SYNSEA Report

Application and Screening of Lactic Acid Bacteria Isolated from Cobia Intestine

SYNBIO TECH INC.

"Probiotic solutions for antibiotic free farming era"

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Better Probiotic Better Life

Cobia (*Rachycentron canadum*) is an economically important aquaculture species in tropical and sub-tropical areas because of its good-quality meat and high growth rate. Since the successful development of cobia aquaculture techniques in 1990, cobia has been extensively cultured in Taiwan, and it has become one of the highest priority species in large-scale commercial aquaculture. However, high-density farming in aquaculture can lead to severe photobacteriosis infections, resulting in substantial economic losses. Although antibiotics and vaccines have been used to prevent or treat these infections, their application has been inconsistent, and their use is often accompanied by unwanted drug residues and drug resistance. Therefore, this approach does not provide a workable solution. To combat aquaculture infections and avoid the use of antibiotics, it is necessary to develop green farming techniques. Based on the results of earlier studies, increasing the activity of microorganisms present in the intestine through feed intake is effective for promoting aquaculture health and disease resistance. Thus, the effects of probiotics, either mixed with feed or directly sprayed in aquaculture water, has become a highly anticipated issue in the fishery industry.

Probiotics applied to aquaculture have been shown to have multiple health and economic benefits. For example, through colonization, some probiotics can reduce the quantity of pathogens and some produce metabolites that inhibit pathogen growth. Probiotics can also improve the intestinal structure and promote nutrient absorption by producing enzymes that are beneficial for the host. Some probiotics may also increase host resistance through physiological or immunological modulation. Lactic acid bacteria (LAB) that reside in the intestine have been shown to effectively colonize intestinal cells, produce bacteriocins, and improve gastrointestinal health. Thus, LAB are the largest group of microorganism used as probiotics.

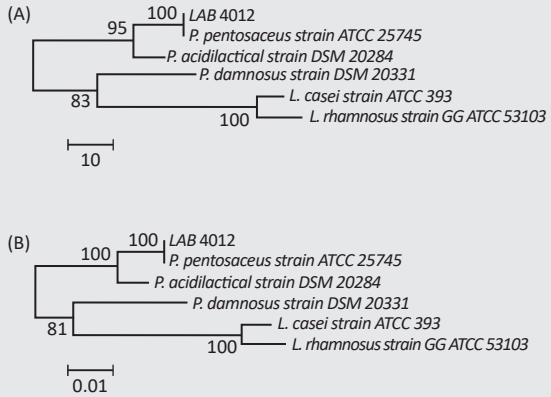
The most common mistake when selecting appropriate LAB strains is ignoring the differences in the effects of strains from different origins on the target host species. Ideally, strains from the intestine of species that are closely related to the target host species should be screened. However, only 1–10% of enteric bacteria exhibit probiotic potential. For use aquaculture, there must be sufficient experimental data about the LAB strain to understand its characteristics. Then, multiple examinations of their function and application should be performed. Only after functional validation and economic value assessment, can a strain be effectively applied. In cooperation with the science and technology departments at Penghu University and Taiwan University, SYNBIO TECH, INC. has developed the SYNTEK® thorough system for screening, selecting, and optimizing probiotics isolated from the intestines of cobia for use in aquaculture.

Suitability for aquatic animals

When assessing strains for use in aquaculture, first, the origin of the strain should be considered, because freshwater and seawater environments are very different. There are differences in water conductivity, carbonate hardness (alkalinity; KH value), salinity, and so on, and these environmental conditions are very strict. Therefore, strains originating from the intestines of seawater fish can be widely applied in fish of saltwater origin and should be less affected by the environment. The microflora in the intestines of fish is easily altered by environmental changes, cultivation conditions, and feed ingredients. Thus, the selected strains should be dominant microorganisms. During research and development, more than 400 strains from the intestines of adult cobia were screened by testing their tolerance to conditions in the digestive tract and their adsorption ability. Finally, three strains with outstanding performance were selected. Among these, strain LAB 4012 displayed excellent characteristics in terms of inhibiting the growth of *Photobacterium damselae* subsp. *Piscicida* (*Pdp*)and promoting increased numbers of immune cells to eliminate pathogens. Thus, this strain was chosen as a candidate fish-origin probiotic.

Strain LAB 4012 was identified and its functions were assessed by 16S ribosomal DNA (rDNA) sequencing and comparison to reference strains from GenBank in phylogenetic analyses using the minimum evolution and maximum parsimony methods (bootstrap value = 100). The analyses revealed that strain LAB 4012 was *Pediococcus pentosaceus*, of the order *Lactobacillales* in the family *Lactobacillaceae* (Fig. 1). The sequence was deposited in GenBank (Accession number, JN674456). After commercialization of this strain, the name was changed to PP4012.

Fig. 1. Phylogenetic analyses of strain LAB 4012 isolated from cobia intestine and related reference strains.
(A) Results of the maximum parsimony analysis.
(B) Results of the minimum evolution analysis.
Bootstrap value = 100.



Metabolites produced by PP4012 with anti-photobacteriosis activity

Metabolites produced by LAB, such as organic acids, bacteriocins, and anti-microbial peptides, can inhibit pathogens. The data shown in Fig. 2 demonstrate that bacteriocin production by PP4012 (LAB 4012) in the culture supernatant is significantly correlated with pH. At pH 6.2, there was little or no inhibitory activity against *Pdp* in the LAB 4012 culture supernatant. In contrast, in the presence of lactic acid, *Pdp* growth was partially inhibited, and LAB 4012 culture supernatant at pH 4.1 further inhibited *Pdp* growth, but neither could completely inhibit *Pdp* infection. Based on these results, we surmised that PP4012 produces high amounts of lactic acid; however, it may also produce other substances that inhibit the growth of *Pdp*, including other organic acids and acidic substances.

Fig. 2. The effects of LAB 4012 culture supernatant and lactic acid on the growth of *Pdp*.

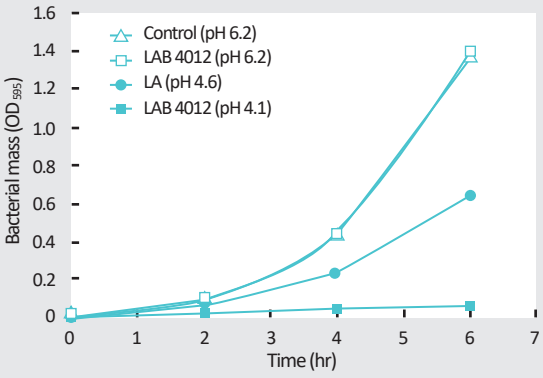
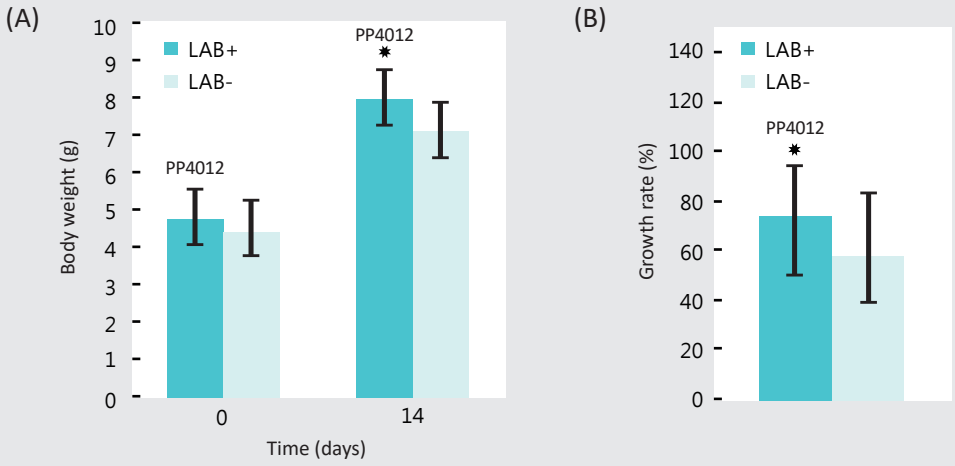


Fig. 3. The effect of feeding LAB 4012 to cobia for 2 weeks on growth.
(A) The average body weight of cobia at the beginning and end of the feeding test.
(B) The growth rate of cobia, calculated as the gain in body weight divided by the starting body weight.
*, P < 0.05. Each value is the mean ± SD from triplicate sets of 17 fish per set.



PP4012 promotes the growth of cobia larvae and modulates immunity

For aquatic animals, the hatching or culturing period is the stage at which the highest losses are incurred because of the undeveloped intestine, which is the largest immune organ. Without a fully developed immune system for defense, the animals in aquaculture are very sensitive to their environment. We propose treating the aquaculture with probiotics to establish an early defense against infection and promote the growth performance of cobia.

Before the feeding trial and analysis of cobia intestine, we did not detect PP4012 in the intestine of larvae, which was isolated from an adult cobia. To evaluate the effect of PP4012 on the growth performance and resistance of fish fry, PP4012 was introduced into the intestine of an undeveloped, young cobia. At the end of a 2-week feeding trial, PP4012 was detectable in the intestine of the cobia. This result demonstrates that PP4012 was unaffected by the gastric acid, bile, and anti-bacterial substances (bacteriocins) in the digestive tract. In addition, the immune response can be evaluated by measuring the respiratory burst (RB) activity of peripheral blood leukocytes (PBL) after challenge with *Pdp*, as the RB activity represents the resistance capability of PP4012 against pathogens or infectious disease.

The data shown in Fig. 4 demonstrate that PP4012 promotes the elimination of pathogens and enhances the resistance to *Pdp* infection, resulting in increased survival. This demonstrates that PP4012 produces bacteriocins that modulate immunity and promote disease resistance.

Fig. 4. The effect of feeding LAB 4012 to cobia for 2 weeks on the respiratory burst of PBL. Cobia were fed LAB 4012 (LAB+) for 2 weeks. Data are the average ± SD of 6 fish. ***, P < 0.001.

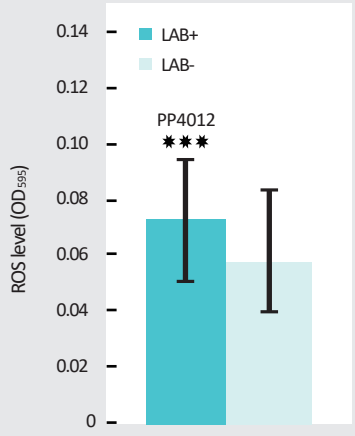


Fig. 5. The efficacy of feeding LAB 4012 to cobia for 2 weeks on protection against *Pdp* infection. Cobia were fed with LAB 4012 (LAB+) or without (LAB-) for 2 weeks, and were then immersion-challenged with Pdp (2×10^4 CFU mL-1). Cumulative mortality was recorded 10 days post challenge. Each value represents the average ± SD from triplicate sets of 17 fish each. ***, P < 0.001.

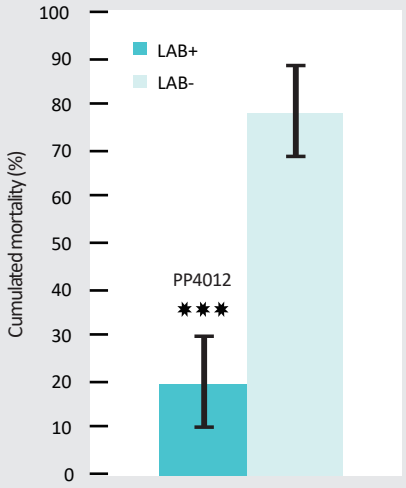


Fig. 6. Levels of *Pdp*-specific immunoglobulin (Ig) in immunized cobia fed LAB 4012. V+LAB+ cobia were immunized with inactivated *Pdp* and fed with LAB 4012 beginning on day 2 post immunization. The total feeding period was 5 weeks. V+LAB- cobia were immunized with inactivated *Pdp*, but were not fed LAB 4012. V-LAB- cobia were not immunized or fed LAB 4012. *Pdp*-specific Ig levels were determined by ELISA. Each value is the average ± SD from triplicate sets of 12 fish each.

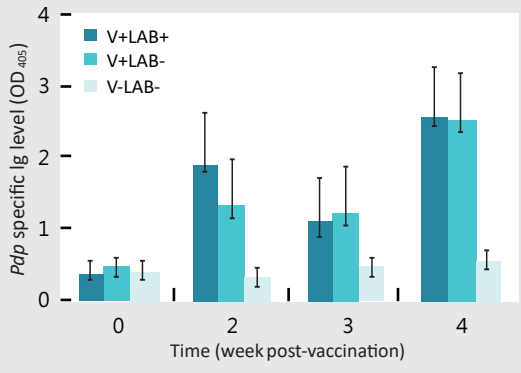
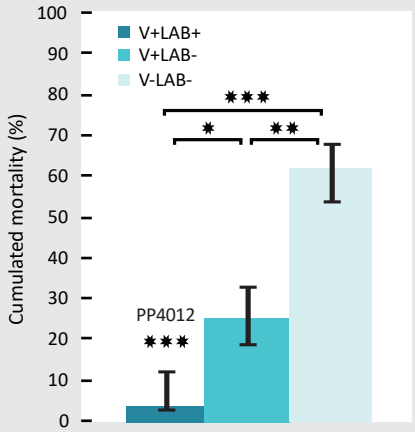


Fig. 7. Cumulative mortality of immunized cobia with or without LAB 4012 feeding (for 5 weeks) after Pdp challenge. V+LAB+ cobia were immunized with inactivated *Pdp* and fed LAB 4012 beginning on day 2 post immunization. V+LAB- cobia were immunized with inactivated *Pdp* and were fed LAB 4012. Control cobia were not vaccinated and were not fed LAB 4012. Each value is the mean ± SD from triplicate sets of 12 fish each. *, P < 0.05; **, P < 0.01; ***, P < 0.001.



PP4012 promoted protection by the *Pdp* vaccine

Farmers vaccinate aquacultures to increase resistance against diseases. LAB are often used together with vaccines to help fish produce more antibodies. We tested whether feeding PP4012 to cobia for 4 weeks could improve the immunity of vaccinated fish (Fig. 6). Our findings showed that feeding with PP4012 for a 2-week period significantly promoted the antibody response in vaccinated cobia, which means that PP4012 enhanced the immune response against *Pdp* in vaccinated fish. Therefore, administration of vaccines and PP4012 more efficiently promoted the disease resistance of fish than the vaccine alone.

After 4 weeks of feeding, all fish were challenged with *Pdp* to evaluate the immune response to infection and survival. The survival rates were, from highest to lowest: vaccinated and PP4012-fed, vaccinated, and control (without vaccination or PP4012) fish (Fig. 7). The survival rate of the vaccinated and PP4012-fed group was consistent with the results

shown in Fig. 6. Administration of a vaccine and PP4012 together significantly increased the survival rate, likely due to the increased antibody response.

All experimental data in this study were published in Fish & Shellfish Immunology in 2013. PP4012, developed jointly with academic institutes, was isolated from the intestines of cobia in Taiwan, and a certificate of invention patent was successfully obtained (No. I433651) based on its special and proven functions. SYNBIO TECH developed SYNTEK® thorough a development procedure, including strain identification, functional validation, and strain commercialization, that systematically improves the properties of probiotics during industrialization, to continuously and deeply develop LAB for use in various fields. SYNBIO TECH is the first biotech company in Taiwan specializing in the research and manufacture of LAB for applications in poultry, livestock, aquaculture, plants, humans, etc.

PP4012 Patent & Publication

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