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Mitigating Vibrio-Induced Skin Ulceration in Sea Cucumbers Using Probiotic Strains

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Abstract: Sea cucumbers are valuable in aquaculture, but their cultivation faces challenges from diseases such as skin ulceration syndrome caused by *Vibrio alginolyticus* (VA). This study aimed to isolate and identify probiotics capable of combating VA and improving sea cucumber's growth performance. Pathogenic VA was identified, through 16S rDNA sequencing, confirming its high genetic similarity (>99%) to *Vibrio alginolyticus*. Two *Bacillus* strains, *Bacillus licheniformis* YB-1, and *Bacillus megaterium* YB-2, were isolated as potential probiotics, with identification supported by 16S rDNA phylogenetic analysis and deposition in microbial culture collections. They demonstrated strong antibacterial activity against VA in vitro without exhibiting antagonism when combined. Probiotic tolerance to environmental stressors was observed, while feeding trials revealed significant growth improvements in sea cucumbers, with the highest specific growth rates observed at 1×10^6 CFU/mL for both strains. Immersion challenge tests showed that sea cucumbers treated with probiotics exhibited reduced symptoms of rotten skin syndrome and higher survival rates. The optimal combination of YB-1 and YB-2, with viable bacteria concentrations of 5×10^7 CFU/mL each, achieved a 55% survival rate after a VA challenge, demonstrating their synergistic efficacy. These findings suggest that YB-1 and YB-2 offer promising probiotic solutions for enhancing sea cucumber health and resistance to VA infections in aquaculture.

Keywords: sea cucumber; aquaculture; probiotic; *Vibrio alginolyticus*; rotten skin syndrome

Key Contribution: This study identifies two probiotic Bacillus strains, YB-1 and YB-2, with strong antibacterial activity against *Vibrio alginolyticus* (VA), the causative agent of skin ulceration syndrome in sea cucumbers. The probiotics demonstrated synergistic efficacy, improving survival rates and growth performance in sea cucumbers, offering a sustainable solution for disease management in aquaculture.

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1. Introduction

The aquaculture of *Apostichopus japonicus*, a high-value sea cucumber species, has expanded significantly in China, driven by surging consumer demand. This species now represents a cornerstone of the marine industry in northern China, with over one million acres dedicated to farming and a production value exceeding USD 120 million annually [1]. However, this rapid expansion, coupled with non-standardized farming practices, has led to an alarming increase in disease outbreaks. Since 2003, farms have reported widespread

cases of ulceration syndrome in *A. japonicus*, characterized by muscle tissue ulcers, tumescence around the peristome, autolysis, and high mortality rates. These issues threaten the sustainability of the industry and limit further expansion potential [2].

Sea cucumber aquaculture faces significant challenges due to diseases like skin ulceration, which have been reported in species such as *Holothuria scabra* and *Apostichopus japonicus* [3]. *H. scabra* populations in Australia, New Caledonia, and Madagascar have experienced outbreaks of ulceration-related diseases, while intestinal parasites have been documented in Galápagos Sea cucumber larvae [4]. In China, various pathogens have been implicated in disease epidemics in *A. japonicus*, including *Vibrio splendidus*, *Vibrio tapetis*, *Marinomonas dokdonensis*, *Aeromonas salmonicida*, and *Aeromonas media*, identified through molecular techniques [5].

Studies indicate that *Vibrio* species, including *V. splendidus* and *V. tapetis*, play a critical role in skin ulcer syndrome and related pathologies [6]. These pathogens often coexist with environmental stressors, creating a complex interplay that exacerbates disease severity. Isolated cases, such as those in Rizhao and Yantan (Shandong, China), identified *Aeromonas* spp. as the dominant causative agent, highlighting regional variability in pathogenic profiles [7]. Drugs commonly used to treat *Vibrio alginolyticus* infections, including oxytetracycline hydrochloride, florfenicol, erythromycin, and others, are often reported as ineffective in fully controlling skin ulceration. Inefficacy arises from the emergence of multidrug-resistant strains and the environmental persistence of pathogens under stress [8]. The overuse and misuse of these antibiotics not only disrupts the intestinal microbiota of affected sea cucumbers but also contributes to the development of antibiotic-resistant bacteria in aquaculture systems [9]. Residual antibiotics accumulating in the aquatic environment and within the tissues of sea cucumbers further amplify ecological risks and pose significant threats to human health and food safety through the seafood supply chain. This underscores the urgent need for sustainable treatment approaches, such as the use of probiotics or antimicrobial peptides, to mitigate the limitations of conventional antibiotics [10].

Recent advancements in microbiology and microecology have shifted the focus towards probiotics as a sustainable alternative to antibiotics for disease control in aquaculture [11]. Probiotics such as *Bacillus*, *Lactobacillus*, *Saccharomyces cerevisiae*, *Clostridium butyricum*, and photosynthetic bacteria are gaining widespread application due to their ability to enhance immunity and disease resistance in aquatic animals [12]. Among these, *Bacillus subtilis* is the most extensively studied, with *Bacillus licheniformis* and *Bacillus coagulans* also demonstrating significant benefits in improving host immunity and resilience against pathogens [1]. Lactic acid bacteria such as *Lactococcus lactis*, *Lactobacillus plantarum*, and *Enterococcus faecium* have been shown to boost immunity and growth performance, while *Lactobacillus delbrueckii* and *Lactobacillus casei* exhibit protective effects against bacterial pathogens like *Aeromonas hydrophila* [13]. Research indicates that multispecies probiotic formulations are often superior to single-strain probiotics due to their synergistic effects, integrating functions that enhance overall host health and disease resistance [14]. This approach not only reduces the dependence on antibiotics but also minimizes the risks of antimicrobial resistance and environmental contamination. By leveraging the diverse functional properties of probiotics, aquaculture can achieve sustainable growth while mitigating disease impacts, making probiotics a promising strategy in the industry.

The objective of this study is to evaluate the antibacterial activity and growth-promoting effects of *Bacillus licheniformis* YB-1 and *Bacillus megaterium* YB-2 on sea cucumbers, with a focus on their ability to mitigate *Vibrio-alginolyticus*-induced rotten skin syndrome. This will be achieved through 16S rDNA sequencing for bacterial identification, in vitro antibacterial assays, and growth performance experiments. Additionally, we will

assess the synergistic effects of combined probiotics on sea cucumber survival rates and resistance to *Vibrio alginolyticus* under challenging conditions.

2. Materials and Methods

2.1. Experimental Chemicals and Sources

In this study, a comprehensive range of materials and reagents was employed to ensure the precision and consistency of experimental outcomes. Tryptic Soy Broth (TSB), bile salts, trypsin and agar powder, sourced from Solarbio (Beijing, China), served as the primary media for microbial cultivation. Essential growth nutrients were supplied through peptone and yeast extract from Oxoid (Basingstoke, UK). Glycerol obtained from Kemio (Shanghai, China) was used as a cryoprotectant during sample storage. For molecular biology procedures, Ex Taq polymerase and universal primers were acquired from Takara Bio (Dalian, China). DNA fragments were visualized and analyzed using Golden View stain and DNA marker from Solarbio, with 50× TAE buffer and loading buffer from the same supplier. Bacterial microbiochemical identification kits and antibiotic susceptibility discs, supplied by Hangzhou Microbial Reagent Co., Ltd. (Hangzhou, China), were used to facilitate bacterial identification and characterization.

2.2. Isolation and Identification of Pathogenic Bacteria and Probiotic Bacteria

The diseased sea cucumbers were rinsed three times with sterile distilled water before dissection. Infected tissue from the body wall was collected, and sterile PBS buffer was added. The tissue was thoroughly homogenized for 10 min using a tissue homogenizer. The homogenate supernatant was collected and spread evenly on a TCBS identification medium, followed by incubation at 28 °C for 48 h in a constant-temperature incubator. A single colony was picked up and resuspended in 200 μL of sterile PBS, and the procedure was repeated thrice. The pathogenic bacterium VA was ultimately isolated and stored at −80 °C. Furthermore, samples of sea cucumber culture water and intestinal tissue were separately collected and soaked in sterile PBS buffer for 24 h. The supernatant was collected and evenly spread onto a 2216E solid medium. The plates were incubated in a constant temperature incubator at 28 °C for 48 h. Single colonies were selected and resuspended in 200 μL of sterile PBS. This process was repeated three times to ensure purity. Two probiotic strains, YB-1 and YB-2, were successfully isolated and stored in a −80 °C refrigerator for further analysis.

The morphological characteristics of the isolated strains were evaluated using scanning electron microscopy (SEM). For 16S rRNA sequence analysis, a single bacterial colony was transferred into 100 μL of sterilized ultrapure water using a sterile toothpick. The sample was heated in a 100 °C water bath for 10 min, centrifuged at 5000× *g* for 3 min, and the supernatant was used as the DNA template.

PCR amplification of the 16S rRNA gene was performed using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′), targeting a 1450 bp sequence. The 50 μL reaction mixture included 2 μL of DNA template, 1 μL of each primer, 25 μL of Ex Taq DNA polymerase, and 21 μL of sterile ultrapure water. The PCR conditions were initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 S, annealing at 55 °C for 30 S, and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 10 min, followed by a hold at 4 °C. The amplified product was analyzed using 1% agarose gel electrophoresis and subsequently sent to Shanghai Sangon Bioengineering Co., Ltd., for NGS sequencing. The obtained 16S rRNA sequences were analyzed using BLAST and Clustal alignment tools, and a phylogenetic tree was constructed in MEGA 6.0 software with bootstrap analysis (1000 replicates) to ensure robustness and accuracy [15,16].

2.3. Transmission Electron Microscopy Observation of Pathogenic Bacteria

The purified bacterial strain VA was cultured in a 2216E liquid medium until it reached the logarithmic growth phase. The culture was centrifuged at 5000× *g* for 3 min at 4 °C, and the supernatant was discarded. The resulting bacterial pellet was resuspended in sterile PBS. A 200-mesh carbon-coated copper grid was used to capture the bacteria by gently immersing it in the bacterial suspension. The grid was then placed on filter paper to remove excess liquid and allowed to dry. Subsequently, the grid was negatively stained with 2.5% (*w*/*v*) phosphotungstic acid for approximately 3 min. After drying in a fume hood, the grid was examined under a Hitachi HT7700 transmission electron microscope (Hitachi, Tokyo, Japan) to observe the morphology of the bacterial cells [17].

2.4. Antibacterial and Antagonistic Activity of Probiotics

To evaluate the antibacterial activity of probiotics, VA at a concentration of 1×10^7 CFU/mL was evenly spread onto a 2216E solid medium. Once the surface dried, Oxford cups (inner diameter 6 mm, outer diameter 8 mm, height 10 mm) were placed vertically on the agar. Each cup was filled with 1×10^9 CFU/mL bacterial suspension of YB-1 and YB-2, respectively. A control group was treated with an equal volume of 2216E liquid medium. The plates were incubated at 28 °C for 18 h, and the diameter of the inhibition zones was measured to assess antibacterial activity. For the antagonistic activity assay, circular filter paper pieces were soaked in 1×10^8 CFU/mL suspensions of YB-1 and YB-2, respectively. The soaked filter papers were placed on 2216E solid medium surfaces evenly coated with either YB-2 or YB-1. The plates were incubated at 28 °C for 18 h, and the presence of inhibition zones around the filter papers was observed to determine probiotic antagonism. Each experiment was repeated three times.

The biological properties of probiotics YB-1 and YB-2 were evaluated under varying conditions, including pH, temperature, bile salt concentration, and trypsin concentration, to assess their adaptability and growth. Both strains were cultured in an MRS liquid medium at 28 °C for 24 h to prepare the test solutions. To study the effect of pH, the strains were inoculated into an MRS medium with pH levels ranging from 6 to 10 and a salinity of 30%, incubated at 25 °C for 24 h, and growth was monitored. Temperature tolerance was examined by inoculating the strains into MRS medium (salinity 30%, pH 8) and incubating at 10 °C, 20 °C, 30 °C, 40 °C, and 50 °C for 24 h. For bile salt tolerance, bile salts at concentrations of 0, 1, 2, 4, and 6 g/L were added after pre-culturing the strains at 25 °C for 24 h in MRS medium (salinity 30%, pH 8), followed by 60 min of additional incubation. Similarly, trypsin tolerance was evaluated by adding trypsin at the same concentrations under identical conditions. Observations revealed the growth response of YB-1 and YB-2 to these varying conditions, demonstrating their resilience and potential for survival in diverse environmental and gastrointestinal settings.

2.5. Acute Challenge of Sea Cucumbers with Probiotics and Vibrio alginolyticus

Ten healthy sea cucumbers from each group were raised in 10 L of filtered seawater under controlled conditions: water temperature of 16 °C, pH 7.5, dissolved oxygen 5.5 mg/L, and salinity 30 g/L [18]. According to Cai's method, the bacteria liquid is frozen and dried. The specific cryoprotectants were sucrose 7.5 (*w*/*v*)%, skim milk powder 10.0 (*w*/*v*)% and maltodextrin 12.5 (*w*/*v*)% [19]. In order to prevent prolonged freezing from reducing the activity of probiotics, this part was completed within one week after freeze-drying. The first group received 100 mg/L YB-1, while the second group received 10,000 mg/L YB-1. Similarly, the third and fourth groups were treated with 100 mg/L and 10,000 mg/L YB-2, respectively. The fifth group served as the control and was supplemented with an equal volume of sterile PBS. For the first four groups, the YB-1 or YB-2 treatments were

replenished every 24 h, while the control group received sterile PBS. Symptoms related to sea cucumber poisoning were recorded at intervals of 1 h, 4 h, 12 h, 24 h, 48 h, 72 h, and 96 h. For the VA challenge, sea cucumbers were randomly divided into groups, each containing 10 individuals, with three replicates per group. The breeding conditions were consistent with the probiotic challenge setup. In the cultured seawater of the experimental group, the final concentration of the suspensive bacteria solution was 1×10^3 CFU/mL, 1×10^4 CFU/mL,1×10^5 CFU/mL, 1×10^6 CFU/mL, 1×10^7 CFU/mL,1×10^8 CFU/mL and 1×10^9 CFU/mL. The control group was added with PBS. Incidence rates and mortality were recorded daily for 10 d. Bacteria from deceased sea cucumbers were re-isolated and identified using 16S rDNA sequencing. Survival curves were generated using Prism 8 software.

2.6. Probiotic Feeding and Challenge Experiments

Sea cucumbers were divided into nine groups, each with 30 individuals, and reared for 30 days under control conditions. Feeding was conducted daily at a fixed time, with the feeding amount set at 3% of body weight. The specific experimental groups are shown in Table 1. At the end of the experiment, growth performance indicators, including weight gain (WG) and specific growth rate (SGR), were calculated using the following formulas:

$$\text{Weight Gain (g)} = W_2 - W_1,$$

where W_2 is the final wet weight and W_1 is the initial wet weight.

$$\text{Specific Growth Rate (\%/d)} = 100 \times (\ln W_2 - \ln W_1)/T,$$

where T is the number of feeding days.

Table 1. Specific growth rate groupings.

Group	Experimental Content
Group 1	1 mL YB-1 at 1×10^4 CFU/mL
Group 2	1 mL YB-1 at 1×10^6 CFU/mL
Group 3	1 mL YB-1 at 1×10^8 CFU/mL
Group 4	1 mL YB-2 at 1×10^4 CFU/mL
Group 5	1 mL YB-2 at 1×10^6 CFU/mL
Group 6	1 mL YB-2 at 1×10^8 CFU/mL
Group 7	1 mL of a mixture of YB-1 and YB-2 at 1×10^6 CFU/mL (1:1 volume ratio)
Group 8	1 mL of a mixture of YB-1 and YB-2 at 1×10^8 CFU/mL (1:1 volume ratio)
Group 9	1mL PBS

After the feeding period, 20 sea cucumbers from each group were transferred to 10 L of filtered seawater and exposed to VA at a final concentration of 1×10^7 CFU/mL. Mortality rates were monitored daily for 10 days under the same conditions, without feeding or additional treatments during the observation period.

2.7. Statistical Analysis

In this study, all data were subjected to statistical analysis, and standard errors (s) were calculated for all results. Data are presented as mean ± standard deviation ($\bar{x} \pm s$). A one-way ANOVA was employed to assess significant differences in mean values among the groups, with p-values < 0.05 considered significant and p-values < 0.01 considered extremely significant. All statistical analyses were conducted using GraphPad Prism 5.0 or SPSS software (version 22.0).

2.8. Ethics Statement

All research was conducted according to the recommendations in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by Liaoning Province, China. All experimental animal protocols were approved by the Biological and Medical Ethics Committee of Dalian University of Technology. The approval number is DUTSBE241223-02.

3. Results

3.1. Isolation and Identification of Pathogenic Bacteria

As shown in Figure 1A, the VA colony on the TCBS plate appeared dark yellow, medium-sized, round, and slightly elevated, with a smooth surface. The colony was slightly sticky, making it difficult to stir, and exhibited a light-yellow annular halo around it, consistent with the typical characteristics of VA. The genome of the dominant isolated pathogenic strain VA, obtained through screening, was extracted, and its 16S rDNA was amplified using Universal primers (27F and 1492R). The sequencing results revealed that the amplified VA 16S rDNA fragment was 1191 bp in length. The sequence was compared with known sequences in GenBank using the NCBI BLAST tool. A phylogenetic tree was constructed using the VA 16S rDNA sequence and representative strains, as shown in Figure 1B. The analysis confirmed that strain VA belongs to the genus *Vibrio*, sharing over 99% homology with *Vibrio alginolyticus*. High-homology sequences were selected to construct the phylogenetic tree, which showed that strain VA clustered closely with *Vibrio alginolyticus* strains 20-1-1 and Xmb025. Based on these findings, strain VA was conclusively identified as *Vibrio alginolyticus*. Furthermore, through transmission electron microscopy observation of pathogenic bacteria, as shown in Figure 1C, it was observed that strain VA is rod-shaped, approximately 1.0 μm in size, with polar single flagella and bluntly rounded ends, consistent with the electron microscopy characteristics of *Vibrio alginolyticus*.

3.2. Isolation and Molecular Identification of Probiotics

The genomes of strains YB-1 and YB-2, obtained through screening, were extracted and amplified using specific 16S rDNA primers. The amplified products were analyzed via 1% agarose gel electrophoresis and subsequently sent to Shanghai Sangon for sequencing. The sequencing results revealed that the 16S rDNA fragment length was 1100 bp for YB-1 and 1114 bp for YB-2. The sequences were compared with known sequences in GenBank using the NCBI BLAST tool. Phylogenetic trees were constructed with the 16S rDNA sequences of YB-1 and YB-2 using MEGA 6.0 software, as shown in Figure 2A,B. Strain YB-1 was identified as belonging to the genus *Bacillus* and showed more than 81% homology with *Bacillus licheniformis*. High-homology sequences were used to construct the phylogenetic tree, which revealed that YB-1 clustered with *Bacillus licheniformis* strains AWE22 and AWC48. This strain was deposited in the China Microbial Culture Collection Center under accession number CGMCC No. 25456. Strain YB-2 was also identified as belonging to the genus *Bacillus* and demonstrated 65% homology with *Bacillus megaterium* strain OKF05. The phylogenetic tree indicated that YB-2 clustered with *Bacillus megaterium* strains HX-2 and IRHB1-27. This strain was deposited in the China Microbial Culture Collection Center under accession number CGMCC No. 25455.

3.3. Antibacterial Activity and Compatibility of Probiotics

The antibacterial effects of YB-1 and YB-2 against *Vibrio alginolyticus* VA are depicted in Figure 3A, where (a) represents YB-1, (b) represents YB-2, and (c) represents the control. Both YB-1 and YB-2 demonstrated strong antibacterial activity in vitro, effectively inhibiting the growth of VA, while no antibacterial activity was observed in the control group.

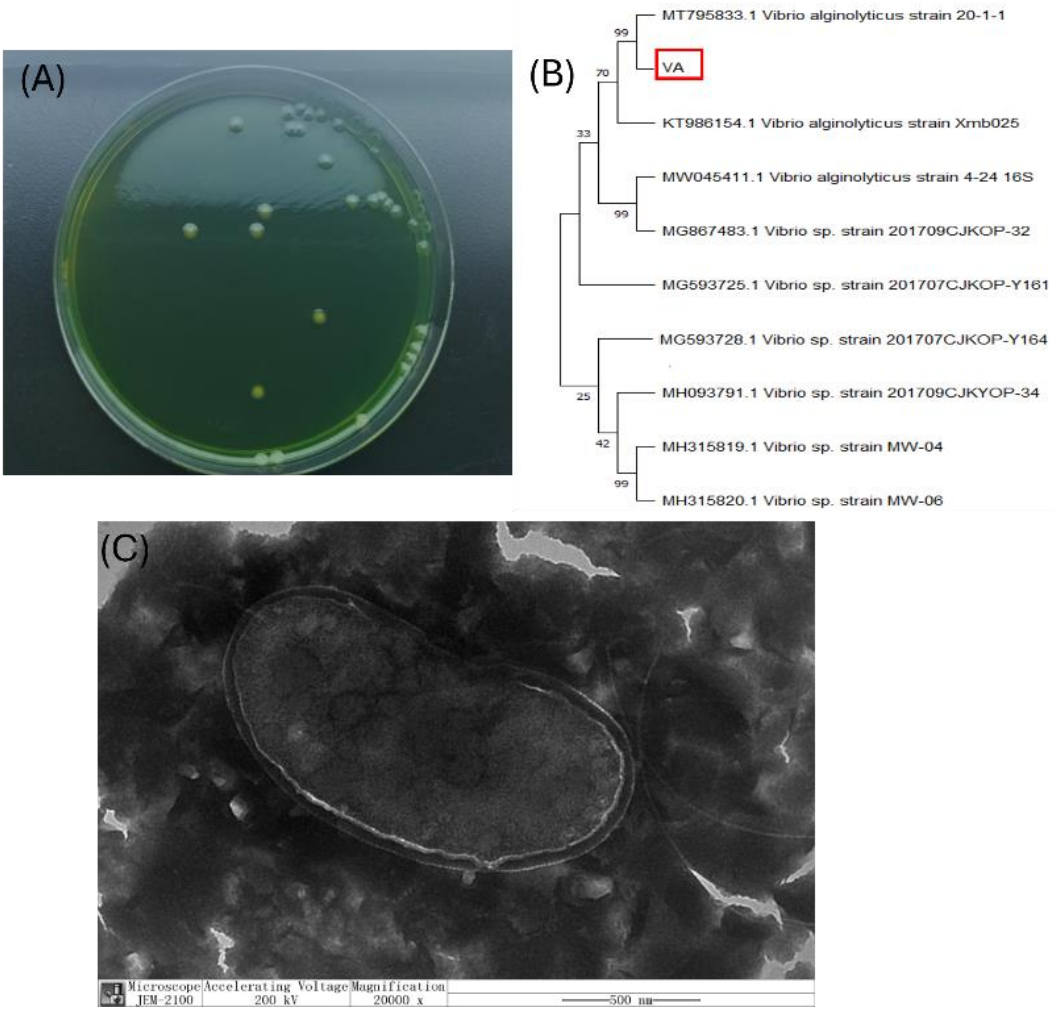

Figure 1. (**A**) Colony morphology of VA on TCBS selective medium; (**B**) VA evolutionary tree; (**C**) colony morphology of VA under a transmission electron microscope (20,000×).

The colony morphology results are shown in Figure 3B,C. Figure 3B illustrated the placement of filter paper containing YB-2 on 2216E culture medium pre-coated with YB-1, while Figure 3C depicted filter paper containing YB-2 placed on 2216E culture medium pre-coated with YB-2. After incubation, no obvious inhibition zones were observed around the filter papers containing YB-1 or YB-2. These findings suggest that *Bacillus* strains YB-1 and YB-2 exhibit no significant antagonistic effects and can be effectively used together in combined applications.

3.4. Biological Properties of Probiotics

The growth characteristics of *Bacillus licheniformis* YB-1 and *Bacillus megaterium* YB-2 under various conditions are shown in Table 2. While the bacteria could grow in a pH range of 6–9, their tolerance to alkaline environments was limited. Both strains exhibited normal growth within a temperature range of 20–40 °C, with optimal growth observed between 20 and 30 °C. At a bile salt concentration of 6 g/L, the lowest viable count recorded was 9.8×10^5 CFU/mL, indicating that both strains can tolerate moderate bile salt concentrations. Furthermore, after treatment with trypsin at a concentration of 6 g/L for 30 min, the viable count of YB-1 was 5.31×10^7 CFU/mL, demonstrating that trypsin has minimal impact on the growth of YB-1. However, after YB-2 was treated with trypsin, the concentration of bacterial solution changed greatly, indicating that YB-2 was sensitive to trypsin.

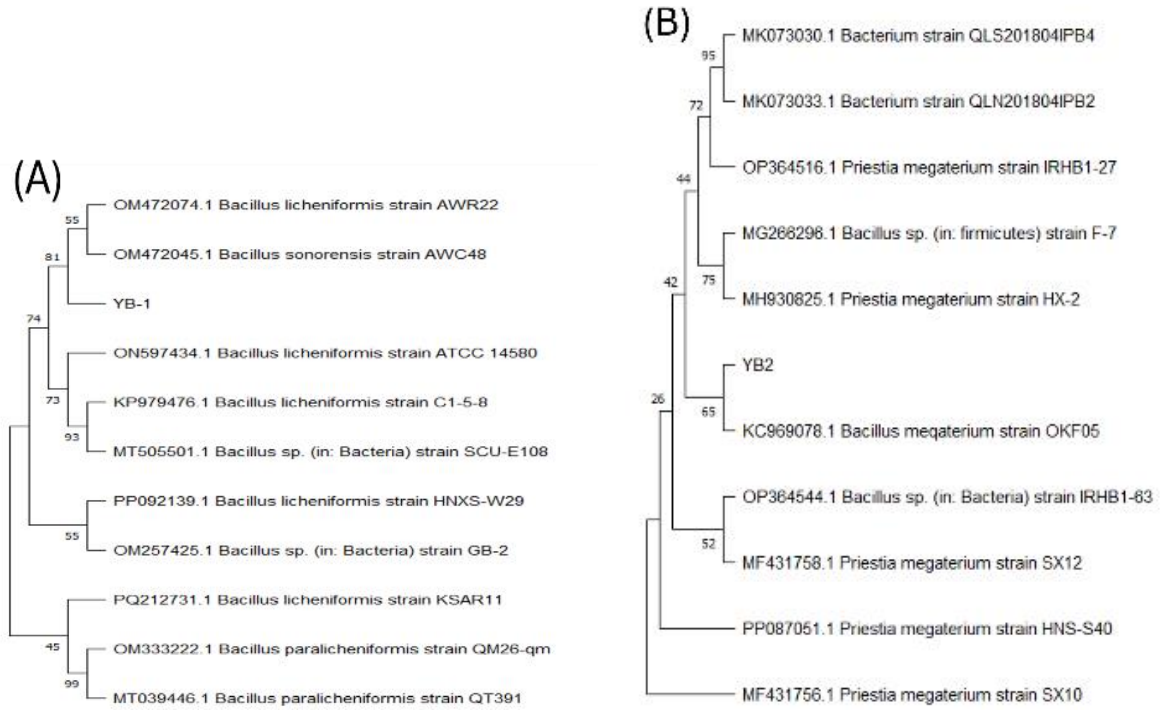

Figure 2. (**A**) Phylogenetic tree of YB-1 (*Bacillus licheniformis*); (**B**) phylogenetic tree of YB-2 (*Bacillus megaterium*).

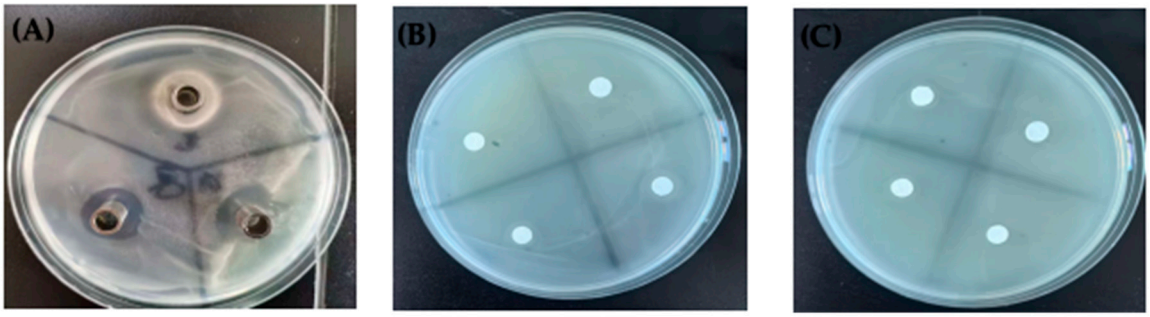

Figure 3. (**A**) In vitro antibacterial results of YB-1 and YB-2 on *Vibrio alginolyticus* VA, a is YB-1, b is YB-2, and c is the control; (**B**) a filter paper piece containing YB-2 was placed on the 2216e culture medium coated with YB-1; (**C**) a filter paper piece containing YB-1 was placed on the 2216e culture medium coated with YB-2.

Table 2. Growth of strains investigated under different culture conditions, among which *Bacillus licheniformis* was recorded as YB-1 and *Bacillus megaterium* was recorded as YB-2.

pH	6	7	8	9	10
YB-1 (10^7 CFU/mL)	2.11 ± 0.16^{b}	6.60 ± 0.23^{a}	5.91 ± 0.54^{a}	2.15 ± 0.27^{b}	0.91 ± 0.03^{c}
YB-2 (10^7 CFU/mL)	2.32 ± 0.33^{b}	5.41 ± 0.91^{a}	4.93 ± 0.74^{a}	1.75 ± 0.11^{b}	0.75 ± 0.07^{c}
Temperature (°C)	10	20	30	40	50
YB-1 (10^6 CFU/mL)	4.51 ± 1.13^{c}	66.63 ± 8.17^{a}	66.01 ± 1.73^{a}	52.11 ± 4.31^{b}	0.02 ± 0.01^{d}
YB-2 (10^6 CFU/mL)	4.71 ± 0.14^{b}	73.44 ± 3.65^{a}	74.32 ± 5.02^{a}	70.81 ± 4.21^{a}	0.05 ± 0.01^{c}
Bile salts (g/L)	0	1	2	4	6
YB-1 (10^7 CFU/mL)	6.32 ± 0.65^{a}	6.12 ± 0.11^{a}	5.49 ± 0.01^{b}	2.31 ± 0.74^{c}	0.98 ± 0.03^{d}
YB-2 (10^7 CFU/mL)	5.74 ± 0.64^{a}	4.41 ± 0.14^{b}	2.10 ± 0.64^{c}	0.93 ± 0.11^{d}	0.48 ± 0.16^{d}
Protease (g/L)	0	1	2	4	6
YB-1 (10^7 CFU/mL)	6.69 ± 1.12^{a}	6.12 ± 0.88^{ab}	5.83 ± 0.65^{b}	5.57 ± 0.41^{b}	5.31 ± 0.61^{b}
YB-2 (10^7 CFU/mL)	7.28 ± 0.65^{a}	5.33 ± 0.97^{b}	3.77 ± 0.32^{c}	1.66 ± 0.02^{d}	1.31 ± 0.15^{d}

Values with different superscript letters (a–d) in the same row are significantly different ($p < 0.05$).

3.5. Experimental Results of Acute Challenge of Sea Cucumbers with Probiotics and Vibrio alginolyticus

Sea cucumbers were exposed to concentrations ranging from 0 to 10,000 mg/L of YB-1 or YB-2 in standard dilution water. Even at the highest concentration of 10,000 mg/L, which significantly exceeds the levels typically encountered in experiments and real-world applications, no signs of poisoning or mortality were observed. This demonstrates that YB-1 and YB-2 are non-toxic to sea cucumbers. Additionally, the 96 h LD50 for both strains was found to be greater than 10,000 mg/L, classifying them as virtually non-toxic.

In contrast, when sea cucumbers were exposed to varying concentrations of VA, symptoms appeared rapidly, as illustrated in Figure 4A. Within one day of exposure to 1×10^9 CFU/mL, the sea cucumbers exhibited reduced food intake and head-shaking behavior. By the second day, the groups exposed to 1×10^6 CFU/mL to 1×10^9 CFU/mL developed symptoms including vomiting, blackheads, and skin ulcers, which eventually led to death. Mortality rates reached 100% in the 1×10^7 CFU/mL, 1×10^8 CFU/mL, and 1×10^9 CFU/mL groups. Dead sea cucumbers displayed unresponsiveness, inability to contract their mouths, and severe skin ulceration or autolysis. Pure cultures isolated from the epidermis and body cavity fluid of infected sea cucumbers were identified as VA through 16S rDNA sequence analysis, matching the original infecting strain. Meanwhile, sea cucumbers in the control group showed normal feeding behavior and exhibited no symptoms throughout the experiment.

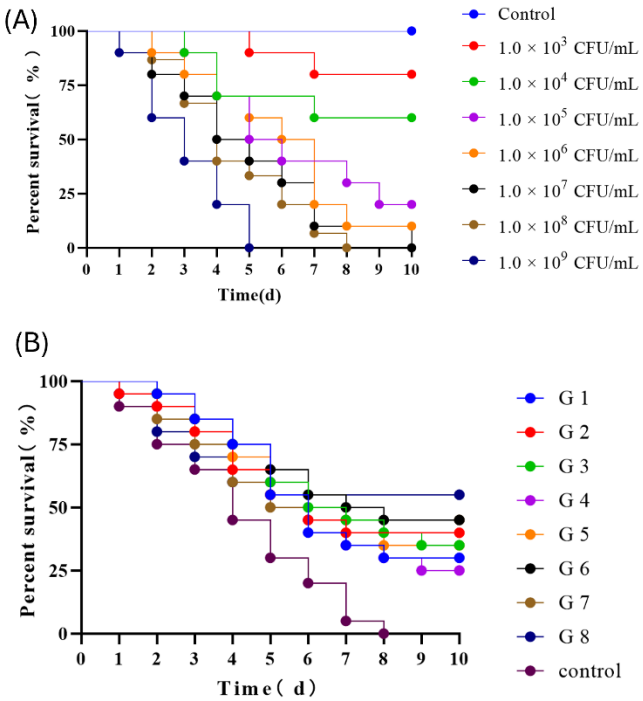

Figure 4. (**A**) Acute challenge of sea cucumbers with probiotics and VA; (**B**) survival rate % of sea cucumbers after challenging experiment.

3.6. Probiotic Feeding Experiment

As summarized in Table 3, feeding sea cucumbers with YB-2 resulted in a significant correlation between different concentrations of probiotics and the final specific growth rate ($p < 0.05$). The highest specific growth rate was observed at a concentration of 1×10^6 CFU/mL of YB-2. Similarly, feeding with YB-1 also showed a significant correlation between probiotic concentration and specific growth rate ($p < 0.05$), with the optimal concentration being 1×10^6 CFU/mL of YB-1.

Table 3. Probiotic feeding experiment.

Experimental Groups	Average Initial Weight/g	Average Final Weight/g	Specific Growth Rate/d
Group 1	4.38 ± 0.13	7.960 ± 0.03	0.625 ± 0.04 ^{b}
Group 2	5.35 ± 0.02	11.50 ± 0.25	0.979 ± 0.97 ^{de}
Group 3	4.20 ± 0.19	9.03 ± 0.09	0.804 ± 0.91 ^{cd}
Group 4	4.52 ± 0.20	7.92 ± 0.02	0.597 ± 0.05 ^{b}
Group 5	5.08 ± 0.05	10.84 ± 0.12	0.881 ± 0.10 ^{d}
Group 6	4.05 ± 0.15	8.38 ± 0.05	0.735 ± 0.07 ^{c}
Group 7	4.68 ± 0.18	11.50 ± 0.20	0.968 ± 0.67 ^{d}
Group 8	4.87 ± 0.14	10.78 ± 0.33	0.836 ± 0.77 ^{cd}
Group 9	5.12 ± 0.13	8.01 ± 0.13	0.493 ± 0.06 ^{a}

Values with different superscript letters (a–d) in the same row are significantly different ($p < 0.05$).

When comparing the two species, YB-2 demonstrated a more pronounced effect on weight gain than YB-1. Additionally, the combination of the two strains, at both 1×10^6 CFU/mL and 1×10^8 CFU/mL, exhibited an effective synergistic impact, resulting in enhanced growth performance in sea cucumbers.

3.7. Post-Feeding Challenge Experiment

Sea cucumbers challenged with VA via immersion exhibited symptoms such as vomiting, swollen mouth, skin ulceration, and eventual death. The survival rates after a 10-day challenge are illustrated in Figure 4B. The LD50 value in 96h is 3.697×10^6 CFU/mL. The results indicate that probiotics provided notable preventive and therapeutic effects against rotten skin syndrome. Among the probiotics tested, YB-2 showed the best antibacterial effect at 1×10^6 CFU/mL, while YB-1 was most effective at 1×10^8 CFU/mL. The optimal outcome was achieved with a mixed bacterial solution containing equal volumes (1:1 ratio) of YB-2 and YB-1, where the concentration of viable bacteria for each strain was 5×10^7 CFU/mL. This combination resulted in a survival rate of approximately 55%, demonstrating the synergistic efficacy of the mixed probiotic treatment in enhancing resistance to VA.

4. Discussion

Vibrio alginolyticus is a pathogenic bacterium widely recognized as a significant threat to marine aquaculture, including sea cucumber farming [20]. This pathogen is known to cause "rotten skin syndrome", characterized by symptoms such as vomiting, skin ulceration, blackheads, and eventual mortality in sea cucumbers (*Apostichopus japonicus*). The infection often leads to a rapid onset of symptoms, with high mortality rates observed in severely affected populations [21]. Pathogenicity is largely attributed to the bacterium production of virulence factors such as hemolysins, extracellular enzymes, and toxins, which disrupt host cellular integrity and immune responses [22]. Environmental stressors, such as elevated water temperatures and poor water quality, exacerbate *V. alginolyticus* infections by weakening the host immune defenses, creating favorable conditions for pathogen proliferation [23]. Previous studies have shown that outbreaks can decline aquaculture stocks, leading to substantial economic losses. This study provides compelling evidence supporting the use of probiotics *Bacillus licheniformis* YB-1 and *Bacillus megaterium* YB-2 as effective measures against *Vibrio alginolyticus*-induced infections in sea cucumbers (*Apostichopus japonicus*). The isolation, identification, and characterization of these probiotics, along with their antibacterial activity and biological properties, align with previous research that underscores the critical role of probiotics in sustainable aquaculture. *Vibrio* infections are among the most challenging issues in aquaculture as they cause significant mortality and economic loss. Probiotics offer an environmentally friendly alternative to antibiotics by modulating host immunity and improving gut microbiota balance [24].

The robust in vitro antibacterial activity observed in YB-1 and YB-2 supports earlier findings reported by the authors of [25], who highlighted that *Bacillus* species produce an-

timicrobial substances that inhibit pathogens. The compatibility tests further demonstrated that these strains can be used synergistically without antagonistic effects, a result consistent with studies on multispecies probiotics, which exhibit enhanced growth and survival outcomes in aquatic organisms [26]. Additionally, the optimal growth characteristics of YB-1 and YB-2 under a wide range of temperatures, moderate bile salt concentrations, and enzymatic exposure suggest their resilience and adaptability to gastrointestinal environments, corroborating the findings [27].

The probiotic feeding experiments revealed a significant increase in specific growth rates (SGRs), especially at concentrations of 1×10^6 CFU/mL, with synergistic effects observed in mixed treatments. This aligns with previous reports showing improved growth performance in fish and shrimp fed with probiotics [28,29]. Furthermore, the post-feeding challenge experiments demonstrated notable reductions in mortality rates following *V. alginolyticus* infection. The mixed probiotic solution yielded a survival rate of approximately 55%, reflecting the efficacy of probiotics in enhancing host resilience to pathogens. The results of *Bacillus atrophaeus* on potato scab showed that *Bacillus atrophaeus* reduced the incidence of scab caused by Streptomyces scabies from 87.32% to 60.77%, and the disease index from 58.89% to 25% [30]. In aquaculture, *Bacillus gigantium* can protect animal health by inhibiting the hemolytic activity of *Vibrio Harveyi* and preventing it from binding with the body surface and gastrointestinal surface mucosa of fish and shrimp [31]. In terms of inhibiting *Vibrio para-hemolyticus*, Jiang et al. screened five probiotics in water, including *Bacillus inaquosorum*, *Bacillus subtilis* and *Bacillus velezensis*, and their inhibition rates against *Vibrio para-hemolyticus* ranged from 32.61% to 74.67% [31]. Probiotics can upregulate antioxidant enzymes such as catalase and GPx, mitigating reactive oxygen species (ROS) and enhancing cellular resilience, as seen in our study and previous work [32]. The reduced pathogenicity of *V. alginolyticus* in probiotic-treated groups can also be attributed to competitive exclusion mechanisms, where beneficial microbes outcompete pathogens for nutrients and binding sites [33]. Overall, our findings strongly support the integration of *Bacillus* probiotics into aquaculture systems to mitigate *Vibrio* infections and enhance host health. The dual benefits of pathogen inhibition and growth promotion make YB-1 and YB-2 promising candidates for sustainable aquaculture practices.

5. Conclusions

Our findings demonstrate that *Bacillus licheniformis* YB-1 and *Bacillus megaterium* YB-2 exhibit strong antibacterial activity against *Vibrio alginolyticus*, with no antagonistic effects when used together. The probiotics significantly enhanced sea cucumber growth performance and resistance to Vibrio-induced rotten skin syndrome, with optimal effects observed at specific concentrations. While the combination of probiotics showed promising synergistic effects, further research is needed to explore their long-term application, scalability, and impact on aquaculture systems to improve disease management and sustainability.

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