Immunostimulant Qualities of Probiotic Bacteria

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

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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 to 0.5 m and UV-sterilized, with temperature maintained at 28° C. Shrimp were fed 50%-protein, commercial pellets sterilized daily. The aquariums were inoculated using the logarithmic phase of each strain at 10^7 bacteria/ml/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-g shrimp in each 50-l 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)/g 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% of the water was exchanged. On the fourth day of the experiment, *V. harveyi* (S2) was inoculated at 10^7 CFU/ml for 24 hours before restarting the water exchange. Interaction percentages were evaluated by counting the CFU/g of HP in Lb agar (2% 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 g were distributed 10 animals each in 50-l aquariums and fed commercial pellets at 3% biomass twice daily for 25 days.

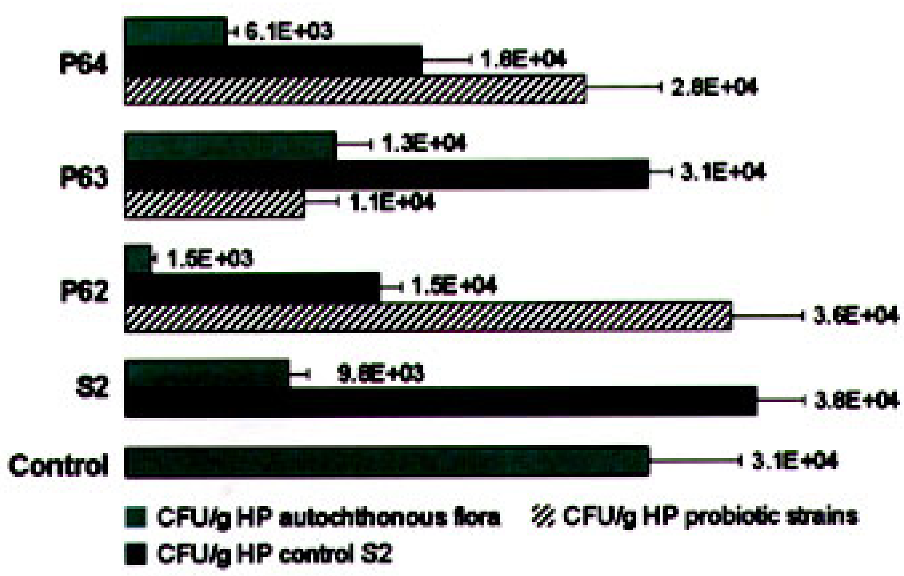

Figure 1. *Bacterial concentration reached by* Vibrio *P62,* Vibrio *P63, and* Bacillus *P64 in an interaction experiment against* Vibrio harveyi *(S2) in* Penaeus vannamei.

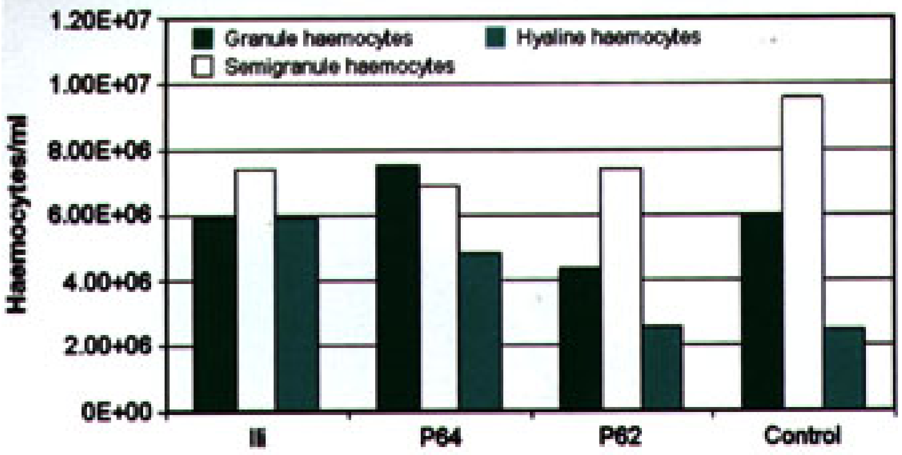

Figure 2. *Differential haemocyte count in shrimp treated with* Vibrio *P62,* Bacillus *P64, and* Vibrio alginolyticus *(Ili).*

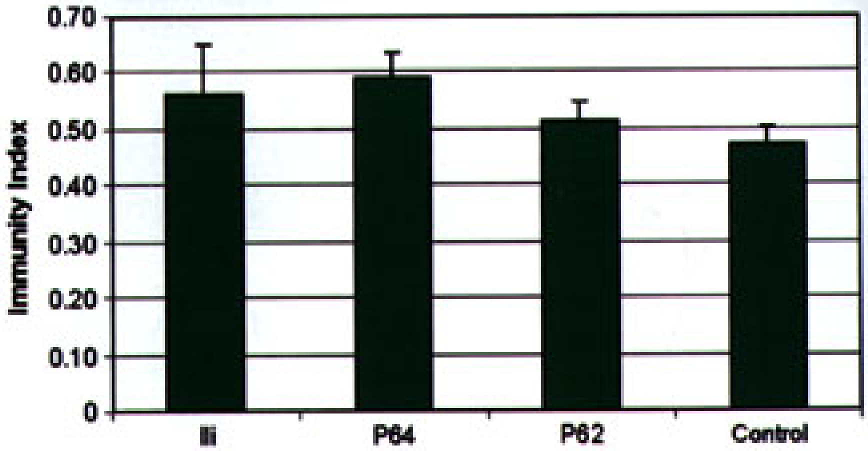

Figure 3. *Immunity index of shrimp treated with* Vibrio alginolyticus *(Ili strain),* Bacillus *P64, and* Vibrio *P62 after 25 days of experiment.*

The inoculation took place every two days during 10 days with 50% 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%, inhibiting the in vivo growth of *V. harveyi*, and not causing histological damage in inoculated shrimp at a concentration of 10^7 CFU/ml.

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/g 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/g HP, but the colonization percentage reached by *Vibrio* P62 was 83%, demonstrating a stronger antibacterial effect than *Vibrio* P63.

In the case of shrimp inoculated with *Bacillus* P64, the total bacterial number was significantly higher – $5.3 \times 10^4 \pm 7.6 \times 10^3$ CFU/g HP. Although the 58% colonization reached by this strain was similar to the one reached by the *Vibrio* P63 (60%), 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% with S2 inoculation, and up to 80% with the inclusion of probiotics. *Vibrio* P62 achieved the largest inhibitory effect, reducing by 60% 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% of inhibition. The *Bacillus* P64 inhibited the natural flora and competed with the pathogen, reducing by 34% the S2 penetration (Figure 1).

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 (Figure 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. 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* (Figure 3). Mean shrimp weights in the probiotic groups were significantly higher ($p < 0.05$) than the control.

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

Immunitary Values	*V. alginolyticus*	*Vibrio* P62	*Bacillus* P64	Control
Total haemocytes (cells/ml)	$2.2x7 \pm 9.9x10^6$	$1.7x7 \pm 4.9x10^6$	$2.1x7 \pm 3.1x10^6$	$2.0x7 \pm 3.9x10^6$
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