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Probiotic Effects of Lactic Acid Bacteria Against *Vibrio Alginolyticus* in *Penaeus* (Fenneropenaeus) *Indicus* (H. Milne Edwards)

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Abstract

Cell free extracts of four strains of Lactic acid bacteria (LAB) *viz. Lactobacillus. acidophilus, Streptococcus.cremoris, Lactobacillus bulgaricus* –56 and *Lactobacillus bulgaricus* –57 inhibited growth of *Vibrio alginolyticus* in nutrient broth. The antagonism of LAB to *Vibrio alginolyticus* was further confirmed by streak plating wherein suppression of growth of *Vibrio* was obtained.

Juveniles of *Penaeus indicus* (average weight 0.985 ± 0.1 g) on administering orally a moist feed base containing 5×10^6 cells·g of the four LAB probionts for a period of four weeks showed better survival (56 to 72%) when challenged with *V. alginolyticus* by intra-muscular injection of 0.1 ml containing 3×10^9 cells·ml. Animals maintained on a diet devoid of bacterial biomass exhibited 80% mortality. No external or internal pathological changes were observed in shrimp fed with the LAB incorporated diets. Results showed inhibition of *V. alginolyticus* by LAB and stimulation of the non-specific immune response resulting in resistance to disease in the shrimp fed on LAB incorporated diets.

Introduction

The rapid growth of the penaeid shrimp culture industry has been accompanied by an increased awareness of the negative impact of disease. Reports of infections and disease caused by *Vibrios* have been by far the

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most numerous of the reported bacterial agents of penaeid shrimp and reported to constitute the majority of bacteria present in the normal microflora of cultured and wild penaeid shrimp (Yasuda and Kitao 1980; Sakata and Taruno 1987; Dempsey et al. 1989; Hameed,1993; Gomez-Gil et al. 1998; Leano et al. 1998; Singh et al. 1998). These opportunistic pathogenic microbes apparently establish lethal infections as a result of other primary conditions that might include other infectious diseases, nutritional diseases, extreme environmental stress and wounds. Infections by these bacteria display massive colonization of the appendages and foregut followed by infection of the mid gut, hepatopancreas and a terminal septicemia.

In addition to good husbandry and culture conditions, Vibriosis is traditionally controlled with chemotherapeutic agents (Mohney et al.,1989). Frequent use of chemotherapeutic agents, especially antibiotics, leads to the emergence of resistant strains (Akoki 1975) pathogenic to the animals. Vaccinations to prevent infections have been successful in laboratory scale but yet to be proved under field conditions.

An alternative prophylactic treatment would be to support the natural non-specific host microbial and therapeutic defense mechanism by administration of live bacteria with demonstrable inhibitory effects upon pathogens as probiotics. This concept has already been proved to be successful in terrestrial animals and humans (Conway 1989) and is being tried in aquaculture. It has been shown that the alimentary tract of penaeids is a congenial environment for vibrios to multiply and, activation of any stress factors in the culture system may make the animal susceptible to the invasion of pathogenic strains of the genus (Singh et al. 1998). Therefore the concept of altering this microbial flora dominated with Vibrios by the application of an antagonistic probiont sounds promising.

Therefore a study was undertaken to elucidate the antagonistic property of some LAB probionts on *Vibrio alginolyticus in vitro*. This along with the immuno stimulatory property were further confirmed by *in vivo* challenges of juveniles of commercially important shrimp *P. indicus* after feeding them with biomass incorporated diets for four weeks

Materials and Methods

Microorganisms

In comparison to the wealth of information available on lactic acid bacteria the most commonly used pobiotic organisms in endothermic animals, only few studies demonstrate the use of the same in aquaculture. Thus for the present study four commonly encountered LAB strains viz.*Lactobacillus acidophilus* NCIM 2285, *Streptococcus cremoris* NCIM 2285, *Lactobacillus bulgaricus* NCIM 2285(2056)and *Lactobacillus bulgaricus* NCIM 2285 (2057) Hansens strain obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India were thus used as the probiotic organism. They were revived on NCIM enrichment

media containing glucose, lactose, liver extract yeast extract and salts (pH 7.6) and maintained as slant cultures on MRS agar and subcultured every week. The authenticity of the cultures was verified by subculturing and identification based on their morphological and biochemical characteristics (Bergey's Manual of Systematic Bacteriology 1989). Pure cultures were stored at 4°C in agar slants with subculturing every 3 to 4 weeks. Pure culture of *V. alginolyticus* obtained from the collection maintained at the Microbiology Laboratory, Centre for Fish Disease Diagnosis and Management, School of Environmental Studies, Cochin University of Science and Technology was used as the test organism. The cultures were subcultured every week and stored at 4°C as TSA slants containing 1.0% sodium chloride.

Production of bacterial biomass

L. acidophilus, S. cremoris, L. bulgaricus –56 and *L. bulgaricus* –57 were grown aseptically in 10 ml of nutrient broth for 24 hr at room temperature (28 ± 2°C). Five ml of log phase culture was then transferred under aseptic conditions into 250 ml of MRS broth and placed on a rotary shaker at 150 rpm for 24 hr at 28 ± 2°C. The cells of each strain were harvested separately by centrifuging at 10, 000 rpm under aseptic conditions for 15 min at 4°C. The supernatants were saved and used for testing *in vitro* the antagonistic property of *vibrio* while the accumulated cell biomass was dispensed in 5 ml of PBS in individual sterile glass vials and used for incorporating in feed.

In vitro experiments

The cell free supernatant saved after sedimentation of biomass was filter sterilized through Celtron filters having a pore size of 0.2 mm (Merck) and pH adjusted to 6.8. Five ml replicates of nutrient broth were individually supplemented with 2 ml of each of the individual LAB strain supernatants. The tubes were then inoculated with freshly grown culture of *V. alginolyticus* and incubated at 28 ± 2°C for 48 hr and growth was recorded by measuring the optical density at 540 nm. Control tubes comprised of nutrient broth inoculated with *alginolyticus* alone.

All the four LAB strains were streaked on Tryptone Soya Agar (TSA) plates containing 1.0% sodium chloride and incubated at 28 ± 2°C for 48 hr. Freshly grown culture of *alginolyticus* was streaked perpendicular to this growth and after incubation at 28 ± 2°C observed for antagonism.

Feed preparation

A moist compounded feed base was prepared fresh every week as per the specification of New (1989). A preweighed quantity of freshly extracted fish oil (sardine) procured from the extraction mill was added to each of the individual LAB biomass and the whole remixed thoroughly over a vortex mixer before being incorporated into the feed to give a final concentration of

5×10^6 cells·g of diet. The control feed contained only PBS and oil. The feeds were stored at 4°C and viability of the bacterial cells determined at regular intervals of 24 hr by standard plate count using MRS agar.

Feeding experiments

Juveniles of *P. indicus* averaging 0.985 ± 0.1 g in weight and 7.5 ± 1 cm in length, were procured from the ponds of the Central Institute of Brackishwater Aquaculture, Narakkal. They were transported to the laboratory in oxygen bags and acclimatized for a week in 500 l capacity fiber glass tanks supplied with aeration at 28 ± 2°C prior to commencement of the experiment. The shrimp were randomly segregated into 15 groups so that three replicates were available for each of the four treatments and the control groups. The animals were maintained in plastic tubs containing 40 l of filtered and uv light irradiated seawater. They were maintained on a pelleted diet at 12% of body weight per day in two divided doses at 10 and 16 hr daily. The physicochemical parameters of the rearing water were found to be remaining within a range of 32 ± 2‰ salinity; 4.5 ± 0.5 mg·l oxygen; 8 ± 0.5 pH and 28 ± 1°C temperature during the entire experiment. The feeding experiment was carried out for 4 weeks before the animals were challenged with the pathogen in the form of intra-muscular injections.

In vivo challenge experiments

After 4 weeks of feeding with the respective LAB probionts, 15 shrimps from each of the treatments were injected with 0.1 ml of an aliquot of V. *alginolyticus* containing 3×10^9 cells·ml. This infective dose was selected based on the results of some of our associated studies on diseases in penaeid shrimp. Subsequently they were maintained in segregation but under the same experimental conditions being fed with a compounded pelleted feed every second day. Animals were observed for a week for signs of vibriosis and morbidity. At the end of seven days surviving animals of each inoculated group were sacrificed and hepato pancreas and tail muscle dissected out under aseptic conditions. Serial dilutions were carried out and were plated onto Trypticase soya agar (TSA) plates containing 1.0% sodium chloride and incubated at 28 ± 2°C. Ten colonies were isolated randomly and were identified. The remaining uninoculated animals from each group were sacrificed, and hepato pancreas and tail muscle were dissected out and processed for histological observations.

Analytical methods

Proximate composition of the feed ingredients and experimental feeds was evaluated as per AOAC (1990). Total plate count (TPC) of gut micro flora, feed and water was carried out as per standard methods (APHA 1976). Histopathological observations were carried out as per Bell & Lightner (1991).

Results

The antagonism of the four LAB strains *viz. L. acidophilus, S. cremoris, L. bulgaricus* –56 and *L. bulgaricus* –57 to *V. alginolyticus* was ascertained by tube tests as well as by cross streaking on TSA plates (Plate I).Nutrient broth containing cell free supernatants of the four strains failed to record turbidity after inoculating with the pathogen and incubating for 72 h implying inhibition (Fig 1). Turbidity measured as optical density was obtained in all the control tubes while tubes containing the LAB supernatants recorded low optical densities of < 0.2. The above tests demonstrated the production of antagonistic principles by the lactic acid bacteria which inhibited *V. alginolyticus in vitro.*

Feeds D_1, D_2, D_3 and D_4 incorporated with biomass of the four LAB strains *viz.. L. acidophilus, S. cremoris, L. bulgaricus* –56 and *L. bulgaricus* –57 respectively were readily consumed by the experimental shrimp throughout the experimental period. Their proximate composition showed them to have a protein content ranging between 55 to 55.10%, lipid to be ranging between 4 to 4.16%, fibre 2.02 to 2.10% and ash 11 to11.67% all of which met the nutritional requirements specified for shrimp. Growth and feed conversion ratios were superior in these four groups of feed incorporated with lactic acid bacteria in comparison to the group of shrimp fed the diet devoid of bacterial biomass (DO). The viability of the cells as determined every second day showed that the bacteria in the feeds remained viable up to 6 days at 4°C, the duration for which every batch of feed was stored at 4°C in the present study.

Intra-muscular injections of 0.1 ml of an inoculum containing 3×10^9 cells·ml of *V. alginolyticus* recorded low mortality rate ranging between 20% for D2 (the group fed *S. cremoris)* and 40% for D4 (the group fed *L. bulgaricus* –57) during a period of 6 days after the challenge (Fig 2). The group of shrimp maintained on a feed devoid of bacterial biomass (DO) recorded high mortality (80%) within 48 h of challenge with *V. alginolyticus*. The control group of animals exhibited extensive colonization of the pathogen with the foregut and midgut regions as observed upon plating and in few specimens septicemia was observed at the site of injection. Though TPC of the homogenates of the hepato pancreas and tail muscle regions of the surviving animals of the treatment groups showed colonization of *V. alginolyticus* the count was very low compared to those obtained in group DO (Table 1).

Administration of LAB incorporated feeds to *P. indicus* juveniles for four weeks, however failed to elicit any structural variations in the hepato pancreas and tail muscle regions.

Discussion

The use of probionts as feed additives has been preferred over that of antibiotics as they do not exhibit any of the undesirable effects associated in

Table 1. Colonization of the hepatopancreas and tail muscle regions by *Vibrio spp*.in the experimental and control of *P. indicus* juveniles (average of three replicates).

Feed number	Tail muscle (colony forming units·g)	Hepatopancreas
DO	6 X 10^5	5 X 10^3
DI	2 X 10^4	1 X 10^4
D2	1 X 10^3	3 X 10^4
D3	2 X 10^4	1 X 10^3
D4	1 X 10^4	2 X 10^4

the use of antibiotics *viz.* toxicity, allergy, residues in food, bacterial drug resistance and indiscriminate suppression of intestinal microflora. Metchnikkoff (1907) worked with Bulgarian bacillus, an organism closely related to lactobacillus starter of yoghurt (*L. delbreuckii* subspecies *bulgaricus*), and to this day lactobacilli have remained the most commonly used probiotic organisms.

However, in comparison to the wealth of information available on the LAB in endothermic animals, only few studies demonstrate the use of the same in aquaculture. The first reported study by Schrøder et al. (1980)

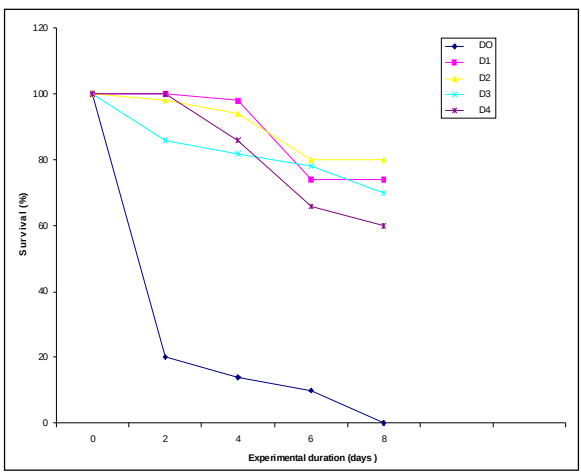

Fig. 1. Growth rate of the *in vitro* test tube experiments

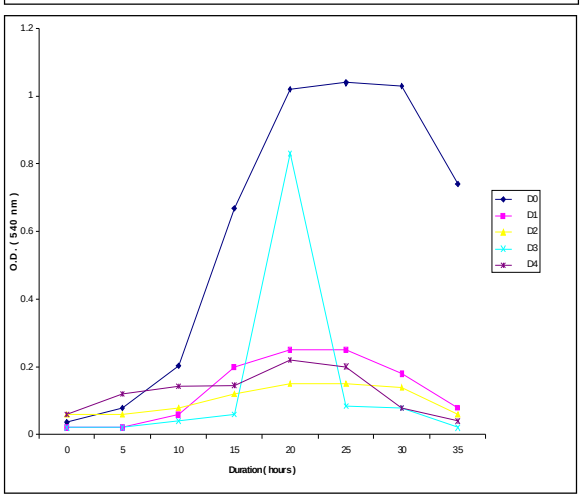

Fig. 2. Survival of *P. indicus* juveniles challenged with *V. alginolyticus*

showed that *L. plantarum* isolated from Saithe, produced inhibitors against *Vibrio sp.* when the lactobacillus was grown in the presence of culture filtrate of *Bacillus thurigiensis.* However, there was no inhibitory effect without the culture filtrate showing the limit of such *in vitro* antagonism test.

Growth of fish pathogen *V. anguillarum* was strongly inhibited by both culture filterates and viable bacteria of LAB isolated from salmon intestines (Strøm 1988). An opportunistic strain of Vibrio spp. was reported to cause mass mortality of turbot larvae, scophthalmus maximus, during the period of feeding on rotifers. The treatment of rotifers with antibiotics or feeding them with probiotics (lactic bacteria) and spores of Bacillus toyoi, was proposed. Although the feeding of probiotics did not improve the survival rate of turbot, their mean weight increased (Gatesoupe,1991). Though there are many reports about the antibacterial activity of LAB, most are against Gram positive bacteria (Piard and Desmazeaud 1992). Lewus et.al. (1991) showed the activity against *A. hydrophila* of 19 LAB strains including *Carnobacterium piscicola* and *L. plantarum*. In the present study the role of LAB probionts orally administered via a moist feed base in suppressing disease and enhancing resistance to pathogens in juvenile *P. indicus* was evaluated *in vitro* as well as *in vivo* challenge experiments.

Inhibition of *vibrio* by the four LAB cultures such as *L. acidophilus, S. cremoris, L. bulgaricus* –56 and *L. bulgaricus* –57 respectively obtained in this study is in agreement with those obtained with LAB culture filtrate and by LAB in mixed culture against *A. salmonicida* (Gildberg et al. 1995).

Very few challenge studies have been carried out so far in aquatic animals. The *Lactobacillus/Carnobacterium* strain isolated from rotifers increased the resistance of turbot larvae against a pathogenic *Vibrio sp.* at day 9. The total viable cell count of LAB had a decisive effect on survival rate, of turbot larvae and the optimum was between 10^7 and 2 X 10^7 colony forming units daily added per ml of the enrichment medium (Gatesoupe 1994). The addition of freeze-dried *C. divergens* to compounded feed did not improve the resistance of salmon fry challenged against pathogenic *A. hydrophila* (Gildberg et al. 1995). However, a similar dietary addition reduced the mortality rate of Atlantic cod fry when challenged against *V. anguillarum* (Gildberg et al. 1997).

The effect of LAB in increasing disease resistance to *Vibrio* pathogens was tested experimentally in challenge studies by Griffith (1995) and Gomezgil (1995) who reported on the beneficial effects of nutritional probiotics in developing shrimp of high immunity. In this study also high percent survival with no external disease manifestations were detected in LAB fed *P. indicus* juveniles on challenging with intra-muscular injections of *V. alginolyticus,* whereas 80% mortality was obtained in animals maintained on feed devoid of LAB probiotic. The studies of Garriques and Arevelo (1995) also agree with these observations and recommend the use of probiotics in increasing the disease resistance of animals. Austin et al (1995) challenged Atlantic salmon of 25 g average weight with a dose of 2xLD 50 of *Vibrio harveyi* after feeding a probiotic feed for 7 days and reported 56% mortality in the control group as compared to 24% mortality in the probiotic fed

group. The results of the present study also clearly demonstrate the beneficial effects of LAB administered through feed on growth as well as resistance to diseases.

Bacillus S11 bacterium isolated from black tiger shrimp habitats was added to shrimp feed as a probiotic in three forms: fresh cells, fresh cells in normal saline solution, and a lyophilized form (Rengpipat et.al. 1998). After a 100-day feeding trial with probiotic supplemented and nonsupplemented (control) feeds, *P. monodon* (from PL30) exhibited no significant difference ($p>0.05$) in growth, survival nor external appearance between all three probiotic treatments, but significant differences ($p<0.05$) occurred between probiotic and control groups. After challenging shrimps with a shrimp pathogen, *V. harveyi*, by immersion for 10 days, all probiotic treatment groups had 100% survival; whereas the control group had only 26% survival. In addition, the control group had an unhealthy external appearance, and deformed texture of the hepatopancreas and intestine, while treatment group shrimp appeared healthy and normal. Probionts may therefore be modifying the intestinal microflora of prawns and providing immuno stimulants which stimulate the nonspecific immune mechanism (Vanbelle et al. 1989).

Besides production of antimicrobial substances, a great variety of mechanisms have been proposed for the action of probiotics eg. competition for adhesion receptors in the intestine, competition for nutrients and immuno stimulation. Further investigations on these lines would throw more light into the actual mechanism of probiotic action in shrimp.

Conclusion

It appears that LAB do offer ample scope as probionts showing antagonism towards *Vibrio* pathogens. As these probionts were able to suppress *Vibrio* growth *in vitro* and *in vivo,* it can be hypothesized that they have the ability to colonize the gastrointestinal tract of shrimp, which however, merits further confirmation. Consequently, they may prove to be suitable candidates for oral administration to shrimp, in commercial ventures to improve health and to protect them from *Vibrio* infections.

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References

A.O.A.C. 1990. Official methods of analysis of A.O.A.C., Vol 1, 15th edn. K. Helrich (ed). Association of Analytical Chemists, Inc., Arlington. VA: 684.

Aoki, T.1975. Effects of chmotherapeutics on bacterial ecology in the water of ponds and the intestinal tracts of cultured fish, Ayu (*Plecoglossus altivelis)* Japanese Journal of Microbiology 19:7-12.

APHA.1976. Compendium of methods for the microbiological examination of foods. (Speck, M. L. ed.) American Public Health Association, New York, 701 p.

Austin. B., L.F. Stuckey., P.A.W.Robertson, I. Effendi and Griffitth, D.R.W. 1995. A probiotic strain of *Vibro alginolyticus* effective in reducing disease caused by *Aeromonas salmonicida, Vibro anguillarum and Vibrio ordalii.* Journal of Fish Diseases 18, 93 – 96.

Conway, L.C. 1989.*Lactobacilli*: fact and fiction,p 263-282.In: The regulatory and protective role of the normal microflora, R.Grubb, T.Midtvedt. and E.Norin(eds). Macmillan Press Ltd., London.

Culling, C.F.A., R.T. Allison, and W.T. Barr.1985.Cellular Pathology Technique.VI Edn, Butterworth,London,pp.391.

Garriques, D. and G. Arevalo.1995. An evaluation of the production and use off five bacterial isolates to manipulate production of *P. vannamei* post larvae in Eucador. World Aquaculture meeting, 1-14.

Gomezgil, R. 1995.The use of bacteria as probiotics in shrimp larviculture. Larvi 95, Fish and shellfish larviculture symposium, Belgium. Pp 479-480.

Griffith, D.R.W. 1995.Microbiology and role of the probiotics in Eucadorian shrimp hatcheries. Larvi95, Fish and shellfish larviculture symposium, Belgium. Pp 478-481.

Gildberg, A.A., J. Johansen and J. Bøgwald. 1995.Growth and survival of Atlantic salmon (*Salmo salar) fry* given diets supplemented with fish protein hydrolysate and lactic acid bacteria during a challenge trial with *Aeromonas salmonicida.* Aquaculture 138:23-34.

Gildberg, A., H. Mikkelsen, E. Sandaker and E. Ringø.1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua).*Hydrobiologia, 352,279-285.

Gatesoupe,-F.J. 1991. Bacillus sp. spores: A new tool against early bacterial infection in turbot larvae, Scophthalmus maximus Larvi-'-91. Lavens,-P.;Sorgeloos,-P.;Jaspers,-E.;Ollevier,-F. no. 15 pp. 409-411.

Gatesoupe, F.J. 1994.Lactic acid bacteria increase the resistansc of Turbot larvae,*Scopthalmus maximus*,against pathogenic vibrio. Aquatic living resources. 7:277-282.

Lewus, C.B., A. Kaiser and T.J. Montville. 1991. Inhibition of food –borne bacterial pathogens by bacterial pathogens by bacteriocincins from lactic acid bacteria isolated from meat. Applied Environmental Microbiology 57:1683-1688.

Mohney, L.L., D.V. Lightner and R.R. Williams. 1990. Bioencapsulation of therapeutic quantities of the antibacterial Romet-30 in nauplii of the brine shrimp *Artemia* and in the nematode *Panagrellus redivivius*. Journal of the World Aquaculture.Society 21:186-191.

Piard, J.C. and M. Desmazeaud. 1992. Inhibiting factor produced by lactic acid bacteria.2.Bacteriocins and other antibacterial substances. Lact., 72: 113-142.

Rengpipat,-S., W Phianphak., S. Piyatiratitivorakul. and P. Menasveta. 1998.Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167(3-4): 301-313

Schrøder,K., E. Clausen, A.M. Sandberg and J. Raa. 1980.Psychrotrophic *Lactobacillus plantarum* from fish and it's ability to produce antibiotic substances. In:Advances in Fish Science and Technology, Connell,J.J.(Ed) Fishing News Books, Farnham, Surrey, England, pp 480-483.

Singh, I.S.B., P. Lakshmanaperumalsamy and D. Chandramohan. 1998. Bacterial flora of pond reared *Penaeus indicus* (Milne Edwards). Journal of Aquaculture in theTropics13(2):133-142.

Strøm, E.1988. Melkesyrebakterier i fisketarm.Isolasjon karakterisering og egenskaper. MSc thesis. The Norwegian College of Fishery Science. 88 p.

Yasuda, K. and T. Kitao. 1980. Bacterial flora in the digestive tract of prawns, *Penaeus japonicus* Bate. Aquaculture. 19:229-234.

Sakata,T., N. Taruna. 1987. Ecological studies on microflora of digestive tract of prawn *Penaeus japonicus* Bate. Aquaculture. 35:147-151.

Dempsey, A.C., C.L. Kitting and R.A. Rosson. 1989. Bacterial variability among individual penaeid shrimp digestive tracts. Crustaceana 56(3): 267-278.

Hameed, A.S.S. 1993. A study of the aerobic heterotrophic bacterial flora of hatchery –reared eggs, larvae and post-larvae of *Penaeus indicus*. Aquaculture 117: 195-204.

Gomez-Gil, B.L. Tron-Mayen, A. Roque, J.F. Turnbull, V. Inglis and A.L.Guerra-Flores. 1998. Species of vibrio isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei*. Aquaculture 163:1-9.

Leano, E.M., C.R. Lavilla- Pitogo and M.G. Paner. 1998. Bacterial flora in the hepatopancreas of pond –reared *Penaeus monodon* juveniles with luminous vibriosis. Aquaculture 164: 367-374

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