Screening and Identification of *Bacillus amyloliquefaciens* from the Gut of Aquatic Animals

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Abstract: Probiotics have the dual effects of inhibiting pathogenic bacteria and promoting growth, while being green and environmentally friendly, and will not cause problems such as increased drug resistance, veterinary drug residue, and meat quality deterioration, making them to be good substitute for antibiotic. In order to realize healthy and ecological culture and provide good microbial resources for probiotics, this experiment isolated probiotics from the intestinal tracts of cultured Procambarus clarkii and crab. One strain LPB-5 was identified according to traditional colony morphological characteristics observation, physiological and biochemical tests and 16S rDNA sequence analysis. The results showed that the strain had the closest relationship with Bacillus amyloliquefaciens MPA 1034, the 16S rDNA sequence similarity was 99%, and the physiological and biochemical reaction spectrum was consistent with the 16S rDNA sequence analysis, which identified the strain as *Bacillus amyloliquefaciens*. Procambarus clarkii and crab are important aquaculture animals. The probiotics isolated from the intestinal tract of procambarus clarkii have their own biosafety and high efficiency, which lays a foundation for the later development of probiotics application in aquatic feed.

1 INTRODUCTION

There are a very large number of microorganisms in the intestinal tract, which are generally divided into neutral bacteria, probiotics and harmful bacteria. In the long-term evolution of intestinal flora, as the host adapts to the environment and natural selection, the microbial community, host and environment depend on each other to form a system of mutual restriction, which is always in a state of relative dynamic balance (Yan, 2017). The relationship between the host and its intestinal microbes also has a very important impact on the health of aquaculture animals (Chaiyapechara, 2012. Liu, 2011). A large number of studies have found that intestinal flora can help and promote the body's antagonism to pathogenic bacteria, enhance the body's immunity and absorb nutrients (Ravi, 2007. Olmos, 2011).

Using probiotics to replace antibiotics and other drugs with great side effects to achieve true ecological aquaculture has become the trend and research hotspot of the entire aquaculture (Chauhan, 2019). So far, some probiotics have been used as alternative antibiotics or disease control agents for the prevention and control of diseases in aquaculture animals (Das, 2008. Kuebutornye, 2019. Waldroup, 2003). Probiotics inhibit the growth of harmful bacteria in the gut (Hazel, 2020). The experiment of Li et al. verified the inhibitory effect of competition between two kinds of pathogenic bacteria and five kinds of probiotics on the gastrointestinal mucosa of fish (Li, 2012). Probiotics can produce non-specific immune regulatory factors to stimulate the host's immune function, effectively increase the concentration of immunoglobulin in the host, significantly enhance the activity of macrophages and interferon, and strengthen the host's immunity to

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viruses, bacteria, fungi and parasites (Elsabagh,2018. Douglas, 2008. Opiyo, 2019). Abarike ED et al. showed that Nile tilapia can increase the activity of relevant enzymes in their bodies and increase their own immunity by consuming a commercial probiotic called BS (a mixture of *Bacillus subtilis* and *Bacillus licheniensis*) (Abarike, 2018). Probiotics can secrete various digestive enzymes (amylase, protease, lipase, etc.) in the body of aquaculture animals, promote the absorption and transformation of nutrients in the feed of aquaculture animals, and improve feed digestibility, which may play a role in animal nutrition and feed utilization rate (Das, 2019. Hai, 2009. Balcáza, 2006). Probiotics can also improve the constitution and meat quality of aquatic animals, and improve their tolerance to aquatic environment (Valente,2016. Wang, 2020). Studies have shown that after feeding Western King prawns with probiotics, the water content of the shrimp body decreases, while the protein content increases, and their constitution and meat quality are significantly improved (Hai, 2009).

In this study, procambarus clarkii and crab were used as experiment subjects and their intestinal microorganisms were selected, separated and identified. The isolated probiotics have great potential in developing probiotics for aquaculture feed due to their biosafety and high efficiency.

2 MATERIALS AND METHODS

2.1 Test Raw Materials and Media

Five alive procambarus clarkii and five crabs, which were purchased from dajiang Aquatic Market in Huai 'an, were used as raw materials.

Nutrient broth medium (per liter): 10 g peptone, 3 g beef extract, 5 g sodium chloride, pH was adjusted to 7.2-7.4. Nutrient broth agar medium: 17 g agar was added on the basis of the above. It was sterilized at 121℃ for 20 min in a high pressure steam sterilizer.

Soluble starch medium (per liter): 10 g peptone, 5 g sodium chloride, 5 g beef extract, 2 g soluble starch. It was sterilized at 115℃ for 30 min in a high pressure steam sterilizer.

Gelatin liquefication medium (per liter): 10 g peptone, 3 g beef extract, 5g sodium chloride, 12g gelatin; the pH value was adjusted to 7.0 and sterilize at 115℃ for 30 min in a high-pressure steam sterilization pot.

Sugar fermentation medium (per liter): 5 g peptone, 5 g tryptone, 5 g sodium chloride, 5 mL Tween-80, 1.4 mL 1.6% Bromocresol violet solution, 10 g sugar, 6 g agar. It was sterilized at 115℃ for 30 min in a high pressure steam sterilizer.

2.2 Incubation and Isolation of the Strain

Procambarus clarkii and crabs were washed with sterile water and the body surfaces were disinfected with 75% ethanol. The intestinal tracts were dissected in a super clean table under sterile conditions. They were placed in test tubes with 10 mL sterilized normal saline, shaken and mixed, and left for 10 min. The supernatant was inoculated in the nutrient broth medium and placed in a constant temperature and oscillation incubator for 24 h at 37℃ and 200 rpm. The cultured bacteria solution was placed in a water bath at 80℃ for 15 min to kill the non-spore cells, and then inoculated again into the nutrient broth medium for enrichment and cultured for 24 h. Appropriate gradient dilution was performed on the bacterial solution, which was coated in nutrient broth agar medium, and the colonies with growth advantage were selected for repeated lineation separation until pure strains were obtained.

2.3 Physiological and Biochemical Identification of Strain

The isolated strains were selected and inoculated on the nutrient broth agar medium and placed in a constant temperature incubator at 37℃. Colony morphological characteristics were observed after 12 h culture, and the bacteria were selected for gram staining test, contact enzyme test, starch hydrolysis test and other physiological and biochemical tests.

2.3.1 Gram Stain Test

Gram dyeing test generally includes four steps: initial dyeing, medium dyeing, decolorization and redyeing. The specific operation methods are as follows: 1) Take strains, smear them and fix them on the slide. 2) Stain with crystal violet of ammonium oxalate for 1.5min and rinse with fine water. (3) Cover the coated surface with iodine solution and dye it for about 1min. Rinse with fine water and absorb the water with absorbent paper. 4) Add a few drops of 95% alcohol, and gently shake for decolorization, after 20 seconds, wash with water to absorb the water. (5) After dyeing with sand yellow

solution for 1min, rinse with distilled water. Dry and examine under microscope.

2.3.2 Contact Enzyme Test

The single colonies were inoculated on the medium for 24 h, and then inoculated on the glass with 3% hydrogen peroxide by inoculation ring. If there were bubbles, it was positive; otherwise, it was negative.

2.3.3 Starch Hydrolysis Test

Single colonies were inoculated on soluble starch medium and incubated at 37℃ for 48 h. Then the iodine solution was dropped on the petri dish. If the colonies formed a transparent circle around them, they were positive; if they remained blue and black, they were negative.

2.3.4 Gelatin Liquefaction Test

The strains were punctured and inoculated into the test tubes filled with gelatin liquefactional medium, and the uninoculated test tubes were used as blank control, and cultured in 37℃ incubator for 24h. Then the test tube was placed in cold water, and the phenomenon was observed after the medium in the blank control group solidified. If the experimental test tube liquefaction, it was positive, otherwise it was negative.

2.3.5 Sugar Fermentation Experiment

The strains cultured for 24 h were punctured and inoculated into a test tube containing sugar fermentation medium, and then inverted small glass tubes were added into the test tube. Each sugar was divided into three parallel groups. The uninoculated group was cultured in 30℃ incubator for one week. The phenomenon was observed and recorded, if acid and ga were produced (from purple to yellow), it was positive and no change was negative.

2.4 Identification of Strains by Molecular Biology

Using the kit method, the bacterial DNA was extracted according to the operation steps of the bacterial genome extraction kit of Sangon Biotechnology Company, and then the bacterial 16S rDNA universal primers 27F/1492R (Table 1) were used for amplification. Each sample consisted of 50 μL solution containing DNA template, forward primers, reverse primers, sterile water (Table 2). The 5 μL PCR product was tested by 1% agarose gel electrophoresis, and the purified PCR sample was sent to Shanghai Biotechnology Co., LTD for sequencing. According to sequences returned by sequencing companies, phylogenetic trees were constructed using MEGA 5.0 software.

Amplification procedure: 1) pre-denaturation at 94℃ for 3 min; Denaturation at 94℃ for 1 min; 2) Annealing at 55℃ for 1 min; 3) Extended at 72℃ for 2 min for 25 cycles; 4) Extend at 72℃ for 6 min; 5) Store at 4 ℃.

Table 1: Universal primers for bacterial 16S rDNA.

Prime	Genetic sequence
27F	5'-AGA GTT TGA TCC TGG CTC AG-3'
1492R	5'-GGT TAC CTT GTT ACG ACT T-3'

Table 2: PCR amplification system.

Compositio	Volume /μL
Taq Master Mix	25
Primer 1	1
Primer 2	1
DNA template	2
Sterile water	21

3 RESULTS AND ANALYSIS

3.1 Screening Results

After being treated at 80℃ for 15 min, a total of 5 strains were inoculated on the medium, and the dominant strain LPB-5 was selected as the experimental strain. The strain grew round colonies with creamy white opacity, neat edges, smooth and moist surface on nutrient broth agar medium (FIG. 1). The microscopic results of gram staining are shown in Figure 2. The sand yellow staining solution cannot stain the bacteria red, so the bacteria is gram-positive.

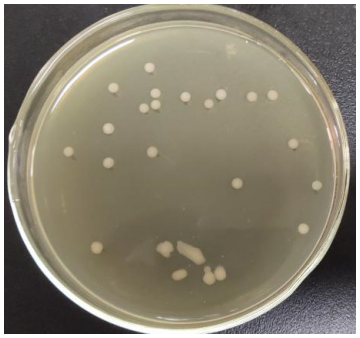

Figure 1: Colony morphology of the strain.

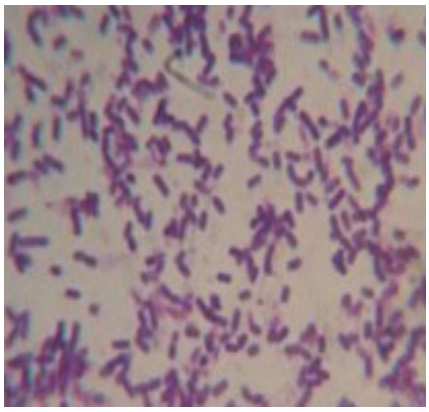

Figure 2: Microscopic diagram of gram stain of strain (Dye according to the four steps of initial dyeing, medium dyeing, decolorization and redyeing, and then observe under the 1000x oil microscope).

3.2 Physiological and Biochemical Identification

The physiological and biochemical identification of strain LPB-5 (contact enzyme test, starch hydrolysis test, gelatin liquefication test, Glucose fermentation test, sucrose fermentation test, maltose fermentation test) were carried out, and the results were shown in Table 3. According to Berger's Manual of Systematic Bacteriology, the strain could be preliminarily identified as Bacillus.

Table 3: Physiological and biochemical characteristics of LPB-5("+" means a positive result, "-" means a negative result.).

Project	Result
Gram stain test	+
Contact enzyme test	—
Starch hydrolysis test	—
Gelatin liquefaction test	+
Sterile water	(Glucose)+
	(Sucrose)+
	(Maltose)+

3.3 Molecular Identification

The PCR purified product of this strain was sent to Shanghai Shenggong Bioengineering Technology Service Co., LTD for sequencing. The agarose gel electrophoresis of this strain was shown in Figure 3. According to the 16S rDNA gene sequence returned by the company, Blast comparison was performed with the nucleic acid sequence database in GenBank, and the phylogenetic tree was constructed by neighbor-joining method with MEGA 5.0 software (FIG. 4). Strain LPB-5 was in the same branch with *Bacillus amyloliquefaciens* MPA 1034, and had the highest homology with the closest evolutionary distance and the similarity was 99%. Therefore, the strain was identified as *Bacillus amyloliquefaciens*.

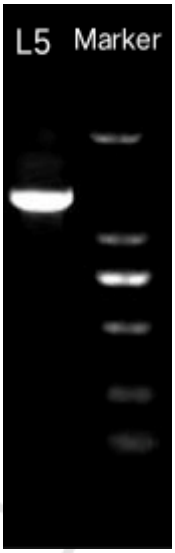

Figure 3:16S rDNA electrophoresis results (Condition: Voltage =120V, time =30min).

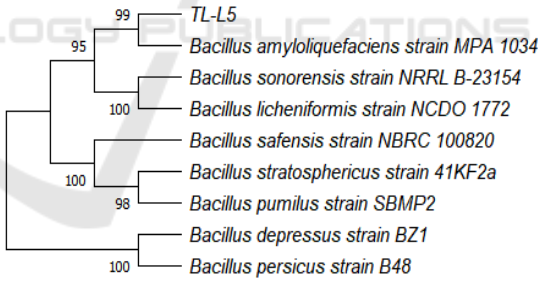

Figure 4: Phylogenetic tree of strain LPB-5(LPB-5 had the closest relationship with Bacillus amyloliticus MPA 1034, and the similarity was 99%.).

4 DISCUSSIONS

In this study, nutrient broth, a common medium, was used to isolate strains from the intestines of healthy cultured procambarus clarkii and crabs. It was identified as *Bacillus amyloliticus* by morphological observation, physiological and biochemical tests and 16S rDNA sequence analysis. In strain identification, if a single application of molecular

biology of the strains identified is likely to affect the accuracy of the appraisal result, eventually lead to the wrong conclusion, so the paper also combines the bacteria morphology observation and physiological and biochemical test evaluation, to ensure the accuracy of the appraisal results and academic.

In order to avoid the problems caused by the use of antibiotics such as the enhancement of drug resistance, veterinary drug residues, and the deterioration of meat quality, and to realize healthy ecological aquaculture, the aquaculture industry has an increasingly strong demand for good alternatives to antibiotic feed (Watts, 2017.Didier, 2020). Probiotics can improve feed digestibility, promote growth, inhibit pathogens, improve immunity, and improve water quality, etc., and are considered as the most potential alternatives (Wang, 2020.El Saadony Mohamed T, 2021). In a report in 1893, Kozasa used spores of Bacillus Toyota as feed additives for yellowtail fish and found that the growth rate of yellowtail fish was improved, which was the first application of probiotics in practice and proved the beneficial effect of probiotics in aquaculture (Cruz, 2012).

In recent years, the aquaculture industry has started to add some probiotics to the feed, and practice has proved that it is feasible to use probiotics to prevent and control the diseases of cultured fish and shrimp, and the use of probiotics in aquaculture can significantly reduce the need for antibiotics (Opiyo, 2019. Abarike, 2018). Sun et al. exposed the fertilized zebrafish larvae to perfluorinated butanesulfonic acid (PFBS) and applied the probiotic lactobacillus rhamnosus during this period (Sun, 2021). The results showed that probiotics supplement effectively inhibited the growth retardment caused by PFBS, Probiotics can secrete various digestive enzymes (amylase, protease, lipase, etc.) in the body of aquaculture animals, promoting the absorption and transformation of nutrients in the feed of aquaculture animals, improving feed digestibility, and promoting individual growth (Das, 2019. Hai, 2009). After entering the digestive tract of the host, probiotics mainly prevent the growth of pathogens on the intestinal surface by producing antibacterial substances, regulating intestinal pH and competing with harmful bacteria for adsorption sites (Kuebutornye, 2019. Kuebutornye, 2020. Chabrillon, 2005). Studies have shown that maintaining a high level of probiotics in fish ponds can not only reduce the accumulation of dissolved organic carbon and granular organic carbon during the growing season, but also balance the production of phytoplankton (Cruz, 2012. Kazun, 2019).

Most of the probiotics that make up the probiotics used in aquaculture come from the digestive tracts of mammals, or soil and seawater, etc. The probiotics used in aquaculture were originally commercial probiotics designed for land animals. In the development of probiotics for aquaculture, the probiotics in the intestinal tract of aquaculture animals are good raw materials, because they are not only biosafe, but also can effectively improve the survival rate and colonization rate of probiotics in the intestinal tract (Ramlucken, 2020). In this study, only one strain with this characteristic was screened, but the development of relevant probiotic preparation products still needs to consider various factors such as hemolysis, antibiotic resistance, compliance analysis, pH and bile salt tolerance. (Balcázar, 2008.). In addition, in some studies, to test the safety of probiotics, probiotics were injected directly into fish intraperitoneally to study mortality without inducing severe pathogenic symptoms (El-Rhman, 2009.Aly, 2008). In any case, safety is always the most important, so further experiments are needed to verify the addition of this strain as a probiotic preparation to aquatic feed.

5 CONCLUSIONS

In this study, a probiotic strain with quantity advantage was screened and isolated from the intestinal tract of procamblus clarkii and crab which were purchased from dajiang Aquatic Market of Huai 'an city. The strain was identified by observation of colony morphology, physiological and biochemical tests and 16S rDNA sequence analysis. The results showed that the strain had the closest relationship with *Bacillus amyloliquefaciens* MPA 1034. The 16S rDNA sequence similarity was 99%, and the physiological and biochemical reaction spectrum was consistent with the 16S rDNA sequence analysis. The biosafety and high efficiency of the strain in aquaculture feed can improve the excellent microbial resources for further development of probiotics and other products.

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