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Editors

I.S. Bright Singh
S. Somnath Pai
Rosamma Philip
A. Mohandas

CENTRE FOR FISH DISEASE DIAGNOSIS AND MANAGEMENT
SCHOOL OF ENVIRONMENTAL STUDIES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
FINE ARTS AVENUE, KOCHI 682 016, KERALA, INDIA

Efficacy of gut probionts in enhancing growth in *Penaeus indicus* post larvae

Manpal Sridhar, R. Paul Raj
Physiology, Nutrition and Pathology Division, Central Marine Fisheries Research Institute, PB 1603, Kochi - 682 014, Kerala, India

1. Introduction

Diseases exert heavy economic losses in fish and shrimp culture due to mortality, morbidity, poor product quality and costs associated with chemotherapy. Aquaculturists are, therefore interested in developing cost effective management strategies that would prevent the outbreak and /or reduce the severity of epizootics. The control regimes so far in practice for quelling opportunistic bacteria have centred on vaccination, acidic culture mediums for vibrionaceae and other alkalinophiles (Gatesoupe, 1990) and antibiotics, but replete with drawbacks. Indiscriminate use of broad- spectrum antibiotics may alter the normal gut flora and cause an overgrowth of antibiotic resistant pathogenic bacteria. In agriculture the value of probiotics notably Gram positive bacteria such as *Lactobacillus*, has come to be appreciated as an alternative to antibiotics in disease control strategies (Fuller, Turvey 1971; Parker 1974; Roach, Tannock 1980; Fuller 1978; Smoragiewicz et al., 1993).

The state of the art concerning probiotics is not as advanced in fish and shrimp as it is in homoeothermic vertebrates and many lacunae remain. Lactic acid bacteria have been used in some studies but as their presence in all fish and shrimp is still not ascertained and their use in aquaculture remains controversial. Use of probiotics could be a significant management tool in aquaculture. However, their efficacy depends on the total number of viable cells introduced, their capability to multiply in the intestine and to interact with the intestine flora. In this light, the present study was undertaken to isolate from the intestine of *P. indicus* an organism which would serve as gut probiont on administering orally and to evaluate its efficacy in enhancing growth.

2. Materials and methods

2.1. Isolation of putative probionts from the gut

Penaeus indicus juveniles and adults procured from the wild were sacrificed in batches of five animals each under strictly aseptic conditions after exposure to an overdose of anaesthetic (MS 222,Sigma, Poole, England). The entire gut was aseptically dissected from.each animal, pooled and homogenised with 0.85% saline in glass vials. Following 10-fold dilution, 0.1ml aliquots were plated on ZoBell's marine agar, incubated at 25°C for 48 h and the colonies developed were enumerated. Isolates were identified based following Baumann et.al., 1984.

2.2. In vitro antagonism studies

The isolated strains were screened for antagonism against three fish pathogens viz. *Aeromonas salmonicida, Vibrio anguillarum and V. harveyi.* Pure cultures of the putative probionts and pathogens

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were streaked onto TSA media plates containing 1.0% NaCl. After 48 h incubation, a loop full each of the fresh cultures of both were diluted with 9 ml of 0.85% saline and vortexed. Disruptions in the growth of pathogens were observed by both cross-streaking as well as drop plate technique.

2.3. *Toxicity studies of the putative probionts in vivo*

The bacterial strains, which showed maximum antagonism to the three pathogenic cultures, were tested for toxicity *in vivo* before being selected for the feeding trials. Groups of six *P. indicus* juveniles with an average weight 2.56 ± 0.342 g were maintained in separate groups in 10 litre capacity covered plastic tubs under aeration at an average temperature of 25°C±2. Fresh cultures of each of the selected strains of putative probiotic bacteria were diluted with PBS to yield final concentrations containing 10^7 cells/ml^{-1} and 0.1ml were injected into the shrimp. They were fed a compounded feed and water was exchanged daily and observed for pathological signs and mortality over a period of 14 days. In case of mortalities the shrimps were dissected immediately and after observing for any pathological signs the tail muscle and gut were aseptically plated on TSA plates and microbial flora ascertained after incubation at 25°C for seven days. Surviving shrimp were killed after exposure to an overdose of anaesthetic (MS 222,Sigma, Poole, England) and examined for both external and internal pathological changes.

2.4. *Feed preparation and feeding trials with selected probionts*

Fresh cultures of each of the selected probionts were inoculated into 100ml of TSB containing 1.0% NaCl. After incubation at 25°C for 48 h, each culture was centrifuged at 4°C for 15 min at 10,000 rpm. The supernatant was discarded and the pellet resuspended in 5ml of PBS. Five ml fish oil was added to each and mixed thoroughly in a vortex mixer. One hundred gram compounded feed prepared as per the formulation given in Table1 was added to each and mixed thoroughly in order to obtain evenly coated pellets of each feed containing $5x10^7$cells/g^{-1} of diet. The control feed contained only PBS and oil. The feeds were stored in sterilized conical flasks and viability of the bacterial cells determined periodically.

Late post larvae of *P. indicus* were used for the feeding trials, which were carried out in triplicates for both the probionts and control group with fourteen animals per tub. The experimental set up was the same as described earlier. The animals were fed the probiotic feeds at 20% of their body weight in two divided doses at 10.00 and 16.00 hr daily. Upon termination of the experiment after thirty two days, the length and weight of the animals were measured after which they were sacrificed. The guts of six animals from each group were observed for intestinal colonisation of the probionts.

Table 1. The Percentage composition of compounded feed base used in the experiment for-coating with probionts.

Ingredient	% incorporation
Prawn meal	25
Fish meal	15
Soyabean meal	10
Groundnut oil cake	15
Wheat flour	15
Tapioca	12.5
Vitamins	1.0
Minerals	1.5
Cholesterol	0.5
Soya lecithin	0.5
Oil[a]	4.0
Total	100

[a] *The fish oil and vegetable oil used was in a 1:1 combination.*

2.5. Colonization of the gut by probiotic bacteria

Gut samples were homogenized in 9 ml volumes of physiological saline (PS) in a mortar and pestle under aseptic conditions and 10-fold dilutions were prepared to 10^{-5} in PS, and 0.1 ml volumes were spread onto triplicate plates of tyrptone soy agar supplemented with 1.0% NaCl and incubated for 24-72 h at 30°C. A representative of each colony type was purified by streaking and restreaking on fresh media. Pure cultures were stored at 4°C in agar slants with subculturing every 6-8 weeks and were identified.

2.6. Analytical methods

The proximate composition of the feeds and feed ingredients was determined using standard procedures (AOAC, 1990). Moisture was determined by drying in an oven at 80°C ±2. Total nitrogen content was determined by the microkjeldahl method using the conversion factor 6.25. Lipid in samples was determined by Soxhlet extraction method. Crude fibre was determined by acid (H_2SO_4 1.25N) and alkali (NaOH 1.25N) digestion and washing with acetone. Ash content of samples was estimated by ignition in a muffle furnace at 600°C±5 for 6 h. Carbohydrate content determined as nitrogen free extractives (NFE) was obtained by difference. Data obtained in the feeding experiments was subjected to statistical analysis (Snedecor and Cochran, 1973).

3. Results

Of the thirty two bacterial isolates from gut only six exhibited maximum antagonism to the three fish pathogens. The biochemical characteristics of these strains are given in Table 2. However, only two strains caused no mortality and pathological signs in the injected animals and they were thus selected as putative probionts. These were identified as *Bacillus* and *Micrococcus,* designated as PB and PM respectively. The proximate composition of the control and experimental probiotic feeds are recorded in Table 3. The incorporation of bacterial biomass into the feed base caused an increase in protein particularly in the case of feed PMII (incorporated with *Micrococcus*). Both probiotic feeds recorded good water stability as compared to the control feed, loosing only about 23–25% dry matter at the end of five hours of immersion in water. The results of feeding *P. indicus* post larvae with the two probiotic feeds are given in Table 4.

Table 2. The characteristics of the six probiotic bacteria isolated from the gut and selected for the feeding trials.

Strain		Gram Stain	Mobility	Shape	Pigment	Doubling time (min)
Micrococcus[a]	P1	+	–	Cocci	Yellow	70
Bacillus[a]	P2	+	+	rods	–	69
Bacillus	P3	+	+	rods	–	22
Pseudomonas	P4	–	+	rods	–	48
Pseudomonas	P5	–	+	rods	–	63
Micrococcus	P6	+	–	Cocci	Light yellow	56

[a] *The strains which exhibited maximum antagonism to the pathogens and selected for the feeding experiments.*

Table 3. Proximate composition of control and probiotic incorporated feeds.

Parameter % dry matter basis	Control Feed	Feed PBI [a]	Feed PMII [b]
Dry matter	93.05	93.09	93.18
Moisture	6.95	6.91	6.82
Crude protein	37.84	39.25	43.75
Crude Fat	5.51	5.84	6.16
Crude Ash	12.13	12.31	12.18
Crude Fiber	1.60	2.10	2.02
Acid Insoluble ash	1.15	1.20	1.12
NFE [c]	35.98	33.61	29.07

[a] Feed incorporated with biomass of *Bacillus* Sp. [b] Feed incorporated with biomass of *Micrococcus* sp.
[c] Nitrogen Free Extractives calculated by differnece.

Table 4. Growth performance of *P. indicus* juveniles fed with the control and the two putative probiont coated compounded feeds for a period of 32 days.

Parameters	Control	GROUPS PBI	PMII
Average initial length (cm)	29.46 ± 2.67	27.89 ± 3.10	29.77 ± 2.98
Average initial weight (g)	0.459 ± 0.049	0.612 ± 0.060	0.566 ± 0.059
Average final length (cm)	35.97 ± 3.82	43.015 ± 1.838	47.35 ± 2.195
Average final weight (g)	0.553 ± 0.103	1.130 ± 0.0925	0.909 ± 0.094
% increase in length	20.48	84.47	60.145
% increase in weight	46.54	108.16	82.77
Specific growth rate [a]	0.204	0.846	0.605
Survival (%)	86	96.5	82.5

[a] *Specific growth rate*

The PBI group showed higher increase in weight, specific growth rate ($P<0.05$) and increased survival in comparison to the control. While the PMII group also showed higher increase in weight and specific growth rate ($P < 0.05$) in comparison to the control, the survival rate was lower. Colonization of the gut by *Bacillus* sp. in treatment group PBI was marginally higher at 6.3×10^6 cfu/ shrimp compared to *Micrococcus* sp. fed group PMII which recorded 4.5×10^6 cfu/ shrimp.

4. Discussion

Our results here indicate that *Bacillus* sp. could be beneficial as probiotic to improve shrimp health in culture systems. Comparing the performance of the two groups

of larvae fed with the two selected probiotics, *Bacillus* and *Micrococcus* sp., the response of the larvae fed with the former was overall better in all parameters studied. Moriarity (1998) reported the benefit of adding selected strains of *Bacillus* or probiotic bacteria to control luminescent vibriosis in shrimp ponds in Indonesia. *Penaeus monodon* fed with a probiotic *Bacillus* sp. for 100 days survived (100%) challenge with *Vibrio harveyi* in comparison to only 26% survival in group not fed with the probiotic (Rengpipat et.al.,1998). Other than *Bacillus* sp, lactic acid bacteria and *V. alginolyticus* have also been shown to have probiotic effects in *P. indicus* and Atlantic salmon (Fernandez, 1997; Austin et.al.,1995). Vibrios are shrimp pathogens, and their use as probiotics for protection of these animals should be made with caution.

Mixing the probiotic bacteria with compounded feed did not result in their loss of viability in this study but the possibility of freeze drying the probiotic is worthy of further consideration, as this would allow more efficient application to aquatic animals via bioencapsulation, such as with rotifers or *Artemia*. There is increasing evidence for the potential of probiotics in aquaculture (Douilett and Langdon, 1994). They can be used at length and at a high dose in the feed, in order to prevent digestive disorders and/ or to increase the zootechnical performances (Vanbelle et al., 1989).

This study indicates that long term feeding of probiotics would not cause any harm to the shrimps. It would be interesting to study the vibrio or other shrimp pathogen load in the shrimp body and rearing water when fed with these probiotics. It has been proposed that mucus may serve as a source of nutrients and may enhance colonisation by serving as an initial attachment site for bacteria or as a matrix for permanent bacterial attachment. The mucus layer may also be in some instances an effective barrier, providing protection against penetration by invading microorganisms (Ringo and Gatesoupe, 1998). More detailed study as to the retention time of these organisms in the gut of the animals and whether they exhibit antagonism in vivo would prove extremely useful. Austin et al. (1995) could recover the probiont from the intestine, but not from the stomach, kidney or spleen of salmonids within 1 h of exposure by immersion. Sridhar and Austin (1998) obtained extensive colonization of the gut of rainbow trout (*Oncorhyncus mykiss*, Walbum), by seven strains of probiotic bacteria administered by mixing with a lipid oraliser.

The present study suggests that it is possible to maintain artificially the gut bacterial populations at high level by regular intake with feed. Seeding the gut with these harmless bacteria, which occupy the attachment sites, may prevent infection by pathogenic bacteria. However, further investigations in the form of challenge studies of these probiont reared animals with common fish and shrimp pathogens, and as sources of immunostimulants are warranted.

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