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Global Aquaculture Advocate

Health & Welfare

Immunostimulant qualities of probiotic bacteria

Sunday, 1 December 2002
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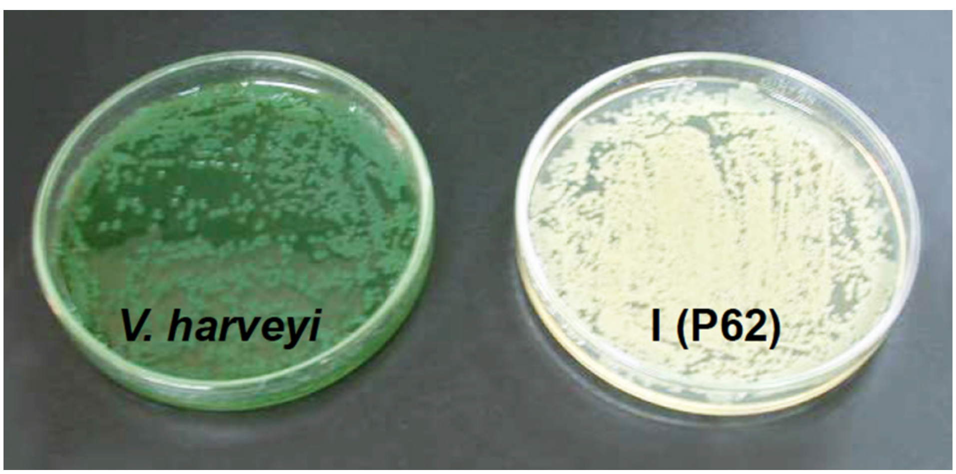
Vibrio P62, *Bacillus* P64 can be used as probiotics for *L. vannamei*



Agar plates show colony-forming units by some of the bacterial strains in the study.

The use of probiotic bacteria and immunostimulants are two promising and controversial methods recently implemented for the prevention and management of infectious shrimp diseases. One of the main challenges in developing probiotic bacteria is using appropriate selection and colonization methods. Probiotics should not inhibit shrimp growth or induce resistance to the probiotics used.

Bacterial strains

A study isolated bacteria with both probiotic and immunostimulant capacity. The probiotic strains used in the study were isolated from wild, adult (*Penaeus vannamei)* shrimp collected at Manglaralto, Ecuador. Half of each shrimp was used for histological analysis, while the hepatopancreas (HP) was extracted for bacterial isolation.

Probiotic strains were selected for their in vitro inhibition properties against *Vibrio harveyi* (S2), following Ruiz et al. (1996). Of 80 strains isolated, three came from healthy shrimp and inhibited in vitro growth of S2. By phenotypic observation AFLPs and 16S RNA gene sequencing, two strains were identified as *Vibrios* and another as *Bacillus*.

Experimental setup

For the three experiments described below, animals were acclimated for 15 days, with continuous water exchange and aeration in the culture system. Seawater was filtered and UV-sterilized, with temperature maintained at 28 degrees-C. Shrimp were fed 50 percent protein, commercial pellets sterilized daily. The aquariums were inoculated using the logarithmic phase of each strain at 107 bacteria per milliliter per aquarium.

Colonization capacity of bacteria

To test the colonization capacity of the bacteria, the experimental design was completely randomized with five replicates of eight treatments, with 20 1-gram shrimp in each 50-liter aquarium. *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 bacteria were used, with one control without bacteria.

After acclimation, only one bacterial inoculation was applied. Done without water exchange, the exposure time was 24 hours. For the recovery isolation, pools of 10 HP were used in replicate and five 1/10 serial dilutions were performed. Colonization percentages were evaluated by counting the colony-forming units (CFU) per gram HP based on colony morphology and identifying the strains by arbitrarily primed polymerase chain reaction using three primers. The health condition of the shrimp was determined by histology.

Interaction against V. harveyi

In a test of competitive interaction against *V. harveyi*, the strains *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 were used, as well as a negative control and pathogen control. Stock density, shrimp weight, and inoculum dose were the same as in the first experiment. The strains were inoculated for three consecutive days.

After 20 hours of exposure, 50 percent of the water was exchanged. On the fourth day of the experiment, *V. harveyi* (S2) was inoculated at 107 CFU per milligram for 24 hours before restarting the water exchange. Interaction percentages were evaluated by counting the CFU per gram of HP in Lb agar (2 percent NaCl), differentiating strains on morphological characteristics by AP-PCR and monoclonal antibodies against S2.

Immune system stimulation

Vibrio P62 and *Bacillus* P64 were used in an evaluation of the bacteria as immune system stimulants, with the strain *V. alginolyticus* (Ili strain) as a probiotic control. Shrimp of 1.5 ± 0.2 grams were distributed 10 animals each in 50-liter aquariums and fed commercial pellets at 3 percent biomass twice daily for 25 days.

The inoculation took place every two days during 10 days with 50 percent water exchange after 20 hours of exposure. After inoculation, haemolymph was extracted from each shrimp in intermolt stage. The stimulating effects of the strains were evaluated using haemogram counts, quantification of reactive oxygen intermediates, measurement of phenoloxidase activity, antibacterial activity quantification, and measurement of plasma protein concentration. The results of these tests were used to calculate the immune index.

Results

Of the 80 strains isolated from the HP of wild shrimp, two fulfilled the conditions of originating from healthy shrimps, reaching colonization percentages above 50 percent, inhibiting the in vivo growth of *V. harveyi*, and not causing histological damage in inoculated shrimp at a concentration of 107 CFU per milliliter.

Competitive exclusion power

The colonization experiment demonstrated the capacity of the strains to enter the HP and their competitive exclusion power. This effect was remarkable for *Vibrio* P62 and *Vibrio* P63, with the total quantity of CFU per gram HP not significantly different from the control ($p > 0.05$), indicating the high capacity of both *Vibrios* to inhibit autochthonous bacteria or carry out competitive substitution. The mean bacterial count reached at these treatments was 4.2 x 104 ± 8.5 x 103 CFU per gram HP, but the colonization percentage reached by *Vibrio* P62 was 83 percent, demonstrating a stronger antibacterial effect than *Vibrio* P63.

In the case of shrimp inoculated with Bacillus P64, the total bacterial number was significantly higher – 5.3 x 104 ± 7.6 x 103 CFU per gram, HP. Although the 58 percent colonization reached by this strain was similar to the one reached by the *Vibrio* P63 (60 percent), P63 was more efficient in performing the competitive substitution of the autochthonous flora. No histological damage was registered in the inoculated shrimp after 12 hours.

Competitive interaction

In the competitive interaction experiment, the total bacterial concentration increased by 64 percent with S2 inoculation, and up to 80 percent with the inclusion of probiotics. *Vibrio* P62 achieved the largest inhibitory effect, reducing by 60 percent the S2 penetration, while displacing the autochthonous microflora of the HP. The inhibitory effect of *Vibrio* P63 was greater on the natural flora than the pathogen S2, on which it achieved only 19 percent of inhibition. The *Bacillus* P64 inhibited the natural flora and competed with the pathogen, reducing by 34 percent the S2 penetration (Fig. 1).

Fig. 1: Bacterial concentration reached by *Vibrio* P62, *Vibrio* P63, and *Bacillus* P64 in an interaction experiment against *Vibrio harveyi* (S2) in Penaeus vannamei.

Immune system stimulation

The total haemocyte count did not show significant differences between the treatments. The haemocyte population distribution was less in the shrimp inoculated with *V. alginolyticus* (Ili) and *Bacillus* P64 than in the control (Fig. 2). The phagocyte stimulation rate was low for all treatments, not registering significant differences ($p > 0.05$) of the reactive oxygen intermediate production (Table 1).

The phenoloxidase activity was significantly higher ($p < 0.05$) in shrimp stimulated with *Bacillus* P64, Vibrio P62, and *V.*

Fig. 2: Differential haemocyte count in shrimp treated with *Vibrio* P62, *Bacillus* P64, and *Vibrio alginolyticus* (Ili).

alginolyticus (Ili). The antibacterial inhibition percentage of the plasma was lower than the control. The quantity of plasmatic proteins stayed within the normal range (Table 1). The global immune index was significantly greater ($p < 0.05$) in the shrimp stimulated with *Bacillus* P64 and *V. alginolyticus* (Fig. 3). Mean shrimp weights in the probiotic groups were significantly higher ($p < 0.05$) than the control.

Gullian, Mean immunological values of control and probiotic treatments, Table 1

Immunitary Values	*V. alginolyticus*	*Vibrio* P62	Bacillus P64	Control
Total haemocytes (cells/ml)	2.2x7 ± 9.9x10^6	1.7x7 ± 4.9x10^6	2.1x7 ± 3.1x10^6	2.0x7 ± 3.9x10^6
NBT rate	1.18 ± 0.08	1.15 ± 0.11	1.20 ± 0.09	1.11 ± 0.05
Antibacterial activity (%)	8.5 ± 5.6	25.4 ± 13.7	20.6 ± 8.7	30.0 ± 9.0
Plasmatic protein (mg/ml)	112.1 ± 8.1	97.7 ± 6.0	102.6 ± 3.9	104.3 ± 8.6
Phenoloxidase activity (O.D.)	670 ± 0.02[a]	661 ± 0.07[a]	738 ± 0.08[a]	449 ± 0.05[b]

Table 1. Mean immunological values of control and probiotic treatments. [ab] indicates significant difference ($p < 0.05$).

Conclusion

Results of this study demonstrated that the isolated beneficial bacteria of the natural microflora are potential competitors of pathogenic bacteria. The interaction of *Vibrio* P62 and *Bacillus* P64 with *V. harveyi* (S2) confirmed it is possible to diminish the colonization of this strain in the hepatopancreas.

The inoculation of *Vibrio* P62 and *Bacillus* P64 during 10 days improved the general health of the shrimp. The total number of haemocytes and total plasmatic protein concentrations in the three treatments remained within normal values, indicating the administration did not deteriorate shrimp health. However, the immune index evaluation showed *Bacillus* P64 and *V. alginolyticus* (Ili) were more effective than *Vibrio* P62 at stimulating the shrimp immune response.

Results demonstrated that *Vibrio* P62 and *Bacillus* P64 can be used as probiotics in the prevention of *P. vannamei* diseases. The main objective in their use would be to limit the appearance of pathogenic bacteria in culture ponds. We believe this occurs by competitive exclusion or by stimulation of a defense reaction in the host.

Note: Cited references are available from the authors.

(Editor's Note: This article was originally published in the December 2002 print edition of the Global Aquaculture Advocate*.)*

Fig. 3: Immunity index of shrimp treated with *Vibrio alginolyticus* (Ili strain), *Bacillus* P64, and *Vibrio* P62 after 25 days of experiment.

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