Health & Welfare

Culture water pre-inoculation of probiotics in Pacific white shrimp nurseries

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Longer inoculation of probiotics improves postlarvae growth, survival

This study showed that a longer inoculation of probiotics will improve shrimp postlarvae growth and survival, and that a new technique developed for more accurately estimating postlarvae weight can contribute to significantly reduce the food that must be supplied.

The term probiotic, initially coined in 1974 to define “organisms and substances that contribute to intestinal microbial balance,” was redefined in 1989 as a “live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance.”

By growing in the intestinal tract and adhering to its mucosa, they prevent harmful microorganisms from settling and performing their negative functions, acting as a barrier that prevents their colonization by pathogens.

Introduced into the culture environment, they have proven effective in competing with pathogenic bacteria and controlling their proliferation, as well as promoting the growth of cultured organisms. In addition to competing and excluding harmful microorganisms, probiotics exert a positive effect on the handling and transformation of sediments in culture tanks, which is why they have become one of the best tools in the production of shrimp larvae.

Antibiotics – used in large quantities to control outbreaks of Vibrios and other pathogenic bacteria – have proved to be, in many cases, ineffective, or have resulted in an increase in the virulence of pathogens, as well as being a concern for promoting the transfer of resistance in human pathogens. Therefore, the use of probiotics as an alternative to control pathogens is considered a preferential option to the use of antibiotics.

Action mechanisms

Although not yet fully elucidated, the mechanisms of action of the bacteria that are used as probiotics are:

- **Competition for binding sites:** also known as “competitive exclusion,” where probiotic bacteria attach to the binding sites in the intestinal mucosa, forming a physical barrier that prevents the connection of pathogenic bacteria.
- **Production of antibacterial substances:** probiotic bacteria synthesize compounds such as hydrogen peroxide and bacteriocins, which have antibacterial action, mainly in relation to pathogenic bacteria. They also produce organic acids that reduce the pH of the environment of the gastrointestinal tract, preventing the growth of several pathogens and the development of certain species of *Lactobacillus*.
- **Competition for nutrients:** the lack of available nutrients that can be used by pathogenic bacteria is a limiting factor for their maintenance.
- **Stimulation of the immune system:** some probiotic bacteria are directly related to the stimulation of the immune response, increasing the production of antibodies, the activation of macrophages, the proliferation of T-cells and the production of interferon.

Experimental evaluation of water maturation time with probiotics

The use of probiotic microbes in aquaculture is now widely accepted and are several probiotic preparations that are commercially available as feed additives or for incorporation into pond water for the culture of fish, shrimp and molluscs. According to manufacturers’ claims, these products are safe and effective to keep aquatic animals healthy, although there are also conflicting opinions regarding the general concept of probiotics and manufacturers’ claims, so it is justified to investigate further the intestinal microbiology and the effective preparation and evaluation of the safety of probiotics.

In Ecuador, a common practice in shrimp postlarvae (PL) operations in circular tanks or raceways involves the addition of probiotics to water just hours before stocking the animals (with a maximum of 24 hours in advance). Is that enough time to make the most of the potential benefits of probiotics?

To answer this question, a recent study carried out at the Technical University of Machala as a thesis project in Aquaculture Engineering has established that the inoculation of probiotics in water – with a longer period of time in advance of stocking – positively influences and benefits shrimp PL health, promoting their best development during the culture period, that this longer period is three to four times greater than the one generally used in these production procedures. This article summarizes this study and is adapted from the June 2017 issue of *Aquacultura* (Ecuador).

This test used a mixture of two commercial probiotics with a composition from the first manufacturer that includes *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp. and an organic vehicle, and a guaranteed concentration of not less than 3×10^{12} CFU/kg. For the second manufacturer, the composition included *Bacillus* sp., *Enterococcus* sp., *Pediococcus* sp., *Thiobacillus* sp., *Paracoccus* sp. and an organic vehicle, and with a guaranteed concentration of not less than 2×10^{12} UFC/kg. The ratio of the mixture was 5 ppm of the first probiotic and 2 ppm of the second.

In order to establish if the time of inoculation of the probiotic in the water has a significant effect on the productive parameters, a completely randomized experimental design was formulated (4 x 2 random blocks) consisting of eight 200-liter tanks with water from the same source. Three treatments with a replicate were evaluated for inoculation times of 48, 72 and 96 hours prior to the stocking of the PL.

As controls, two tanks were used as controls where the probiotic was inoculated 24 hours in advance. After reaching the different inoculation times, larvae (PL8) were stocked simultaneously in each tank at a density of 50 PL/L (1,000 PL/tank). In order to simulate the growing conditions typical in commercial operations, the same amount of probiotic was added every 24 hours in each tank.

The culture test lasted 12 days. At the time of stocking and then every four days, random samples of 15 to 30 PL were collected from each tank, and their size and weight were determined individually. At the end of the study, a total count of larvae was done to determine the overall survival in each tank.

Estimation larvae biomass with individual data

To determine the weight of the larvae in each sample, the animals were sacrificed by adding a volume of formalin to the water in the container of each sample until reaching a concentration of 10 percent. Each larva was grabbed with a fine-tipped clamp, placed on a filter paper to absorb the water adhered to the body, and immediately weighed on a 0.01 mg analytical balance

After determining the weight, each PL was placed on an adhesive tape attached to a sheet of graph paper, with numbered boxes to identify the correlative order of each individual. Each sheet of paper with the PL was photographed in order to have a digital image with a millimeter scale.

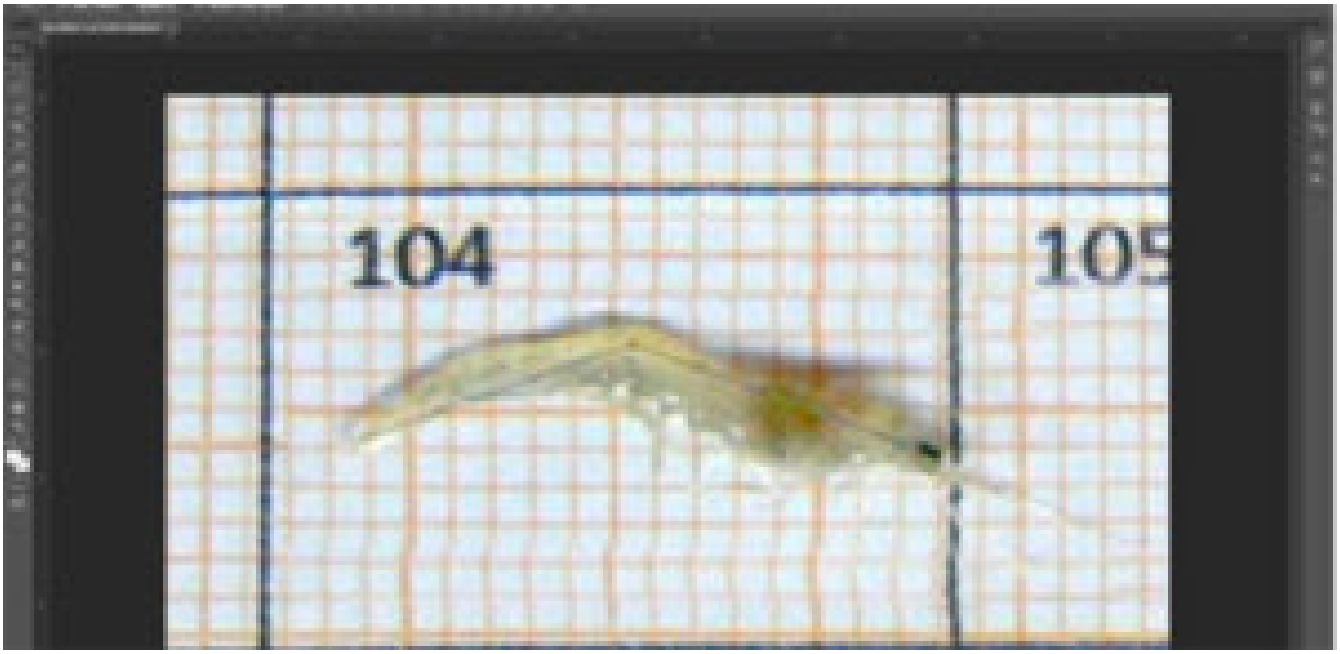

Fig. 1: Example of the determination of the length of each PL with Adobe Photoshop's measuring tool.

To determine the length of each PL, the images were digitally processed with ADOBE Photoshop CS6 software, using the measurement tool and defining a custom scale in which the equivalence between the number of pixels and the logical unit of measurement was established, which in our study was 10 mm. Thus, it was possible to measure the length of each PL by moving its body over the magnified image from the base of the eye to the terminal end of the telson, and in this manner fix evident points and minimize possible confusion in case the image was not perfectly focused (Fig. 1).

To establish the length-weight relationship, the data were analyzed by linear regression following the least squares method. To determine the effect of each treatment on PL growth rate, the regression lines established between length vs. time were compared, contrasting the slopes of the linear regressions by analysis of variance. To determine the existence of dependence between survival and treatments, the Chi square independence test was used.

Results

The daily record (every 6 hours) of the physicochemical data during the test showed fairly stable average values in all the experimental tanks: water temperature: 26.20 ± 0.54 degrees-C; salinity: 27 Ups; pH: 8.82 ± 0.39; ammonium: 0.61 ± 0.25mg /L; and dissolved oxygen: 7.06 ± 0.07mg/L.

The multivariate analysis of variance for PL weights between treatments and replicates showed no statistical differences between the replicas for each treatment ($P > 0.05$) but did show differences between treatments ($P < 0.00001$). The interaction was also not significant between main effects ($P > 0.05$).

The Multiple Ranges test allowed the identification of three homogeneous groups with no differences in average weights of PL between treatments 1 and 2 (24 and 48 hr. of inoculation) but did show differences between these and treatments 3 and 4 (72 and 96 hr. of inoculation, respectively) where the averages were higher (Treatment 4 > Treatment 3 > Treatment 2 = Treatment 1). Fig. 2 illustrates these differences by indicating the mean ± the confidence interval for the mean at 95 percent, in each case.

Fig. 2: Average ± confidence interval for the average weight of PL between treatments.

Analysis of variance for differences in PL lengths between treatments and replicates showed no statistical differences between the replicas for each treatment ($P > 0.05$) but between treatments ($P < 0.00001$). The interaction was also not significant among main effects ($P > 0.05$). Fig. 3 illustrates these differences by showing the mean ± the 95 percent confidence interval for the mean in each case.

Fig. 3: Average ± 95 percent confidence limits for PL length between treatments (1): 24 hours, (2): 48 hours; (3): 72 hours; and 4 (96 hours).

As with the weight factor, the Multiple Ranges Test (Fisher's LSD) showed that there are no differences in PL length between treatments 1 (24 hours of inoculation) and 2 (48 hours of inoculation); but there were differences between the first two and treatments 3 (72 hours of inoculation) and 4 (96 hours of inoculation). In order of better results: Treatment 4 > Treatment 3 > Treatment 2 = Treatment 1).

Fig. 4: Regression lines for length (A) and weight (B) of PL vs. time.

Regarding the growth rate in size and weight, the comparison by analysis of variance of the slopes of the regressions adjusted to the linear model revealed that with the treatment of 96 hours of pre-inoculation, the highest slope was obtained in relation to the control group, which demonstrated higher growth rate in this treatment (Fig. 4). Fig. 5 shows the survival percentage of the PL in each treatment.

Fig. 5: Survival of shrimp PL in each treatment.

Perspectives

The results obtained in this study showed that, on an experimental scale, that the inoculation of probiotics in the water prior to stocking *L. vannamei* PL had a positive effect on their growth. This favored the accelerated growth of the PL, which was demonstrated through statistical analyses with highly significant differences ($P < 0.001$) of these parameters between treatments, as well as better survival rates. Between the time periods for water maturation with the probiotics (24 to 96 hours), the PL reached larger lengths and weights when they were stocked in the tanks with longer maturation times with the probiotic.

It is important to note that inoculating the culture water with probiotics, 72 to 96 hours before stocking does not represent a significant additional cost, except for electrical energy to maintain the aeration and the continuous circulation of water in the system.

An interesting aspect in our study was the possibility of establishing the length-weight relationship from individual data of the PL to obtain a regression equation adjusted to the potential model. With this equation, the individual weight of each larva can be estimated from its length, and thus yields a more precise estimate of the PL biomass in the system. With a more accurate biomass estimate, it is possible to adjust the food rations to more adequate percentages.

Fig. 6 shows the relationship between length vs. weight obtained for *L. vannamei* PL after 12 days of culture, with potential regression adjustment. The equation that describes this function is: Weight = 0.0081L2.8986 with a coefficient of determination $R2 = 0.93$ that indicates that the adjusted model explains 93.27 percent of the variability of the weight as a function of the variation of the length of the PL.

Fig. 6: Length vs. weight relationship obtained for L. vannamei postlarvae after 12 days of rearing in this study.

When performing the calculations using this equation to determine the average biomass from a representative sample of the PL stock, it was found that the estimated ration differed considerably from the amount of food recommended by the tables from feed manufacturers. In fact, based on these estimates, the total daily food supply was reduced by at least 40 percent.

With this simple adjustment, the larvae not only have the required amount of food, but it is also possible to keep the rearing tanks much cleaner, with less sediment, thus increasing the potential of the probiotics that act by transforming these sediments into compounds more efficiently that do not generate problems for the animals. In addition, this adjustment helps keep low ammonium levels and a pH close to neutral in the culture tanks.

During normal shrimp PL production, the expense in food represents between 12 to 15 percent of the total costs and, therefore, adjusting the ration to the actually required by the PL would not only lead to a saving but would avoid maintaining an unnecessary excess of balance in the system. This would provide better quality in the cultivation system, which could contribute to reducing stress and its associated consequences, all of which would ultimately result in a faster growth of the PL and a greater survival, allowing, in addition, savings in food expenses.

It is important to note that the recommendations presented here based on experimental tests have been put to the test at the commercial PL production operation in raceways of the company Crisanticlub S.A. There they have reduced the total daily amount of food to be supplied by at least 40 percent. They have also obtained a sustained PL production of excellent quality and with average survival of at least 90 percent during 18 runs carried out during the year 2017.

Therefore, it is possible to conclude from our study that a longer period beforehand in the inoculation of probiotics will improve PL growth and survival, and that the development of a new technique for more accurately estimating PL weight can contribute to significantly reduce the food that must be supplied.

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