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Preliminary screening, identification and biological characteristic analysis of *Bacillus* probiotics isolated from *Cynoglossus semilaevis*

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Abstract

To screen local probiotic strains to promote antibiotic-free farming, two potential probiotic strains (S3, S5) were recognized among 89 cultivable bacterial strains isolated from the intestine of healthy *Cynoglossus semilaevis*. The two potential probiotic isolates were analyzed in terms of their morphology, physiology, biochemistry, the similarity of 16S rDNA sequences, growth characteristics, enzyme production capacity, bacterial antagonism, and safety in *C. semilaevis.* The results revealed that the bacterial morphology and physiological and biochemical characteristics of S3 and S5 were similar to those of *Bacillus subtilis*. The 16S rDNA sequences had 99.9 % similarity to that of *Bacillus subtilis* MH 145363.1. Therefore, S3 and S5 were identified as *B. subtilis*. In addition, we found that S3 and S5 had a strong ability to secrete amylase, protease, and lipase. During the safety tests of S3 and S5 in *C. semilaevis* with high concentrations, *C. semilaevis* in immersion, injection, and feeding groups remained in good condition without falling ill or dying. Moreover, we found that S3 and S5 exhibited superior growth at 25~50℃, salinities of 10 to 40, and pH values of 5 to 9. Furthermore, S3 and S5 had significant bacteriostatic activity against *Vibrio anguillarum*, *Aeromonas salmonicida,* and *Shewanella algae*, which are the main pathogenic bacteria of mariculture fish. In summary, S3 and S5 showed superb inhibition of the pathogenic bacteria of marine fish, rapid growth, eurythermal and euryhaline features, and suitability for the intestinal environment of *C. semilaevis*. Thus, strains S3 and S5 have excellent commercial development potential. These results provide a basis for ecological disease prevention strategies and are also valuable for developing and utilizing probiotics.

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Introduction

Cynoglossus semilaevis Günther, commonly known as "Longli" and "Tami", is an important mariculture fish in coastal areas of Northern China, especially in the Yellow and Bohai Seas (Luo et al., 2022; Li XP et al., 2021). *C. semilaevis* is a popular food fish because of its high meat yield, smooth taste, and high nutritional and economic value. Because of the few natural resources of this species and its mild characteristics, and low residual bite injury in cultivation, this fish is prevalent among fish farms. In recent years, *C. semilaevis* farming has decreased, mainly due to diseases and deteriorating environmental conditions, which have caused serious economic losses (Tan et al., 2020; Zhao N et al., 2021). In traditional disease control, the application of antibiotics has an immediate effect. Still, frequent use of antibiotics can easily result in drug residues and drug-resistant bacteria, causing severe environmental hazards and many other adverse consequences. Moreover, national bans on antibiotic use as growth promoters in animal feeds have been issued, such as those of the European Union nations in 2006 (the European Parliament and Council Regulation EC No. 1831/2003) and of China in 2020 (Bulletin No. 194/2019 by Ministry of Agricultural and Rural Affairs of People's Republic of China). Therefore, increasing efforts have focused on alternatives that could reduce or replace antibiotics. Recently, the goal of antibiotic-free farming has been widely applauded and implemented worldwide (Wang JY et al., 2019; Wang et al., 2021).

The term probiotics was first proposed by Parker in 1974. Due to their positive effects on the host, probiotics are considered environmentally friendly alternatives to antibiotics. These organisms are widely used as biological control agents in aquaculture, agriculture, food processing, and other industries and have recently been applied in biotechnology (Li et al., 2018; Wang RD et al., 2019; Chen et al., 2020; Zhao JB et al., 2021). With the development of the aquaculture industry and the promotion of intensive aquaculture, the aquacultural environment has deteriorated seriously, and aquatic animal diseases occur frequently. Aquatic probiotics are widely used in aquaculture for their environmental friendliness and safety. As the primary probiotic candidate, *Bacillus* has been revealed to affect cultured animals' growth, immunity, and culture environment. To date, the growth-promoting and immune-regulating effects of *Bacillus* have been thoroughly studied in farming fish, shrimp, and shellfish. Liu et al. (2017) found that dietary *B. subtilis* improved the growth and promoted the digestion of *Oreochromis niloticus*. Liu et al. (2021) studied the ability of *B. subtilis* WH1 to purify aquacultural water and found that WH1 could significantly reduce the contents of NO_2^--N, NH_3-N, and COD in the water. However, in practical applications, probiotics should not only have excellent effects on aquatic animals but also tolerate the harsh living environments of the digestive tract (i.e., the presence of gastric acid and bile salt). Therefore, selecting probiotics from aquatic animals is significant for healthy aquaculture. However, studies on applying local probiotics in *C. semilaevis* aquaculture have not yet been documented.

This work aimed to screen potential local probiotics from the gut of healthy *C. semilaevis* based on morphological and biochemical characteristics and molecular biological identification. In addition, the enzyme production capacity, bacteriostasis, and safety of the strains were analyzed. This study aimed to screen out strains with strong beneficial, antagonistic, and growth characteristics. To the best of our knowledge, this is the first report of local probiotics from the mariculture fish *C. semilaevis*. These results could provide a basis for ecological disease prevention strategies and are also valuable for developing and using probiotics.

Materials and Methods

Experimental fish

Healthy *C. semilaevis* from a marine fish farm in Qinhuangdao with an average weight of 21.3 ± 2.4 g were randomly grouped and acclimatized in tanks (70 cm×30 cm×35 cm in size) containing filtered and ozone-sterilized seawater for seven days. During the experiment, the temperature was maintained at 20~22℃, and a 1/3 volume of seawater was replaced daily.

C. semilaevis is not a protected species; individuals were bought from a local farmer. All procedures involving collecting and sampling *C. semilaevis* during this study were approved by the Animal Care and Use Committee of Hebei Normal University of Science and Technology.

Experimental materials

Culture plates for screening isolates: 2216E agar plates, lactobacillus selective (LBS) agar plates, MRS (de man, rogosa and sharpe) agar plates, brain heart infusion agar (BHIA) plates, photosynthetic bacteria agar plates, yeast extract peptone dextrose (YPD) agar plates, *Bacillus* agar plates, trypticase soy agar (TSA) plates, and general nutrient agar culture plates were purchased from Luqiao Biotechnology Co., Ltd.

Shanghai Sangon Bioengineering Service Co., Ltd synthesized the universal primers for bacterial 16S rDNA. The DNA extraction kit was purchased from Tiangen Biochemical Technology Co., Ltd. The Gram staining kit was purchased from Solarbio Co., Ltd.

Vibrio anguillarum, *Aeromonas salmonicida*, and *Shewanella algae* were isolated and preserved by our laboratory (Key Laboratory of Preventive Veterinary Medicine, Hebei Normal University of Science and Technology).

Culture and isolation of bacteria

The intestine was clipped and homogenized with sterilized NaCl solution (1.5 %) and cultured on each agar medium plate using the streaking inoculation method. All plates were incubated at 28 ℃ for 24 h to 48 h to observe colony morphology. Colonies with identical forms and the most significant area on the medium were considered dominant bacteria and selected for purification culture.

Morphological observation and physiological and biochemical tests of the isolates

The isolated strains were inoculated on TSA agar medium containing 1.5 % NaCl to assess the morphological characteristics of the colonies. Single colonies were selected for Gram staining. In addition, single colonies were determined by scanning electron microscopy (SEM). Physiological and biochemical characteristics of the bacterial strains were identified by bacterial biochemical reaction microtubes purchased from Qingdao Haibo Biological Technology Co., Ltd. The classification was determined by referring to the Manual of Common Bacterial System Identification (Dong and Cai, 2001) and Bergey's Manual of Bacterial Identification (Buehanan and Gibbons, 1974).

Sequence analysis of 16S rDNA of the isolates

The isolated strains grown on each medium were identified through 16S rDNA sequencing and alignment. The primers for 16S rDNA were as follows: 27F (5'-AGAGTTTGATCCTGGCTC AG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). A single colony was picked and placed into 50 μL deionized water. After even vibration, the sample was placed in a metal bath and incubated at 99 ℃ for 10 min, followed by centrifugation at 12,000 rpm for 1~2 min. The supernatant was collected and used as a PCR template. PCR was performed in a 50 μL reaction system consisting of 25 μL of 2× PCR mix, 0.8 μL of each primer, 4 μL of template DNA, and 19.4 μL of ddH_2O.

Briefly, after an initial denaturation step at 94 ℃ for 5 min, amplifications were carried out with 30 cycles at a melting temperature of 94 ℃ for 40 s, an annealing temperature of 57 ℃ for 40 s, and an extension temperature of 72 ℃ for 70 s. Finally, an extra elongation step was performed at 72 ℃ for 10 min, followed by preservation at 4℃. The PCR product was subjected to 1.0% agarose gel electrophoresis and recovered by an agarose gel DNA recovery kit. Shanghai Bioengineering Co. sequenced the amplification products, Ltd. Sequencing results were blasted in the NCBI database. The isolates with high sequence homology were selected for phylogenetic tree construction by Mega 6.0 using the neighbor-joining method.

Enzyme production activity determination of preferred isolates

The preferred strains were inoculated on starch medium, skim milk powder medium, and oil medium and incubated at 28℃ for 48 h. Whether the strains could produce amylase, protease and lipase was determined based on the hydrolytic starch circle, hydrolytic protein circle, and lipolytic red spot on the culture medium, respectively.

Detection of antibacterial activity

According to Liu et al. (2021), *V. anguillarum*, *A. salmonicida*, and *S. algae* were added to the nutrient broth medium. After cultivation for 24 h at 28 ℃, a small number of indicator bacteria were taken, diluted with sterilized normal saline, and evenly coated on nutrient agar plates drilled in Oxford cups. The presumed probiotics were inoculated into a nutrient agar medium, and the concentration of the bacteria was adjusted to 1×10^8 CFU/mL after culturing at 28 ℃ for a given period. A volume of 150 μL of the bacterial solution was added to 3 Oxford cups. The inhibition zone was observed around the holes, and the diameter of the inhibition zone was measured after incubation at 28 ℃ for 24~48 h.

Safety evaluation of preferred strains

Healthy *C. semilaevis* individuals were randomly divided into 18 groups, with 10 in each group, and the safety of selected strains was evaluated by injection, feeding, and immersion tests. Injection test: The control group was injected with 0.1 mL 0.9 % normal saline, and the experimental group was injected with 5×10^8 CFU/mL bacterial solution of the same volume (Abarike et al., 2018). Feeding test: The control group was fed a routine diet of 0.5 % of the fish weight every morning and evening. The experimental group was given an equal amount of feed with a concentration of the added bacterial solution of 6×10^5 CFU/g (Liu et al., 2017). Immersion test: The bacterial solution was added to the cultivation water every 7 days in the experimental group to achieve a concentration of up to 1×10^8 CFU/L (Talib et al., 2017). Normal saline was added to the control group in the same volume. The experiment lasted for 30 days; the status and death of the fish were observed and recorded every day.

Growth characteristics of preferred strains

According to Shi et al. (2018), the preferred strains were inoculated into 300 mL sterilized nutrient broth liquid medium, and the cultures were placed in an oscillating incubator at 15 ^{0}C, 20 ^{0}C, 25 ^{0}C, 30 ^{0}C, 35 ^{0}C, 40 ^{0}C, 45 ^{0}C, 50 ^{0}C, 55 ^{0}C, and 60 ^{0}C. There were 3 replicates for each temperature and strain. The OD600 values for each temperature were measured after incubation at 180 r/min for 24 h, and the optimal temperature of the preferred strains was determined.

Referring to Wang et al. (2018), nutrient broth liquid media established a series of salinities of 10, 15, 20, 25, 30, 35, 40, 45, and 50. The preferred strains were inoculated in 300 mL of the sterilized liquid media mentioned above. There were three replicates for each salinity and strain. The OD_{600} values at each salinity were measured after incubation at 180 r/min for 24 h, and the optimal salinity of the preferred strains was determined.

According to Kavitha et al. (2018), a series of pH values of 3, 4, 5, 6, 7, 8, 9, 10, and 11 were established in nutrient broth liquid media using 1 mol/L NaOH and 1 mol/L HCl. The preferred strains were inoculated in 300 mL of the sterilized liquid media mentioned above. There were 3 replicates for each pH and strain. The OD_{600} values for each pH were measured after incubation at 180 r/min for 24 h, and the optimal pH of the preferred strains was determined.

Statistical analysis

Data are presented as the means ± standard deviations (SDs). One-way ANOVA ($p<0.05$) was performed to determine the significant differences in the growth characteristics of preferred strains. Statistical analyses were executed by using SPSS 16.0 software.

Results

Morphological characteristics of isolated strains

In this experiment, 89 isolates were isolated from the different culture media. According to the results of 16S rDNA sequence analysis, two strains of *Bacillus* isolated from the intestine of *C. semilaevis* were selected as potential probiotics and were recorded as S3 and S5. The colony characteristics of S3 and S5 were observed after being cultured at 28 ℃ for 24 h. These two strains were grayish-white on TSA plates; their surfaces were dry, dull, and opaque. Their shape was approximately round, with a protruding middle fold and irregular edge. In addition, the bacteria were long rods with blunt round ends under the microscope. The dyeing results showed that the gram staining was positive and uniform, and there was no noticeable expansion of the sporocyst or parasporal crystal under a scanning electron microscope (**Figure 1**).

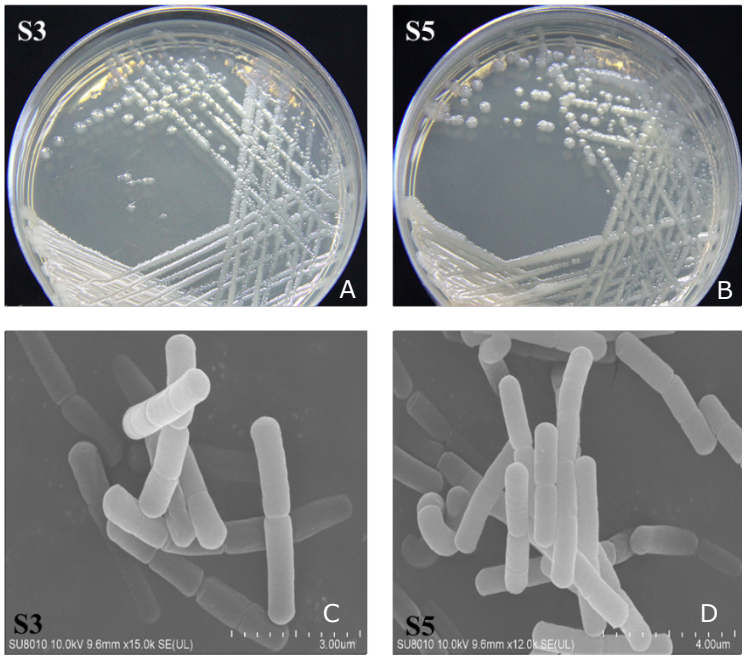

Figure 1 Colony morphology and SEM photos of strains S3 and S5
A, B: The colony morphology of S3 and S5 on TSA plate.
C, D: SEM photograph of strain S3 and S5.

Physiological and biochemical identification of isolated strains

The physiological and biochemical characteristics results showed that S3 and S5 were Gram-positive. Both could grow at 30 ℃ and 40 ℃, and two strains could decompose and use mannitol, glucose, arabinose, and maltose and hydrolyze starch. The results of the biochemical reaction of the contact enzyme test, gelatin liquefaction test, V-P test, propionate test, and citrate test results were positive. The physiological and biochemical characteristics of the strains were consistent with those of other previously reported Bacillus. According to the characteristics of the Bacillus genus in "The Manual of Systematic Identification of Common Bacteria (Dong and Cai, 2001)" and "Bergey's Manual for the Identification of Bacteria (Buehanan and Gibbons, 1974)", these two strains were preliminarily identified as belonging to Bacillaceae.

Analysis of 16S rDNA sequences and construction of the phylogenetic tree

These two strains were highly homologous with *Bacillus*, showing 99.9% homology with *Bacillus subtilis* MH 145363.1 based on the NCBI database. The 16 strains with the most similar gene sequences were selected from the National Center for Biotechnology Information (NCBI) database to construct the phylogenetic tree. **Figure 2** shows that strains S3 and S5 were clustered with *Bacillus subtilis* MH145363.1*. Comparing their 16S rDNA gene sequences and phenotypic characteristics shows that* S3 and S5 were identified as *Bacillus subtilis* (**Figure 2**)*.*

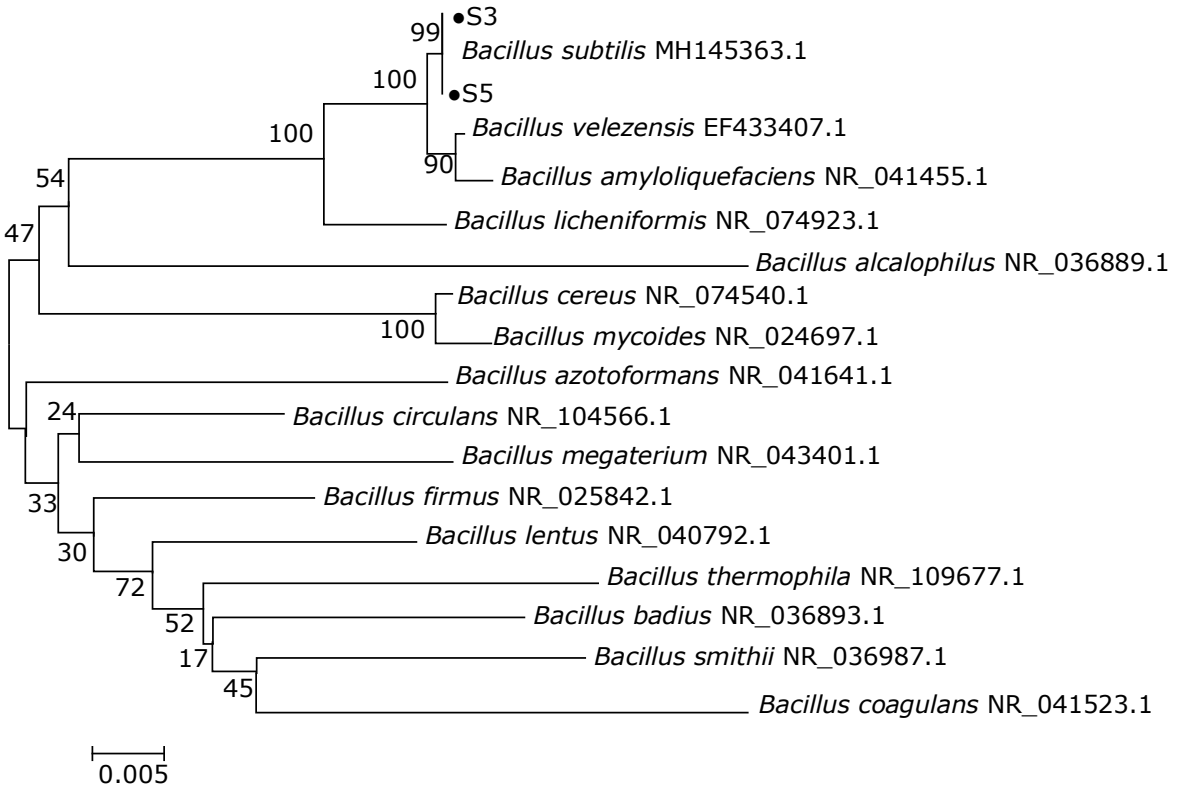

Figure 2 Phylogenetic tree of strains S3 and S5 based on 16S rDNA sequences

Enzyme production activity of S3 and S5

The enzyme production activities of S3 and S5 were detected using the starch medium, skim milk powder medium, and oil medium. The results showed that transparent circles appeared in starch and protein media, and red spots were produced in an oil medium (**Figure 3**). This indicated that S3 and S5 could decompose starch, protein, and oil and produce amylase, protease, and lipase. The hydrolytic circle diameters from the production of amylase and protease by S3 and S5 were 23.0±1.3 mm and 27.9±0.4 mm and 15.1±0.3 mm and 16.9±1.6 mm, respectively.

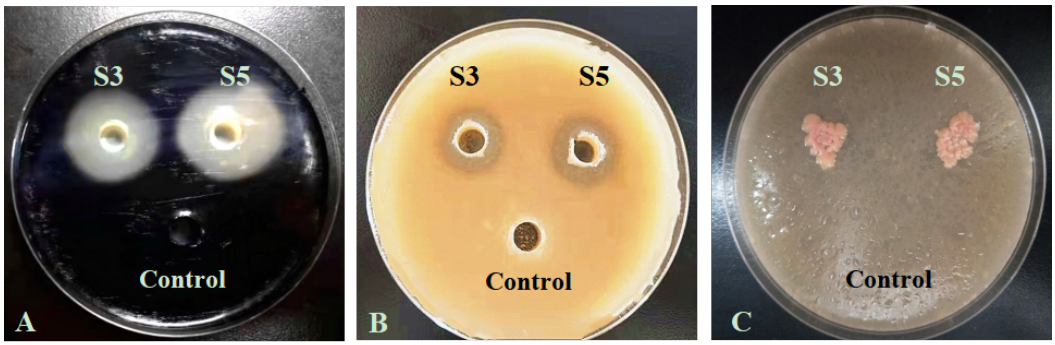

Figure 3 Test of the enzyme production ability of strains S3 and S5
A, B and C are amylase, protease, and lipase test results, respectively

Detection of antibacterial activity

The Oxford cup test showed that the inhibition zones of S3 and S5 were 16.5±3.3 mm and 17.3±3.2 mm against *V. anguillarum,* 11.6±2.5 mm and 14.4±3.6 mm against *A. salmonicida*, 15.2±2.3 mm and 12.3±5.6 mm against *S. algae*, respectively. There was no significant difference ($P>0.05$). The results indicated that S3 and S5 had strong bacteriostatic effects against the pathogenic bacteria of mariculture fish.

Safety test of S3 and S5

The safety of S3 and S5 was tested by intramuscular injection, feeding, and immersion. The results showed that the growth and survival of *C. semilaevis* were not significantly affected by injection with 5×10^8 CFU/mL, feeding with 6×10^5 CFU/g, or immersion with 1×10^8 CFU/L of S3 and S5, indicating that these two strains were safe for *C. semilaevis*.

Growth characteristics

The growth of S3 and S5 at different temperatures is shown in Figure 4. With the blank medium as a control, S3 and S5 grew at 20 ^{0}C ~ 50 ^{0}C. When the temperature was lower than 25 ℃, the growth rate of the two strains was slower, but an obvious growth advantage was seen in the range of 25 ^{0}C ~ 50 ^{0}C, and the fastest growth rate was in the range of 30 ^{0}C ~ 45 ^{0}C.

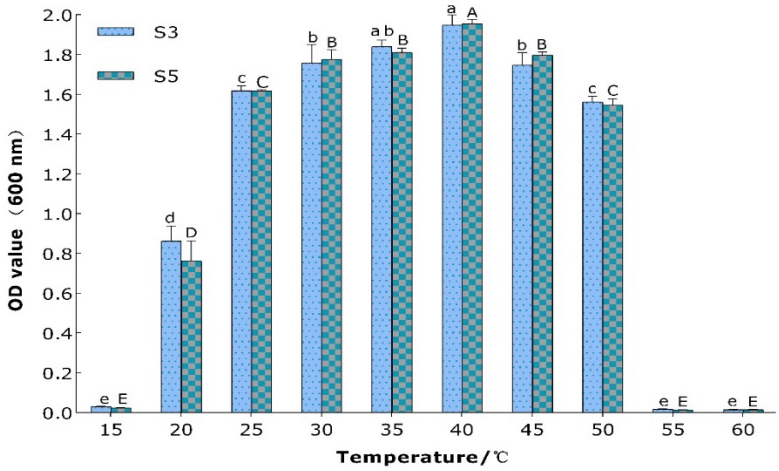

Figure 4 Growth of strains S3 and S5 at different temperatures
Note: Different letters a, b, c, d, e and A, B, C, D, E indicate significant differences among different temperatures of S3 and S5 at $p < 0.05$ (Mean ± SD, n=3), respectively.

The growth of S3 and S5 at different pH values is shown in **Figure 5**. With the blank medium as a control, S3 and S5 did not grow when the pH was 3.0 or higher than 10.0. When the pH was lower than 4.0, the growth of the two strains was slow, and their growth was better in the pH range of 5.0 to 9.0, with significantly higher growth than that of the other groups. These two strains had the fastest growth rate when the pH was 8.0.

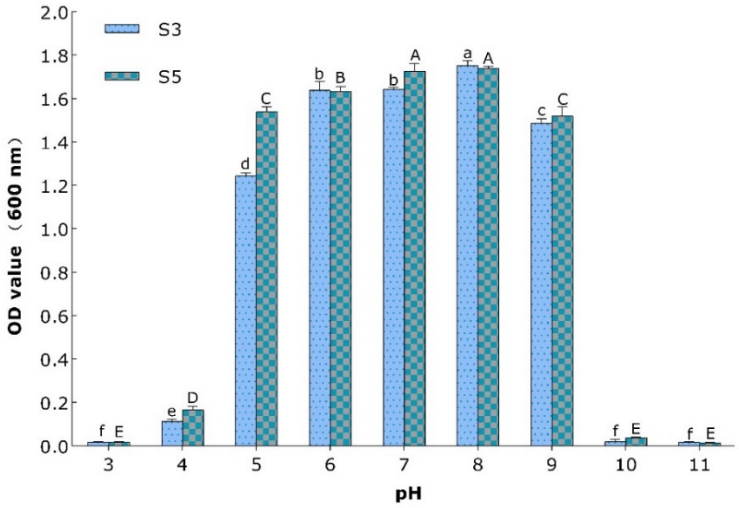

Figure 5 Effect of different pH values on the growth of strains S3 and S5
Note: Different letters a, b, c, d, e, f and A, B, C, D, E indicate significant differences among different pH values of S3 and S5 at $p < 0.05$ (Mean ± SD, n=3), respectively.

The growth of S3 and S5 at different salinities is shown in **Figure 6**. With the blank medium as a control, both S3 and S5 grew in the salinity range of 10~50. With the increase in salinity, the general growth trend of S3 and S5 increased first and then decreased. The optimum salinities of S3 and S5 were 20.

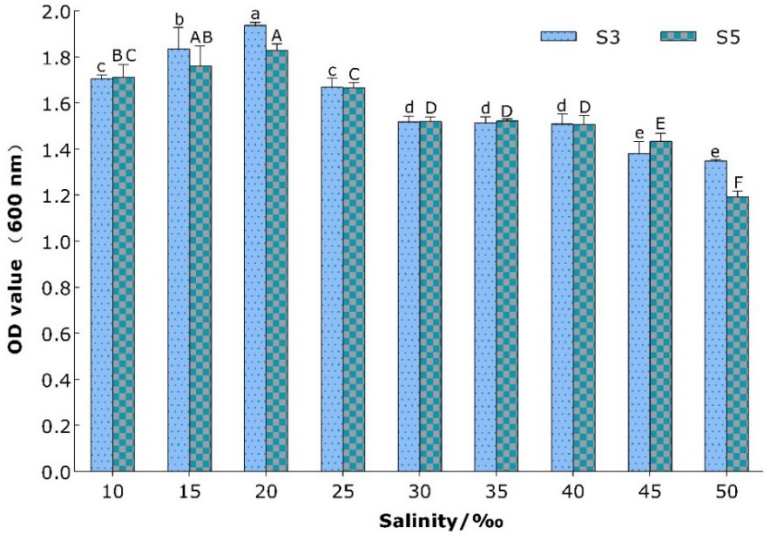

Figure 6 Growth of strains S3 and S5 under different salinities
Note: Different letters a, b, c, d, e and A, B, C, D, E, and F indicate significant differences among different salinities of S3 and S5 at $p < 0.05$ (Mean ± SD, n=3), respectively.

Discussion

Screening probiotics is the first step to obtaining adequate and safe probiotic products. The adhesion of probiotics in the intestinal tract of the host is specific. In some cases, commercial probiotics did not come from fish and could not survive effectively in the intestinal tract of host fish. Therefore, the application effects of some non-homologous probiotics in aquaculture are not satisfactory. In this study, two potentially probiotic bacillus strains (S3 and S5) were isolated from the intestine of *C. semilaevis.* The formula feed used in the Qinhuangdao fish farm sampled or during the 7-day acclimatization was produced by Santong Biological Engineering Co., Ltd without any probiotics added. In addition, the follow-up research indicated that they could rapidly reproduce and become the dominant bacteria in the intestinal tract of *C. semilaevis*. Consequently, the S3 and S5 B. subtilis isolates were believed to derive from *C. semilaevis*.

Strains S3 and S5 were identified as *B. subtilis* based on their characteristics and morphological observations, biochemical properties, 16S rDNA sequencing, and phylogenetic tree analysis. *B. subtilis* has good probiotic properties and has been frequently studied in the intestinal tracts of aquatic animals, such as *Carassius auratus* (Ba et al., 2017), *Epinephelus lanceolatus* × *E. fuscoguttatus* (Wang CQ et al., 2019), and *Ctenopharyngodon idellus* (He et al., 2012). However, *B. subtilis* had not yet been reported from the intestinal tract of *C. semilaevis*. The results of this study showed that two strains of *B. subtilis* (S3 and S5) screened from the intestinal tract had sound antagonistic effects on the three bacterial pathogens of mariculture fish, which could help reduce the incidence of disease in the culture of *C. semilaevis* and had great potential in the prevention and control of disease as a substitute for antibiotics. These two strains grew fast in the temperature range of 25~50 ℃, salinities of 10 to 40, and pH range of 5 to 9, better than the four strains of *Bacillus* isolated from aquatic feed by Shi et al. (2018). Furthermore, these two strains from the intestine of *C. semilaevis* have the advantages of fast growth, broad temperature tolerance, and broad salt tolerance.

They are especially suitable for the intestinal environment of *C. semilaevis*; thus, they are conducive to developing probiotics for use in producing these fish.

The activity of digestive enzymes in fish affects their digestion and absorption ability. It serves as an essential index reflecting physiological digestive function with a particular influence on growth, development, and reproduction. Increasing evidence indicates that dietary supplementation with *Bacillus* can promote the growth performance and/or immunity of aquatic animals (Cao et al., 2019; Tang et al., 2019). In addition to digestive function, the intestine is the largest immune organ and barrier for the defense against pathogen infection. Fish growth relies on nutrient utilization, which is closely related to digestive enzyme activities, including amylase, lipase, and pepsin in the intestine of fish (Wang et al., 2021). Li YZ et al. (2021) reported that dietary supplementation of *Lactobacillus plantarum* and *B. subtilis* could improve the growth performance, innate immune response, antioxidant capacity and digestive enzyme activity of *Paralichthys olivaceus*. Liu et al. (2016) demonstrated that dietary administration of *Bacillus subtilis* HAINUP40 could improve the protease and amylase activity of *Oreochromis niloticus*. Li et al. (2019) and Ju and Li (2018) added different concentrations of *B. subtilis* to the feeds of *E. lanceolatus* × *E. fuscoguttatus* and *C. idellus*, respectively. At the end of the experiment, the activities of amylase, lipase, and trypsin in the intestinal tract of fish were significantly increased with the addition of *B. subtilis*. The two strains selected in this research have a more vital ability to produce amylase, protease, and lipase, indicating that they can potentially improve the activities of these enzymes in the intestinal tract of *C. semilaevis*. Therefore, these strains can further improve nutrition, digestion and absorption to benefit the growth of this species.

As a biological control agent, probiotics have achieved initial results in improving water quality and inhibiting the growth of pathogenic bacteria. The safe use of probiotics and their safety to the ecological environment are also primary considerations in their selection process. Overuse of probiotics can also cause diseases in aquatic animals. For example, *B. thuringiensis* can cause the massive death of *Trionyx sinensis* (Chen et al., 2014), and *B. cereus* can cause ascites and the death of *Ictalurus punctatus* (Zhang et al., 2019). Therefore, the safety of S3 and S5 was determined by the high-concentration stress test in this study. During the whole experiment, there were no signs of disease or death in the immersion, injection or feeding groups and no adverse effects on the growth and survival of *C. semilaevis*, indicating that these two strains are safe for use in *C. semilaevis* at the conventional dosage.

Conclusion

In conclusion, *B. subtilis* S3 and S5 displayed most of the properties of a good probiotic. *B. subtilis* S3 and S5 had strong enzyme production ability, wide growth temperature range, and strong antibacterial ability. They could rapidly reproduce and play a role in the intestinal tract of *C. semilaevis*. Additionally, the strains promoted disease resistance against *V. anguillarum*, *A. salmonicid*a, and *S. algae*. These studies will help identify excellent probiotics for further developing fishery probiotics products and demonstrate that the administration of *B. subtilis* S3 and S5 is ideal for increasing immune resistance in *C. semilaevis*.

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References

Abarike ED, Cai J, Lu YS, Yu H, Chen LH, Jian JC, Tang JF, Jun L, Kuebutornye FKA, 2018. Effects of a commercial probiotic BS containing *Bacillus subtilis* and *Bacillus licheniformis* on growth, immune response and disease resistance in Nile tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol.* 82, 229-238. https://doi.org/10.1016/j.fsi.2018.08.037

Ba CY, Zhang LB, Zhang PJ, Zhao FG, Li YH, 2017. Isolation, identification and characterization of two strains of *Bacillus subtilis. J South Chin Agric Univ.* 38(3), 46-51. doi: 10.7671/j.issn.1001-411X.2017.03.008.

Buehanan RE, Gibbons NE. Bergey's Manual of Determinative Bacteriology, 1974. 8th ed. Baltimore, Md.: Williams & Wilkins Co. ISBN:9780683006032

Cao HZ, Yu RH, Zhang YY, Hu BQ, Jian SQ, Wen CG, Kajbaf K, Kumar V, Yang G, 2019. Effects of dietary supplementation with β-glucan and *Bacillus subtilis* on growth, fillet quality, immune capacity, and antioxidant status of Pengze crucian carp (*Carassius auratus* var. Pengze). *Aquaculture,* 508, 106-112. https://doi.org/10.1016/j.aquaculture.2019.04.064

Chen FY, Li XL, Dong YQ, Li JH, Li YX, Li H, Chen L, Zhou M, Hou HB, 2020. Biodegradation of phthalic acid esters (PAEs) by *Cupriavidus oxalaticus* strain E3 isolated from sediment and characterization of monoester hydrolases. *Chemosphere,* 266, 129061. https://doi.org/10.1016/j.chemosphere.2020.129061

Chen JS, Zhu NY, Kong L, Bei YJ, Zheng TL, Ding XY, He ZY, 2014. First reported fatal *Bacillus thuringiensis* infections in Chinese soft-shelled turtles (*Trionyx sinensis*). *Aquaculture*, 428-429,16-20. https://doi.org/10.1016/j.aquaculture.2014.02.018

Dong XZ, Cai MY. Identification Manual of Common Bacteria System, 2001. Science Press. *Beijing.* ISBN: 9787030084606

He GL, Liu LH, Zhang H, 2012. Study on bacillus from grass carp (*Ctenopharyngodon idellus*) intestine and its zymogenicities*. Fres Fish.* 42(4), 3-8.

Ju SY, Li JS, 2018. Effect of dietary *Bacillus subtilis* on growth performance, digestive enzymes activity and anti-oxidation function of *Ctenopharyngodon idellus. Fres Fish.* 48(5), 99-105. DOI: 10.13721/j.cnki.dsyy.2018.05.016

Kavitha M, Raja M, Peruma P, 2018. Evaluation of probiotic potential of *Bacillus* spp. isolated from the digestive tract of freshwater fish *Labeo calbasu* (Hamilton, 1822). *Aquac Rep.* 11, 59-69. https://doi.org/10.1016/j.aqrep.2018.07.001

Li C, Ren YC, Jiang SH, Zhou S, Zhao JS, Wang RJ, Li YM, 2018. Effects of dietary supplementation of four strains of lactic acid bacteria on growth, immune-related response and genes expression of the juvenile sea cucumber *Apostichopus japonicus* Selenka. *Fish Shellfish Immun.* 74, 69-75. https://doi.org/10.1016/j.fsi.2017.12.037

Li JL, Yang QH, Tan BP, Dong XH, Chi SY, Liu HY, Zhang S, 2019. Effect of low-fish meal diet with *Bacillus subtilis* on growth performance, digestive enzyme activity, antioxidant enzyme activity and its mRNA expression of juveniles *Epinephelus lanceolatu*♂ × *Epinephelus fuscoguttatus*♀*. J Fish Chin*. 43(4), 1126-1137. DOI: 10.11964/jfc.20180211186

Li XP, Zhang J, 2021. Tongue sole (*Cynoglossus semilaevis*) interleukin 10 receptors are involved in the immune response against bacterial infection. *Dev Comp Immunol.* 115, 103885. https://doi.org/10.1016/j.dci.2020.103885

Li YZ, Yang YM, Song LM, Wang JL, HuYR, Yang Q, Cheng P, Li JH, 2021. Effects of dietary supplementation of *Lactobacillus plantarum* and *Bacillus subtilis* on growth performance, survival, immune response, antioxidant capacity and digestive enzyme activity in olive flounder (*Paralichthys olivaceus*). *Aquac Fish.* 6, 283-288. https://doi.org/10.1016/j.aaf.2020.10.006

Liu HT, Wang SF, Cai Y, Guo XH, Cao ZJ, Zhang YZ, Liu SB, Yuan W, Zhu WW, Zhang Y, Xie ZY, Guo WL, Zhou YC, 2017. Dietary administration of *Bacillus subtilis* HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol.* 60, 326-333. https://doi.org/10.1016/j.fsi.2016.12.003

Liu XY, Wang LL, Luan HN, Zheng W, Hou SY, Liu ZH, Zhang SH, 2021. Isolation and identification of a *Bacillus subtilis* strain for water purification. *Microbiol Chin.* 48(2), 449-461. DOI: 10.13344/j.microbiol.china.200182

Luo Z, Hao S, Bai XH, Zhang ZG, Ma YF, Feng SM, Zhang DF, 2022. Identification and genomic analysis of *Mycobacterium ulcerans* ecovar Liflandii from the farmed Chinese tongue sole, *Cynoglossus semilaevis* Günther*. Aquaculture,* 548, 737614. https://doi.org/10.1016/j.aquaculture.2021.737614

Shi SQ, Jiang W, Yuan L, Qi KZ, Tu J, Song XJ, 2018. Isolation, identification and biological characteristics analysis of four *Bacillus* strains. Jiangsu Agr Sci. 46(2), 112-115. doi: 10.15889 /j.issn.1002-1302.2018.02.031

Talib A, Onn KK, Chowdury MA, Din WMW, Yahya K, 2017. The beneficial effects of multispecies *Bacillus*, as probiotics in enhancing culture performance for mud crab *Scylla paramamosain* larval culture. *Aquac Int.* 25(2), 849-866.

Tan J, Hu XC, Lv AJ, Liu XX, Sun JF, Niu YC, 2020. Skin proteome profiling of tongue sole (*Cynoglossus semilaevis*) challenged with *Vibrio vulnificus*. *Fish Shellfish Immunol.* 106, 1052-1066. https://doi.org/10.1016/j.fsi.2020.09.020

Tang Y, Han L, Chen XX, Xie MQ, Kong WG, Wu ZX, 2019. Dietary supplementation of probiotic *Bacillus subtilis* affects antioxidant defenses and immune response in grass carp under *Aeromonas hydrophila* challenge. *Probiotics Antimicrob Proteins.* 11, 545-558. DOI: 10.1007/s12602-018-9409-8

Wang CQ, Wang JY, Huang BS, Li BS, 2019. Study on *Bacillus subtilis* from hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂) intestine and its zymogenicities*. J Fish Res.* 41(5), 366-373. DOI: 10.14012/j.cnki.fjsc.2019.05.002

Wang JY, Li B, Wang YG, Liao MJ, Rong XJ, Zhang Z, 2019. Influences of immersion bathing in *Bacillus velezensis* DY-6 on growth performance, non-specific immune enzyme activities and gut microbiota of *Apostichopus japonicus*. *J Oceanol Limnol.* 37(4), 1449-1459. https://doi.org/10.1007/s00343-019-8119-8

Wang JY, Li B, Wang YG, Liao MJ, Rong XJ, Zhang Z, Niu YY, Ning LG, 2018. Screening and characteristic of *Bacillus velezensis* derived from ponds for sea cucumber (*Apostichopus japonicus*). *J Fish Sci Chin.* 25(3), 567-575. DOI: 10.3724/SP.J.1118.2018.17383

Wang RD, Liu SN, Wang Y, Zhao YN, Xue Y, 2019. Analysis on quality characteristics and dominant strains of bacteria in dried salted Spanish mackerel. *Prog Fish Sci.* 40(2), 141-147. DOI:10.19663/j.issn2095-9869.20180814002

Wang Y, Wang QK, Xing KZ, Jiang P, Wang JR, 2021. Dietary cinnamaldehyde and *Bacillus subtilis* improve growth performance, digestive enzyme activity, and antioxidant capability and shape intestinal microbiota in tongue sole, *Cynoglossus semilaevis*. *Aquaculture*, 531, 735798. https://doi.org/10.1016/j.aquaculture.2020.735798

Zhang HY, Wang HB, Zhang ZW, Zhao MJ, Xia L, 2019. Toxicological safety assessment of a potential probiotic strain *Bcillus cereus* D7 isolated from shrimp. Prog. *Veterinary Med.* 40(3), 59-65. DOI:10.16437/j.cnki.1007-5038.2019.03.010

Zhao JB, Zhang ZH, Li W, Zhao WJ, Han YS, 2021. Immune mechanism of probiotic preparation regulating the intestinal health of livestock and poultry and the interaction of microorganisms in digestive tract. *Chin Feed.* 3, 7-11. DOI:10.15906/j.cnki.cn11-2975/s.20210302

Zhao N, Jia L, He XX, Zhang B, 2021. Proteomics of mucosal exosomes of *Cynoglossus semilaevis* altered when infected by *Vibrio harveyi*. *Dev Comp Immunol*. 119, 104045. https://doi.org/10.1016/j.dci.2021.104045