



Use of *Bacillus subtilis* D9 to purify coastal aquaculture wastewater and improve grass carp resistance to *Vibrio* infection

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ABSTRACT: In coastal areas of China, high-density aquaculture has caused environmental problems and fish health concerns. *Bacillus subtilis* D9 is a new strain isolated from coastal soils which could be used in aquaculture to improve the water environment. We investigated the effect of *B. subtilis* D9 on the purification of coastal aquaculture wastewater and the resistance of grass carp *Ctenopharyngodon idellus* to pathogenic *Vibrio* infection. Three inoculation levels of *B. subtilis* D9 were used (5.5×10^7 , 5.5×10^8 and 5.5×10^9 cfu ml⁻¹ as BD7, BD8 and BD9, respectively), together with sterilized saline water without *B. subtilis* D9 as the Control. *B. subtilis* D9 at the inoculation level of BD8 showed the best performance with 81, 87, 91, 52 and 86% removal of NH₄⁺-N, NO₃⁻-N, total nitrogen (TN), NO₂⁻-N and turbidity, respectively, after 25 d of treatment. These values were significantly higher than at the BD7, BD9 and Control levels. Under aeration (AIR) conditions, *B. subtilis* D9 at the inoculation level of BD8 showed removal efficiency of 93, 91, 95, 76 and 89%, respectively. In contrast it was only 26, 29, 16, 10 and 57% in an inactivated bacteria liquid (IBL) treatment. After 22 d of infection by *Vibrio parahaemolyticus*, significant differences were found in weight gain, specific growth rate and relative percentage of survival among grass carp grown on AIR, BD8 or IBL wastewater. In summary, *B. subtilis* D9 with aeration has beneficial effects on the purification of coastal aquaculture wastewater and on the resistance of grass carp to disease caused by *V. parahaemolyticus*.

KEY WORDS: *Bacillus subtilis* D9 · Aeration · Coastal aquaculture wastewater · Purification · Grass carp disease · *Ctenopharyngodon idellus* · *Vibrio parahaemolyticus*

1. INTRODUCTION

Coastal aquaculture relieves the enormous pressure on ocean resources caused by sea fishing. However, the feasibility, sustainability and potential effectiveness of coastal aquaculture are heavily debated (Di Trapani et al. 2014, Ferreira et al. 2014). The direct discharge of coastal aquaculture wastewater can cause serious deterioration of the marine environment, leading to an increase in pathogenic

microbes, the acceleration of pathogenic propagation and eutrophication (Kijjoa et al. 2004, Cho et al. 2019). In recent years, the use of antibiotics in coastal aquaculture has brought serious consequences to humans and aquaculture, such as producing drug-resistant strains and causing endogenous infections (Hlongwane et al. 2019, Lulijwa et al. 2019).

Microbial ecological agents have become the most promising substitute for antibiotics due to eco-friendliness, low cost, extensive adaptability and

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non-secondary pollution (Martínez Cruz et al. 2012, Oliveira et al. 2012). These preparations contain large amounts of probiotics made of microbial thalli extracted from natural environments (Dong et al. 2010, Akhter et al. 2015). Ai et al. (2013) investigated the effects of microbial ecological agents on the immunocompetence of loach, and found that they increased lysozyme activity and superoxide dismutase activity. In another study, Gelfand et al. (2003) reported that NH_3 concentration and pH value of the water were significantly decreased while the increase of nitrite was inhibited in the presence of microbial ecological agents, resulting in increases in the weights of individual white shrimp and the total yield.

Bacillus subtilis is one of the most commonly used and characteristic microbial ecological agents (Shao et al. 2020). This bacterium mainly exists as a spore, with high stability, stress resistance and antibacterial activity (Pepi et al. 2016, Subtil et al. 2019). *B. subtilis* can secrete multiple exoenzymes, including protease, lipase, cellulase and soft phospholipase (Lu et al. 2018, Cho et al. 2019), which may improve water quality, and can inhibit the growth and reproduction of harmful microbes. Ding et al. (2012) reported removal of $\text{NH}_3\text{-N}$, $\text{NO}_2\text{-N}$ and sulfide by *B. subtilis* WH-5 as 80.89, 61.72 and 47.19%, respectively. Similarly, Shao et al. (2016) found that *B. subtilis* removed $\text{NH}_4\text{+N}$, $\text{NO}_2\text{-N}$, total nitrogen (TN) and total phosphorus more effectively than water spinach *Ipomoea aquatica* did, with maximum removal of 68.06, 86.49, 49.96 and 58.82%, respectively.

B. subtilis is often used as a feed additive. Its nutritional metabolites such as amino acids and vitamins promote animal growth, improve intestinal microflora and regulate immunity (Liu et al. 2010, Shao et al. 2016). Several studies have reported that *B. subtilis* improved the activities of digestive enzymes, immunity and antioxidative function in white shrimp (Liu et al. 2010, Wang et al. 2019), yellow croaker (Hossain et al. 2015), laying hens (Lee et al. 2014) and tilapia (Zokaeifar et al. 2014). *B. subtilis* showed no pathogenic and toxic effects on cultured animals, and was easily produced and stored (Chen et al. 2017).

Vibrio parahaemolyticus is found in marine environments and estuaries. It is a key enteropathogenic bacterium and poses a threat in mariculture (Shen et al. 2013, He et al. 2019). Large *Vibrio* populations in water are a potential threat to aquatic organisms. Hu et al. (2015) reported that cultured grass carp *Ctenopharyngodon idellus* can be infected with pathogenic vibrios, among which *V. parahaemolyticus* contributes to the most severe disease outbreaks and fish mortality.

B. subtilis D9 is a newly discovered strain isolated in a coastal area from soil continuously-cropped with *Artemisia selengensis* infected with *Fusarium* (95% of plants) (Chen et al. 2016, 2017). It has the characteristics of *B. subtilis* in terms of metabolic and antibacterial activities (Chen et al. 2017). Effects of *B. subtilis* D9 on wastewater purification and disease resistance of fish in coastal aquaculture have not been reported to date. In this study, the performance of *B. subtilis* D9 in wastewater treatment and in improving the resistance of grass carp to disease caused by pathogenic *V. parahaemolyticus* was tested with the aim of proving the potential applicability of *B. subtilis* D9 to intensive coastal aquaculture.

2. MATERIALS AND METHODS

2.1. Wastewater samples

Wastewater samples for the laboratory experiments were collected from a coastal white shrimp pond in Dongtai City, Jiangsu Province, China, and stored under cool conditions (4°C) before use. Samples were analysed for dissolved oxygen (DO), turbidity and nitrogen in triplicate (mean \pm SD follow). DO was $1.0 \pm 0.1 \text{ mg l}^{-1}$ and turbidity was 7.8 ± 0.4 nephelometer turbidity units. TN, $\text{NH}_4\text{+N}$, $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ concentrations were 53.34 ± 4.67 , 15.90 ± 2.74 , 6.84 ± 1.42 and $0.77 \pm 0.54 \text{ mg l}^{-1}$, respectively. Based on the nitrogenous forms, >50% of TN comprised reduced organic nitrogen.

2.2. Microbe strains and culture

Bacillus subtilis strain D9 was isolated in a coastal area from soil continuously-cropped with *Artemisia selengensis* infected with *Fusarium* (Chen et al. 2016, 2017) and was stored in the China General Microbiological Culture Collection Center (CGMCCC) (under accession no. 9170). The selected *B. subtilis* strain was cultured for enrichment at 37°C for 24 h in nutrient broth which contained peptone (10 g l^{-1}), beef extract (3 g l^{-1}) and NaCl (5 g l^{-1}), pH 7.2–7.5, and autoclaved at 121°C for 30 min. Bacterial cell density of the incubation solution was adjusted to $5.5 \times 10^{11} \text{ cfu ml}^{-1}$. The inactivated bacteria liquid (IBL) of *B. subtilis* D9 was made by autoclaving it at 110–125°C for 30 min.

The *Vibrio parahaemolyticus* strain (accession no. 43305) was provided by the CGMCCC and cultured with a bacterial cell density of $4.65 \times 10^7 \text{ cfu ml}^{-1}$ in the incubation solution.

2.3. Experimental design

2.3.1. Wastewater purification

The experiments were conducted in a greenhouse at temperatures of 29–40°C (day) and 15–30°C (night) in Nanjing, China, for 25 d. Twelve glass jars of 0.1 m³ containing 10 l of wastewater were used in the experiments with 4 treatments. In each treatment, 100 ml of *B. subtilis* D9 solution with 5.5×10^9 , 5.5×10^{10} and 5.5×10^{11} cfu ml⁻¹ and sterilized saline water were added to the jars and mixed well. This yielded *B. subtilis* D9 concentrations of ca. 5.5×10^7 (BD7), 5.5×10^8 (BD8) and 5.5×10^9 cfu ml⁻¹ (BD9), together with the Control. For the wastewater purification test, each treatment (n = 3), was intermittently shaken without additional aeration. The quality indices for NH₄⁺-N, NO₃⁻-N, TN, NO₂⁻-N and turbidity for wastewater were determined 1, 2, 4, 6, 8, 10, 15, 20 and 25 d after the initial inoculation. The amount of *B. subtilis* D9 in the wastewater was measured for the *B. subtilis* D9 inoculation treatments on Day 25. The % removal of pollutants (Y) was calculated as follows:

$$Y = (C_0 - C_t)/C_0 \times 100 \quad (1)$$

where C_t and C_0 are the final and initial pollutant concentrations, respectively, and t is the duration of the experiment in days.

2.3.2. Aeration (AIR) and IBL treatment

B. subtilis D9 solution (100 ml) and IBL with 5.5×10^{11} cfu ml⁻¹ together with sterilized saline water was added into 0.1 m³ jars containing 10 l of wastewater and mixed well. Air was supplied to the jars using a four holes high-power aeration pump at a flow rate of 2.0 l min⁻¹, and the DO concentration was maintained at 5–6 mg l⁻¹ for AIR and IBL treatments as well as for Control treatments to which no *B. subtilis* D9 was added. Each treatment was performed in 3 replicates. The experimental process and conditions were the same as those described in Section 2.3.1. NH₄⁺-N, NO₃⁻-N, TN, NO₂⁻-N and turbidity were determined on Days 1, 2, 4, 6, 8, 10, 15, 20 and 25 after inoculation.

2.3.3. Grass carp infection experiment

Grass carps (n = 240) with an average (\pm SD) length and weight of 13.56 ± 1.25 cm and 10.67 ± 0.97 g were selected to be acclimatized for 7 d in fish jars. The fish were divided into 4 experimental groups

including Control, BD8, AIR and IBL as described in the above experiments (Control and BD8 treated as in Section 2.3.1; AIR and IBL treated as in Section 2.3.2). One ml of *V. parahaemolyticus* with bacterial colonies of 4.65×10^7 cfu ml⁻¹ was added into grass carp culture jars of 0.7 m³ containing 100 l of aquaculture wastewater. Each treatment had 3 replicates, and the experiment lasted 22 d. Food was provided at 09:00 and 17:00 h every day, and 5% of water (treated for 25 d) was supplemented every 2 d to top up the volume. The feeding quantity was 5–7% of the fish body weight. Weight and mortality of the grass carp were recorded every 2 d. Percentage of weight gain (PWG) (Hao et al. 2014), specific growth rate (SGR) (Liu et al. 2017) and relative percentage of survival (RPS) (Marinho-Soriano et al. 2011) were calculated with the following equations:

$$\text{PWG} = [(W_t - W_0)/W_0] \times 100 \quad (2)$$

$$\text{SGR} = [(\ln W_t - \ln W_0)/W_0] \times 100 \quad (3)$$

$$\text{RPS} = [1 - (P_t - P_c)/W_0] \times 100 \quad (4)$$

where W_t and W_0 are the final and initial fish weight, respectively; t is the experimental duration in days; P_t is the percentage of fish mortality in treated groups; and P_c is the percentage of fish mortality in the Control group. The populations of *V. parahaemolyticus* were determined for each experimental group on Day 22 post infection.

2.3.4. *In situ* aquaculture pond water purification

In situ purification of intensive white shrimp pond water was conducted at an aquaculture farm located near the coast in Dongtai City, Jiangsu Province, to compare the purifying effects of *B. subtilis* D9 with EM, a commercial aquaculture water purification agent. Three treatments were set up as follows: (1) Control: no EM or BD9 in the aquaculture pond; (2) EM: EM at the concentration of about 5.5×10^8 cfu ml⁻¹ in the pond; and (3) BD8: *B. subtilis* D9 at the concentration of about 5.5×10^8 cfu ml⁻¹ in the pond. 6670 ml of EM or *B. subtilis* D9 containing 5.5×10^{11} cfu ml⁻¹ was used per 6.67 m² of each pond at about 1.0 m depth; they were mixed with aquaculture water at a volume ratio of 1:1000 and uniformly applied to the breeding pond via an electric spray. Water samples at a depth of 60 cm were taken in 3 different places on Day 25 of each treatment. Numerical values of the water quality indicators for NH₄⁺-N, NO₃⁻-N, TN, NO₂⁻-N and turbidity were determined and compared.

2.3.5. Water quality indicators and biomass

The biomass of *B. subtilis* D9 and *V. parahaemolyticus* was determined using the plate count method (Yang et al. 2012). Nessler's reagent spectrophotometry was used for NH_4^+ -N determination (Yu 2015). N-(1-naphthyl) ethylene diamine dihydrochloride spectrophotometric method was used for NO_2^- -N determination (Liu et al. 2017). A YSI 550A portable DO meter was used to measure DO (Yang et al. 2012), and UV spectrophotometry was applied to determine NO_3^- -N (Yang et al. 2012). TN was measured with alkaline potassium persulfate digestion UV spectrophotometry (Li et al. 2009), and turbidity was determined with a WGZ-1B portable turbidity meter (Yang et al. 2012).

2.4. Statistical analysis

One-way ANOVA followed by Tukey's post hoc test was used to compare water quality parameters in the *B. subtilis* D9-based wastewater purification experiment, as well as fish weight and mortality indicators in the infection experiments among treatments and the Control groups. Paired-sample *t*-tests were used to compare AIR and IBL treatments in the water quality experiments. Prior to statistical analysis, raw data were assessed for normality of distribution and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests, respectively (Zar 2010). Data are presented as means \pm SD ($n = 3$). All statistics were conducted in SPSS 19.0. Results were considered statistically significant when $p < 0.05$. Figures were drawn with Origin Pro 8.0 scientific graphing and data analysis software.

3. RESULTS

3.1. Effects of the concentration of *Bacillus subtilis* D9 on wastewater purification

Initially, on the first day, concentrations of NH_4^+ -N, NO_3^- -N and TN in the BD7, BD8 and BD9 treatments increased after *B. subtilis* D9 was added, whereas they did not change significantly in the Control treatment (Fig. 1). Generally, however, the concentrations of NH_4^+ -N, NO_3^- -N, TN, NO_2^- -N and turbidity decreased over time, reached a stable value on Day 8 and then continued to decrease slowly (Fig. 1a–e). DO in all *B. subtilis* D9 treatments showed a decreasing trend at the beginning (Fig. 1f) and a rapid increase after Day 8. DO values in the BD8 treatment

were significantly higher than in the other BD (Tukey's test, $p = 0.013$) and Control (Tukey's test, $p = 0.010$) treatments after Day 10.

Percent removal of NH_4^+ -N, NO_3^- -N, TN, NO_2^- -N and turbidity was calculated on Days 8 and 25 (Table 1). Results showed that the BD8 treatment had the best removal efficiencies, of 81, 87, 91, 52 and 86 % for NH_4^+ -N, NO_3^- -N, TN, NO_2^- -N and turbidity, respectively, on Day 25; significantly higher than those in the other *B. subtilis* D9 treatments (Tukey's test, $p = 0.010$) and Control (Tukey's test, $p = 0.008$) (Table 1). On Day 25, DO in the BD8 treatment reached 3.47 mg l^{-1} , which was significantly higher than in the other *B. subtilis* D9 treatments (Tukey's test, $p = 0.018$) and the Control (Tukey's test, $p = 0.021$) (Table 1). Furthermore, the biomass of viable bacteria in BD8 and BD9 treatments ranged between 1.4×10^8 and $4.9 \times 10^8 \text{ cfu ml}^{-1}$ on Day 25, which was significantly reduced (*t*-tests, $p < 0.05$) compared to those in the initial inoculation, while the viable bacteria in the BD7 treatment were increased (*t*-tests, $p < 0.05$) (Fig. 2).

3.2. Effects of AIR and IBL of BD8 on wastewater quality

Concentrations of NH_4^+ -N, NO_3^- -N and TN in the AIR, IBL and Control treatments initially increased after bacterium injection (Fig. 3). After 1 d, the concentrations of NH_4^+ -N, NO_3^- -N, TN, NO_2^- -N and turbidity in wastewater in the treatments decreased gradually with the reaction time, reached a stable value after 6–10 d, and then decreased again slowly. On Day 8, % removal of NH_4^+ -N, NO_3^- -N, TN, NO_2^- -N and turbidity in the AIR treatment reached 93, 91, 95, 76 and 89 %, respectively, which was much higher (*t*-tests, $p < 0.05$) than in the IBL treatment (9, 5, 6, 10 and 27 %, respectively) and Control treatment (21, 28, 30, 29 and 36 %, respectively) (Table 2). On Day 25, the % removal of all pollutants in the AIR treatment remained almost unchanged (*t*-tests, $p > 0.05$) compared to Day 8. However, the % removal in IBL and Control groups on Day 25 was significantly higher (*t*-tests, $p < 0.05$) than those on Day 8 except for NO_2^- -N. Compared with the BD8 data in Table 1, the relevant removal rates of pollutants were ranked as AIR > BD8 > Control > IBL on Days 8 and 25 (*t*-tests, $p < 0.05$).

3.3. Grass carp infection experiment

The final weight, weight gain, PWG and SGR of grass carp were significantly higher in all groups and

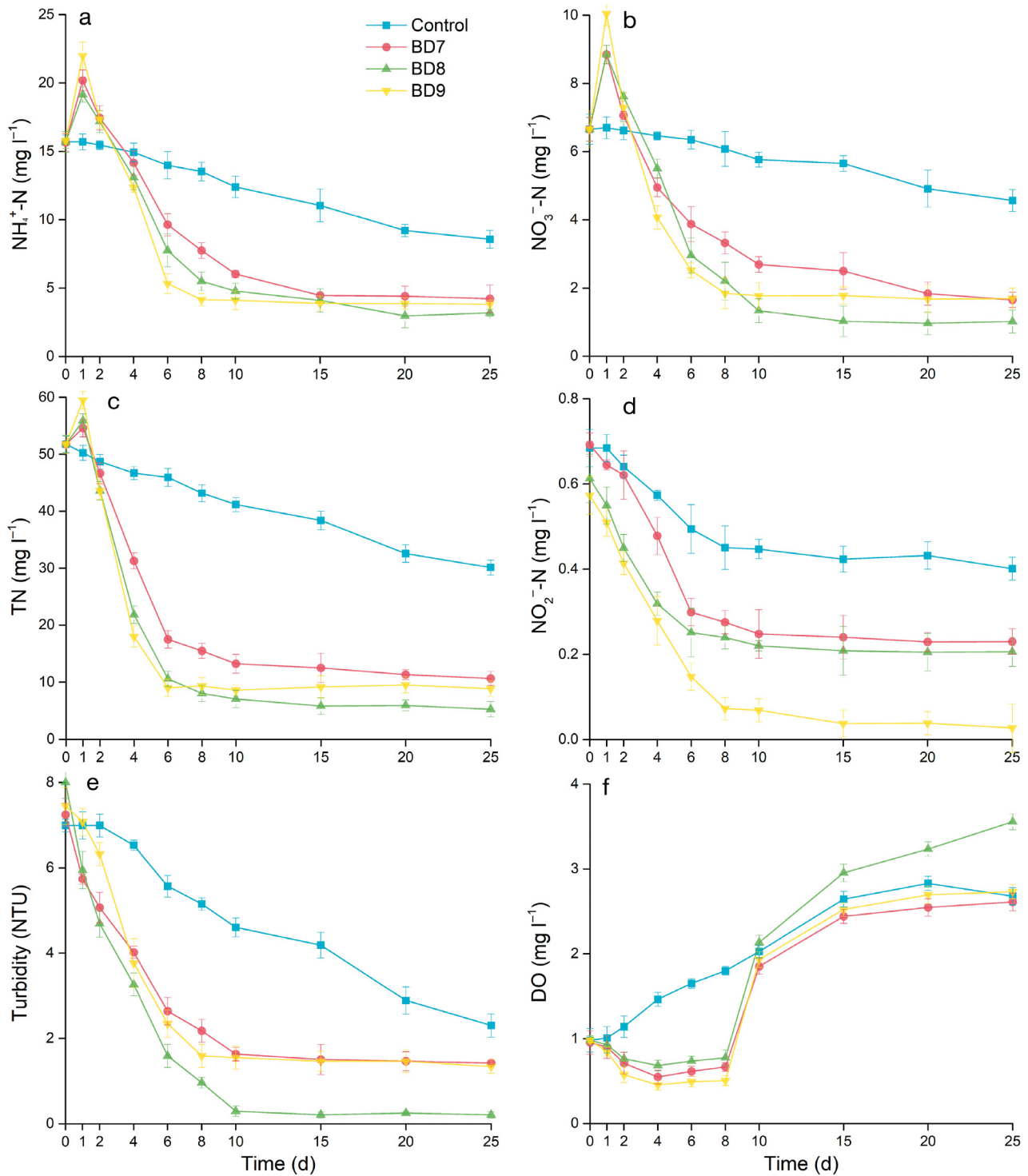


Fig. 1. (a) $\text{NH}_4^+\text{-N}$, (b) $\text{NO}_3^-\text{-N}$, (c) total nitrogen (TN), (d) $\text{NO}_2^-\text{-N}$, (e) turbidity and (f) dissolved oxygen (DO) variation curves under different treatments. Vertical bars represent \pm SD of the means (n = 3). BD7/BD8/BD9: inoculation levels of *Bacillus subtilis* D9 at 5.5×10^7 / 5.5×10^8 / 5.5×10^9 cfu ml^{-1}

ranked as AIR > BD8 > Control > IBL (Table 3). The RPS of grass carp was higher in the AIR group (ANOVA, Tukey's test, $F_{3,8} = 12.528$, $p = 0.001$) com-

pared to the BD8 group (ANOVA, Tukey's test, $F_{3,8} = 8.517$, $p = 0.008$) and IBL group (ANOVA, Tukey's test, $F_{3,8} = 6.814$, $p = 0.013$). The final weight, weight

Table 1. Effect of *Bacillus subtilis* D9 inoculum on the removal of nitrogenous pollutants, turbidity and dissolved oxygen (DO) concentration in wastewater after 8 and 25 d. Data are means \pm SD. BD7/BD8/BD9: inoculation levels of *B. subtilis* D9 at $5.5 \times 10^7 / 5.5 \times 10^8 / 5.5 \times 10^9$ cfu ml⁻¹; TN: total nitrogen. Within columns, means with the same superscript letters are not significantly different (Tukey's post hoc test, $p > 0.05$)

Day	Treatment	Removal (%)		Removal (%)	Removal (%)		DO (mg l ⁻¹)
		NH ₄ ⁺ -N	NO ₃ ⁻ -N		TN	NO ₂ ⁻ -N	
8	Control	14 \pm 0.6 ^c	18 \pm 0.2 ^c	20 \pm 0.1 ^c	21 \pm 0.5 ^c	31 \pm 0.4 ^c	1.81 \pm 0.07 ^a
	BD7	66 \pm 0.7 ^b	61 \pm 0.5 ^b	74 \pm 0.8 ^b	39 \pm 0.3 ^b	69 \pm 0.8 ^b	0.79 \pm 0.03 ^c
	BD8	70 \pm 0.9 ^{ab}	81 \pm 0.8 ^a	86 \pm 0.7 ^a	48 \pm 0.3 ^a	84 \pm 0.9 ^a	0.87 \pm 0.05 ^b
	BD9	76 \pm 0.8 ^a	75 \pm 0.3 ^a	83 \pm 0.6 ^{ab}	36 \pm 0.4 ^b	68 \pm 0.5 ^b	0.73 \pm 0.08 ^c
25	Control	45 \pm 0.7 ^c	40 \pm 0.3 ^c	45 \pm 0.5 ^b	27 \pm 0.4 ^c	67 \pm 0.7 ^c	2.67 \pm 0.21 ^b
	BD7	75 \pm 0.8 ^{abc}	78 \pm 0.9 ^b	80 \pm 0.7 ^a	42 \pm 0.4 ^b	73 \pm 0.8 ^b	2.68 \pm 0.18 ^b
	BD8	81 \pm 0.7 ^a	87 \pm 1.2 ^a	91 \pm 1.3 ^a	52 \pm 0.6 ^a	86 \pm 0.9 ^a	3.47 \pm 0.33 ^a
	BD9	78 \pm 0.9 ^{ab}	78 \pm 0.6 ^b	83 \pm 0.9 ^a	39 \pm 0.4 ^{bc}	69 \pm 0.4 ^c	2.78 \pm 0.22 ^b

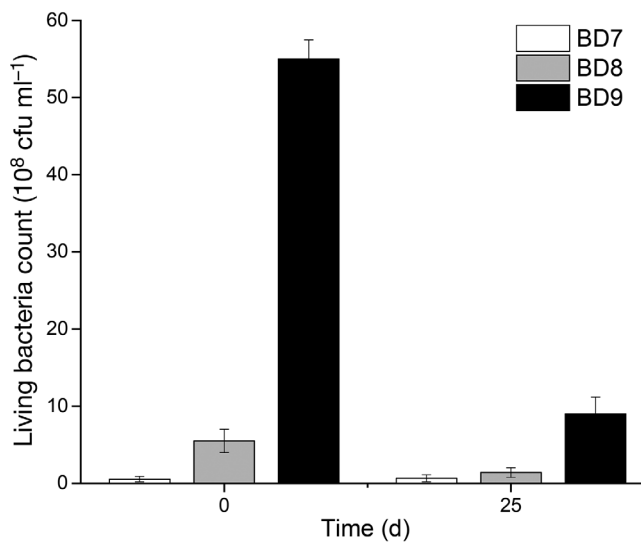


Fig. 2. Biomass of viable *Bacillus subtilis* D9 in different treatments on Days 0 and 25. Vertical bars represent \pm SD of the means ($n = 3$). BD7/BD8/BD9: inoculation levels of *Bacillus subtilis* D9 at $5.5 \times 10^7 / 5.5 \times 10^8 / 5.5 \times 10^9$ cfu ml⁻¹

gain, PWG and SGR of grass carp in the IBL group were lower than those in the Control group, but the differences were not significant (t -tests, $p > 0.05$). The RPS value in the IBL group (ANOVA, Tukey's test, $F_{3,8} = 13.927$, $p = 0.001$) was negative, and was significantly lower than those in the BD8 group (ANOVA, Tukey's test, $F_{3,8} = 9.927$, $p = 0.004$) and the Control group (ANOVA, Tukey's test, $F_{3,8} = 8.152$, $p = 0.010$).

3.4. In situ aquaculture pond water purification

Spraying of *B. subtilis* D9 into the shrimp pond at 5.5×10^8 cfu ml⁻¹ significantly lowered the concen-

trations of NH₄⁺-N, NO₃⁻-N, TN, NO₂⁻-N and turbidity compared with the Control and EM treatments ($p < 0.05$) on Day 25 (Table 4). Percent removal of NH₄⁺-N, NO₃⁻-N, TN, NO₂⁻-N and turbidity in the BD8 treatment reached 81, 87, 85, 62 and 82%, respectively, which was significantly higher than that in EM and Control treatments.

4. DISCUSSION

In the present study, the concentrations of NH₄⁺-N, NO₃⁻-N and TN in the *Bacillus subtilis* D9 treatments increased during the first day after bacterium injection to wastewater, possibly due to short-term accumulation of nitrogen from the transformation of organic biomass in the wastewater reaction system. This result is consistent with reports by Boopathy et al. (2015) and Chen et al. (2017), who used other microbial agents for wastewater treatment. Our results indicate that *B. subtilis* D9 can effectively reduce the concentrations of NH₄⁺-N, NO₃⁻-N, TN, NO₂⁻-N and turbidity in wastewater, similarly to other microbial ecological agents (Lu et al. 2012). The highest removal of NH₄⁺-N, NO₃⁻-N, TN, NO₂⁻-N and reduction in turbidity were recorded in the BD8 group. The decrease in various forms of N could be attributed to *B. subtilis* D9 degrading NH₄⁺-N, NO₃⁻-N and NO₂⁻-N through nitrification and denitrification and possibly assimilating NH₄⁺-N, NO₂⁻-N and NO₃⁻-N as cell components during the process (Chen et al. 2017, Yun et al. 2019). The reduction of turbidity in the *B. subtilis* D9 treatments could be attributed to flocculation and microbial decomposition (Lu et al. 2018, Cho et al. 2019). DO in the 3 BD treatments decreased during the first 4 d, which differed from the Control treatment. This could be due

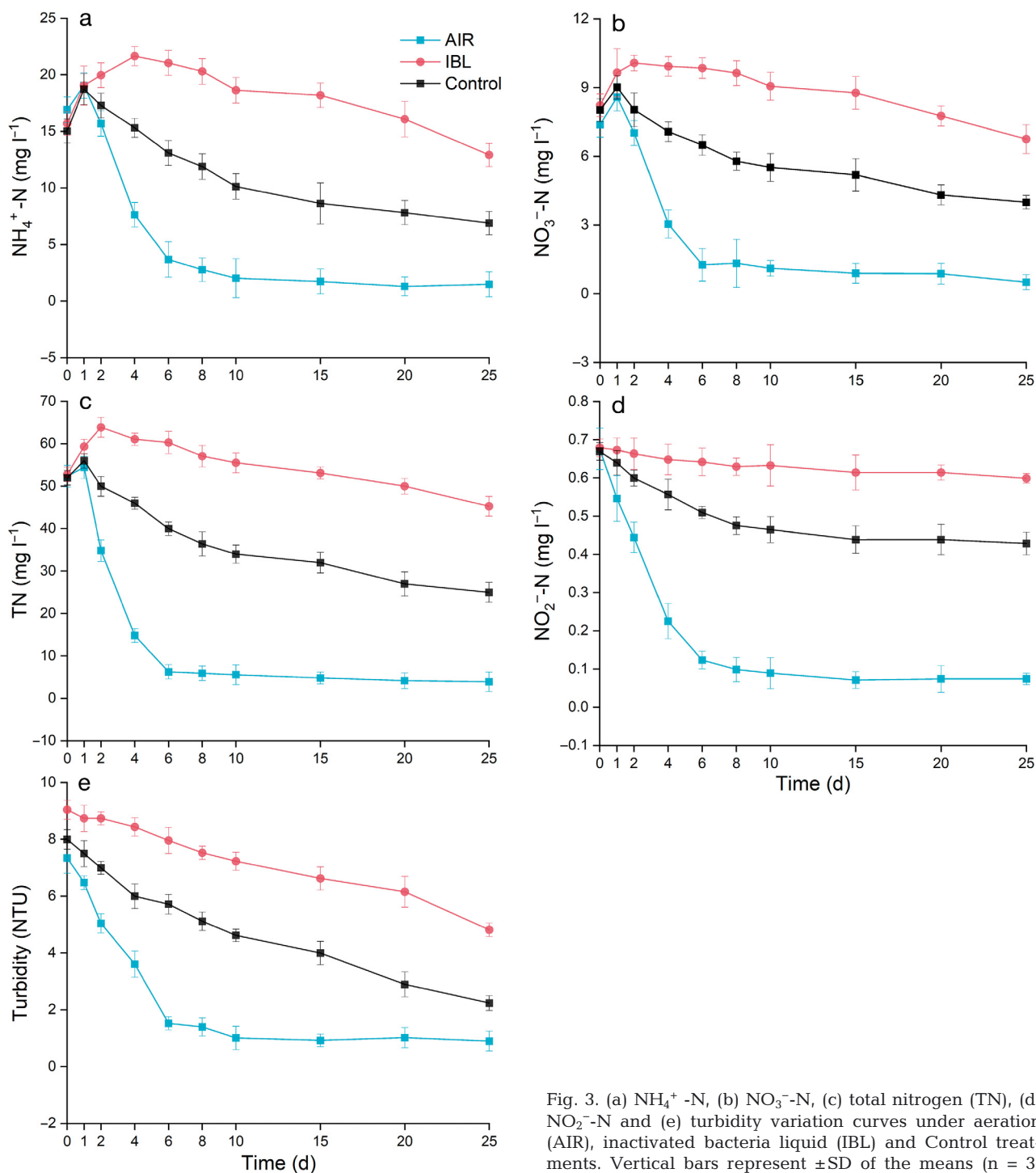


Fig. 3. (a) $\text{NH}_4^+\text{-N}$, (b) $\text{NO}_3^-\text{-N}$, (c) total nitrogen (TN), (d) $\text{NO}_2^-\text{-N}$ and (e) turbidity variation curves under aeration (AIR), inactivated bacteria liquid (IBL) and Control treatments. Vertical bars represent \pm SD of the means (n = 3)

to the fact that *B. subtilis* is a Gram-positive, aerobic bacterium that consumes a large amount of oxygen (Lu et al. 2012). The increase in DO after 4–8 d may be due to the decomposition of a high amount of reduced organic substances and the reduction of viable bacteria in wastewater. Observed removal of $\text{NO}_2^-\text{-N}$ was lower, indicating that $\text{NO}_2^-\text{-N}$ oxidation

caused by *B. subtilis* D9 was impeded at a low concentration of DO (Ding et al. 2012). Removal of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, TN, $\text{NO}_2^-\text{-N}$ and reduction of turbidity in the AIR treatment were significantly higher than in the IBL treatment (Table 2), suggesting that the bacterial activity of *B. subtilis* D9 is key for wastewater purification. Similar findings of the effect of *B. sub-*

Table 2. Removal of nitrogenous pollutants and turbidity under aeration (AIR) and inactivated bacteria liquid (IBL) treatments with inoculation levels of *Bacillus subtilis* D9 at 5.5×10^8 cfu ml⁻¹ (BD8) after 8 and 25 d. Data are means \pm SD. Within columns, means with the same superscript letters are not significantly different (Tukey's post hoc test, $p > 0.05$)

Time	Treatment	Removal (%)				
		NH ₄ ⁺ -N	NO ₃ ⁻ -N	TN	NO ₂ ⁻ -N	Turbidity
8 d	AIR	93 \pm 1.2 ^a	91 \pm 1.1 ^a	95 \pm 1.2 ^a	76 \pm 0.6 ^a	89 \pm 0.8 ^a
	IBL	9 \pm 0.5 ^b	5 \pm 0.3 ^b	6 \pm 0.2 ^b	10 \pm 0.1 ^b	27 \pm 0.7 ^b
	Control	21 \pm 0.4 ^c	28 \pm 0.5 ^c	30 \pm 0.6 ^c	29 \pm 0.8 ^c	36 \pm 0.8 ^c
25 d	AIR	94 \pm 1.5 ^a	92 \pm 1.3 ^a	96 \pm 1.5 ^a	79 \pm 0.8 ^a	91 \pm 0.9 ^a
	IBL	19 \pm 0.5 ^b	19 \pm 0.3 ^b	16 \pm 0.2 ^{cb}	11 \pm 0.1 ^b	57 \pm 0.7 ^b
	Control	54 \pm 0.5 ^c	49 \pm 0.8 ^c	52 \pm 0.6 ^c	36 \pm 0.4 ^c	72 \pm 0.7 ^c

tilis on lightly polluted water were observed by Chen & Hu (2011), who reported that the removal of NH₄⁺-N and NO₂⁻-N reached 96.7 and 82.0%, respectively, 9 d after bacterium injection. Furthermore, the removal of pollutants in the AIR treatments was higher than in the BD8 treatment without artificial aeration (Table 1), indicating that aerobic conditions are beneficial for wastewater purification (Chen et al. 2017). Similar to the results of Lu et al. (2012) and Liu et al. (2017), our experiment also suggested that the IBL treatment reduced water purification efficiency and even aggravated water pollution. The total number of viable bacteria in BD7, BD8 and BD9 treatments on Day 25 was basically on the same order of magnitude, but there was a significant difference compared to that at the beginning of experiments (Fig. 2), which was also observed by Chen & Hu (2011). The current study showed that BD8 could be regarded as an optimum concentration of *B. subtilis* D9, and aeration was beneficial for wastewater purification.

Moreover, in the AIR treatment, the final weight, weight gain, PWG, SGR

and RPS of grass carp were significantly higher, and the populations of pathogenic *Vibrio parahaemolyticus* were reduced compared to the BD8, Control and IBL groups. Our results are in agreement with reports by Liu et al. (2010) and Kuebutornye et al. (2019), who reported that *B. subtilis* could enhance disease resistance, reduce stress, improve gastrointestinal morphology and promote growth of cultured fishes. Shen et al. (2013) observed that *B. subtilis* in diets could promote the growth of black carp and decrease the feed conversion ratio.

Similarly, Chen et al. (2017) reported that *B. subtilis* in diets could improve the immunity and antioxidative function of Nile tilapia *Oreochromis niloticus*. Our *in situ* purification test demonstrated that BD8 significantly lowered the concentrations of NH₄⁺-N, NO₃⁻-N, TN, NO₂⁻-N and reduced turbidity and was more effective than EM.

B. subtilis D9 may also be potentially used as a feed additive in aquaculture to inhibit pathogenic bacteria colonization and regulate fish immunity. Possible mechanisms of *B. subtilis* D9 affecting the disease resistance of grass carp are summarized in Fig. 4. On the one hand, *B. subtilis* D9 may modulate the secre-

Table 4. *In situ* aquaculture pond water purification, reported as the removal (%) of nitrogenous pollutants on Day 25 (mean \pm SD). EM: commercial aquaculture water purification agent; BD8: inoculation levels of *Bacillus subtilis* D9 at 5.5×10^8 cfu ml⁻¹. Within columns, means with the same superscript letters are not significantly different (Tukey's post hoc test, $p > 0.05$)

Treatment	NH ₄ ⁺ -N	NO ₃ ⁻ -N	TN	NO ₂ ⁻ -N	Turbidity
Control	24 \pm 0.6 ^c	28 \pm 0.2 ^c	30 \pm 0.1 ^c	11 \pm 0.5 ^c	31 \pm 0.4 ^c
EM	55 \pm 0.8 ^b	48 \pm 0.9 ^b	60 \pm 0.7 ^b	42 \pm 0.4 ^b	73 \pm 0.8 ^b
BD8	81 \pm 0.7 ^a	87 \pm 1.2 ^a	91 \pm 1.3 ^a	62 \pm 0.6 ^a	86 \pm 0.9 ^a

Table 3. Growth performance of grass carp challenged with *Vibrio parahaemolyticus* for 22 d. Data are means \pm SD. BD8: inoculation levels of *Bacillus subtilis* D9 at 5.5×10^8 cfu ml⁻¹; AIR: BD8 under the aeration treatment; IBL: BD8 with an inactivated bacteria liquid; PWG: percentage of weight gain; SGR: specific growth rate; RPS: relative percentage of survival. Within columns, means with the same superscript letters are not significantly different (Tukey's post hoc test, $p > 0.05$)

Treatment	Initial weight (g)	Final weight (g)	Weight gain (g)	PWG (%)	SGR (%)	RPS (%)	<i>Vibrio</i> (10 ² cfu ml ⁻¹)
Control	15.64 \pm 0.10	20.63 \pm 0.28 ^c	4.99 \pm 0.21 ^c	30.20 \pm 1.21 ^c	1.20 \pm 0.04 ^c	0	3.20 \pm 0.14 ^c
BD8	15.36 \pm 0.11	24.67 \pm 0.35 ^b	9.31 \pm 0.38 ^b	61.98 \pm 2.68 ^b	2.19 \pm 0.08 ^b	42.79 \pm 8.62 ^b	1.45 \pm 0.06 ^b
AIR	15.65 \pm 0.12	32.56 \pm 0.58 ^a	16.91 \pm 0.48 ^a	108.02 \pm 2.48 ^a	3.50 \pm 0.30 ^a	80.10 \pm 4.31 ^a	0.50 \pm 0.02 ^a
IBL	15.51 \pm 0.20	18.86 \pm 0.26 ^c	3.35 \pm 0.09 ^{bc}	21.62 \pm 0.52 ^c	0.89 \pm 0.02 ^c	-26.87 \pm 14.93 ^c	3.49 \pm 0.22 ^c

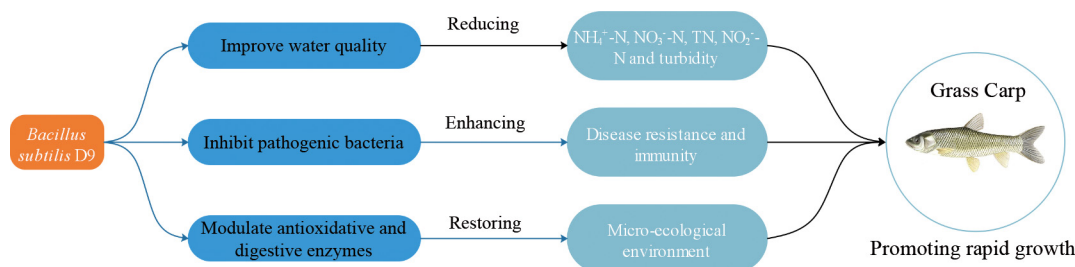


Fig. 4. Proposed mechanisms of *Bacillus subtilis* D9 affecting disease resistance of grass carp

tion of antioxidative and digestive enzymes, thus promoting the rapid growth of grass carp. On the other hand, probiotics produced by *B. subtilis* D9 effectively restore the micro-ecological environment and reduce the quantity of toxic materials, thus enhancing the disease resistance and immunity of grass carp.

5. CONCLUSIONS

Laboratory and *in situ* experiments showed that *Bacillus subtilis* D9 application significantly improved the quality of wastewater in terms of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, TN, $\text{NO}_2^-\text{-N}$ and turbidity. The optimum applied concentration was BD8 (5.5×10^8 cfu ml^{-1}). We found significant differences among AIR, BD8, Control and IBL groups in PWG, SGR and RPS. We conclude that BD8 of *B. subtilis* D9 with aeration significantly improved water quality of the wastewater and increased the disease resistance of grass carp to *Vibrio parahaemolyticus*. Therefore, *B. subtilis* D9 could be used as a novel microbial agent with a great application potential in the aquaculture industry for wastewater purification and disease resistance.

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