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Assessing the impact of bacteriophages in the control of Vibrios in *Litopenaeus vannamei*

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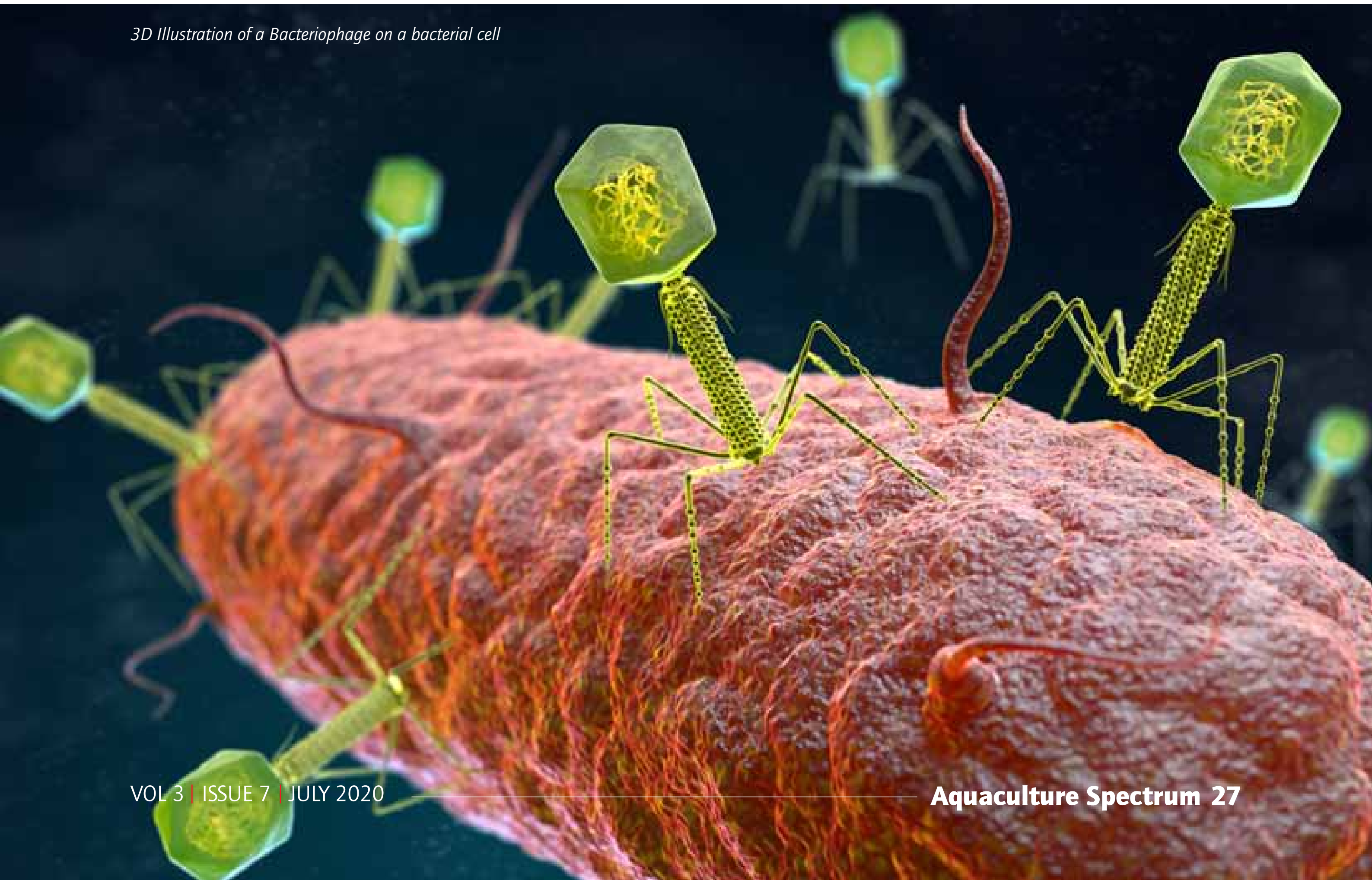
The shrimp aquaculture industry ranks as the highest foreign exchange earner among the marine product exports from India. Countries like India, Ecuador, Vietnam and Indonesia have emerged as global leaders in shrimp production. In spite of being the most rapidly expanding sector in food production, aquaculture is vulnerable to losses caused by disease. Current growth in aquaculture production is parallel with the increasing number of disease outbreaks, which can affect the production, profitability and sustainability of the industry worldwide. Among the groups of pathogenic microorganisms, Vibrios are well-known causatives of massive mortalities in farmed shrimp, fish and shellfish resulting in severe economic losses. An intensive mode of culture with high stocking densities have resulted in an increase in the incidence


3D Illustration of a Bacteriophage on a bacterial cell

of diseases. To maintain productivity in such intensive aquaculture production systems, massive use of antibiotics had been employed. The spread of antibiotic resistance from aquaculture settings to the natural environment is increasing. About 70% of the Vibrios isolated from aquaculture settings are multi-drug resistant. Bacterial infections, including multi drug-resistant strains, have been recognized as an important limitation to the development of the aquaculture production.

Another concern associated with the use of antibiotics is the problem of residues, which has resulted in rejection of farmed shrimp consignments, containing traces of antibiotics by seafood importing countries. Alternative strategies must be developed to control diseases in aquaculture. These strategies should reduce the risk of developing and spreading microbial resistance and be more environment friendly. Unless concerted action is taken to curb antibiotic overuse and misuse, in humans and animals, the world may be heading for a post-antibiotic age.

Bacteriophage therapy

Bacteriophage therapy is currently considered as one of the most viable alternatives to antibiotics for the treatment of bacterial infections in aquaculture systems. Bacteriophages attach to specific bacterial hosts and kill them by internal replication and bacterial lysis. The use of bacteriophages to control bacterial infections in aquatic food production systems has greater potential to address the twin problem of controlling bacterial infections and at the same time avoiding residue contamination. The application of phages in aquaculture, thus has good advantages over the use of antibiotics.

The use of phages for the biological control of pathogens of cultured shrimp has developed interest in recent years since no drug residues or drug toxicity is associated with this type of therapy. Bacteriophages are the most abundant organisms in the environment, with the total number of phages on earth estimated to be between 1030 and 1031, approximately 10 times higher than their bacterial hosts. Phages are natural predators of bacteria, self-limiting and self-replicating in their host cell, and can adapt to resistant bacteria. They are commonly found in large numbers wherever their hosts live; in sewage, in soil, in hatchery, in deep thermal vents, or in natural bodies of water. Several studies have demonstrated that bacteriophages can effectively control the bacterial infections in shrimp, fish and other animals.

In an attempt to assess the potential advantages of using bacteriophages against vibrios in shrimp culture, a study was initiated in a *Litopaeneus vannamei* aquaculture farm. A commercial formulation of Bacteriophages against Vibrios namely ELIXIR manufactured by Aristogene Biosciences Private Limited, Bangalore, India has been used to evaluate the effectiveness of bacteriophages against vibrios in shrimp culture. The results of which are presented herein.

Description of the study area

This study was carried out at SK Aquafarms, Ganapavaram, Karlapalem mandal, Andhra Pradesh. Spread across a total area of 45 acres, this farm has 4 sections – A, B, C and D comprising of 7, 7, 5 and 4 ponds each respectively. A and B sections with 7 ponds each (water spread area ranging between 0.6 to 1.7 acres), stocked with *L. vannamei* at a density of approximately 15 pcs/m^2, were selected for the present study. Aeration was provided using paddle wheel aerators @ 4 HP (min) per metric ton biomass in the pond. Salinity ranged between 12-18 ppt all through the culture period. Treated creek water as well as borewell water was used. Feed of a reputed brand was used.

Pond preparation and shrimp stocking

Ten-day old *Litopaeneus vannamei* post larvae (PL10) were sourced from a reputed commercial hatchery in the Marakkanam coast (Tamil Nadu). The PLs were stocked in 14 ponds in the farm, namely A1-A7 and B1-B7 after adequate acclimatization. The stocking density was around 15 pcs/m^2 in all the ponds except B7 which was stocked at a density of 24 pcs/m^2. The shrimp were fed based on the demand, four times daily (06:30, 10:30, 14:30 and 19:30 hours). The duration of culture in this study was on an average of 174 days.

Water quality parameters were monitored throughout the rearing period. Water temperature, dissolved oxygen and pH were measured daily, while parameters such as nitrate, nitrite and ammonia were measured weekly/

Satellite view of the study area

bimonthly. Operational water column in all ponds was 120 -150 cm. Water exchange was done 2-3 times during the culture period based on the requirement.

Four ponds namely B1, B2, B3 and B4 were assigned for the experiment wherein ELIXIR was mixed at the rate of 5g/kg of feed, one meal per day from 42 DOC (days of culture). Elixir was mixed with the feed using a proven non-toxic binder, dried in shade and applied. Other 10 ponds namely A1-A7, B5, B6 and B7 served as control where a probiotic containing a mixture of enzymes and live stabilized microbial mixture was used at the rate of 10g/kg of feed one meal per day.

Weekly samples of the grown shrimp were caught from each pond to estimate the periodical growth performance and the total biomass. The average weight of the shrimp sampled in each pond was determined and the amount of feed provided to the shrimp was adjusted accordingly. At the end of the experiment, all the ponds were harvested, the total yield (biomass of shrimp) was recorded, and the final survival rates were estimated. The other parameters such as, average daily gain (ADG), production per hectare, productivity per lakh of seed and feed conversion ratio (FCR) were calculated.

Measurement of growth performance, feeds utilization parameters and economic values

- Feed conversion ratio (FCR) = Total feed consumed (kg) / Total harvested biomass (kg)
- Production per hectare = (Production in kg/unit area acre) x 2.47
- Production per lakh seed = (Production in kg/ Quantity of seed stocked) x 100000
- Average daily gain (ADG) in g = (Average body weight of Shrimp (g)/ Total of days of culture
- Survival Rate (SR %) = (No. of shrimp harvested / No. of seed stocked) × 100

Statistical analysis

The results ADG, Survival, production, FCR, were assessed by one-way analysis of variance (ANOVA) model. The existence of significant differences between the control (treated with probiotics) and ELIXIR was performed at a level of $P < 0.05$ significance.

Table 1: Effect of ELIXIR treatment on growth performance of marine shrimp (*Litopaeneus vannamei*) reared in earthen grow-out ponds

Pond No	Area (Acres)	Date of Stocking	No. of Seed Stocked	Stocking density (Pcs/sqm)	Date of Harvest	DOC at Harvest	Quantity Harvested (Kgs)	Survival Rate (%)	ABW at Harvest (g)	Feed Consumption (Kgs)	FCR	Production per Ha (kg)	Production per lakh seed (kg)	ADG (g)
A1	1.27	29.10.2019	76000	14.96	22.04.2020	176	2010.80	83.87	31.55	3471	1.73	3912.4	2645.8	0.15
A2	1.04	29.10.2019	60000	14.42	22.04.2020	176	1738.90	92.74	31.25	2964	1.71	4131.6	2898.2	0.16
A3	1.18	29.10.2019	71000	15.04	21.04.2020	175	1904.60	86.38	31.06	3447	1.81	3988.4	2682.5	0.15
A4	1.12	29.10.2019	77000	17.19	19.04.2020	173	1891.90	75.77	32.43	3488	1.84	4174.0	2457.0	0.14
A5	1.38	29.10.2019	93000	16.85	21.04.2020	175	2125.10	78.15	29.24	3944	1.86	3805.2	2285.1	0.13
A6	1.42	08.11.2019	80000	14.08	19.04.2020	164	1853.50	69.18	33.49	3083	1.66	3225.4	2316.9	0.14
A7	1.31	08.11.2019	70000	13.36	20.04.2020	165	1010.00	53.39	27.03	2407	2.38	1905.1	1442.9	0.09
B5	1.72	29.10.2019	103000	14.97	23.04.2020	177	1870.90	67.21	27.03	3654	1.95	2687.8	1816.4	0.10
B6	1.01	29.10.2019	60000	14.85	22.04.2020	176	1598.60	79.93	33.33	2490	1.56	3911.0	2664.3	0.15
B7	0.63	29.10.2019	60000	23.81	20.04.2020	174	1285.90	79.30	27.03	2498	1.94	5043.6	2143.2	0.12
B1	1.02	29.10.2019	60000	14.71	24.04.2020	178	1777.20	90.64	32.68	2973	1.67	4305.4	2962.0	0.17
B2	1.02	29.10.2019	60000	14.71	24.04.2020	178	1821.60	89.56	33.90	2827	1.55	4412.9	3036.0	0.17
B3	1.35	29.10.2019	81000	15.00	22.04.2020	176	2310.20	85.56	33.33	3454	1.50	4228.5	2852.1	0.16
B4	1.42	29.10.2019	85000	14.96	23.04.2020	177	2146.80	85.11	29.67	3588	1.67	3735.7	2525.6	0.14

B1, B2, B3 and B4 - ELIXIR applied ponds; A1-A7, B5-B7 - Control ponds (probiotics applied)

The results of the effect of application of ELIXIR (bacteriophage formulation) on marine shrimp on the survival percentage, FCR, Production and ADG

The average survival percentage of ELIXIR treated ponds and that of control were 87.72% ± 2.79 and 76.59% ± 11.12 respectively with significant differences ($P < 0.05$) among the treatments. The average FCR of ELIXIR treated ponds and that of control were 1.60 ± 0.09 and 1.84 ± 0.23 respectively with significant differences ($p < 0.05$) among the treatments. The average production per hectare showed a significant difference (ANOVA, $p <0.1$) with the average of ELIXIR treated and that of control being 4170.63 kg ±299.63 and 3678.43 kg ± 873.49, respectively. The average production per lakh of seed also differed significantly (ANOVA, $p <0.05$) with 2843.94 kg ± 225.24 and 2335.22 kg ± 463.87 for Elixir and control, respectively. Also, the ADG differed significantly (ANOVA, $p <0.05$) between ELIXIR and control treatment with the values of 0.16 g/pc/day ± 0.01 and 0.13 g/pc/day ± 0.03.

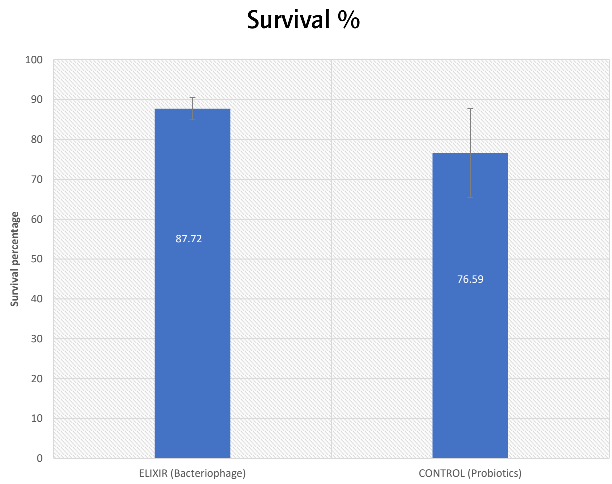
White Faecal disease was seen in ponds A6, A7, A1 and A2. These were the ponds where ELIXIR was not used.

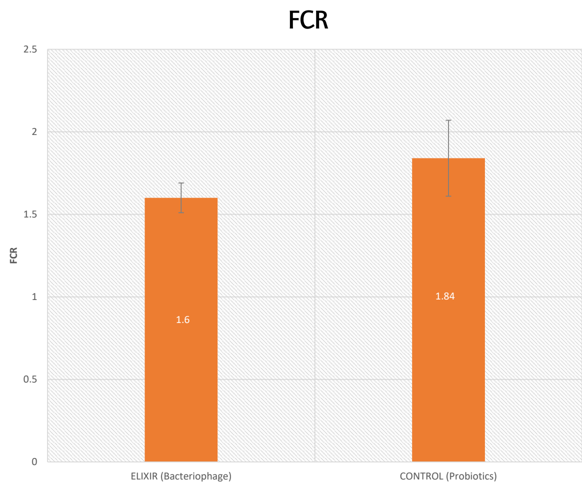
White faecal disease seen in control ponds

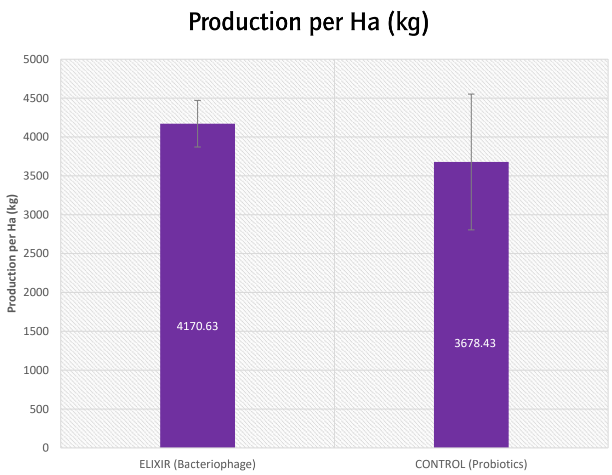
Table 3. Economic analysis of production per acre using ELIXIR (bacteriophage formulation) Vs Probiotics

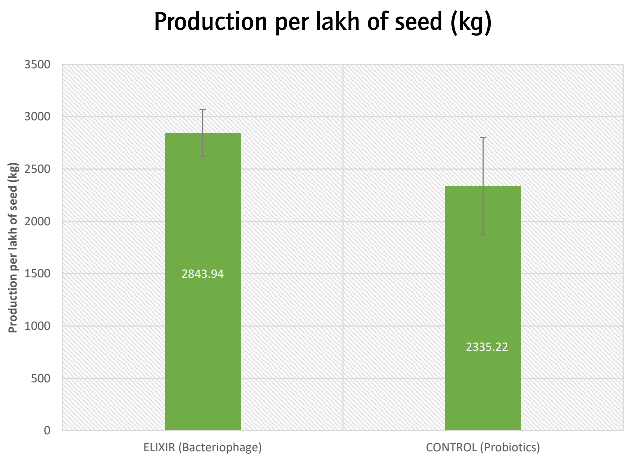
Economic indicators	ELIXIR	Control
Gross Revenue (GR) INR/acre	624560	491238
Total Cost (TC) INR/acre	461995	462605
Benefit-Cost Ratio = GR/TC Ratio	1.35	1.06

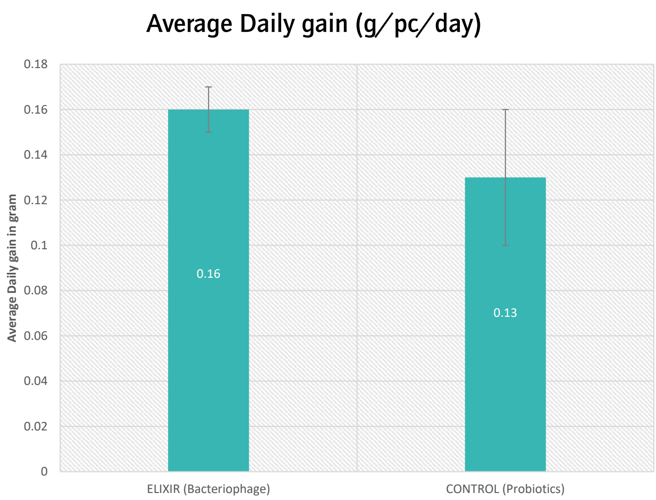
Effect of Application of ELIXIR on Survival, FCR, production per hectare, production per lakh of seed and Average daily gain











Whereas the ponds treated with ELIXIR did not have any incidence of white fecal disease.

From the above-mentioned results, it could be concluded that use of ELIXIR, a bacteriophage-based formulation against vibrios resulted in better growth, survival, FCR and production of marine shrimp *Litopenaeus vannamei*. The benefit-cost ratio also is higher in ELIXIR treated ponds.

Also, in this study some of the ponds which were not treated with ELIXIR did develop white faecal disease which was not observed in any of the treated ponds. One of the possible causes for white faecal syndrome is a combination of EHP (*Enterocytozoon hepatopenaei*), other enteric pathogens like vibrio and unknown environmental stress. Effective control of pathogenic Vibrios in the bacteriophage treated ponds possibly is the reason for the absence of WFS in these ponds.

Phages and phage-based technologies are promising approaches for the treatment and biocontrol of bacterial diseases, including drug-resistant pathogens.

Conclusion

The widespread acquisition of antibiotic resistance by bacteria necessitates new strategies for combating drug-resistant bacteria. The results of research on bacteriophages, indicating that they can be an alternative means of eliminating pathogens posing a threat to humans and animals, justify its continuation, particularly in view of increasing drug-resistance in bacteria and restrictions on the use of antibiotics. Results of our study has demonstrated the potential of vibrio-specific phages to significantly reduce the impacts of vibrios, with a concomitant positive effect on shrimp survival. Also, this study further demonstrates that use of bacteriophages gives higher benefit to cost when compared to probiotics. Therefore, phage therapy may be a realistic alternative approach for controlling pathogenic bacteria in aquaculture owing to its several advantages over the conventional antibiotics and other methods against pathogenic multiple drug resistant (MDR) bacteria.