INVESTIGATIONS ON THE EFFECT OF PROBIONTS AS A TOOL AGAINST BACTERIAL INFESTATION IN *Penaeus (Fenneropenaeus) indicus* (H. MILNE EDWARDS) JUVENILES

DISSERTATION SUBMITTED BY

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DEDICATED TO MY BELOVED PARENTS

AND

LOVING SISTERS

CERTIFICATE

Certified that the dissertation entitled **"INVESTIGATIONS ON THE EFFECT OF PRO-BIONTS AS A TOOL AGAINST BACTERIAL INFESTATION IN *Penaeus Fenneropenaeus indicus* (H.MILNE EDWARDS) JUVENILES** is a bonafide record of work done by **Miss AJITHA.S.** under our guidance at the Central Marine Fisheries Research institute during the tenure of her **M.F.Sc.** (Mariculture) Programme of 1995-97 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

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DECLARATION

I hereby declare that this thesis entitled **INVESTIGATIONS ON THE EFFECT OF PROBIONTS AS A TOOL AGAINST BACTERIAL INFESTATION IN *Penaeus (Fenneropenaeus) indicus* (H. MILNE EDWARDS) JUVENILES** is based on my own research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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सारांश

0.985 ± 0.1 ग्रा माध्य भार के पी. इन्डिकस किशोरों को डी, $डी_2$, $डी_3$, और $डी_4$ नाम के एस. क्रिमोरिस, एल. असिडोफिलिस, एल. बलगारिकस - 56 और एल. बलगारिकस-57 समविष्टित आहार दिया था । निरीक्षण प्राणियों को जीवाणुज जैवमाग्र रहित आहार दिया था । इन प्राणियों को प्रति टब में दस के स्टॉकिंग धनत्व में वातन युक्त 17 लिटर जल में पालन किया था । इस प्रकार के 15 टब में उपचार किया था । इन प्राणियों को प्रति दिन 10^6 कोश / मि लि सान्द्रता के जीवाणुज जैवमात्रा, गीले गुंधा हुआ आटा खाद्य में जोडकर शरीर भार के 12 % दर में दिये थे । उपचार किये गये प्राणियों की बढती और अतिजीवितता निरीक्षण प्राणियों की तुलना में अच्छी थी ।

निरीक्षण प्राणियों के रूधिरलसीका प्रोटील में जोन ए में केवल चार बैंड थे जब कि उपचार किये गये प्राणियों में जोन - ए में प्रोटीन बैंडों की संख्या में वृद्धि दिखाई पडी थी और जोन - बी में बैंडों की चौडाई भी अधिक थी । यह अधिकतः एल. असिडोफिलिस खिलायी गयी प्राणियों में देखी गयी थी ।

लाक्टिक आसिड बासिलस से विब्रियो रोगाणु के पात्रे और जीवे चैलेंज अध्ययन चलाया गया था । कोश रहित अधिप्लवी एवं व्यष्टिगत संवर्धों को विब्रियो जातियाँ रोगाणु के साथ संरोपण किया था और बढती में दमन देखा गया था ।

प्रोबियन्ट पर खिलाये गये प्राणियों को तीन दिन से अधिक अवधि ($\times 10^9$ कोश / मि लि) की कोश सान्द्रता में इन्ट्रापेरिटोनियली विब्रियो जाति रोगाणुओं के साथ अतः क्षिप्त करके निरीक्षण करने पर संक्रमण देखा गया था । निरीक्षण जातियों में 80% नश्वरता टिकाई की गयी थी जहाँ उच्च विब्रियो काउन्टस और सेप्टीसीमिया रोग कारण थे । लेकिन प्रयोगार्थ समुह स्वस्थ देखे गये और इनमें 3 दिनों के संक्रमण के बाद कोई रोगलक्षण भी प्रकट नहीं था ।

यकृतग्नाशय और पुच्छ पेशी में नियंत्रण प्राणियों की तुलना में कोई विशेष परिवर्तन नहीं दिखाया पडा ।

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PREFACE

(The family Vibrionacea plays an ambiguous part in marine fish and shellfish as it includes bacterial strains that have been reported to proliferate and cause mass mortalities in semi intensive and intensive culture systems.) Gram negative bacterial septicemias disease or Vibriosis has been observed in captured as well as cultured marine crustaceans exposed to stress where it has resulted in severe mortalities. Systemic infection or septicemis have also been reviewed. The infections are usually caused by Vibrio parahaemolyticus, Vibrio alginolyticus or the odd Aeromonas spp. Although there are many reports of vibriosis among marine crustaceans none have been studied in detail.

The general view is that gram negative organisms that predominate the marine environment are facultative pathogens, part of Ubiquitous indigenous microflora which give rise to Vibriosis, when a portal of entry is effected in a compromised (stressed) host and the defence mechanism cannot cope up with the invasion by eliminating bacteria or limiting growth.

It is obvious that culture methods for marine animals are becoming increasingly complex and more and more dependent on the artificial environment. So, a pressing need for disease control is already developed in hatcheries and grow out systems for the profitable culture both of fin fish and shell fish.

(Among the bacterial disease, vibriosis, is responsible for mortalities of 90% or even greater.) In the culture system

control of this disease has so far been accomplished using chemo-therapeutants and vaccines. A variety of antibiotics have also been used in order to protect the culture organisms from the pathogens. The reckless use of antibiotics in aquaculture has however, posed a threat to the quality of the yield and the efficacy of the organism to develop natural resistance against the pathogens. The use of antibiotics as a growth factor in culture systems has, therefore, been restricted since 1969. Also, the ineffectiveness of most antibiotics when dissolved in sea water, severely limits their usefulness for mariculture purposes as they result only in high environmental pollution.

Immunization, is another prophylactic treatment against vibriosis where killed bacteria is used.There is no specific tool like vaccination against these opportunistic bacteria during the larval stages of the culture organisms as the available treatments are aimed at all Vibrio.

Due to the constraints faced in the use of antibiotics and vaccination in the control of disease in culture system an alternative was proposed in feeding the animals with probionts. Probiotics is a food additive containing lactic acid bacillus or spores. It is stated that these probionts contribute to the internal microbial balance or that they are the natural intestinal bacteria which after oral administration in effective doses are able to establish, themselves in the digestive tract. They eventually colonize to keep or increase the natural flora of the digestive tract; prevent colonization of other pathogenic

organisms and promote optimal utilisation of the feed. (The probionts are likely to release antibiotics, but their deciding advantags over chemicals lies, in the fact, that they work at very low concentrations as well as their medium is not affected by any drugs.)

In the light of the afore mentioned facts it was thought pertinent to carry out a study in order to investigate the effect of probiotics as a tool against bacterial infestation in Penaeus indicus, a commercially important prawn on the Indian context.

The present study was carried out in juvenile P.indicus using four strains of lactic acid bacteria (LAB) with the following objectives:

1. Investigate the effect of LAB additives monitored orally via compounded feed on growth and survival of Penaeus indicus juveniles for a period of 30 days.

2. Assess the possible influence of LAB additives on a few blood parameters in the experimental animals.

3. Challenge study of the probiont fed animals to the pathogens (Vibrio sp) both in vitro and in vivo in order to study their role in disease resistance .

4. Examine histopathological changes, if any, in the hepatopancreas and tissues of the experimental animals.

INTRODUCTION

Today's intensive farming regimes aim at securing the highest yield per unit area within a short time span, with very little concern for the environment. This proves highly stressful to the shrimp, leaving them highly susceptible to infection and disease outbreaks.

Traditionally, farmers have used electrolytes and antibiotics to combat disease and infection, but pressure is mounting for much greater control over the use of antibiotics and thus, farmers are but forced to look for alternative methods for healthier and quicker growth and disease control. In this context probiotics are being considered as suitable alternatives.

Use of probionts in shrimp feeding

Garriquer and Arevelo (1994); Griffith (1995) and Gomezgil (1995) outlined various developments made in the nutritional use of probiotics in developing shrimp of high immunity. The use of international probiotics promote natural resistance of fish to disease and simultaneously accelerate the growth rate .

Very few studies have been carried out on the effect of probiotics in shrimp culture. Mohammed (1995) used live heterotrophic bacteria as 50% replacement to microalgae as diet of *P.monodon* larvae. Survival and growth rate were markedly improved upon feeding *Pseudomonas* and *Micrococcus* species (Mohammed 1990).

Further studies on this aspect by Sridhar and Chandrasekar (1996) evaluated the effects of feeding five strains of bacterial biomass (comprised by two strains of Bacillus, one strain of Pseudomonas and two strains of Micrococcus respectively) to larvae of P.indicus. Both Bacilli BTM-01 and BTM-05 and the micrococcus (BTM-12) promoted survival rates from 64-70%. Groups of P.indicus larvae fed with 100% Micrococcus BTM-12 strain showed good growth, initially which, however declined with advancement. Though complete feeding with Bacillus spp and the Pseudomonas spp yielded poor development with total mortality the study revealed a possibility that bacteria could be used for feeding larval shrimp.

Marine shrimp and wheat bran fermented with B.licheniformis and Beauveria spp. fungi compounded and fed to post larvae of P.indicus at different concentrations for a period of 45 days also showed that probiotics enhanced growth and survival in shrimp (Sridhar and Chandrasekar, 1996). Probionts may provide growth factors and inhibit the proliferation of pathogen and stimulate the non specific immune response (Vanbelle et al., 1989).

Except for the few studies conducted on shrimp regarding this aspect much of the work has been done on teleosts. In teleosts probiotic studies have been done by feeding the animals both directly and indirectly. Indirect feeding is done through live food organisms like artemia nauplii and rotifers.

The treatment of rotifer with antibiotics or feeding them with probionts, lactic acid bacteria and spores of Bacilli toyoi was also proposed (Gatesoupe 1989).

Gatesoupe (1991a) used Bacilli species spores as food additive for rotifer Brachionus plicatilis and used them for the improvement of the bacterial environment and dietary value of larval turbot Scophthalmus maximus.

When live lactic bacteria were given to rotifers together with bakers yeast, vitamins and cod-liver oil the production rate of rotifers and growth rate of turbot (Scophthalmus maximus) were improved (Gatesoupe, 1989). Lactic acid bacteria (Wolter 1986 and Bacillus toyoi (Kozasa, 1988) are known to modify the gut microflora. These LAB cultures when introduced into the food chain improved the survival rate of turbot with more suitable method than antibiotic treatment.

Turbot larvae were fed with rotifers given Acosil, Adjulact and the spores of B.toyoi (toyocerin) and paciflora (Gatesoupe 1989, 1990, 1991 b) and the efficiency of Acosil was also tested on Japanese flounder (Gatesoupe et al.1989) and there was increase in the growth rate of larval fish.

Bogaert et.al. (1988) isolated thirty bacterial strains from the rotifer tanks while studying the effect of probiotics on the growth of rotifers and reported some bacterial strains to repeatedly increase rotifer growth rate. Significant effects were obtained with Enterococcus faecium, a probiotic for

terrestrial organisms. It has been stated that rotifer growth promotion can be induced with Enterococcus faecium.

Use of probionts in Disease resistance

Probiotics being proposed as an alternative to antibiotics, selected concentrated viable counts of lactic acid bacteria often composed of Lacto bacillus, acidophilus and Streptococcus faecium or strains of Bacilli were used at length and at a high dose in feed, in order to prevent digestive disorders and/or to increase zoo-technical performances (Vanbelle et al., 1989).

They also listed the effect of probiotics as - to produce metabolites which inhibit the proliferation of pathogen, or the production of toxins, other metabolities may detoxify and may also stimulate the immunological system of the host,

Use of L.plantarum and L.acidophilis in the nutritional probiotic application inhibiting pathogenic gram positive and gram negative bacteria improved not only nutritional utilisation of feed but also stimulated non specific immune response (Mohammed 1995).

A recent study conducted in Eucador by adding a non pathogenic strain of Vibrio alginoliticus to Penaeus vannamei larval culture media which helped in controlling other pathogenic Vibrio spp (Garriques and Arevalo,1995). When one LAB strain was introduced daily into the enrichment medium of rotifers,fed to turbot (S.maximus) LAB were retrieved in large

amounts in turbot and a significant limitation of larval mortality was observed when turbot were challenged with pathogenic Vibrio (Gatesoupe 1994).

Atlantic salmin (Salmo salar) fry given diets supplemented with fish protein hydrolysate and lactic acid bacteria and then challenged with Aeromonas salmonicida showed unexpectedly high mortality in fish given the diet containing lactic acid bacteria, (Gildberg et.al. 1995).

Bacillus species spore were also used for their probiotic efficiency against bacterial infestation in turbot larvae Scophthalmus maximum (Gatesoupe 1991).

In view of this limited amount of reliable information on the real effect/mode of action of probiotic preparations in shrimp culture, the present study on investigations on the effect of probiotics on the bacterial infestation of P.indicus juveniles was deemed necessary.

MATERIALS AND METHODS

1.1. MICROORGANISMS

Pure strains of LAB Lactobacillus acidophilus NCIM 2285 (ATCC 4963), Streptococcus cremoris NCIM 2285 (ATCC 2402), Lacto bacillus bulgaricus NCIM 2285 (2056) and Lacto bacillus bulgaricus NCIM 2285 (2057) (ATCC 8000), (Hansens strain) were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune.

The cultures were maintained as agar slope cultures using enrichment media (Table 1) as per the composition given by NCIM.

Table 1. Enrichment media for LAB cultures(NCIM)

Nutrient	Level of Incorporation
Glucose	0.5 percent
Lactose	0.5 percent
Liver extract	1.0 percent
Sodium acetate	600 mg
Yeast extract	500 mg
Salt A*	0.5 ml
Salt B*	0.5 ml
Agar	2.0 percent

Salt A and B were prepared as a bulk and kept in the refrigerator as stock solution. The composition of Salt A and B are as follows.

Salt A*	Salt B*
KH_2PO_4 - 25 g	$Mg\ SO_4\ 7H_2O$ - 10 g
KH_2PO_4 - 25 g	Nacl - 500 mg
	$Mn\ SO_4\ 7H_2O$ - 500 mg

Salt A and B were dissolved in distilled water and volume made upto 50 ml separately 0.5 ml of each was taken.

The cultures were subcultured every second day initially for accumulation of biomass and thereafter every weak (Plate 1).

1.2 Production of Bacterial Biomass for Feeding Experiment

The four strains viz. L.acidophilus, S.cremoris, L.bulgaricus 56 and L.bulgaricus 57 were individually and asceptically grown in 10 ml of nutrient broth for 18 hours at room temperature (28+2^{o}C). Five ml of the log phase cultures were then asceptically transferred into 250 ml of the enrichment media and kept in a rotary shaker at 150 rmp for 18 hours at room temperatures (Plate 2). The cells were harvested by centrifugation (Remi R.23 Centrifuge) at 4,000 rpm for 15 minutes and adjusted to a final concentration of 10^{6} cells/ml using sterile physiological saline (0.85% Nacl) after repeated washing with the same and stored at 4^{o}C. This biomass (Plate 3)was used directly i feeding experiments for feeding shrimp after incorporation into the feed mix.

1.3 Biochemical analysis of LAB cultures

About 2 ml of the log phase culture was centrifuged at 4000 rpm for 15 minutes and the pellets were washed in physiological saline 250 ul of a mix containing 10% SDS and 2% mercaptoethanol was added to lyse the cells and vortexed vigorously for 5 minutes. Protein (Lowry et.al., 1951) Fig.1, lipid (Folch et.al., 1957) and reducing sugar (DNS method, Miller, 1959) Fig.2 in the mixtures were estimated. Dry matter was determined by drying the cells to constant weight at 70^{o} $+5^{o}C$ for 24 hours in hot air over (AOAC, 1990).

1.4 Feed preparation

Feed was formulated (Table 2) using the specifications given by New (1989).

The experimental feeds D_1. D_2, D_3 and D_4 were prepared as moist dough every week based on the above mentioned formulation. Daily the 4 individual Lactic acid bacterial (LAB) biomass was incorporated into the preweighed quantity to be fed at a concentration of 10^{6} cells/ml, extruded into strands and fed in two divided doses to the experimental animals at 10 a.m. and 4 p.m. respectively. Feeding was carried out at 12% of the body weight/day.

Feed DO was based on the same formulation but was mixed, pelletised, dried and stored in air tight containers. this feed devoid of bacterial biomass served as feed for the Control Group of animals.

Fig. 1. Standard curve for protein

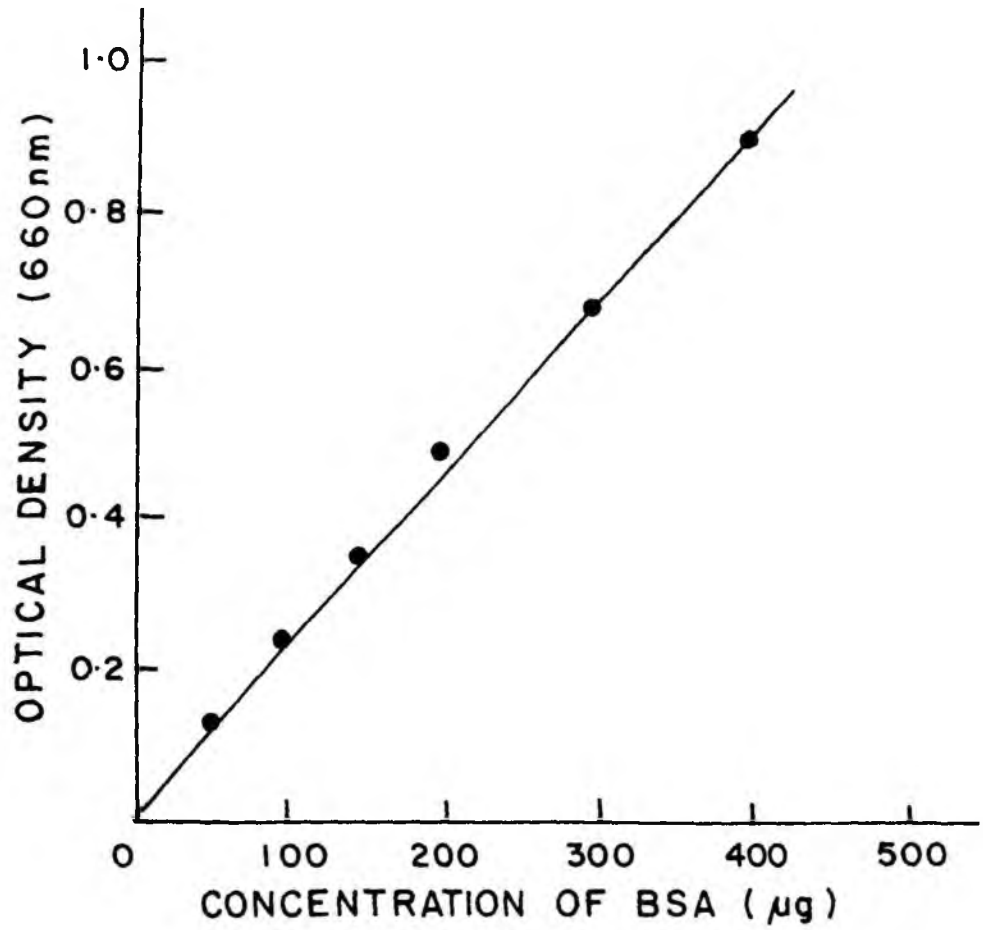
OPTICAL DENSITY (660nm)
1·0
0·8
0·6
0·4
0·2
0
100
200
300
400
500
CONCENTRATION OF BSA (μg)

Fig. 2. Standard curve for glucose

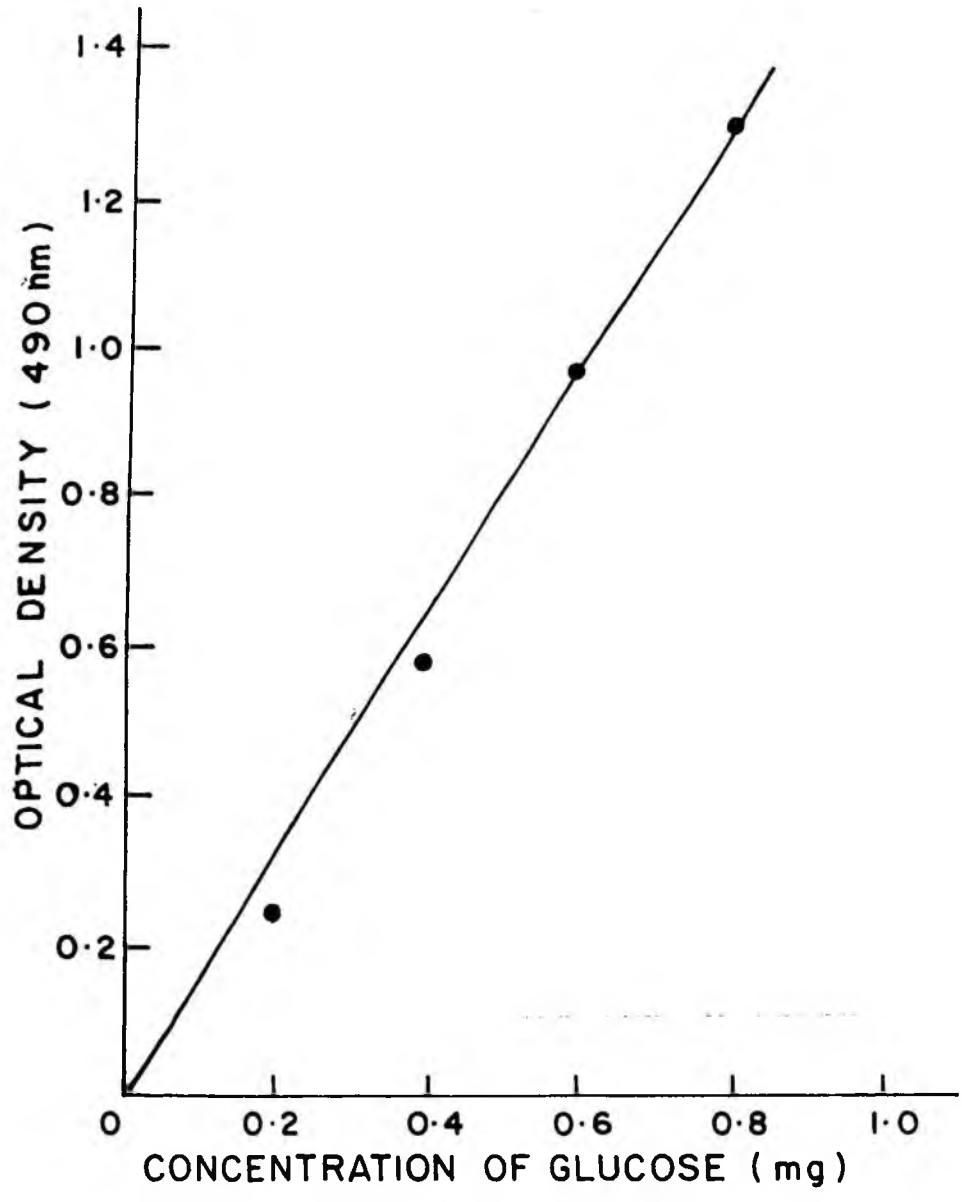
OPTICAL DENSITY (490 nm)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0·2
0·4
0·6
0·8
1·0
CONCENTRATION OF GLUCOSE (mg)

Plate 1. The four LAB cultures viz. *L.acidophilus*, *S.cremoris*, *L.bulgaricus* 56 and *L.bulgaricus* 57 used in the presen study.

Plate 2 : The LAB cultures grown in enrichment media for collection of cell biomass.

Plate 3: The concentrated biomass of the four LAB cultures used for the feeding trials.


S. CAGH
ORS
L. ACID
OPHILUS
L. BULGA
RICUS
56
L. BULG
RICUS
57

Table 2. PERCENTAGE COMPOSITION OF INGREDIENTS USED IN THE FEED FORMULATION

Ingredients	Percentage of Incorporation
Fish meal	15.0
Soybean meal	36.0
Shrimp waste meal	10.0
Wheat flour	20.0
Rice bran	12.0
Fat*	2.0
Binder	2.0
Vitamin mixture**	0.5
Mineral mixture***	0.5
Dicalcium phosphate	1.0
Coated vitamin	0.038

* A combination of 1:1 ratio of cod liver oil and vegetable oil

** As per M.B. New (1987)

*** Stand USP XVII mixture supplied by SISCO chemicals

2.1 Experimental set up

About one hundred and fifty numbers of juveniles of *Penaeus indicus* of average size (length 7,5 ± 1 cm and weight (0.985 ± 0.1 g) were collected from the ponds of the Central Institute of Brackishwater Aquaculture (CIBA) and transported to C.M.F.R.I. in oxygen bags.

Plate 4: A view of the experimental set up for the shrimp feeding trial.

The animals were then segregated and maintained in 15 plastic tubs of 40-50 l capacity arranged in vertical racks (Plate 4). The experiment was carried out in triplicates. Each tub was provided with individual aeration from aerators and covered with nylon nets in order to prevent escape of animals. The animals were acclimatized for a week on the control feed prior to start of experiment.

The sea water used for the experiment was collected from the open sea off Cochin and transported from the place of collection to CMFRI in mobile van and stored in plastic bins.

Prior to use for the experiments the water was sterilized thoroughly. It was first filtered by using bolting silk of 40 U mesh size and then through sterilized absorbent cotton; which was changed every 10 minutes. This filtered water was then irradiated with ultra violet light for half an hour.

The culture system was cleaned every day by siphoning out the feacal matters and feeding waste and one fourth of the water was exchanged with fresh filtered sea water. Complete water exchange was done once in two days.

Physico chemical parameters of the water were monitored for the entire experimental duration and the range for the various parameters is given in Table 3.

Table 3: The range of Physico chemical parameters maintained during the experiments.

Parameter	Range
Salinity	32 - 34 ppt
Oxygen	3 - 4 mg/l
pH	7.5 - 8.5
Temperature	27-29^{o}C

3. Analytical methods

3.1 Proximate composition

Proximate composition of the experimental feed as well as the experimental animals were evaluated as per the standard procedure given by AOAC (1990). Moisture content was determined by drying at 80^{o}C. Total nitrogen content was determined by the Kjeldahl method using the conversion factor 6.25. Lipid in samples was determined by soxhlet extraction method crude fibre was determined by acid (H_2SO_4-1.25%) and alkalie (Na Ol _+1.25%) digestion. Ash content was estimated by ignition in a muffle furnace at 600^{o}C _+ 5^{o}C for 6 hr. Nitrogen Free Extraction (NFE) was obtained by calculation.

3.2 Total plate count of gut microflora and water

Total plate count of the gut microflora of the animals as well as the water samples were carried out as per the standard methods (FDA 1984).

3.2.1. Total plate count of gut microflora

A few animals of the control and experimental groups were dissected and the gut along with the hepatopancreas excised and removed and the contents squeezed out. The gut was then washed repeatedly in 0.85% physiological saline. A suitable aliquot (0.50) of gut tissue pooled was taken and mixed thoroughly. Serial dilution were prepared using sterile saline and suitable dilution were plated on Zobell's marine agar using pour plate technique and after inoculation at room temperature for 12 hr. the developed colonies identified.

3.2.2. Total plate count of sea water

TPC of the water samples were done in order to elucidate the inherent microflora. About 100 ml of the water was taken in a sampling bottle and after thorough mixing 1 ml was pipetted from it and serial dilution carried out using sterile aged sea water. Suitable dilutions were plated using Zobell's marine agar and for 12 hr. the developed colonies identified after incubation at room temperature.

3.3 Challenge study

3.3.1. In vivo challenge to pathogen

After four weeks of feeding with probionts, six *P.indicus* juveniles from each treatment tank were transferred to laboratory. They were stocked in suitable beakers and held under the same conducive condition of the experiment. The shrimps were

challenged with Vibrio spp. Each day two animals from each treatment were given extraperitonial injectious (Volume of 0.01 ml) (Plate 5) of Vibrio sp. (supplied by the Environmental microbiology laboratory, School of environmental Studies, Cochin University)at a concentration of 3×10^9 cells/ml and isolated. This was repeated over three days. A set of animals was also injected daily with buffer to serve as an additional control. The animals were placed under observation and the survival in the control and different treatment groups observed.

3.3.2. In vitro challenge study with pathogen

The four lactic acid bacterium (LAB) were grown for 2 days at $30^{\circ}C$ in standard liquid hydrolysate medium (enrichment medium) and centrifuged at (4000 rpm for 10 minutes)at4 . The cell free supernatent was pH adjusted from 5.7-6.8 which was the original pH of the medium filter sterilized and used as 5, 10 and 20% (U/V).

Tube test- The supernatent collected was used to test the bactericidal and bacterostatic property of the extracellular metabolites produced by the organism .For this 5ml nutrient broth was supplemented with 2ml supernatent prepared as described above and inoculated with nutrient agar slant grown Vibrio. As control nutirent broth alone also was inoculated with the culture. One of the inoculated tubes contaning the mixture of nutrient broth and the culture supernatent served as the test for the sterility of the extract. The preparation was incubated at 28_+0.4$^{\circ}C$ for 48 hrs and turbidity was read as the sign of growth.

Plate 5: Intraperitoneal injections of the experimental shrimp with *Vibrio* spp. pathogen.

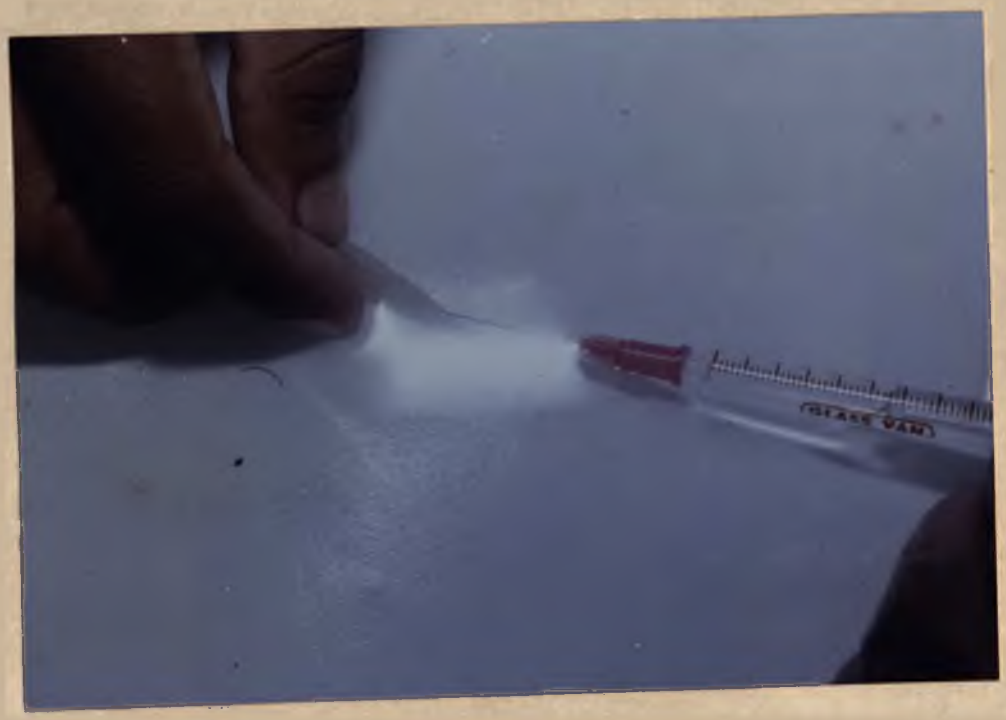

Plate test

The culture of lacto-bacilli and streptococci were streaked on agar plates and inoculated till good growth was obtained. Subsequently the Vibrio spp was streaked perpendicular to the growth of Lactobacilli and streptococci and incubated at 28 + 0.4°C for 48 minutes incubation of growth of Vibrio spp near to the already grown lactobacilli and streptococci was accounted for their antagonistic property.

3.4. Electrophoresis

Electrophoresis of the haemolymph proteins of both the experimental and control groups was undertaken as per the method of Davis (1964) with minor modifications.

Standardisation of the methodology to be adopted in this study was to evolve a suitable standard electrophoresis procedure to obtain better separation and resolution of general protein mixture containing the haemolymph of Penaeus indicus.

Haemolymph preparation:

The animals used for the study were taken and haemolymph extracted by inserting a 22-23 guage needle attached to a 1 ml syringe below the cephalothorax into the heart, the specimen was blotted dry before extraction. The syringe was rinsed with 3% sodium citrate to prevent clotting. The haemolymph was transfered to separate glass tubes.

Gel preparation

Separating gel of 7.5% concentration was used for gel preparation. The separating gels was poured into cassette after mixing with Ammonium per sulphate solution, a few drops of Butanol was added to prevent miniscus formation.

After polymerisation to about 15-30 minutes butanol was poured out and then stacking gel was poured above the polymerised gel. Then cassette was refrigerated for 5 minutes. The circuit of the unit was completed by connecting Whatman (No.1) filter paper between cathodic and anodic tank.

The samples were taken at different dilutions (3, 5 and 10), mixed with sample buffer in 1:1 ratio in a micro titre plate. 65 Ul of sample was carefully loaded using micropipette and current set to 65 MA. When the dye front reaches anodic end of the gel Power supply was cut and gel transferred to staining tray and stain poured over the gel. Gel was stained for 1-1 1/2 hour and then destained it overnight by using a destainer, and the separated protein bands noted. (Plate 6)

3.5 Histopathology of Tissue

In order to discuss any gross anatomical changes in the probiont fed animals in comparison to the control animals, histo-pathology of hepatopancreas and tail muscle tissue was carried out.

The animal used for the investigation were fixed in Davidson's fixative to ensure proper fixation of adult prawn, the

fixative was injected into the body of the specimen at the carapace and abdomen with a hypodermic syringe prior to immersing the whole specimen in the fixative. After fixation for 24 hours the tissue was cut into smaller pieces and transferred to alcohol series of 70%-90% and Absolute alcohol I and II. It was cleared in Xylene and embedded in paraffin wax in hot air oven at 60^{o}C. The prepared blocks were sectioned at 6 u and spread on microslides. Harris haemaloxylene eosin was used to stain the sections after dewaxing with xylene the stained slides was dehydrated in graded alcohol series cleared in xylene and mounted in DPX. The slides examine under a microscope and photographs taken.

RESULTS

Experiments were carried out to evaluate the probiotic effects of four different strains of LAB Viz. *L.acidophilus*, *S.cremoris*, *L.bulgaricus*-56 and *L.bulgaricus*-57 on growth, survival and disease resistance in *P.indicus* and results obtained are presented.

Biochemical Analysis of LAB cultures

The growth and protein yields of the four LAB strains was checked prior to accumulation of biomass for incorporation into feeds (Table 4). *S.cremoris* recorded the shortest and fastest multiplication time (32 min) followed by *L.acidophilus* (46min). Both *L.bulgaricus*-56 and *L.bulgaricus*-57 recorded comparatively longer generation time of 50 min. and 60 minutes respectively. The biochemical characteristics of these four LAB strains are given in Table 5.

S.cremoris recorded the highest protein content (7.62 mg/ml) followed by *L.bulgaricus*-56 (5.10 mg/ml), whereas *L.bulgaricus*-57 showed the lowest protein content (3.49 mg/ml) amongst the four LAB strains. Dry matter content of the four strains ranged from 27.86% (*S.cremoris*) to 19.47% (*L.bulgaricus*-56. *L.acidophilus*, *S.cremoris* and *L.bulgaricus*-57 recorded reducing sugar contents of 409.6, 416.7 and 409.06 mg/ml respectively. *L.bulgaricus*-56 recorded comparatively lower reducing sugar content of 303.13 mg/ml.

5

Table 4: Growth and yield of the four LAB cultures used in the feeding experiments

Sl. No.	Strain Name	NCIM No.	Treatment Number	Optical Density (660 nm)	Protein mg/ml	Generation time (min)
1	L. acidophilus	2285	D_1	0.348	4.20	46
2	S. cremoris	2285	D_2	0.479	7.62	32
3	L. bulgaricus 56	2285	D_3	0.396	5.10	50
4	L. bulgaricus 57	2285	D_4	0.316	3.49	60

Table 5. The Biochemical characteristics of the Lactic Acid Bacterial (LAB) strains used for feeding experiments with *P.indicus* juveniles

Strain Name	Treatment Number	Dry matter (%)	Protein mg/ml	Lipid mg/ml	Reducing sugar mg/ml
L.acidophilus	D_1	22.48	4.20	1.04	409.6
S.cremoris	D_2	27.86	7.62	1.26	416.7
L.bulgaricus 56	D_3	19.47	5.10	1.21	303.13
L.bulgaricus 57	D_4	20.91	3.49	1.11	409.06

Proximate composition of Feed ingredients and Feed

Before formulating the feed all the selected ingredients were subjected to proximate analysis and the results are presented in Table 6.

The control feed D0 was formulated and had a crude protein content of 55.0% . Feeds D_1, D_2, D_3 and D_4 had the four LAB cultures incorporated individually (Table 7) and their proximate composition is as given in Table 8.

Feeding experiments of the LAB biomass incorporated feeds in juveniles of P.indicus

The salient findings with regard to growth performance of the four treatments groups D_1, D_2, D_3 and D_4 fed feed incorporated with L.acidophilus, L.cremoris, L.bulgaricus-56 and L.bulgaricus-57 for a period of four weeks are highlighted in Table 9. All the treatment groups (animals fed diets incorporated with bacterial biomass) showed an increase in weight and length. The highest percentage increase in length (7.5%) was obtained in D_3 (S.cremoris) and lowest increase in length (3.2%) in treatment D_2 (L.acidophilus). The control group D0 recorded an increase in length of only .2.0 % . Maximum increase in weight (77%) was again obtained in treatment group D_2. D_1 and D_3 showed the lowest increase in weight of 28%, (P 0.05). In control animals the increase in weight was 0.21 g corresponding to an increase of 21%.

Table 6: Proximate composition of the ingredients used in diet formulation (% dry matter basis)

Ingredient	Dry matter	Crude protein	Crude lipid	Ash	Crude fibre	*NFE
Soyabean flour	94.76	49.0	9.5	6.5	4.6	25.16
Prawn waste Meal	96.5	66.5	7.5	2.0	16.0	8.00
Fish meal	96.02	58.5	8.0	20.4	1.8	7.32
Rice bran	90.38	10.5	5.0	14.8	15.8	44.8
Wheat flour	98.6	8.75	3.5	0.56	0.05	86.24

* Nitrogen Free Extractives calculated as (100 - % crude protein + crude lipid + ash + crude fibre)

Table 7 . Proximate chemical composition of the experimental (moist) and control feeds used for the feeding experiments with P.indicus juveniles

Dietary Treatment	Percentage					
	Moisture content	Crude* protein	Lipid*	Fibre*	Ash*	NFE**
D0	3.1	55.00	4.000	2.047	11.6	24.253
D_1	26.38	55.042	4.014	2.047	11.6	28.09
D_2	24.96	55.076	4.012	2.047	11.6	28.16
D_3	22.57	55.05	4.012	2.047	11.6	27.03
D_4	24.01	55.035	4.011	2.047	11.6	28.09

* % Dry matter basis

** NFE – Nitrogen Free Extractives calculated as (100 – % crude protein + crude lipid + ash + crude fibre + moisture)

Table 8. Details of the feeding treatments and ingredients inclusion levels adopted in the Feeding Experiment with *P.indicus* juveniles

Sl. No.	Ingredients	Dietary Treatment Code				
		D0	D_1	D_2	D_3	D_4
1	Soybean Flour	36	36	36	36	36
2	Shrimp waste meal	10	10	10	10	10
3	Fish meal	15	15	15	15	15
4	Rice bran	12	12	12	12	12
5	Wheat flour	20	20	20	20	20
	L.acidophilus	-	$\times 10^6$ cells/ ml	-	-	-
	S.cremoris	-	-	$\times 10^6$ cells/ ml	-	-
	L.bulgaricus-56	-	-	-	$\times 10^6$ cells/ ml	-
	L.bulgaricus-57	-	-	-	-	$\times 10^6$ cells/ ml

Table 9. Growth performance of P.indicus juveniles reared on the experimental and control feeds for four weeks.

Growth Parameters	Treatments				
	DO	D_1	D_2	D_3	D_4
Average initial length	6.25	6.25	6.25	6.25	6.25
Average final length (cm)	6.43	6.45	6.7	6.78	6.714
Average initial weight (g)	0.985	0.985	0.985	0.985	0.985
Average final weight (g)	1.195	1.26	1.741	1.258	1.454
Average increase in length (cm)	0.18	0.20	0.45	0.53	0.464
Average increase in weight (g)	0.21	0.275	0.756	0.273	0.469
% increase in length	2.80	3.20	7.2	7.48	7.424
% increase in weight	21.3	27.9	76.75	27.7	47.6
SGR*	0.006	0.008	0.02	0.008	0.013
Survival (%)	98	98	95	92	95

*SGR - Specific Growth Rate

The specific growth rate (SGR) and survival of the control and experimental groups (Table 9) did not show much variation. The highest SGR of 0.02 was observed in group D_2. Highest survival rate of 98% was obtained in D_1 and DO - the control groups. However, survival rate in all treatment groups was above 90%.

The lowest and best FCR of 6.4 was obtained for feed D_2, followed by a value of 15.64 with feed D_4. Feed D_3 and feed D_1 gave feed conversion ratios of 19.93 and 26.39 respectively. The highest (poor) FCR was obtained in the control group (31.3), the group of animals fed a diet devoid of bacterial biomass. Protein efficiency ratios (PER's) values elicited a similar pattern to that of FCR (Table 9). Feed D_2 gave the best PER of 3.8, followed by D_4 and D_3 with values of 3.47 and 2.37 respectively. Comparatively lower PER of 1.69 was obtained in feed D_1. The control feed DO gave a very low PER of 0.08.

After the feeding experiment the animals were sacrificed and carcasses analysed for their proximate composition (Table 10). Protein content in tissues increased in all treatment groups upon feeding the LAB probionts in comparison to the control group DO (39.37%). The highest increase of 55.4% was obtained for group D_2 fed with S.cremoris biomass followed by 48.39% for D_4, 43.75% for D_1 and 42.505% for D_3 respectively.

Lipid content of carcasses also recorded an increase in all treatment group and control group. In control group lipid increased to 4.23% from the initial value of 3.5% and to 5.0%,

Table 10 . Changes in the nutritional quality of the control and treatment groups after feeding probiont feeds for four weeks

Nutrient	Initial	Final				
%		DO	D_1	D_2	D_3	D_4
Dry matter	20	27.3	26.8	32.8	23.3	22.6
Crude protein	38.5	39.375	43.75	55.4	41.565	48.39
Lipid content	4.23	5	5.3	5.12	5.25	3.5
Ash	11.73	17.19	14.32	9	18.78	10.3
Crude fibre	1.6	1.39	4.41	2.2	4.65	5.5
NFE	10.5	5.72	0.55	6.58	7.96	8.67

5.3%, 5.12% and 5.25% for treatment groups D_1, D_2, D_3, and D_4 respectively. Ash carbohydrate and fibre contents failed to reflect any major variatons between the control and experimental groups.

Electrophoretic changes in haemolymph

Haemolymph from both the control and LAB groups D_1, D_2, D_3 and D_4 were subjected to SDS slab gel electrophoresis in order to discern any changes in the haemolymph proteins (Plate 6 and Table 11). A slight change was discerned in the protein bands in the case of haemolymph of animals in group D_1 (L.acidophilus) and group D_2 (S.cremoris) with regard to bands I to VI. Band III was absent in Group D_1 and D_2 while an additional band V (Rm 0.08) was found in group D_2. The other two treatment groups D_3 and D_4 showed a protein profile in (bands I to IV), similar in Relative mobility of 0.01, 0.08, 0.05 and 0.06 respectively to that of the DO control group. The relative mobility of band VII in zone B was much thicker (Rn0.66) in the treatment groups in comparison to 0.60 obtained in the control group of animals.

An additional (band 8) with a relative mobility 0.98 was also present in zone B in the L.acidophilus (treatment D_1) fed animals (Plate 6).

The protein content in the haemolymph of treated animals was determined and compared with that of the control (Table 12) animals. All treatments (D_1, D_2, D_3 and D_4) recorded an increased in protein contents ranging from as high as 15.40 mg.

Table 11 - Electrophoretic pattern of haemolymph of the treated as well as the control animals of *Penaeus indicus* juveniles

Samples	Relative Mobility — Band numbers							
	I	II	III	IV	V	VI	VII	VIII
Control (DO)	0.01	0.03	0.05	0.06	-	0.1	0.60	-
L.acidophilus (D_1)	0.01	0.03	-	0.06	0.08	0.1	0.66	0.98
S.cremoris (D_2)	0.01	0.03	-	0.06	-	0.1	0.66	-
L.bulgaricus -56 (D_3)	0.01	0.03	0.05	0.06	-	0.1	0.66	-
L.bulgaricus -57 (D_4)	0.01	0.05	0.05	0.06	-	0.1	0.66	-

Table 12 : Protein content in haemolymph of the control and LAB fed animals

Samples	Total quantity of haemolymph taken (ml)	Quantity of protein (mg/ml)	% of protein
Control (DO)	0.1	6.80	0.680
L.acidophilus (D_1)	0.1	15.40	1.540
S.cremoris (D_2)	0.1	11.40	1.140
L.bulgaricus-56 (D_3)	0.1	12.00	1.20
L.bulgaricus-57 (D_4)	0.1	13.20	1.320

Plate 6: Electrophoretic separation of the haemolymoph protei
bands of the control juvenile P. indicus

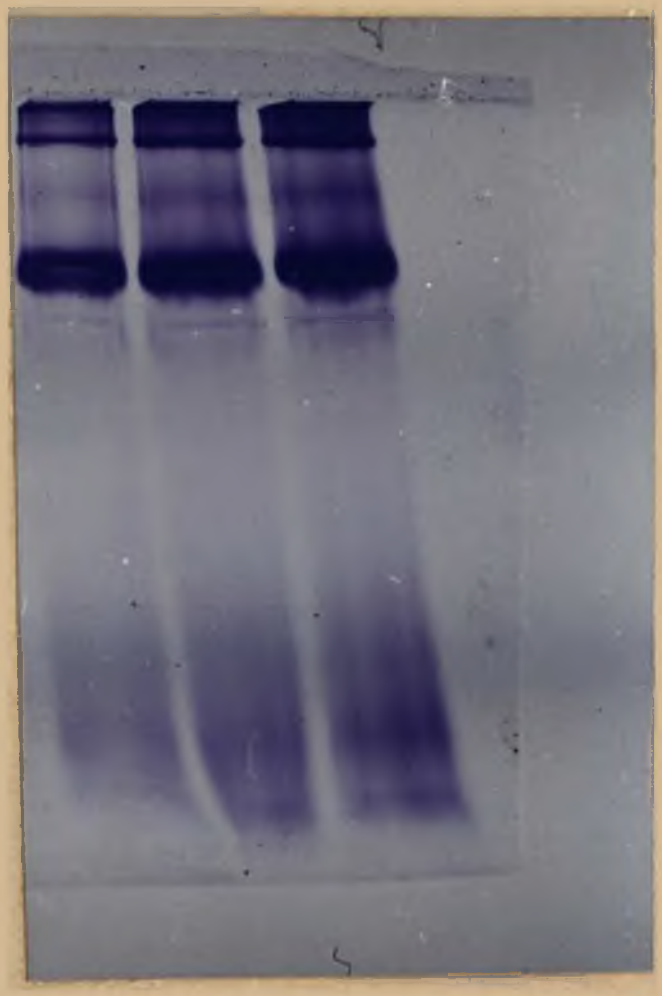

Plate 7: Electrophoretic separation of the haemolymph protein bands of the groups after administering the LAB diets for four weeks.

1 - S.cremoris, 2 - L.acidophilus,

3 - L.bulgaricus 56, 4. L.bulgaricus 57, 5. Control.

For feed D_1 to 11.40 mg in feed D_2. The control group DO recorded a protein content of 6.80 mg only which was much lower than the protein content recorded on all the treatment groups. These results testify to a certain extent that the increase in protein content in haemolymph of the treated group.

Total plate count of gut content

The sea water used in the animal experiments was subjected to TPC. 290 viable counts/ml of Flexibacter spp. enterobactereaceae spp and Pseudomonas spp. formed the dominant bacterial flora in the sea water before subjecting it to sterilization.

The T.P.C. of the gut microflora (Table 13) showed the predominance of LAB in all the treated groups.

In-Vitro and in-vivo challenge study of the LAB culture to Vibrio pathogens

In vitro challange study

The antagonistic properties of the individual Lactic acid bacteria (LAB) to vibrio pathogens was ascertained in tube tests as well as plate test experiments. The supernatent remaining after accumulation of cell biomass of all the four strains of LAB viz. L.acidophilus, S.cremoris, L.bulgaricus-56 and L.bulgaricus -57 was used to test the bactericidal and bacteriostatic property of the extreacellular metabolites produced by the organism (Table 14). All the LAB strains failed to give the turbidity after inoculation with the pathogen indicating the inhibition of the

Table 13 : Bacterial flora of gut wall in the control and experimental animals before challenging them with *Vibrio* pathogen at the end of feeding trial. The bacterial counts are given as colony forming units (CFU)/Shrimp

Treatment number	CFU/Shrimp	Composition
Control (DO)	45	Pseudomonas + Eneterobacteriaceae
L.acidophilus (D_1)	2,10,000	LAB 80%
S.cremoris (D_2)	3,00,000	LAB 95%
L.bulgaricus-56 (D_3)	1,71,000	LAB 100%
L.bulgaricus-57 (D_4)	1,92,000	LAB 92%

Table 14 : The test tube in-vitro challenge study of the pathogen *Vibrio* spp with the experimental group (LAB cultures)

Groups	*L.acidophilus* (D_1)	*S.cremoris* (D_2)	*L.bulgaricus* -56 (D_3)	*L.bulgaricus* -57 (D_4)
Exp.	-	+	-	-
C^+	+	+	+	+
$C_{(s)}$	-	-	-	-

Exp - Experimental group of pathogenic *Vibrio* spp.

C^+ - Control without extract

$C_{(s)}$ - Control without extract, without inoculum

(+) - Indicate turbidity (growth of pathogen)

(-) - Indicate inhibition of growth of pathogen (clear extract)

growth of the pathogen. Turbidity was obtained in all the control tubes inoculated with the LAB cultures alone. The *in-vitro* showed that the LAB probionts produced same metabolites or toxins that inhibited the proliferation of the pathogens.

The result of the tube test were confirmed on plate test. Individual cultures of LAB were streaked on NCIM LAB enrichment agar plates and incubated till good growth was obtained. *Vibrio* spp pathogen were streaked perpendicular to the growth of the LAB culture. Inhibition of the growth of *Vibrio* spp near the areas of LAB confirmed and accounted for their antagonistic property. (Table. 9)

In-vivo challenge studies

Vibrio pathogens diluted to obtain 3 $\times 10^9$ cells were injected (0.01 ml/animal) in to the control and experimental animals at the junction between the last segment and the uropod over a three day period. All the treatment groups D_1, D_2, D_3 and D_4 (animals fed biomass of *L.acidophilus*, *S.cremoris*, *L.bulgaricus*-56 and *L.bulgaricus*-57 did not show any sign of disease even after 120 hrs of injection, while 80% mortality was observed in the case of control DO group (animals not fed bacterial biomass). These results strongly testify the innibition of pathogens and stimulation of the non-specific immune response resulting in the resistance to disease.

Plate 8. Mortality observed in control animals after injecting *Vibrio* sp. pathogen showing septicemia.

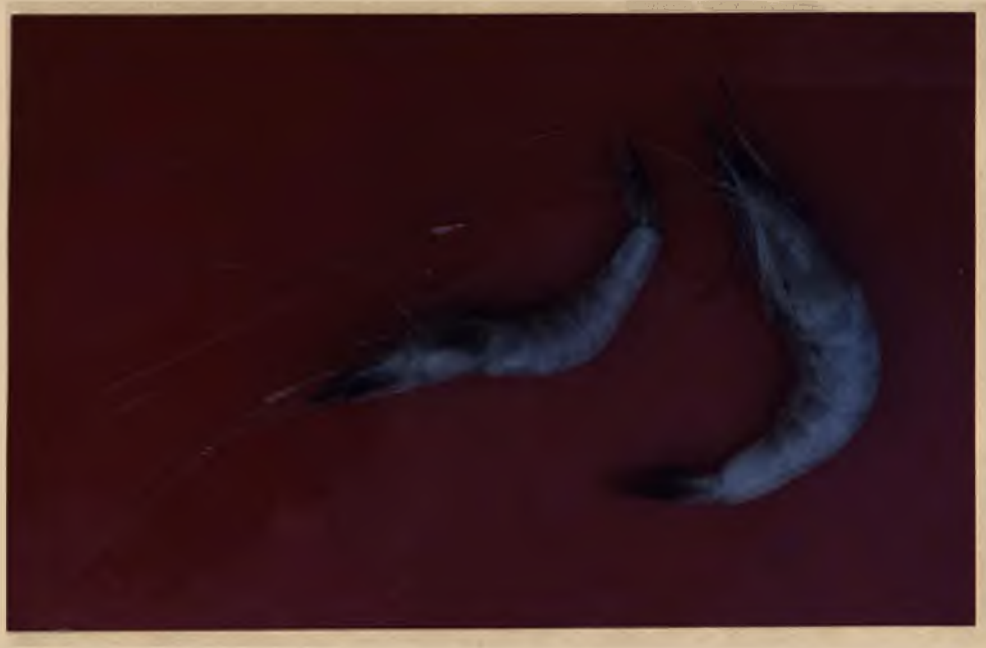

Plate 9. Plate test
i) Control

ii) Showing inhibition of the growth of Vibrio by LAB

Histopathology of Hepatopancreas and Tail muscle

The decapod hepatopancreas, in general, has been composed of four different cell types namely, the E-cells (Embryonalenzellen), the F.cells (Fibrillenzellea), the B-cells (Blazensellen) and the R.cells (Rastzellen). The E-cells, which are generally among the smallest of the hepatopancreatic cells types, are unspecialized cuboidal cells concentrated in the distal portion of the tubules which is the area of proliferation. The F-cells, which appear striated because of an extensively developed rough endoplasmic reticulum, are secretory in function and are present in the mediodistal, medioproximal and proximal portion of the tubules. the B-cells which are secretory and excretory in function are the largest of the hepatopancreatic cell types, seen mainly in the proximal areas of the tubules. The R-cells, the most abundant of the four cell types form about 75% of the total number of cells in a single tubules and are seen in the medio-distal and proximate areas of the tubules (Plate

Exposure of the animals to LAB incorporated feeds failed to elicit any structural variation in the hepatopancreas in any of the treatment groups (D_1, D_2, D_3 and D_4) maintained in the LAB biomass for four weeks.

DISCUSSION

The probiotic role played by LAB cultures in enhancing the growth and disease resistance in juvenile P.indicus via the oral route was evaluated in a series of experiments conducted during this study.

To the best of our knowledge this is in all probaility one of the first works conducted on P.indicus juveniles where the animals have been maintained on feeds incorporated with live LAB biomass. Mohamed (1996) and Sridhar and Chandrasekar (1996) fed larvae of P.monodon and P.indicus respectively with different strains of bacterial biomass and opined that it could very well serve as a partial substitute for microalgae in penaeid shrimp larval rearing. Bacterial biomass offers advantages over conventional plant and animal feed proteins for fish and shrimp nutrition (Tacon, 1990).

Feeding larvae with bacterial biomass alone should poor survival (Mohamed, 1996; Sridhar and Chandrasekar 1996) and this can be attributed to the fact that bacteria lack polyunsaturated fatty acids, sterols and certain aminoacids (Philips, 1984). This was not evident in the present study where the four strains of lactic acid bacteria viz L.acidophilus, S.cremoris, L.bulgaricus 56 and L.bulgaricus 57 recorded a good nutritional profile and any prbable deficiency may have been masked by the supplemental effect of the combination of ingredients used in the compounded feed. Fifty percent of the total 'bio-surface' in

seawater accounted for by bacteria and bacteriovory is widespread among a variety of the larvae of marine organisms. (Azam et al. 1984). Artemia were reared exclusively on a diet of bacteria while Brachionis plicatilis has also been grown with bacteria (Yasuda and Tafa, 1980; Gatesoupe et al. 1989). Intriago and Jones (1993) reported best growth of artemia to preadult stage on a mixture of Flexibacter and algae and concluded that bacteria acted not only as food, but also aided digestion of algae through the presence of exoenzymes. Better growth and feed conversion ratios (FCR) are always obtained on dry pelleted feeds in contrast to moist feeds (New, 1987). Though shrimp were fed the LAB biomass incorporated in moist feeds, the noticeably better growth, PER and FCR obtained in the study provide additional evidence for the conclusion drawn by Intriagoo and Jones (1993).

Carcass protein and lipid increased in all the experimental groups feed with LAB biomass. This increase was maximum in treatment D_2, fed with S.cremoris which had the highest protein content and best nutritional profile among the treatment groups. therefore, it is probable, that apart from other probiotic effects LAB also functions as supplementary feed for shrimp. The level of biochemical constituents in the haemolymph viz proteins (significantly homocyanin), free amino acids, free sugars, total lipids and metallic ions serve as useful indicators of physiological and pathological condition of the particular species. In P.indicus four main components - the slow and fast

hemocyanin, heteroagglutenin and fibrinogen are invariably present along with other simple protein fractions (Laxmilatha, 1991), a maximum numbers of sixteen protein fractions having being recorded. No other similar work has been carried out on haemolymph electrophoretic pattern in order to compare our results on effects of probionts. However, LAB probionts do elicit changes in the haemolymph was confirmed both from the observed changes in protein band pattern as well as the significant increase in haemolymph proteins in all the LAB treated groups in comparison to control. Further investigations on the qualitative assessment of proteins in these bands, would prove the presence of growth factors and/or the stimulation of the nonspecific immune response (Vanbelle et al. 1989) due to probiotics.

It has been shown earlier that growth of fish pathogen Vibrio anguillarum was strongly inhibited by both culture filtrates and viable bacteria of LAB isolated from salmon intestines 1988). There are many reports about the antibacteria activity of LAB, but most against gram positive bacteria (Peard and Desmazeaud, 1992). Lewus et al (1991) have shows the activity against A.hydrophila of 19 LAB strains including Carnibacterium pissicola and L.plantarum. Inhibition of vibrio pathogens obtained both in the test tube culture as well as the plate culture with the fair LAB cultures L.acidophilus, S.cremoris, L.bulgaricus-56, L.bulgaricus-57 in our study is in agreement with those obtained with LAB culture filtrate and by

LAB in mixed culture against A.salomicida (Gildberg et al. 1995).

L.plantarum inhibited the growth of "gut bacteria' in dead fish and decreased the respiration rate of Vibrio spp (Schrlea et al 1980).

Improvement in survival rate of turbot challenged with vibrio pathogen when fed rotifers enriched with LAB is in keeping with one finding of complete survival in all the treatment groups fed with LAB biomass incorporated into moist compounded feed and over 80% mortality in the control animals upon being injected intraperitoneally with vibrio pathogens.

At variance is the study of (Gilberg et al 1995) who observed highest mortality in fish given the diet containing lactic acid bacteria, where as no significant difference was observed between fish given feed supplemented with cod muscle protein and hydrolyzed cod muscle protein.

Besides the production of antimicrobial substances, a great variety of mechanisms have been proposed for the action of probiotics (Montes and Pugh, 1993), eg. competition for adhesion receptore in the intestine, completion free nutrients and immuno stimulation. Further investigations on these lines would throw more insight into the actual mechanisms of probiont action.

The hepatopancreas is one of the most dynamic organs and functions as the main site for digestion and absorption of nutrients, storage of reserve food and excretion (Al-Mohammed and Notl, 1989). The cytology of the decapod hepatopancreas has been

studied extensively by light and electron (microscopy (Gibson and Barner, 1979). In general, 4 types of cells, namely, E-, R-, F- and B cells, have been recognised in most decapods as was also in the present study.

SUMMARY

1. Among the 4 lab cultures used in the present study S. Caemoris recorded the shortest multiplication time (32 min.) and also the higher protein content 7.62 mg/ml. L. acido philis recorded a generation time of 46 min. followed by L. bulgaricus 56 and 57 strain with 50 & 60 min. respectively.

2. The over all best nutrition profile were recorded for S. Caemoris.

3. The experimental diete D1, D2, D3 and D4 showed an increase in weight and length in P. indicus juvenile after feeding for 4 weeks though this increase was not significant (P 0.05) the highest increase in length (7.5%0 and maximum gain in weight 77% was obtained in treatment D2 (The group fed S. Caemoris).

4. The highest specific growth rate (SGR) of .02 was also observed in group D2. Survival rate in all treatment group was above 90%. The highest survival rate of 98% being obtained in D1 and D0 the control.

5. Treatment D2 recorded the best FCR of 6.4 followed by a value of 15.64 with feed D4. Though feeds D3 & D1 recorded slightly higher FCR of 19.93 & 26.3 respectivel. The highest FCR (31.3) was obtained in the control feed. Feed D2 give the best PER of 3.8 followed by D4 and D3 (3.47 & 2.37) respectively).

A comparatively lower PER of 1.69 was obtained in Feed D1. The control feed D0 gave a very low PER of 0.08.

6. Carcass protein of all treated groups increased in comparison to the control. The highest increase of 47.4% was obtained for group D2 fed with S. Cremoris followed by 48.39 %, 43.75% and 42.51% for group D4, D1 and D3 respectively.

Lipid, content of carcasses also recorded an increase in all treatment and control. Ash, Carbohydrate and fibre however failed to reflect any major variation between the control and experimental groups.

7. Variations were discern with regard to the elctrophoretic pattern of haemolymph in the LAB treated groups as compared to control. Band III was absent in group D1 and D2 while an additional band V (Rm. 0.08) was found in Group D2.

The relative mobility of band VII in Zone B was comparatively thicker (Rm. 0.66) In the treatment groups as compared to .66 obtained for the control group.

An additional band (Band VIII Rm. 98) was present in Zone B in L. acidophilus fed animals.

8. All treatments (d1, D2, D3, D4) recorded an increase in protein content ranging from as high as 15.4 mg for feed D1 to 11.40 mg in feed D2. The control group D0 recorded a protein control of 6.80 mg.

9. Invitro tube test and plate test experiments confirmed the bacteriocidal and bacteriostatic property of the extra cellular metabolities produced by L. acidophilus, S. Cremoris, L. bulgaricus 56 & L. bulgaricus 57. While turbidity was obtained in all the control tubes. Tubes inoculated with the pathogen failed to give turbidity.

Individual cultures of LAB treat on agar plates grew well. Vibrio Spp pathogen when streaked perpendicular to this growth recorded inhibition in growth.

10. Intraperitoneal infections of vibrio pathogens 3×10^9 cells failed to develop signs of disease even after 120 hours of injection. 80% mortality was recorded in the case of control D. Group (animals not fed bacterial biomass within 24 hrs of inoculation.

11. TPC of gut microflora revealed localisation of LAB bio mass through out the gut of the experimental animal.

12. Histopathology of hepatopancreas and tail muscle of the animals exposed to LAB incorporated diete failed to elicite any structural variation in comparison to controll.

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