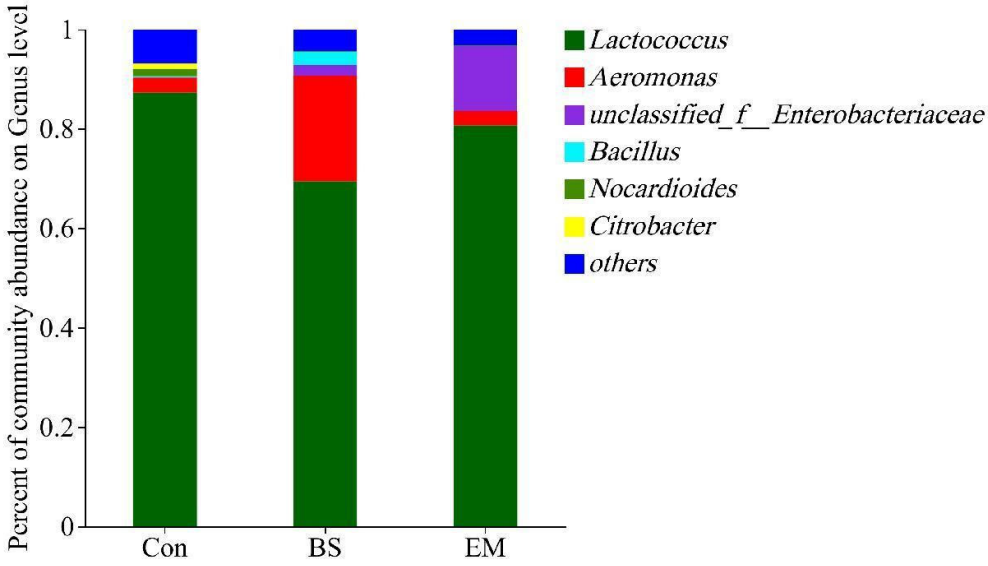

Figure 4. Analysis of dominant flora at genus level in the intestine of *M. rosenbergii*.

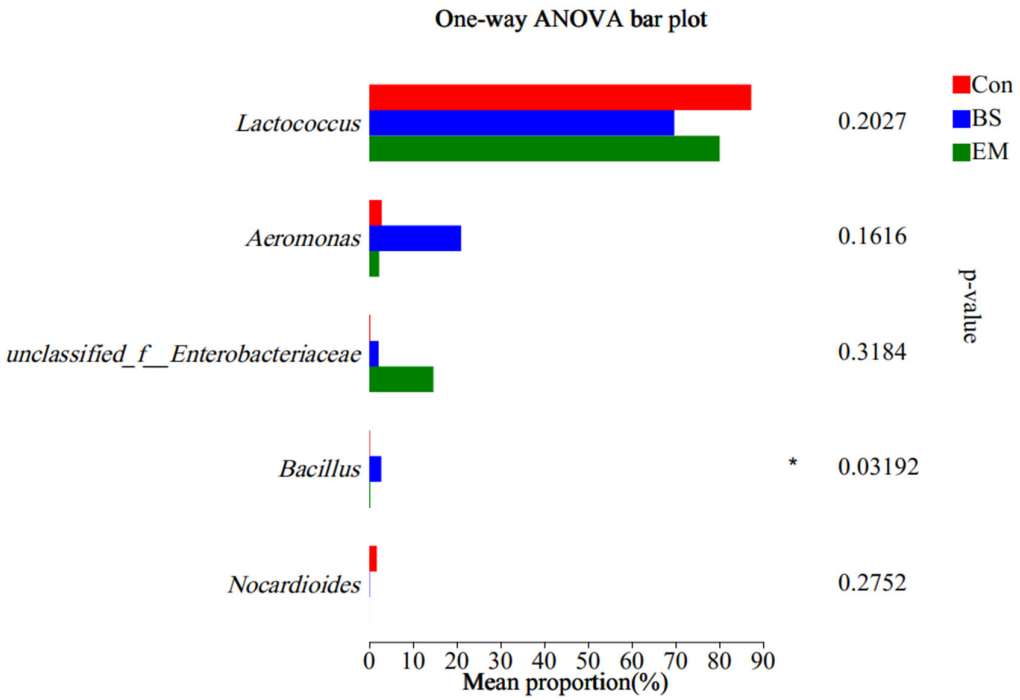

Figure 5. Analysis of significant differences in the dominant flora of the intestinal tract of *M. rosenbergii* at the genus level. Significance ($p < 0.05$) indicated by *.

3.7. Microbial Community Analysis

PICRUSt2 was used to predict the function of intestinal flora at level II. The functional abundance values of global and overview maps, carbohydrate metabolism, amino acid metabolism, membrane transport, energy metabolism, translation, metabolism of cofactors

and vitamins, replication and repair, nucleotide metabolism, and signal transduction in BS group were significantly higher than those in Con group and EM group ($p < 0.05$) (Figure 6).

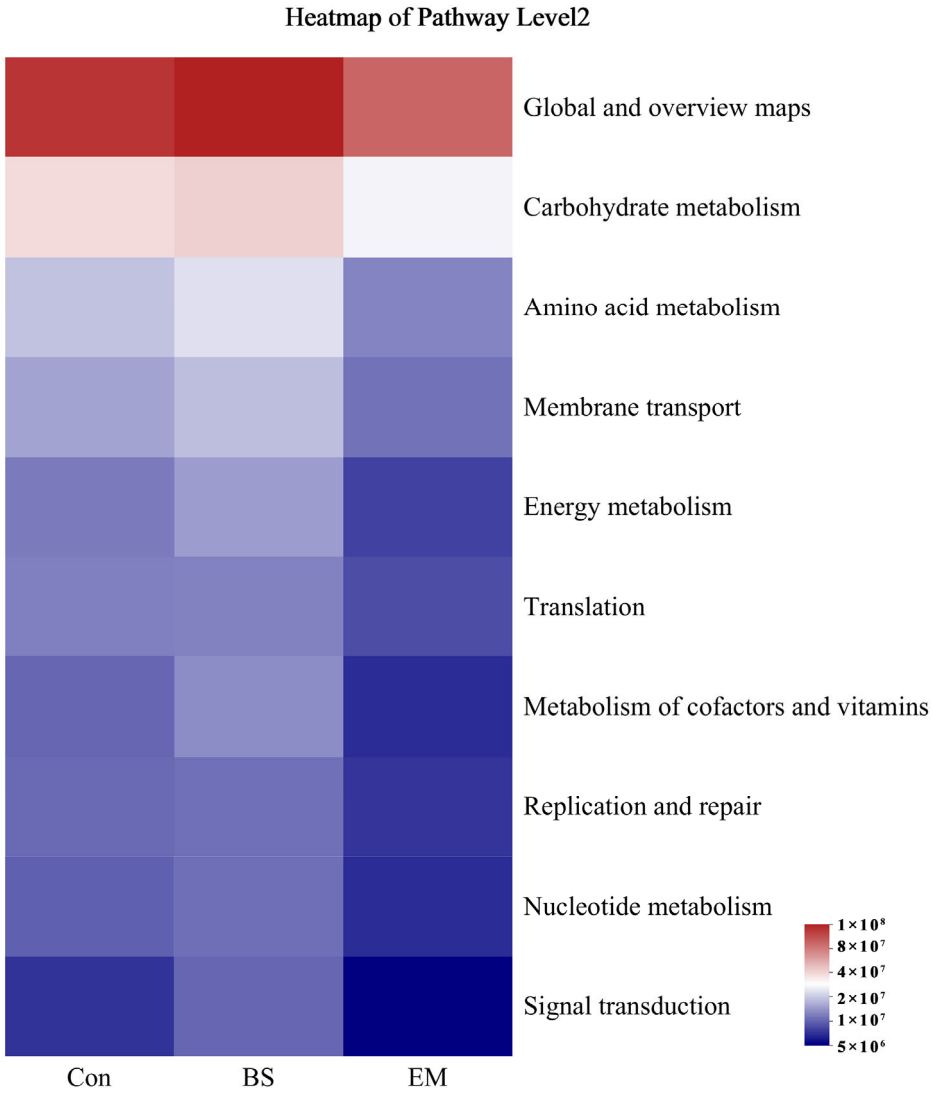

Figure 6. Functional prediction of the intestine microbiome of *M. rosenbergii*.

4. Discussion

During the breeding process, a large number of nutrient elements are not fully utilized by farmed animals and are excluded. Biofloc is rich in amino acids, proteins, fatty acids, and lipids, which enrich the food chain in aquaculture systems [39]. Microorganisms play an important role in the cycling of nutrients. In the biofloc culture mode, almost zero water exchange can be achieved, which avoids the probability of introducing external pathogens into the high-density culture mode and cuts off the pathogen transmission route [40]. Previous studies have shown the importance of bacteria in biofloc [41]. Bacteria affect water quality by degrading organic matter and nitrifying NH^{4+}-N to NO^{3-}-N [42]. Therefore, it is generally believed that the addition of probiotics can improve water conditions or prevent water quality problems [43,44]. However, there are few studies on the effects of probiotics on *Macrobrachium rosenbergii* cultured in a biofloc system.

The health of the water quality is a necessary condition for the survival of all aquatic organisms. Ammonia nitrogen and nitrite nitrogen are important indicators in the aquaculture process [45] to avoid the toxic effects of high concentrations of nitrogen nitrite and ammonia peaks in cultured species in the process of using a biofloc system. Water quality management is crucial in aquaculture production. Existing studies have shown that com-

pared with traditional farming modes, the nitrogen content can be significantly reduced by using biofloc technology in aquaculture ponds [46]. According to research, a small amount of ammonia nitrogen accumulation in water will destroy shrimp growth homeostasis to some extent, resulting in a variety of adverse effects [47]. A significant accumulation of ammonia nitrogen in water will affect the enzyme activity and permeability of the cell membrane of shrimp, and then destroy the excretion system and the balance of osmotic pressure [48].

By adding efficient microbial communities to aquaculture ponds, the material circulation of organic matter in the ponds can be promoted and the clean aquaculture water environment can be maintained. In the current study, total nitrogen and ammonia nitrogen concentrations in water decreased significantly after the addition of *Bacillus subtilis* and effective microorganisms, which is consistent with the research results of Cha et al. [33], who used *Bacillus subtilis* to reduce the concentration of ammonia nitrogen in the Japanese flounder (*Paralichthys olivaceus*) culture system. Similarly, the addition of *Bacillus* and *Lactobacillus* to the catarina scallop (*Argopecten ventricosus*) by Pacheco et al. [49] also significantly improved water quality. Furthermore, we found that the highest concentration of ammonia nitrogen and nitrite appeared between the 18th and the 24th days, owing to the fact that biofloc needed about 18 days, as confirmed by Dou et al. [50], Chen et al. [51], and Miao et al. [52]. The above results showed that the addition of *Bacillus subtilis* and effective microorganisms to biofloc aquaculture water could improve the quality of *M. rosenbergii* aquaculture water.

Biofloc contains a variety of nutrients that can be used as natural bait for aquatic animals, thereby improving the growth performance and survival rate of aquatic animals. Souza et al. [53] reported that the growth and specific growth rate of pink shrimp (*Farfantepenaeus brasiliensis*) could be improved by adding probiotics to the biofloc culture system. Krummenauer et al. [54] proposed that the growth and survival rate of *Litopenaeus vannamei* could be improved by adding probiotics to the biofloc culture system. In this study, the FBW, WG, FCR, and SGR of *M. rosenbergii* were significantly improved by adding *Bacillus subtilis* or effective microorganisms compared to those without adding bacteria, which is consistent with the results of the previous investigations [54]. The reason may be that probiotics contain a large number of nutrients such as protein and vitamins, which can provide nutrition to aquatic animals. In addition, the aquaculture water added with probiotics changed the flora of the water environment, inhibited the growth and reproduction of pathogenic bacteria, and enhanced the immunity of *Macrobrachium rosenbergii*, thereby improving its immunity.

The healthy culture of Macrobrachium rosenbergii depends largely on the control of disease, and the changes of nonspecific immune factors are usually used to measure the size of immune activity. Studies have shown that probiotics can stimulate the immune response of aquatic animals [55]. Because biochemical blood indexes can directly reflect the metabolic process of substances in the body, they are often used as one of the main bases for judging the immunity of aquatic animals. SOD is an important antioxidant enzyme in the body [56], which can repair damaged cells and restore damage caused by free radicals. MDA is a damaging substance produced by lipid peroxidation in the body, which is an important part of the immune response [57]. In our study, the addition of *Bacillus subtilis* to biofloc helped to increase the plasma SOD content and reduce the MDA content in *M. rosenbergii*. This is consistent with the results of Mohd et al. [58], which proved that adding *Enterococcus faecalis* in Indian major carp (*Cirrhinus mrigala*) could increase the SOD content and reduce the MDA content. ACP is an important indicator for measuring the health status and immune function of the body [59]. LZM is an enzyme that hydrolyzes the cell wall of Gram-positive bacteria and plays a key role in resisting pathogenic microbial infections in aquatic animals [35]. In our study, the addition of *Bacillus subtilis* and effective microorganisms to the biofloc significantly improved the plasma ACP of *M. rosenbergii*, and the addition of *Bacillus subtilis* significantly increased the plasma lysozyme content of *M. rosenbergii*. These are consistent with the results of Ghasem et al. [21] on Nile tilapia

(*Oreochromis niloticus*). However, we found that the addition of effective microorganisms to the biofloc led to an increase in the MDA content in *M. rosenbergii* plasma, which may be due to the different effects of probiotics on dosage, the method of addition, and different feeding animals.

The digestive enzyme is an important index that reflects the nutritional physiology of the body [60]. The activity of digestive enzymes directly affects the use of feed by animals. Heterotrophic bacteria in biofloc can secrete extracellular enzymes such as protease and amylase during growth and metabolism. After aquatic animals ingest biofloc, extracellular enzymes enter the intestine and promote the decomposition of proteins and carbohydrates. The addition of probiotics to the biofloc system plays a beneficial role in the digestion of aquatic animals. In this study, the addition of *Bacillus subtilis* significantly increased trypsin activity. Afrilasari et al. [61] showed that the addition of *Bacillus subtilis* significantly increased the protease activity of catfish (*Clarias* sp.). Chang et al. [62] showed that the addition of *Bacillus velezensis* R-71003 significantly increased the protease activity of common carp (*Cyprinus carpio* L.). These may be due to the colonization, proliferation, and metabolism of probiotics in the intestinal tract of aquaculture animals. A variety of extracellular enzymes produced by probiotics enter the intestinal tract, thus improving the activity of digestive enzymes in aquaculture animals [63].

The construction of the flora plays a crucial role in the stability of the intestinal environment, and the diversity of the flora is more conducive to the stability of the intestinal environment. High-throughput sequencing analysis of intestinal flora of *M. rosenbergii* showed that the addition of effective microorganisms significantly decreased the species richness of intestinal flora. However, this is contrary to the results of Leonardo et al. [64], who used probiotics to increase the species richness of the intestinal flora of Nile tilapia (*Oreochromis niloticus*). Such results may be caused by the variety of doses and preparations, for which the specific reasons need to be further studied.

At the phylum level, Chloroflexi is widely distributed in various environments of the biosphere and is an important participant in the biogeochemical cycle of C, N, S, and other elements. Verrucomicrobia can improve intestinal integrity and connective strength [65]. In our study, the addition of probiotics decreased the abundance of Chloroflexi and Verrucomicrobiota, which may be due to changes in the culture environment. Beneficial microorganisms in biofloc had inhibitory effects on aquatic animal diseases [9], and potential probiotics were monitored during biofloc formation to gradually replace conditional pathogens [66]. At the genus level, the abundance of *Bacillus* in the intestinal tract of *M. rosenbergii* was significantly increased after the addition of *Bacillus subtilis* to the biofloc. This is consistent with the addition of probiotics to sea cucumber (*Apostichopus japonicus*) [12], white shrimp (*Penaeus vannamei*) [67], and grass carp (*Ctenopharyngodon idella*) [68] to increase the abundance of genera related to probiotics. The above results indicate that the addition of *Bacillus subtilis* to the biofloc contributes to the reproduction of beneficial bacteria in the intestinal flora of *M. rosenbergii*.

The long-term interaction of intestinal flora determines the function of microbial mediation and is indispensable for the metabolism of the body. In this study, compared with the Con group, the metabolic activity-related pathways of the intestinal flora in the ES group were significantly up-regulated, indicating that the addition of bio-flocs cultured with Bacillus subtilis enhanced the absorption and utilization of nutrients by *M. rosenbergii*.

5. Conclusions

In summary, this study showed that the addition of *Bacillus subtilis* and effective microorganisms in the biofloc culture system had a positive impact on the water body, immunity, and digestion of *Macrobrachium rosenbergii*. In addition, the addition of *Bacillus* in the biofloc could increase the abundance of beneficial bacteria in the intestinal tract of *Macrobrachium rosenbergii*.

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