

Selection of Probiotics for Shrimp and Crab Hatcheries

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ABSTRACT

A study aimed at obtaining a biological control agent against bacterial diseases, specifically luminescent vibriosis, of hatchery-reared shrimps and crabs was done to find an alternative for chemotherapy as a disease prevention and control method. Bacteria were isolated from crustacean rearing environments where luminescent vibriosis was not observed, from natural food, and from various feed ingredients. From hundreds of purified strains, 80 bacterial isolates were tested in one-on-one mixed cultures in seawater for their ability to suppress the growth of luminescent *Vibrio harveyi*. Of the 10 isolates exhibiting that capability, two strains were further studied: C1 from *Chlorella* culture and P9 from a commercial probiotic preparation. However, due to the indigenous nature of C1 strain from the unicellular alga *Chlorella* sp. and the ease in distinguishing it from other bacteria owing to its colony morphology, most tests were done on C1 strain. To determine the suitability of C1, and to some extent P9, as biocontrol bacteria, their pathogenicity against crab larvae and shrimp postlarvae, and their ability to become associated or incorporated into the larvae were determined. Incorporation into the rotifer, *Brachionus*, was also tested. Due to positive results obtained in the incorporation experiments, the growth of strain C1 in microbiological media and unrefined media prepared from agricultural by-products was also tested.

INTRODUCTION

The luminescent bacterium *Vibrio harveyi* is a serious pathogen in shrimp (Lavilla-Pitogo et al., 1990) and crab larval production (Fielder and Heasman, 1999). Together with cannibalism, infection with luminescent bacteria was identified as a major problem causing mortality in hatchery-reared *Scylla serrata* (Quinitio et al., 2001). The limited application of chemotherapy as an effective control measure requires the development of alternative strategies of disease control. Biological control using live bacteria may be an option for bacterial disease prevention and control in crustacean hatcheries. The mechanism involves live bacterial application to promote good health in the hosts by out-competing pathogens in the rearing environment (Nogami and Maeda 1992; Gatesoupe 1999; Skjermo and Vadstein, 1999), or improving the indigenous microflora in the gastrointestinal tract (Gildberg et al. 1997; Rengpipat et al., 1998). Probiotic application is already an accepted practice in poultry and swine industries, but there is still a need to study this approach in aquaculture (Gomez-Gil et al., 2000).

This project aims to test indigenous as well as commercially available bacteria as a biological control agent against microbial diseases, specifically luminescent vibriosis due to *V. harveyi*, affecting hatchery-reared crabs and shrimps. This paper describes the steps taken to find suitable bacteria and appropriate strategies for their application in crustacean larvae production systems.

BACKGROUND

The present rearing system for crab and shrimp larvae is largely based on clean rearing water in which nauplii, unicellular algae and diatoms, zooplankton and other substances are added from their respective production units (Parado-Esteva *et al.*, 1996). This husbandry method creates a niche for opportunistic pathogens, specifically, bacteria. Luminescent vibriosis in larval crustaceans occurred when the hatchery system shifted from one that is ecologically balanced to one that accommodates opportunists (Lavilla-Pitogo and de la Peña, 1998).

Bacterial epizootics due to luminescent bacteria were first recognized in the mid-1980s. Outbreaks were notable because shrimp hatchery operations then had reached industry scale in producing postlarvae needed by the booming grow-out sector. Investigations showed a high incidence of luminescent vibriosis due to *V. harveyi* (Lavilla-Pitogo *et al.*, 1990). Pathogenicity tests showed that exposure of *P. monodon* larvae and postlarvae to 10^2 *V. harveyi* cells/ml resulted in significant mortality within 48 h. Scanning electron microscopy also showed that infected larvae had plaques of bacteria on the mouth and feeding apparatus implying an oral route of entry for the pathogen.

Because chemotherapy induced deformities in treated larvae (Baticados *et al.*, 1990) and chemicals were found generally ineffective, preventive approaches and improved hygiene in the hatchery were tried as measures to prevent disease due to luminescent bacteria in the hatchery system. The sources of luminescent *V. harveyi* in *P. monodon* hatcheries were determined from the different hatchery components (Lavilla-Pitogo *et al.*, 1992) and results showed that aside from nearshore seawater used for larval rearing, spawners, whose midgut bacterial flora contained 16 to 17% luminescent vibrios of its total *Vibrio* population, are significant sources of luminescent vibrios. Interestingly, spawners have been observed to release large amounts of fecal material during spawning, thus facilitating bacterial colonization of newly spawned eggs (Lavilla-Pitogo, 1995). After determining the sources of infection, preventive measures like chlorination of seawater (Baticados and Pitogo, 1990), removal of spawners immediately after spawning (Lavilla-Pitogo *et al.*, 1992), egg washing (Lio-Po *et al.*, 1989), various feed sanitation procedures such as disinfection of zooplankton resting stages prior to hatching (Lio-Po *et al.*, 1989), rinsing of *Artemia* nauplii and other zooplankton, and use of diatoms with inhibitory effects against vibrios (Lavilla-Pitogo *et al.*, 1992; 1998) were seriously considered. The use of microbially matured seawater to select non-opportunistic bacterial flora in the water for rearing marine larvae (Skjermo *et al.*, 1997) and the use of benign bacteria to compete with pathogens (Dopazo *et al.*, 1988; Lemos *et al.*, 1991) are techniques geared towards restoring microbial balance in the rearing system.

‘PROBIOTIC’ APPROACHES IN AQUACULTURE

In aquaculture, bioaugmentation, bioremediation, and probiotic application are terms that are sometimes used interchangeably. Although they are similar in their usage of microbes, they are dissimilar in their manner of application of the microbes or microbial products of choice. Following are their accepted definitions:

Bioaugmentation is the use of selected strains of microbes isolated from the environment to improve some of the processes involved in traditional waste treatment.

Bioremediation is the use of organisms to detoxify and clean up pollution. Techniques are applied in soils and aquifers to remove contaminants by biodegradation. *In situ* bioremediation is the enhancement of the catabolic activity of indigenous microorganisms by adding nutrients and, if necessary, oxygen.

Probiotics are viable monoculture or a mixed culture of organisms that are given with feed to inhabit the intestinal tract and contribute to good health by protecting against disease and providing better nutrition. A good probiotic should adhere to the lining of the gastrointestinal tract and produce substances which fight harmful organisms (Gibson and Fuller, 2000).

In the extensive review done on probiotic bacteria in aquaculture by Verschuere *et al.* (2000), a broader definition of probiotics was proposed to address the objections made on the earlier usage of the term. Thus, it was proposed that “a probiotic is a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment.” Application of commercially available microbial preparations has generated interest among aquaculture practitioners as an alternative to antibiotics in disease control (Moriarty, 1998). Many bacterial products with probiotic value are in the Philippine market to provide biological remedies for environmental problems in aquaculture. The use of at least four of these products was reported by Primavera and co-workers (1993) to provide benefits like pathogen control, waste digestion, sludge clean up, and other waste management problems in shrimp grow-out culture.

Evidence for feasible microbial manipulation in the larval rearing environments of various aquatic species is growing (Dopazo *et al.*, 1988; Nogami and Maeda, 1992; Austin *et al.*, 1995; Garriques and Arevalo, 1995; Riquelme *et al.*, 1997; Skjermo *et al.*, 1997) to effectively control bacterial pathogens in crustacean hatcheries. Recent literature on microbial control against fish diseases include bacteria as probiotic for larvae of *P. monodon* (Rengpipat *et al.*, 1998), microbial manipulation to sustain ecological balance in shrimp hatcheries (Lavilla-Pitogo *et al.*, 1998), probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (Gildberg *et al.*, 1997), siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae (Gatesoupe, 1997), and addition of inhibitor-producing bacteria against bacterial pathogens affecting mass cultures of the Chilean scallop, *Argopecten purpuratus* (Riquelme *et al.*, 2000, 2001). A comprehensive review on the use of probiotics in aquaculture was done by Gatesoupe (1999), and the microbial control techniques used in intensive rearing of marine larvae were discussed by Skjermo and Vadstein (1999). An important review on the use and selection of probiotic bacteria for use in the culture of larval aquatic organisms was done by Gomez-Gil *et al.*,

(2000), focusing principally on results from commercial-scale shrimp larval rearing. These reports were the basis in drawing up criteria for selecting probiotic isolates. All throughout the study, the guidelines and dictums raised by Schisler and Slininger (1997) on microbial selection strategies that enhance the likelihood of developing commercial biological control products were considered.

CRITERIA USED IN SELECTING PROBIOTIC BACTERIA

Strain origin

Bacteria for potential use as biological control agent were isolated from crustacean rearing environments where disease did not occur using standard procedures and commercially available culture media. These sources include the hatchery's natural food cultures where *V. harveyi* did not occur but could. Other potential sources of bacteria included feed ingredients like rice bran and fish meal since earlier samples showed freshly-pelleted artificial feeds also harbored heat-resistant bacterial populations. Results from a previous study showed that wild-caught shrimp postlarvae harbored a population of bacteria in their gut that included relatively few vibrios and an insignificant number of luminous bacteria. Therefore, wild-caught postlarvae were identified as a source of bacterial strains for potential biological control against luminous vibriosis. Bacteria from commercially available probiotic products were also tested in its action against *V. harveyi*. From these sources, strains of bacteria were isolated using general culture media like nutrient agar (NA) and marine agar. From several hundreds of purified bacterial colonies, bacteria were grouped based on colony and cell morphology, oxidation-fermentation reactions and growth on selective media. The number of isolates for the competition experiments was trimmed down to 80 isolates (Table 1).

Table 1. The sources of bacterial isolates used in competition experiments

Source	Number of isolates tested
Crab eggs	15
Crab zoeae	3
Zooplankton	5
Cultured unicellular algae	25
Adult crab hemolymph	5
Commercial probiotics	10
Feed ingredients	10
Wild shrimp postlarvae	7
Total	80

Competition experiments in mixed cultures

Competition experiments between luminescent *V. harveyi* and the candidate biocontrol bacteria were done using mixed cultures in seawater following procedures modified from Lemos *et al.* (1991) and Lavilla-Pitogo *et al.* (1998). The sources of the bacteria used in competition experiments are shown in Table 1. Candidate biocontrol bacteria suppressed growth of *V. harveyi* following two general patters (Fig. 1). Of the 80 strains of bacteria tested, only 10 suppressed growth of *V. harveyi* within 24 h. Subsequent results presented here are based on further studies done on two of the 10 isolates, strains C1 from *Chlorella* sp. culture and P9 from a commercial probiotic product that suppressed growth of *V. harveyi* within 24 h. Bacteria that gave delayed or no suppressive action were discarded.

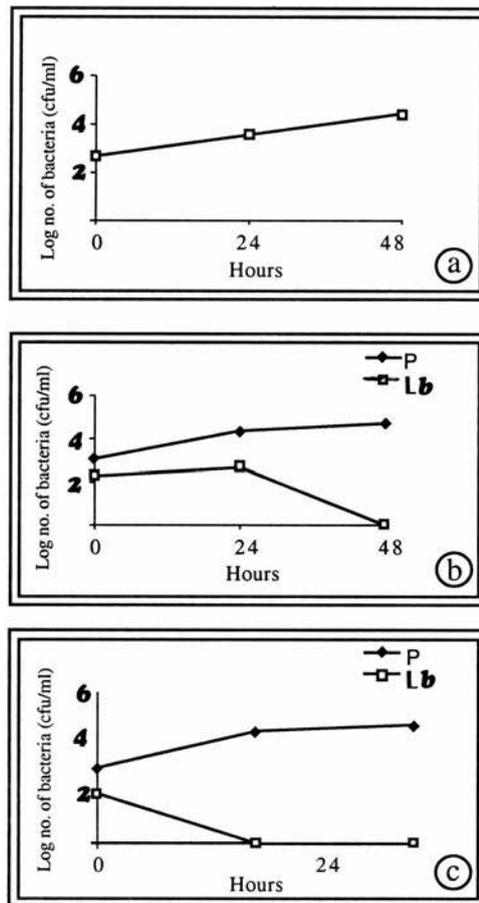


Figure 1. Patterns of bacterial growth obtained in the 48 h competition experiments: a. control - luminous bacteria only; b. inhibition of luminous bacteria after 24 h; c. inhibition of luminous bacteria within 24 h. P = candidate probiotic bacterium; Lb = luminous bacteria

Identification and detection methods

Preliminary identification of isolates was done using standard biochemical tests, although the tests did not classify the strains to genus and species. The general characteristics of isolates C1 and P9 are given in Table 2. The special characters that distinguish these isolates from other bacteria in a similar system are swarming colony for C1 and heat tolerance for P9. The swarming characteristic of strain C1 is especially important when identifying it in samples with mixed bacterial population. To control swarming, nutrient agar medium was prepared with 2% instead of 1.5% agar. The additional agar content, as well as removal of excess moisture on the agar plates by drying in an incubator, controlled the colony of C1 into 5-10 mm diameter with irregular to lobate edge.

Table 2. Characteristics of two candidate probiotic bacteria obtained from *Chlorella* sp. cultures (C1) and from a commercial probiotic (P9)

Characteristics	Bacterial Strain	
	C1	P9
Source	<i>Chlorella</i> culture	Commercial probiotic
Colony on NA*	Swarming	Large, oblate
OF* reaction	Fermentative	Oxidative
<i>Pseudomonas</i> - <i>Aeromonas</i> agar	Colorless	Yellow
TCBS* colony	No growth	Yellow
Special character	Swarming, fast growth	Heat tolerant

* NA = nutrient agar; OF = oxidation-fermentation; TCBS = thiosulfate citrate bile sucrose agar

Pathogenicity

In order for a bacterial strain to become an effective probiotic, it should not cause mortality to the cultured crabs or shrimps. A comparison of published information on the pathogenicity of *V. harveyi* to shrimp larvae and juveniles by static bath challenge is in Table 3. Pathogenicity tests of *V. harveyi* on various stages of crab larvae (from zoea 1 to zoea 5) showed the pathogenic level to be from 10^4 to 10^6 colony-forming-units (cfu) in static bath challenge (Zafran, unpublished). Thus, pathogenicity of the probiotic strains C1 and P9, and the luminescent *Vibrio* sp. (strain CLM3) obtained from crab larval epizootics were conducted on various stages of crab larvae and shrimp postlarvae following protocols described by Lavilla-Pitogo *et al.* (1990).

Table 3. Pathogenicity of *Vibrio harveyi* to shrimp, *Penaeus monodon*, larvae and juveniles by static bath challenge

Host	Bacterial Species	Dose/Duration	Mortality	(Reference %)/Signs
<i>P. monodon</i>	<i>V. harveyi</i>	10 ² - 10 ³ 40 - 48 h	67 - 74 = Z* 69 - 73 = M* 55 - 69 = PL*	Lavilla-Pitogo <i>et al.</i> , 1990
<i>P. monodon</i>	<i>V. harveyi</i>	2.6 x 10 ³	50 % = PL	Karunasagar <i>et al.</i> , 1994
<i>P. monodon</i>	<i>V. harveyi</i>	<10 ² for 1 - 2 days	up to 100=Z up to 78 = M	LeGroumellec <i>et al.</i> , 1995
<i>P. monodon</i>	<i>V. harveyi</i> BP04 strain	10 ³ and 10 ⁴ for 48 h	74.7 = Z 52.9 = M 48.5 = PL	Prayitno and Latchford, 1995
<i>P. monodon</i>	<i>V. harveyi</i>	10 ⁶ - 10 ⁷ 2 h bath	bioluminescence, degeneration of hepatopancreas	Robertson <i>et al.</i> , 1998
<i>P. monodon</i>	<i>V. harveyi</i>	2 h immersion	LD ₅₀ was 1.3 x 10 ⁴ in 14 g juveniles	Saulnier <i>et al.</i> , 2000

* Z = zoea; M = mysis; PL = postlarva

Figure 2 shows that the luminescent bacterium CLM3 is pathogenic to zoea 1 stage *S. serrata* larvae inducing mortality of 63 and 64% with an initial dose of 10² and 10³ cfu/ml. In comparison, 40% mortality was recorded in the control. Note that although no bacteria was inoculated in the control, a mixed bacterial population of up to 10⁴ cfu/ml was enumerated after 24 h. Interestingly, the figure also shows that the initial CLM3 inocula of 10² and 10³ cfu/ml increased to 10⁴ and 10⁵ cfu/ml, respectively, after 24 h.

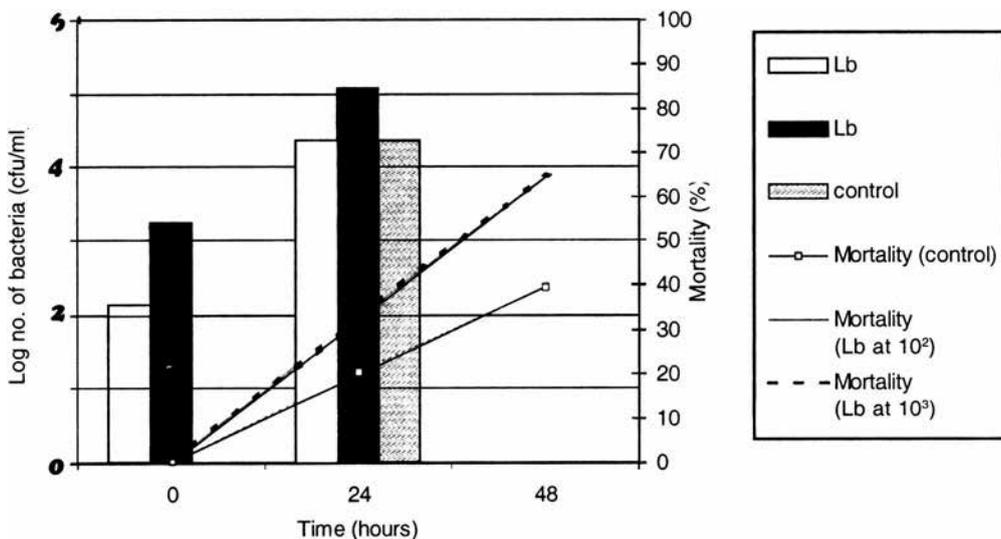


Figure 2. Pathogenicity of *Vibrio harveyi* (strain CLM3) to zoea 1 stage crab (*Scylla serrata*) larvae. Bar graphs represent CLM3 cfu/ml, while line graphs represent percentage mortality of crab larvae. Lb=luminous bacteria

Compared to the above results, Fig. 3 shows that the probiotic bacterial strains are not pathogenic because higher survival rates in crab larvae exposed to C1 and P9 were obtained even at levels of 10⁵ and 10⁶ cfu/ml compared with the control (no bacteria added). Crab larvae in the latter treatment succumbed to luminescent vibriosis due to contamination of test larvae. Although no bacteria were added in the control, 10⁶ cfu/ml were enumerated after 24 h, 9% of which were luminescent. These results highlight the positive effect of C1 and P9 since much higher survival was obtained in those treatments.

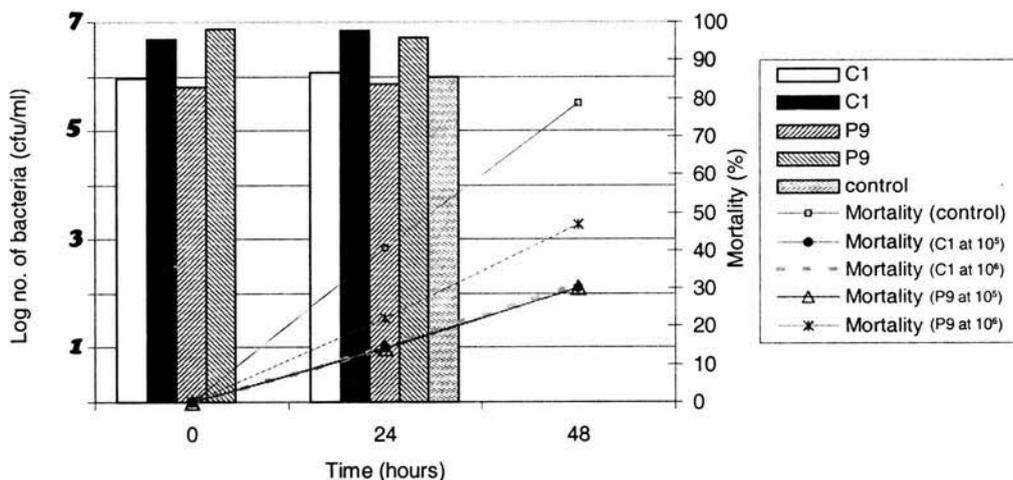


Figure 3. Pathogenicity of probiotic strains C1 and P9 to zoea 3 stage crab (*Scylla serrata*) larvae. Bar graphs represent C1 and P9 cfu/ml, while line graphs represent percentage mortality of crab larvae

Figure 4 illustrates the effect of strains C1 and P9 on shrimp postlarvae. Even at an inoculated dose of 10^5 and 10^6 cfu/ml (which increased to almost 10^6 and 10^7 cfu/ml after 24 h), mortality was 20 % or less in C1 and P9 treatments. Mortality in the control was not significantly different from those of the probiotic strains. Interestingly, the associated bacterial flora of shrimp postlarvae that had been exposed to C1 for 48h showed it to be composed mostly of C1 indicating its probiotic action. It should be noted that pathogenic levels of *V. harveyi* on larvae of *P. monodon* and *Scylla serrata* using static 48 h baths are in the range of 10^2 to 10^4 cfu/ml, which are lower than 10^5 and 10^6 cfu/ml of C1 and P9 used in this study.

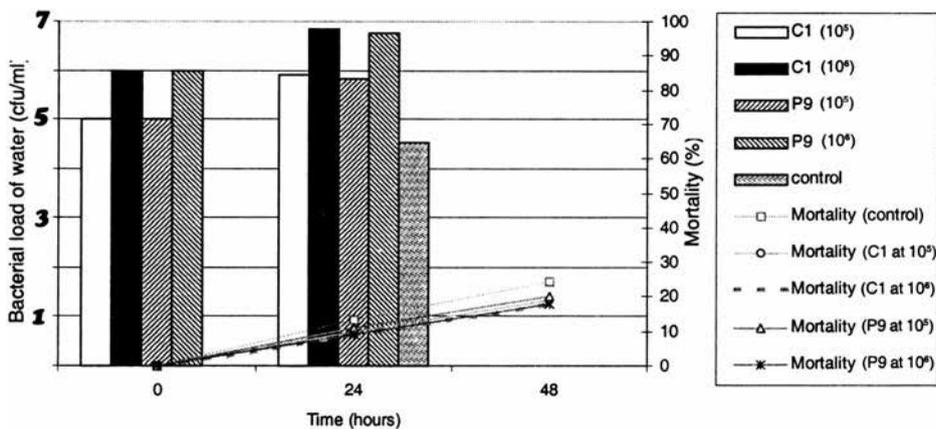


Figure 4. Pathogenicity of probiotic strains C1 and P9 to postlarva 10 stage shrimp (*Penaeus monodon*). Bar graphs represent C1 and P9 cfu/ml, while line graphs represent percentage mortality of crab larvae

TEST ON DELIVERY METHOD

Application of bacteria through live food organisms

The objective of this study was to incorporate probiotic bacterial strain C1 into live zooplankton in order to manipulate the associated bacterial flora of crustacean larvae through feeding. This was done by adding bacteria into pre-washed *Brachionus plicatilis* in sterile seawater (SSW). The animals were allowed to 'starve' in sterile seawater for 2 h after which bacterial suspensions of C1 were added to obtain a final concentration of 10^6 cfu/ml. Bacterial counts in *B. plicatilis* were done on the following periods: right after inoculation with C1 (0 h), 1 h after inoculation, and 2.5 h after inoculation. Determination of bacterial load was done by rinsing the *B. plicatilis* three times in sterile seawater. After removing excess water by blot drying on sterile absorbent paper, the animals were transferred into pre-weighed microcentrifuge tubes and homogenized. Macerated animal suspensions were serially diluted in SSW, plated on NA, Pseudomonas Aeromonas selective agar base (GSP) and thiosulfate citrate bile sucrose agar (TCBS), and incubated at 28-30 C for 18 to 24 h.

Results are presented in Fig. 5. At 0 h, no C1 colonies were associated with the animals. One h after, up to 10^8 cfu of C1/g of *B. plicatilis* was recovered. The C1 bacteria dominated over the initial bacterial population associated with the rotifers prior to inoculation (compare 0 h and 1 h bacterial loads in Fig. 5). After 2.5 h, a reduction in C1 population in the rotifers was observed proving that 1 h is enough period for incorporating C1 probiotic into live *B. plicatilis*. As live rotifers are often considered vectors for bacterial infection (Muroga *et al.*, 1987; Perez-Benavente and Gatesoupe, 1988), the successful incorporation of probiotic bacteria to eliminate potential pathogens from zooplankton or to effectively deliver beneficial bacteria into the culture system provides a window of opportunity for effective biological control. While similar approaches have been tried for *Lactococcus lactis* AR21 strain (Shiri Harzevili *et al.*, 1998) and 4:44 and PB52 strains for first feeding turbot larvae (Makridis *et al.*, 2000), there is a need to develop the technology of C1 application to crustacean larviculture.

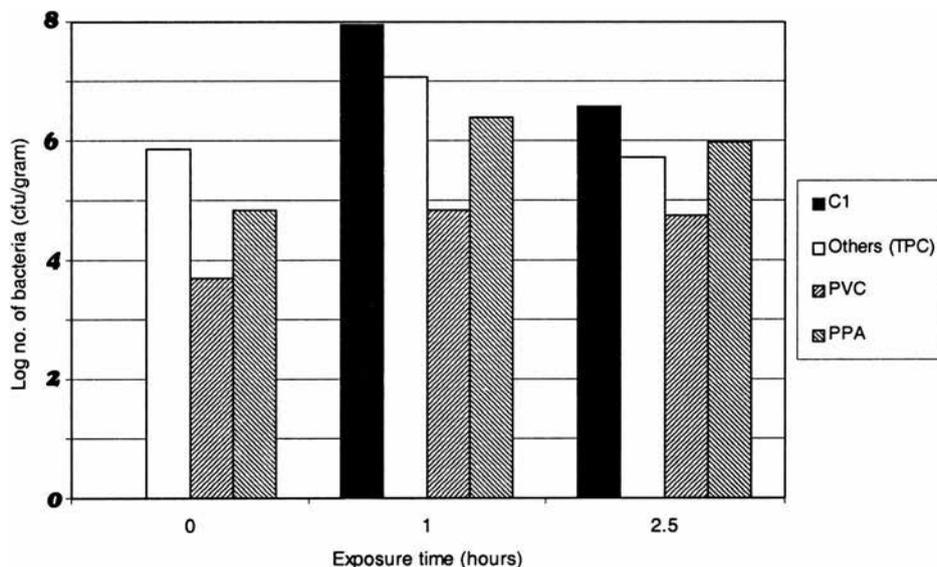


Figure 5. Results of incorporation of probiotic strain C1 into live zooplankton *Brachionus plicatilis*. TPC = total plate count in nutrient agar; PVC = presumptive *Vibrio* count in thiosulfate citrate bile sucrose agar (TCBS); PPA = presumptive *Pseudomonas* and *Aeromonas* count in GSP medium

GROWTH OF BACTERIAL STRAIN C1 IN VARIOUS LIQUID MEDIA

Aside from efficacy, an important criterion to fulfill in the search for a good bacterial probiotic is favorable growth kinetics when grown in commercially feasible liquid media (Schisler and Slininger, 1997). The growth of probiotic bacterial strain C1 in media derived from agricultural by-products like molasses, coconut cream and rice bran was tested. This information is important when large-scale production of bacteria will be needed. For comparison, microbiological grade liquid media like nutrient broth (NB) and brain heart infusion broth (BHIB) were also used to compare the peak bacterial densities obtained.

Figure 6 shows the growth curves of strain C1 in various media. Peak cell densities of 10^8 cfu/ml were obtained on Day 2 in NB and BHIB. Among the media derived from agricultural by-products, high cell densities of up to 9×10^7 were obtained in 10% rice bran extract (pH 7). Growth was not as profuse in crude media using 1% coconut cream and 10% rice bran extract with unadjusted pH of 5. No growth was obtained in 1% molasses medium indicating the inability of C1 to utilize its major component, sucrose, as a nutrient source.

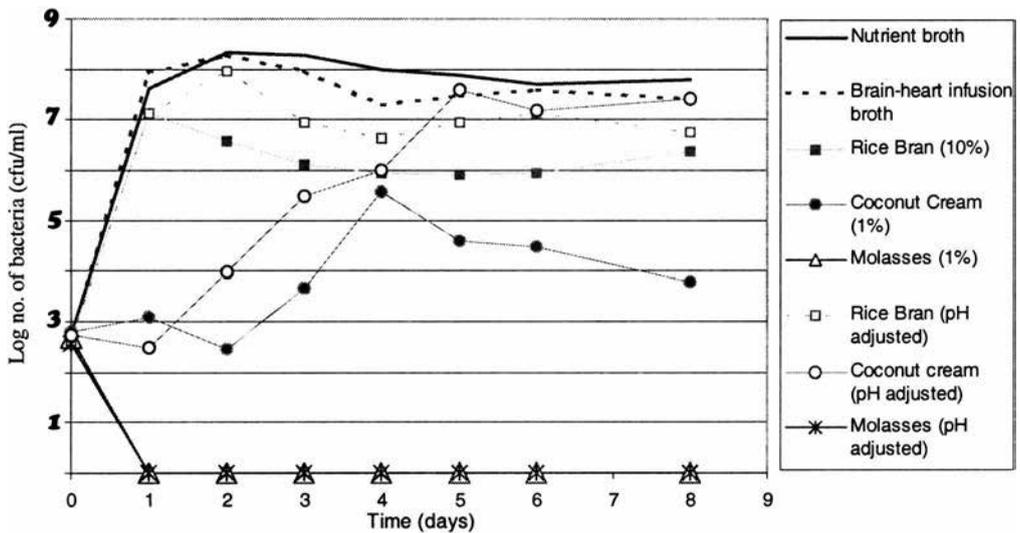


Figure 6. Growth of probiotic strain C1 in various microbiological grade and unrefined media using agricultural by-products

It is clear from this result that probiotic strain C1 can be mass produced using a cheap nutrient source like 10% rice bran extract as long as the pH of the medium is kept within neutral range.

FUTURE PLANS

The above results show promise for bacterial strain C1 as a probiotic. However, a lot more need to be studied regarding its application in crustacean hatcheries. More basic studies to explain the exact mode of action of bacterial probiotics need to be done. A major task ahead is to determine the stability of the microbial environment after C1 application and to develop rearing protocols that will guarantee the attainment of crab and shrimp survival values that are significantly different from those without probiotic application. In addition to improving the survival of hatchery-reared crustacean larvae, more studies for C1 application need to be done to ensure that the associated probiotic bacteria will remain in the animals during grow-out culture.

Although strain C1 lends itself to mass production using a cheap medium of 10% rice bran extract, an important quality control criteria has to be developed to guarantee that no genetic alteration leading to loss of efficacy will occur.

REFERENCES

- Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I. and Griffith, D.R.W. 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J. Fish Dis., 18: 93-96.
- Baticados, M.C.L., Lavilla-Pitogo, C.R., Cruz-Lacierda, E.R., de la Peña, L.D., Suñaz, N.A. 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Dis. Aquat. Org., 9: 133-139.
- Baticados, M.C.L. and Pitogo, C. L. 1990. Chlorination of seawater used for shrimp culture. Israeli J. Aquacult.-Bamidgah, 42: 128-130.
- Dopazo, C.P., Lemos, M.L., Lodeiros, C., Bolinches, J., Barja, J.L. and Toranzo, A.E. 1988. Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. J. Appl. Bacteriol., 65: 97-101.
- Fielder DR and Heasman MP. 1999. Workshop 2. Larval rearing and nursery production. In: Mud Crab Aquaculture and Biology. C.P. Keenan and A. Blackshaw (eds.), pp. 21-24. ACIAR Proceedings No. 78. Australia.
- Gatesoupe, FJ. 1997. Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. Aquat. Living Resour., 10: 239-246.
- Gatesoupe FJ. 1999. The use of probiotics in aquaculture. Aquaculture 180: 147-165.

- Garriques, D. and Arevalo, G. 1995. An evaluation of the production and use of live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador. *In: Proceedings of the Special Session on Shrimp Farming*. C.L. Browdy and J.S. Hopkins (eds.), pp. 53-59. Aquaculture 95, World Aquaculture Society, Baton Rouge Louisiana, USA.
- Gibson, G.R. and Fuller, R. 2000. Aspects of *in vitro* and *in vivo* research approaches directed toward identifying probiotics and prebiotics for human use. *J. Nutr.*, 130: 382S-383S.
- Gildberg, A., Mikkelsen, H., Sandaker, E., Ringo, E. 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.
- Gomez-Gil, B., Roque, A. and Turnbull J.F. 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* 191: 259-270.
- Karunasagar, I., Pai, R., Malathi, G.R. and Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture*, 128: 203-209.
- Lavilla-Pitogo, C.R. 1995. Bacterial diseases of penaeid shrimps: an Asian view. *In: Diseases in Asian Aquaculture II*. M. Shariff, J.R. Arthur and R.P. Subasinghe (eds), pp. 107-121. Fish Health Section, Asian Fisheries Society, Manila, Philippines.
- Lavilla-Pitogo, C.R., Albright, L.J. and Paner, M.G. 1998. Will microbial manipulation sustain the ecological balance in shrimp (*Penaeus monodon*) hatcheries? *In: Advances in Shrimp Biotechnology*. T.W. Flegel (ed.), pp. 185-192. National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand.
- Lavilla-Pitogo, C.R., Albright, L.J., Paner, M.G. and Suñaz, N.A. 1992. Studies on the sources of luminescent *Vibrio harveyi* in *Penaeus monodon* hatcheries. *In: Diseases in Asian Aquaculture I*. M. Shariff, R.P. Subasinghe and J.R. Arthur (eds), pp. 157-164. Fish Health Section Asian Fisheries Society, Manila, Philippines.
- Lavilla-Pitogo, C.R., Baticados, M.C.L., Cruz-Lacierda, E.R. and de la Peña, L.D. 1990. Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture*, 91: 1-13.
- Lavilla-Pitogo C.R. and de la Peña, L.D. 1998. Bacterial diseases in shrimp (*Penaeus monodon*) culture in the Philippines. *Fish Pathol.*, 33: 405-411.
- Le Groumellec, M., Haffner, P., Martin, B. and Martin, C. 1995. Comparative study of bacterial infections responsible for mass mortality in penaeid shrimp hatcheries of the Pacific zone. *In: Diseases in Asian Aquaculture II*. M. Shariff, R.P. Subasinghe and J.R. Arthur (eds.), pp. 163-173. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

- Lemos, M.L., Dopazo, C.P., Toranzo, A.E. and Barja, J.L. 1991. Competitive dominance of antibiotic-producing marine bacteria in mixed cultures. *J. Appl. Bacteriol.*, 71: 228-232.
- Lio-Po, G.D., Fernandez, R.D., Cruz, E.R., Baticados, M.C.L. and Llobrera, A.T. 1989. Recommended practices for disease prevention in prawn and shrimp hatcheries. Aquaculture Pamphlet No. 3, SEAFDEC Aquaculture Department, Tigbauan, Iloilo, Philippines. 14 pp.
- Makridis, P., Fjellheim, A.J., Skjermo, J. and Vadstein, O. 2000. Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. *Aquacult. Int.*, 8: 367-380.
- Moriarty, D.J.W. 1998. Control of pathogenic *Vibrio* sp. in aquaculture ponds by manipulating bacterial species composition. *Aquaculture*, 164: 351-358.
- Muroga, K., Higashi, M. and Keitoku, H. 1987. The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at larval and juvenile stages. *Aquaculture*, 65: 79-88.
- Nogami, K. and Maeda, M. 1992. Bacteria as biocontrol agents for rearing larvae of the crab *Portunus trituberculatus*. *Can. J. Fish. Aquat. Sci.*, 49: 2373-2376.
- Parado-Esteva, F.D., Quintio, E.T. and Borlongan, E.L. 1996. Prawn Hatchery Operations. Aquaculture Extension Manual No. 19. SEAFDEC Aquaculture Department, Tigbauan, Iloilo, Philippines. 45 pp.
- Perez-Benavente, G. and Gatesoupe, F. J. 1988. Bacteria associated with cultured rotifers and *Artemia* are detrimental to larval turbot, *Scophthalmus maximus* L. *Aquacult. Eng.*, 188: 289-293.
- Prayitno, S.B. and Latchford, J.W. 1995. Experimental infections in crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio*: Effect of salinity and pH on infectiosity. *Aquaculture*, 132: 105-112.
- Primavera, J.H., Lavilla-Pitogo, C.R., Ladja, J.M. and de la Peña, M.R. 1993. A survey of chemical and biological products used in intensive prawn farms in the Philippines. *Mar. Pollut. Bull.*, 26: 35-40.
- Quintio, E.T., Parado-Esteva, F.D., Millamena, O.M., Rodriguez, E. and Borlongan, E. 2001. Seed production of mud crab *Scylla serrata* juveniles. *Asian Fish. Sci.*, 14: 161-174.
- Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S. and Menasveta, P. 1998. Probiotics in aquaculture: a case study of probiotics for larvae of the black tiger shrimp (*Penaeus monodon*). In: *Advances in Shrimp Biotechnology*. T.W. Flegel (ed.), pp. 177-181. National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand.
- Riquelme, C., Araya, R. and Escribano, R. 2000. Selective incorporation of bacteria by *Argopecten purpuratus* larvae: implications for the use of probiotics in culturing systems of the Chilean scallop. *Aquaculture*, 181: 25-36.

- Riquelme, C., Araya, R., Vergara, N., Rojas, A., Guaita, M. and Candia, M. 1997. Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). *Aquaculture*, 154: 17-26.
- Riquelme, C.E., Jorquera, M.A., Rojas, A.I., Avendano, R.E. and Reyes, N. 2001. Addition of inhibitor-producing bacteria to mass cultures of *Argopecten purpuratus* larvae (Lamarck, 1819). *Aquaculture*, 192: 111-119.
- Robertson, P.A.W., Calderon, J., Carrera, L., J.R., Zherdmant, M. and Austin, B. 1998. Experimental *Vibrio harveyi* infections in *Penaeus vannamei* larvae. *Dis. Aquat. Org.*, 32: 151-155.
- Saulnier, D., Haffner, P., Goarant, C., Levy, P. and Ansquer, D. 2000. Experimental infection models for shrimp vibriosis studies: a review. *Aquaculture*, 191: 133-144.
- Schisler, D. A and Slininger, P.J. 1997. Microbial selection strategies that enhance the likelihood of developing commercial biological control products. *J. Indust. Microbiol. Biotechnol.*, 19: 172-17.
- Shiri Harzevili, A.R., Van Duffel, H., Dhert, Ph., Swings, J. and Sorgeloos, P. 1998. Use of potential probiotic *Lactococcus lactis* AR21 strain for the enhancement of growth in the rotifer *Brachionus plicatilis* (Muller). *Aquacult. Res.*, 29: 411-417.
- Skjermo, J., Salvesen, I., Oie, G., Olsen, Y. and Vadstein, O. 1997. Microbially matured water - a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. *Aquacult. International*, 5: 13-28.
- Skjermo, J. and Vadstein, O. 1999. Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture*, 177: 333-343.
- Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.*, 64: 655-671.