STUDIES ON THE USE OF PROBIOTICS IN THE LARVAL REARING OF THE SHRIMP *PENAEUS MONODON* (FABRICIUS, 1798)

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Doctor of Philosophy

In Fish and Fisheries Science
(Mariculture)

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Certified that the thesis entitled **"STUDIES ON THE USE OF PROBIOTICS IN THE LARVAL REARING OF THE SHRIMP *PENAEUS MONODON* (FABRICIUS, 1798)"** is a record of independent bonafide research work carried out by **Mrs. Anikumari. N.P** during the period of study from September 1999 to August 2004 under our supervision and guidance for the degree of **Doctor of Philosophy** in **Fish and Fishery Science (Mariculture)** and that the thesis has not previously formed the basis for award of any degree, diploma, associateship, fellowship or any other similar title.

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I hereby declare that the thesis entitled **"STUDIES ON THE USE OF PROBIOTICS IN THE LARVAL REARING OF THE SHRIMP *PENAEUS MONODON* (FABRICIUS, 1798)"** is an authentic record of the work done by me and that no part there of has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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INTRODUCTION

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सारांश

चिंगट पेनिअस मोनोडोन के डिम्भक पालन में प्रोबयोटिक्स के उपयोग पर अध्ययन विषय पर चलाये गये अध्ययन में तीन प्रोबयोटिक जीवों *सकारोमाइसेस बॉलार्डी* , *लाक्टोबासिल्लस प्लान्टारम* और *बासिल्लस सब्टिलिस* के प्रभावों का अध्ययन कर पूरा किया गया । डिम्भकों और पश्च डिम्भकों में इनका संचयन, टिकाऊपन, भार प्राप्ति, अन्य जीवाण्विक वनस्पतिजातों पर इनका प्रभाव और रोगाणु *विब्रियो हारवेयी* की आरे प्रतिरोध निरीक्षण किये गये प्रमुख प्राचल थे । इन प्रोबययोटिक जीवों का आर्टीमिया नॉप्लि में संचयन की इष्टतम अवधि और अधिकतम संचयन स्तरों और आर्टीमिया नॉप्लि के दो ब्रान्डों में संचयन की विभिन्नता जानने के लिए प्रयास किये गये थे। डिम्भकीय एवें पश्चडिम्भकीय दोनों अवस्थाओं में अध्ययन चलाया गया था । डिम्भकीय अवस्था में प्रोबयोटिकों का सीधा प्रयोग किया गया और पश्चडिम्भकीय अवस्था के जीवों को *सकारोमाइसेस बॉलार्डी, बासिल्लस सब्टिलिस* से पोषित आर्टीमिया मेटानॉप्लि खिलाया गया। प्रोबयोटिक्स संचित आर्टीमिया से किये गये परीक्षण में *सकारोमाइसेस बॉलार्डी* के लिए अधिकतम संचयन स्तर 3410, *बासिल्लस सब्टिलिस* के लिए 2093 और *लाक्टोबासिल्लस प्लान्टारम* के लिए प्रति नॉप्लि 105 CFU देखा गया । दो ब्रान्डों के बीच में भी संचयन स्तर विविध था,जैसे *सकारोमाइसेस बॉलार्डी* के प्रयोग में OSI के लिए प्रति नॉप्लि 3410 CFU और लाल ड्रागन के लिए प्रति नॉप्लि 1120 CFU !परीक्षणों में डिम्भक और पश्चडिम्भकों में कहनेयोग्य भार वृद्धि और अतिजीविता दर दिखायी पडी । माइसिस III अवस्था में उच्चतम संचयन स्तर देखा गया । ज़ोइआ से PL 1 तक की डिम्भक अवस्था में देखे गये अधिकतम संचयन *सकारोमाइसेस बॉलार्डी* के लिए प्रति डिम्भक 1535 CFU था और *बासिल्लस सब्टिलिस* के लिए प्रति डिम्भक 2133 CFU लेकिन पश्चडिम्भकीय में यह *सकारोमाइसेस बॉलार्डी* के लिए प्रति डिंभक 1 CFU और *बासिल्लस सब्टिलिस* के लिए प्रति डिम्भक 66 CFU था। लेकिन जीवों के संचयन स्तर अध्ययन में *लाक्टोबासिल्लस प्लान्टारम* अलग नहीं किया जा सका। माइसिस एवं पश्च डिम्भकीय अवस्थाओं में विब्रियो की मात्रा *सकारोमाइसेस बॉलार्डी* और *बासिल्लस सब्टिलिस* के जोड से विचारणीय मात्रा में कम हो गयी थी । समुद्र जल अगार की गणना में *सकारोमाइसेस बॉलार्डी* और *बासिल्लस सब्टिलिस के प्रयोग* से डिम्भकीय अवस्था में कहनेयोग्य प्रभाव नहीं हुआ था परंतु पश्च डिम्भकीय अवस्था में उपर्युक्त के प्रयोग से समुद्र जल अगार की गणना में विचारणीय कमी होती हुई दिखायी पडी। अन्य जीवाण्विक वनस्पतिजातों में *लाक्टोबासिल्लस प्लान्टारम* ने कोई विशेष प्रभाव नहीं उत्पन्न किया । *सकारोमाइसेस बॉलार्डी* और *बासिल्लस सब्टिलिस का प्रयोग डिंभकीय अवस्था में रोगाणु विब्रियो हारवेयी के प्रतिरोध क्षमता को बढा देता है।* पश्चडिम्भक I में रोगाणु *विब्रियो हारवेयी* का ग्रसन रोकने के लिए प्रति मि ली 10^3 CFU *सकारोमाइसेस बॉलार्डी* ब्रोथ और प्रति मि ली 10^4से 10^5 CFU *बासिल्लस सब्टिलिस* कोश अनिवार्य थे। PL I से पाँच दिनों बाद खाद्य रोककर किये गये अध्ययन में *सकारोमाइसेस बॉलार्डी* काउन्ट में 98% और *बासिल्लस सब्टिलिस* काउन्ट में 13% की कमी दिखायी पडी । पश्च डिम्भकों में *बासिल्लस सब्टिलिस का* PL 21 से पाँच दिनों बाद केवल 7% की कमी ही दिखायी दी । प्राबयोटिकों से उपचारित जींवंत नॉप्लि से खिलाये पश्च डिम्भक उपचारित मृत नॉप्लि दिये गये पश्च डिम्भकों की तुलना में प्राचलों के अध्ययन के लिए उचित देखा गया । उपर्युक्त अध्ययन यह सूचना देती है कि *सकारोमाइसेस बॉलार्डी* और *बासिल्लस सब्टिलिस* को चिंगट डिम्भक पालन में प्रभावी प्रोबयोटिक जाति के रूप में उपयोग किया जा सकता है जबकि *लाक्टोबासिल्लस प्लान्टारम इस केलिए उपयुक्त नहीं है* ।

ABSTRACT

The study regarding 'Studies on the use of probiotics in the larval rearing of the shrimp *Penaeus monodon* ' was carried out by studying the effect of three probiotic organisms, *Saccharomyces boulardii, Lactobacillus plantarum* and *Bacillus subtilis.* The parameters monitored were, level of enrichment in larvae and post larvae, survival, weight gain, effect on other bacterial flora and resistance to a pathogenic *Vibrio harveyii* infection. Attempts were made to find out the optimum enrichment duration and maximum enrichment levels of the above probiotic organisms in *Artemia* nauplii and also the difference in enrichment level between two brands of *Artemia* nauplii. The study was carried out in larval as well as in post larval stages. Direct additions of probiotics were carried out in larval stages while *Saccharomyces boulardii* and *Bacillus subtilis* enriched *Artemia* metanauplii were fed to the larvae in post larval section. *Artemia* enrichment experiments revealed the maximum enrichment levels as 3410 for *Saccharomyces boulardii*, 2093 for *Bacillus subtilis* and 105 CFU/larva for *Lactobacillus plantarum*. Enrichment levels also varied between the two brands, 3410 CFU/nauplii for OSI and 1120 CFU/nauplii for Red Dragon brand in the case of *Saccharomyces boulardii*. Significant increase in weight gain and survival rate were noticed in larvae as well as post larvae in the experiments conducted. Highest enrichment levels were noticed for mysis III. Maximum enrichment levels noticed were 1535 CFU/larva for *Saccharomyces boulardii* and 2133 CFU/larva for *Bacillus subtilis* in the larval stages from zoea to PL 1, while in post larvae it was 1 CFU/larva for *Saccharomyces boulardii* and 66 CFU/larva for *Bacillus subtilis*. *Lactobacills plantarum* could not be detected during the enrichment study. Addition of *Saccharomyces boulardii* as well as *Bacillus subtilis* significantly reduced *Vibrio* counts during mysis stage as well as during post larval stages. Effect on sea water agar counts were not significant during larval stages while in post larvae sea water agar counts were decreased with the application of *Saccharomyces boulardii* and *Bacillus subtilis*. Enrichment of *Lactobacillus plantarum* did not produce any significant effect on other bacterial flora. Addition of *Saccharomyces boulardii* and *Bacillus subtilis* increased the resistance of larvae to pathogenic *Vibrio harveyii* infection in larvae as well as post larvae. About 10^3 CFU/ml *Saccharomyces boulardii* broth and 10^4 to 10^5 CFU/ml of *Bacillus subtilis* cells were necessary to surmount *Vibrio harveyii* infection in PL. 1. Post colonization studies after discontinuing the feeding resulted in 98% reduction in *Saccharomyces boulardii* counts and 13% reduction in *Bacillus subtilis* counts after five days from PL 1. In post larvae *Bacllus subtilis* showed only 7% reduction in five days from PL 21. Post larvae fed with live enriched nauplii performed better than those fed with dead enriched nauplii, for the parameters studied. From the above study it is evident that *Saccharomyces boulardii* and *Bacillus subtlis* can be used as effective probiotic species for shrimp larviculture while *Lactobacillus plantarum* is not a suitable species.

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

Aquaculture has gained importance over other fisheries programmes all over the world, as the returns from capture fisheries have become more or less stagnant. Since the 1970's, world aquaculture production has increased with the advent of new technologies, improvement in culture practices, and the introduction of new species. According to FAO statistics (2001) world fish production has reached 130.27mmt. Contribution from aquaculture was around 37.8mmt of which crustaceans were 1.8mmt.

Shrimp farming has become a major industry in tropical and subtropical areas with the current aquaculture production estimated at 1.27mmt valued at US$ 843.2 billion (FAO, 2003). The driving force was the high demand for the product in the export market and the resultant incentive to maximize the output. Like many Southeast Asian countries, in India also the word coastal aquaculture has become synonymous with shrimp farming, largely due to export potential of the commodity and media attention. In the last decade, intensification of the culture started with the adoption of new technologies and India came to fourth in aquaculture production in 1993.

Rapid expansion of the industry and potential for high profit with short pay back period on investment capital encouraged short-term business goals. Intensification had resulted in over exploitation of the natural resources, poor water and sediment quality leading to disease out breaks and loss of production. Over the past few years, research efforts had gone into identification, treatment and control of shrimp diseases and as a result, a wide range of chemicals, therapeutants, vaccines and immunostimulants were manufactured and introduced into aquaculture industry.

There had been significant socioeconomic impact in coastal regions forcing several farm operators to close or abandon their farms. Many adopted low stocking densities, and the number of crops/year were reduced. Farmers started using a large variety of chemo-therapeutants in a desperate attempt to control

mortalities without regard to the potential health hazards. Lack of legislation on the use of chemo-therapeutants and awareness of the importance of health care in aquaculture has led to uncontrolled use and improper selection of chemicals. In many instances, misuse of chemicals often arises from inadequate information or labeling of the products, regarding storage, usage under specific environmental conditions, expiry date and disposal of unused product.

With the intensification of farming activities, hatchery technologies have been developed for the production of post larvae to cope up with the increase in demand. Microbial outbreak of diseases often arises in the early stages of larval rearing, due to a combination of high larval densities and debris from dead larvae. High loads of organic matter and bacteria due to the addition of intensively produced live feed stimulate growth of opportunistic bacteria in the larval tanks. *Vibrios* are among the most important bacterial pathogens of cultured shrimps responsible for a number of diseases, and mortalities up to 100% have been reported due to *Vibrios* (Lightner, 1983). *Vibrio harveyii* has been reported as the causative agent of luminous bacterial infection resulting in mass mortality of *Penaeus monodon* larvae in hatchery and grow out ponds in many countries.

Most of the hatchery operators resort to the routine use of antibiotics both for prophylactic treatment and to control parasitic, fungal and bacterial diseases in hatcheries. Furans, oxytetracycline, erythromycin and treflan have been used with varying degree of success as prophylactic and disease controlling agents. In most cases, determination of actual disease prevalence and accurate diagnosis based on proper monitoring have not been done. During the initial phases, low doses of broad-spectrum antibiotics are applied to the culture water followed by higher doses as the resistance of the disease organism rises, or change to a new antibiotic, sometimes combination of one or more, until the treatment finally becomes ineffective. Such practices result in higher operating costs besides the production of more disease susceptible post larvae. Some chemicals like chloramphenicol, furacin and oxytetracycline, cause mortalities, incomplete moulting, or morphological deformities when applied at reccomended concentrations.

Low concentration of the drugs end up in the environment due to continuous use or through leaching resulting in the development of resistant strains of bacteria (Baticados *et al.* 1990). Resistance to antibiotics results either from mutation or from plasmid mediation. Nearly half of the bacteria isolated from aquaculture facilities during a survey of five Southeast Asian countries in 1993 were resistant to oxytetracycline (Inglis *et al.* 1997). Other adverse environmental impacts include quantitative changes in bacterial flora, toxic effect on wild living organisms and transfer of drug resistance to human pathogens.

In livestock nutrition, growing public concern over the development of antibiotic resistant strains of harmful bacteria, possible residues in the animal products, and cross-resistance with human pathogens had prompted the researchers to adopt the age-old practice of using beneficial bacteria. The probiotic concept was adopted as an alternative therapy to combat disease problems in animal husbandry as well as in human medicine from the 1970's onwards. Probiotics used in livestock nutrition contains selected and viable counts of Lactic acid bacteria (LAB) often composed of strains of *Lactobacillus acidophilus, Streptococcus faecium* or strains of *Bacillus* sp.

Concern over antibiotic residue in farmed shrimp and over the development of increase in antibiotic resistance of pathogens have stimulated wide spread interest in the use of probiotics and bioremediation products. Products containing viable microorganisms have been extensively introduced into aquaculture production facilities as an alternative to antibiotics as a health management strategy to enhance yield and maintain cleaner environment. A number of microorganisms such as *Bacillus* sp, *Vibrio* sp, lactic acid bacteria, pseudomonads and yeasts are being used as potential probiotic organisms. These are administered by inclusion in bio-carriers like *Artemia* nauplii or in feed or directly applied to the culture medium. The application is done in the form of series of doses or inoculations repeated through out the culture period. Possible mode of action of probiotics includes suppression of opportunistic bacteria by production of antibacterial compounds, competition for adhesion sites and nutrients, alteration of microbial metabolism and stimulation of immunity. In comparison, bioremediation agents detoxify unfavourable chemicals, produce enzymes that can break down

metabolic wastes, and inhibit the growth of opportunistic pathogens when applied to water.

In India, a variety of commercial probiotic preparations are being used in shrimp farming operations with the aim of controlling disease outbreaks and increasing production. Farmers depend mostly on international products and use them as per the directions given by the manufacturers, without any actual knowledge of their mode of action. Sometimes when the list of ingredients is provided, there is no guarantee that the product had been tested in the field or proven to be beneficial. Even though laboratory studies show encouraging results, there is little evidence of constant and beneficial result from probiotic applications in commercial aquaculture operations. The difficulty in maintaining adequate number of probiotic organisms in viable form for a long period in the gut may be one of the reasons behind this. The microbial preparations should be added at a high population density and at the right environmental conditions and the efficacy depends on the nature of competition between species or strains of bacteria.

In fish larvae and fingerlings, studies regarding the application of probiotics and their effects were well documented when compared to their use in shrimp hatchery operations. Live food organisms like the rotifer, *Brachionus plicatilis* and *Artemia* nauplii are the most important components of the diet during the larval stages of fishes and shrimps. Because of the convenience in handling and production, *Artemia* nauplii are preferred over other live feeds during larval rearing operations. The demand for *Artemia* cysts had gone up to 1500 mt by 1997 of which over 80% went to shrimp hatcheries. The method of bio-encapsulation also called *Artemia* enrichment had given encouraging results in larval production in terms of their survival, growth, metamorphosis and quality. This method originally developed for encapsulating unsaturated fatty acids has been used for oral delivery of vitamins, chemotherapeutics and recently for vaccines and probiotics.

Addition of live feed organisms into the culture tanks brings in very high levels of pathogenic *Vibrio* species if culture system is not maintained properly. Most of the studies regarding use of probiotics in larval rearing concentrate on live feed enrichment with probiotic organisms in an attempt to overcome this problem

and to provide nutrients in capsule form to avoid dispersion. The addition of lactic acid bacteria to rotifers fed to fish larvae has been shown to improve growth, reduce opportunistic bacterial counts and increase resistance against pathogenic *Vibrios* (Gatesoupe, 1994). Even though commercial shrimp hatchery operations use large quantity of probiotic products only few research papers are available on their effect on shrimp larvae (Garriques and Arevalo, 1995) and few studies on post larvae (Rengipipat *et al.*, 1998) have been reported. Hence the present work was carried out to find the effect of commonly used probiotic organisms on shrimp larvae and post larvae.

The effectiveness of *Sachharomyces boulardii* as a probiotic organism in reducing morbidity and mortality due to *Clostridium difficile* induced pseudomembranous colitis, in animals and the recurrence rate of post antibiotic *C. difficile* colitis in humans were reported (Buts *et al.* 1993). In aquaculture, *Artemia* enrichment study conducted by Patra and Mohamed (2003) with *S. boulardii* has shown beneficial result by increasing its resistance against pathogenic *Vibrio* infection. This had prompted in using the same species for rearing *P. monodon* larvae, where mortality due to *Vibrio* infection is common.

Lactobacillus plantarum had been shown to improve production rate of rotifers, growth rate and resistance of larval turbot against *Aeromonas salmonicida* infection and decrease total aerobic flora (Gatesoupe, 1991, 1994, Strom and Ringo, 1993). *Bacillus* species had been widely used in commercial shrimp aquaculture operations either as a bio-remediator or as a probiotic, with effective results (Moriarty, 1998, Shariff et al. 2001., Devaraja *et al.* 2002). *B. subtilis* is reported to enhance survival and growth rate in common snook, *Mugil cephalus* and spotted trout (Kennedy *et al.*1998).

The present study was carried out to evaluate the efficacy of the yeast *Saccharomyces boulardii, Lactobacillus plantarum* and *Bacillus subtilis* as probiotics organisms for rearing *Penaeus monodon* larvae. The efficacy of these organisms as probiotics was tested by elucidating their effect on growth, survival rate, and the level of assimilation of these organisms by shrimp larvae and their effect on other aerobic micro flora. The following approaches were adopted.

1. Probiotic organisms were fed to *P. monodon* larval stages from Z. 1 to PL. 1 by direct addition to rearing medium.

2. Attempts were made to find out the optimum enrichment duration of these probiotics in *Artemia* metanauplii.

3. Post larvae of *P. monodon* from PL. 3 to PL. 20 were fed with probiotics enriched; live as well as dead *Artemia* to find out the efficacy of the probiotics. Only two probiotic species, *S. boulardii* and *B. subtilis* were used for this study.

4. The success of probiotic feeding was evaluated at the end of each experiment by conducting a *Vibrio* challenge test using pathogenic *V. harveyii*.

REVIEW OF LITERATURE

2.0. REVIEW OF LITERATURE

2.1 Definition of probiotics

The word probiotics is derived from Greek and means "pro life'. Lilly and Stillwell (1965) were the first to define the term probiotics as substances produced by one pathogen that stimulated the growth of another. However, Elie Metchnikoff's work during the beginning of 19th century is regarded as the first research conducted on probiotics (Metchnikoff, 1908). The modern concept of probiotics was put forward by Parker (1974) as "organisms and substances which contribute to intestinal microbial balance". Fuller (1989) gave a widely acceptable and clear definition of a probiotic as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance".

Havennar and Hiusint' Veld (1992) extended the definition of probiotic as "a mono or mixed culture of live organism when applied to animal or man affect beneficially the host by improving the properties of indigenous microflora". As intestinal microbial balance is not studied in most cases, Tannock (1997) modified the term as "living microbial cells administered as dietary supplements with the aim of improving health". Salminen *et al.* (1999) considered a probiotic as any microbial preparation or the components of microbial cells with a beneficial effect on the health of the host.

Most of these definitions were put forward with regard to the probiotic application in terrestrial animals where the influence of external environment is nil or negligible. In aquatic animals the microbial flora of the digestive tract will be similar to that of the surrounding medium (Sugita *et al.*, 1981). Based on this, the probiotic concept in the context of aquaculture was again modified. Maeda *et al.* (1997) suggested the use of probiotics as biological control agents "the antagonism among microbes through which they are killed or reduced in number in the aquatic environment". Moriarty (1998) suggested that the definition of probiotics in aquaculture should include the addition of live naturally occurring bacteria to tanks and ponds in which the animal live. As these definitions varied from the original

concepts Gatesoupe (1999) suggested an alternative definition of probiotics as "microbial cells that are administered in such a way so as to enter the gastro intestinal tract and be kept alive with the aim of improving health". Later Gram *et al.* (1999) broadened the definition to "a live microbial feed supplement which beneficially affects the host by improving its microbial balance", thus removing the need for intestinal microbial balance alone. Irianto and Austin (2002) focusing on the beneficial effect of probiotics on health of the animal described probiotics as "an entire or components of microorganisms that is beneficial to the health of the host".

2.2 Probiotic organisms and their effects

A wide range of probiotic organisms have been used for research purposes as well as for commercial production of finfishes and shellfishes. They are either isolated from aquatic environment or from other environments. Commercial products containing viable preparations of single species or mixture of species are also available in the market. Majority of the genus include Gram positive (*Bacillus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Micrococcus, Streptococcus,* and *Weissella*), Gram negative bacteria (*Aeromonas, Alteromonas, Photobacterium, Pseudomonas* and *Vibrio*), yeasts (*Saccharomyces, Debaryomyces and Phabia*) and microalgae like *Tetraselmis* (Irianto and Austin, 2002).

Bacillus species are known to produce many different antibiotics (Bacitracin, Polymixin, Trycodin, Gramicidin, Circulin are examples) especially in relation to sporulation process (Brock, 1974). Moriarty (1998) suggested the efficiency of the genus to secrete many enzymes that degrade slime and biofilms and allow *Bacillus* and their antibiotics to penetrate slime layers around Gram-negative bacteria. Further more *Bacillus* competes for nutrients and thus inhibits other bacteria from growing rapidly so that any resistant bacteria cannot multiply readily and transfer mutant genes. Sugita *et al.* (1998) isolated a *Bacillus* strain NM-12 and reported that the bacteria produce an antibacterial substance, a heat liable siderophore having a wide antibacterial spectrum against intestinal bacteria of coastal fish, and that the substance might be useful to control bacterial population in both fish intestine and culture medium. Many authors have reported

the antagonistic effect of *Bacillus* species against Gram negative microorganisms like *Vibrio* species, *Coryneyforms, Enterobacteriaceae, Pseudomonas spp., Moraxella spp.* and *Flavobacterium spp.*, (Gatesoupe, 1993; Rengpipat, 1998; Moriarty, 1998; Sugita *et al.* (1998).

Table 1. An account of probiotic organisms, their antibacterial agents and properties of these products.

Organism	Product	Properties	References
Bacillus	Bacitracin, Polymixin, Trycodin, Gramicidin and Circulin (Antibiotics)	Antagonistic against *Vibrio* sp., *Coryneforms, Enterobacteriaceae, Pseudomonas, Morxella* spp. and *Flavobacterium* spp	Brock, 1974; Rengpipat, 1998; Sugita *et al.* 1998; Moriarty, 1998
Lactobacillii	H_2O_2, Lactic acid, Diacetyl and Bacteriocins	Reduce pH of the medium, Compete for adhesion sites with pathogens, lactic acid inhibits proliferation of putrefactive microbe in the gut. Stimulate non specific immune system of the host and inhibit *Vibrios*	Davidson and Hoover, 1993; Montes and Pugh, 1993; Savage, 1987; Schroder *et al* 1980
L. acidophilus	Lactocin, Lactocin-F, Acidolin, Acidophilin	Antagonistic to *Lactobacillus* species	Earnshaw, 1992
L. plantarum	Plantaricin-A, Lactolin and Sakasin A and Reuterin	Antagonistic to Gram negative and Gram positive bacteria and also yeast	Axelsson *et al.* 1987
L. rhamnosus		Effective against	Nikkolskelainen *et al.*

		Furunculosis in rainbow trout	2001 b
L. halveticus	Helvectin-J, Lactolin-27, Nisin, Diplococcin and Lactosperin	Effective against other *Lactobacillus* species	Earnshaw, 1992
Lactococci	Nisin, Diplococcin and Lactosperin	Nisin act as surface active detergent. Effective against *Strepyococci*, *Bacillus* and *Clostridia*	Hall, 1996
Lactococcus lactis	Bacteriocins	Show resistance against infections caused by *Listeria monocytogenes*, *Aeromonas hydrophilla*, *Staphylococcus aureus*. Effective against *Vibrio anguillarum*	Lewus *and Montville,*1991; Harzevilli *et al.* 1998
Pediococcus	Pediocin-A, Pediocin, PA-1 and Pediocin ACH, lactic acid and H_2O_2	Effective against board spectrum of Gram positive organisms	Earnshaw, 1992
Leuconostoc	Bacteriocins	Effective against *Listeria monocytogenes*	Haris *et al.* 1989
Carnobacterium	Carnocin	Antagonistic to *Aeromonas* spp., *Vibrio* spp., *Proteus vulgaris*, *Listeria monocytogenes*, *Flavobacterium* and *Streptococcus milleri*	Joborn *et al.* 1997; Gildberg and Mikkelsen, 1998; Robertson *et al.* 2000

Streptomyces	Streptomycin, Kanamycin, Tobramycin and Gentamycin	Bind to bacterial ribosomes and prevent initiation of protein synthesis	Earnshaw, 1992
Vibrio spp	Siderophores	Inhibit pathogens with low iron intake capacity	Pybus *et al. 1994*
V. anguillarum	Anguibactin related siderophore	Inhibit *V. ordalii*	Austin *et al.* 1995; Garriques and Arevalo, 1995
Aeromonas media	Bacteriocin like inhibitory substance	Effective against *V. tubiashii* infection in Oyster larvae	Gibson *et al.* 1998
Pseudomonas fluorescence AH	Siderophore	Effective against *V. anguillarum* and *A. salmonicida*	Gram *et al.* 1999 and 2001
P. aeruginosa	Pyocyanin	Inhibits *V. harveyii*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela* and *V. vulnificus*	Chythanya *et al.* 2002
Alteromonas strain	2-3 indolinedione (isatin)	Inhibits pathogenic fungus *Laginedium calinectes*	Gil-Turnes *et al.* 1989
Alteromonas strain TJP7	Inhibitory secondary metabolites	Antagonistic to *V. harveyii* and *V. splendidus*	Abraham *et al.* 2001
Saccharomyces boulardii		Putative immunotherapeutic effect against pathogen infections. Found to remove toxin receptors by way of a protease activity	Buts *et al.* 1993
S. cerevisiae	β 1,6 and β 1,3 glucans		Song *et al.*, 1997

Lactobacilli can produce compounds that inhibit the growth of microorganisms (Ringo and Gatesoupe, 1998). *Lactobacilli* produce large amounts of H_2O_2, and also reduces the pH of the medium with the production of organic acids such as lactic acid, acetic acid and diacetyl (Davidson and Hoover, 1993). Once inside the gut of the host, *Lactobacilli* attach to the epithelial cells in the gut wall (Savage, 1987). Competition for adhesion receptors with pathogens might be the first probiotic effect (Montes and Pugh, 1993). Colonization of digestive tract by bacteria capable of producing lactic acid through fermentation inhibits the proliferation of putrefactive microbes in the gut. *Lactobacillus* sp. produces bacteriocins, which have narrow range of antibacterial activity, mostly active against Gram positive bacteria. Lactocin produced by *L. acidophilus* is active against Gram negative and Gram positive bacteria. Acidolin, Acidophilin and Lactocin-F are the other antibacterial compounds produced by *L. acidophilus* which are antagonistic to *Lactobacillus* species only. *L. plantarum* produces three types of bacteriocins, viz., Plantaricin A, Lactolin and Sakasin-A. *L. helveticus* is reported to produce Helvectin-J and Lactolin-27 which are antagonistic against other lactobacillus species. *Lactobacilli* are also reported to play a role in stimulating the non-specific immune system of host. Schroder *et al.* (1980) showed that *L. plantarum* isolated from saithe produces inhibitors against *Vibrio* sp. when the culture filtrate was grown in presence of *Bacillus thuringiensis*. *L. bulgaricus* was able to neutralize the toxin produced by *E. coli* under *in-vitro* conditions. Ruterin produced by *L. plantarum* is antagonistic to Gram positive and Gram negative bacteria, yeast and *Trypanosoma cruzi* (Axelsson *et al.* 1987). The *Lactobacillus/Carnobacterium* strain isolated from rotifers increased the resistance of turbot larvae against a pathogenic *Vibrio* sp. (Gatesoupe, 1994). *L. rhamnosus* was found to be effective against furunculosis in rainbow trout (Nikoskelainen *et al.* 2001b). Price and Lee (1970) found that addition of *Lactobacillus* sp. decreased the count of *Pseudomonas* sp. in oysters.

Lactococci spp. produce three types of Bacteriocins, Nisin, Diplococcin, and Lactostrepcin. Nisin has a narrow spectrum of inhibitory activity affecting only Gram positive bacteria including lactic acid bacteria like *Streptococci*,

Bacillus and *Clostridia*. Possible cause for resistance of microorganism to nisin is the production of the enzyme nisinase. Nisin acts as a surface active detergent (Hall, 1966). The sensitive microbes are adsorbed to the nisin producer followed by cytoplasmic membrane rupture and release of cytoplasmic material. These compounds are antagonistic to Gram positive bacteria only. The bacteriocins produced by *L. lactis* showed some resistance to infections caused by *Listeria monocytogenes*, *Aeromonas hydrophila* and *Staphylococcus aureus* (Lewus and Montville 1991). *L. lactis* enhanced the growth of the rotifer *Brachionus plicatilis* and had an inhibitory effect against *Vibrio anguillarum* (Harzevilli *et al.* 1998). *Pediococcus* is known to produce bacteriocins which are inhibitory to broad spectrum of gram positive microorganisms. The bacteriocins are Pediocin-A, Pediocin-PA1 and Pediocin-ACH. Other inhibitory substances produced by the genus are lactic acid and H_2O_2. Inhibition of pathogens by *Leuconostoc* has been attributed to the production of organic acids such as lactic and acetic acid and diacetyls. Leuconostoc also produces some unknown bacteriocins which are antagonistic against *Listeria monocytogenes* (Haris *et al.* 1989). Ringo *et al.* (1997) demonstrated the presence of *Leuconostoc* sp associated with epithelial mucosa of stomach, small and large intestine of Arctic charr when fed with different dietary polyunsaturated fatty acid. *Carnobacterium* strains produce bacteriocins (Carnocin) or bacteriocin like substances active against other *Carnobacteria* and lactic acid bacteria. Antagonistic activity has been reported against *Carnobacterium spp.*, *Aeromonas* spp., *Vibrio* spp., *Proteus vulgaris, Listeria monocytogenes*, *Flavobacterium psycrophillum, Photobacterium damselae*, .*Streptococcus millieri* (Joborn *et al.*, 1997. Gildberg and Mikkelsen., 1998. Robertson *et al.* 2000).

Probiotic effect of Gram negative species has also been reported. The bacteriocin produced by these species showed specific inhibitory effect against other gram negatives. Some strains of *Vibrio* are known to produce siderophores that have a probiotic effect on the host. Non-pathogenic bacteria which produce siderophores are promising candidate probiotics against pathogens with low iron uptake capacity (Gatesoupe, 1997). Pybus *et al.* (1994) reported that the inhibitory effect of *V. anguillarum* against *V. ordalii* was due to a siderophore. Gatesoupe (1997) reported that probiotic activity of a *Vibrio* species designated as *Vibrio* E was similar to purified bacterial siderophore defroxamine. Antagonistic activity of *Vibrio*

alginolyticus has been reported against *Vibrio* spp., and *Aeromonas* spp. (Austin, 1995. Garriquies and Arevalo, 1995). *Aeromonas media* has been reported to have the ability to produce BLIS (bacteriocins-like inhibitory substance) which was antagonistic in *in-vitro* conditions to a number of aeromonads including *A. hydrophila* and a number of *Vibrio* spp. (Gibson *et al.* 1998). *In-vivo* inhibitory effect of *A. media* has also been demonstrated by the same authors in oyster larvae against *V. tubiashii.*

Several studies have demonstrated that yeast cultures can improve digestive process in rumens. Due to their smaller size, high protein content and relatively low production cost yeasts have been considered as algal substitute for several species of filter feeders like rotifers and (Fukusho, 1980) *Artemia* and bivalve molluscs. Brewers yeast is a good source of nucleic acids, polysaccharides including β1-6 and β 1-3 glucans. The use of yeast for feeding fishes (Li and Gatlin, 2003; Lara-Flores *et al.,* 2003) and crustaceans have been reported.

Saccharomyces boulardii (SB hereafter) is a non pathogenic yeast used as both preventive and therapeutic agent for the treatment of variety of diarrhoeal diseases in humans. Buts *et al.* (1992) reported it's effectiveness in reducing morbidity and mortality in a selected group of infants and children with persistent intestinal symptoms related to toxinogenic *Clostridium difficile* overgrowth. Buts *et al.* (1993) reported increased secretary immunoglobulin A and secretary components of other immunoglobulin in the small intestine of rat treated with *S. boulardii.* McFarland and Bernasconi (1993) confirmed the safety for oral administration. They observed the effectiveness of the species to maintain high stable levels if taken daily. The species was found to be quickly eliminated from the colon once the application discontinued. Klein *et al.* (1993) observed that as the dose increased, the steady state concentration of SB increased significantly in the colon and the percentage recovery from the disease were not dose dependant. *S. boulardii* was found to remove toxin receptors by way of a protease activity and also produces a protease that reduces or removes brush boarder glycoprotein involved in adhesion of pathogens to mucosa (Pothoulakis *et al.*, 1993). Czerucka *et al.* (1994) studied inhibition of the secretion induced by cholera toxin (CT) in rat jejunum with SB. They have observed that SB conditioned medium significantly reduced CT-induced cAMP level in IEC-6 cells. Patra and

Mohamed (2003) used *S. boulardii* for enriching *Artemia* nauplii. They have reported increase in survival and also resistance against pathogenic *V. harveyii* infection.

2.3. Application of probiotics

Many of these organisms are host specific and had been proven beneficial to the host only. Most of the research regarding the use of probiotics focus on their application in larval rearing compared to grow out system since the cultured species is more susceptible to disease attack in larval stages. Application of probiotics in aquaculture varies differently, either by introducing beneficial bacteria to the rearing water or by adding naturally occurring compounds to inert diet which might selectively stimulate beneficial gut bacteria. Introduction of these probiotics is done by introducing bacteria into the food chain by allowing the live feed organisms to graze on the bacteria, feeding either by spray dried bacteria to live feed or by incorporating spray dried bacteria in the formulated feed (Ringo and Birbeck, 1999).

2.4 Probiotic use in finfish

Kozasa (1986) was the first to use probiotics in finfish. He used a probiotic produced for livestock containing *Bacillus toyoi,* against *Edwardsiella* spp. infection in Japanese eel and observed disease resistance and increased growth rate. In marine fish larval rearing probiotic species are normally introduced into the larvae by giving encapsulated live feeds. *Brachionus plicatilis* (Muller) is routinely used as a first feed till *Artemia* nauplii can be introduced (Ringo and Birbeck, 1999). Gatesoupe (1989) observed that the bacteria associated with rotifers have a detrimental effect on the growth and survival of turbot larvae. Gatesoupe *et al.* (1989) used Toyocerin10^{10} containing spores of *Bacillus toyoi* and noticed an improvement in dietary value of rotifers and a slight increase in weight of larval turbot at day 10, when they were fed with enriched rotifers. Nicolas *et al.* (1990) reported a direct co-relation between bacterial population and rotifer production. Recently Chang and Liu (2002) studied the effectiveness of *B. toyoi* against Edwardisiellosis in European eel and observed slight antagonism against *Edwardisiella tarda.* In another experiment Gatesoupe (1991) used Paciflor 9

containing spores of *Bacillus* strain IP 5832 for rotifer enrichment. There was considerable reduction in the number of the pathogen *Aeromonas hydrophilla* in rotifer as well as turbot treatments and he could isolate the spores from turbot gut at day 10.

Many fish species harbour lactic acid bacteria (LAB) in their normal intestinal microbiota. The species include *Lactobacillus, Carnobacterium*, *Streptococcus* and *Leuconostac* (Ringo and Strom, 1994., Ringo *et al.* 1997., Strom, 1998., Sugita *et al.* 1998). The application of LAB as probiotics in fish larvae are well documented (Strom and Ringo, 1993, Ringo and Gatesoupe, 1998., Nikolskelainen, 2001a).

Gatesoupe (1989) noticed an increase in production rate of rotifers and their dietary value for larval turbot with a spray dried additive containing whey and LAB (*Streptococcus thermophilus* and *Lactobacillus helveticus*). Gatesoupe *et al.* (1989) observed decrease in bacterial proliferation in rotifers fed with Ascosil, a spray dried extract of sprouting cereal grains fermented with LAB and enriched overnight with fish oil emulsion. In another experiment, Gatesoupe (1991) cultured rotifer giving three strains of LAB, *Lactobacillus plantarum, L. helveticus* and *Streptococcus thermophilus*. Both the Lactobacillii strains improved the production rate of rotifers and *L. plantarum* was found to be more efficient than *L. helveticus*. The mean weight of larval turbot was significantly increased at day 20. There was significant reduction in the count of total aerobes and *Aeromonas salmonicida* was particularly inhibited.

Garcia de la-Banda *et al.* (1992) added *S. lactis* and *L. bulgaricus* to rotifers and *Artemia* nauplii; and recorded increase in survival rate of larval turbot at the end of weaning period when compared to control group. The study was found to be inconclusive since the group that received dead bacteria showed higher survival when compared to live bacteria, and no difference in larval growth was observed between the rearing groups. In another study Strom and Ringo (1993) isolated *L. plantarum* from cod and added it to rearing water of newly hatched cod larvae. The total aerobic count decreased and probiotic dominated in the treatment tanks. Similarly, Gatesoupe (1994) isolated *L. plantarum*/*Carnobacterium* spp. from

rotifers and noticed an increase in resistance of larval turbot when challenged with a pathogenic *Vibrio*.

Presence of *Carnobacterium* species in fish alimentary tract has been reported in Atlantic salmon (Ringo *et al.*, 1997; Joborn *et al.* 1997 and Strom, 1998), in hatchery reared and wild stock of Arctic charr (Ringo *et al.* 1997) and rainbow trout (Wallbanks *et al.*, 1990 and Spanggaard *et al.* 2000).

Strom (1988) was the first to isolate *Carnobacterium* from the intestine of Atlantic cod. He had originally classified it as *L. plantarum* but later it was identified as *Carnobacterium divergens*. Gildberg *et al.* (1997) used a dry feed of the same species for feeding cod fry. The cumulative mortality of the group fed with *Carnobacterium* was found to be more than that of those fed without it. *Carnobacterium* was found to dominate the intestinal flora of the fish and displaced other potential colonizers. Later Joborn *et al.* (1997) isolated *Carnobacterium* spp. strain K1 from gastrointestinal tract of Atlantic salmon and rainbow trout fingerlings were fed with the strain through feed. The strain survived passage through gastrointestinal tract and adhered non-specifically to intestinal mucous, colonized and multiplied readily in the intestinal mucus and faeces. The bacterium was found to be non virulent and was found to produce inhibitors against *V. anguillarum* and *A. salmonicida* in trout intestinal mucous and feces. Gildberg and Mikkelsen (1998) used two strains of *C. divergens* isolated from Atlantic cod and Atlantic salmon along with immuno-stimulating peptides for feeding Atlantic cod fry. Both the strains were found to colonize the mucous layer of pyloric caeca and intestine and survived the passage through gastrointestinal tract. Except in the beginning of experiment the cumulative mortality of fish supplied with LAB and immuno-stimulating peptides were same in all the groups. *In vitro* experiments showed inhibition against *V. anguillarum*. Robertson *et al.* (2000) used the above strain for feeding rainbow trout fry and Atlantic salmon fingerlings. The strain survived the passage through gastrointestinal tract of both the species and increased in number. After 14 days, challenge test done by cohabitation with infected fishes indicated effectiveness in reducing disease caused by *A. salmonicida, Vibrio ordalii* and *Y. ruckeri* but not *V. anguillarum*.

Harzevilli *et al.* (1998) isolated another LAB species *Lactococcus lactis* AR21 strain from rotifer mass culture. The strain was found to produce diplococcin which had slight inhibitory effect against *L. acidophilus*. Rotifers fed with the strain exhibited an inhibitory effect against *V. anguillarum* when rotifer cultures were maintained at sub optimal regime. The growth rate of rotifers was higher in the treatments receiving *Lactococcus* and *V. anguillarum* than in the treatment where only *V. anguillarum* was added. Another LAB species *Streptoccous lactis* improved production rate of rotifers and larval survival in turbot larvae (Garcia de la Banda *et al.* 1992). In another experiment Chang and Liu (2002) used *Entrococcus faecium* and *B. toyoi* for reducing Edwardsiellosis in cultured European eel. *E. faecium* was found to colonize and multiply in the gut constituting 73% of the total microflora. In a challenge experiment with *Edwardsiella tarda* the survival was significantly higher than the control.

Many species of Gram negative bacteria have been tested for their effectiveness as probiotic species in fish (Austin *et al.*1995., Gram *et al.* 1999).Opportunistic bacterial pathogens, particularly *Vibrio* species are present as part of the normal micro biota of marine fish and have been shown to be causative agents of disease and mass mortality (Horne *et al.* 1977., Austin *et al.* 1995., Muroga, 2001). However, a number of *Vibrio* species are known to inhibit the growth of pathogenic vibrios in fishes by the production of inhibitory substances (Onarheim and Raa, 1990).

Pybus *et al.* (1994) investigated the inhibitory effect of *V. anguillarum* strains against strains of closely related salmon pathogen *V. ordalii*. *V. anguillarum* strain VL4355 was shown to inhibit *V. ordalii*, suggesting the production of anguibactin related phenolate siderophore which were making iron unavailable for the growth of *V. ordalii*. Bergh (1995) isolated bacterial strains capable of inhibiting a pathogenic *Vibrio* from larvae of halibut and found that 93% of the pathogen inhibiting strains belonged to *Vibrio* genera. Ringo *et al.* (1996) isolated *V. pelagicus* from healthy turbot and added this to tank water containing newly hatched out larvae of *Scophthalmus maximus* (L). *V. pelagicus* antigen was detected in the larvae and the strain was able to colonize within 24 hours of inoculation and the species dominated the aerobic flora in treatment tanks. Ringo

and Vadstein (1998) added *V. pelagicus* and *Aeromonas caviae* into the rearing water of healthy turbot and both the species colonized larval guts. The larvae given *V. pelagicus* showed improved larval survival compared to other treatments. Huys *et al.* (2001) used *V. mediterranei* Q40 isolated from the sea bream larvae and few bacterial strains isolated from rainbow trout for their use as potential probionts in turbot larviculture. A non *Vibrio* cluster and *V. mediterranei* Q40 administered at the moment of mouth opening showed a distinctive and reproductive effect on larvae survival. Park *et al.* (2001) injected *V. vulnificus* bacterin to flounder which lead to an antibody response against *V. vulnificus* infection.

Matty and Smith (1978) compared the efficiency of a *Pseudomonas* bacterial protein, yeast protein and single cell algae protein on the growth of rainbow trout fingerlings. The gross conversion efficiency protein conversion efficiency and food conversion efficiency reached an optimum at 25% level for diets containing bacterial protein. Smith and Davey (1993) demonstrated that bathing in a strain of *Pseudomonas fluorescence* reduced subsequent mortality from stress induced furunculosis. In rainbow trout later Gram *et al.* (1999) evaluated *in vitro* and *in vivo* antagonism of antibacterial strain *Pseudomonas fluorescence* AH2 against fish pathogen *V. anguillarum*. The strain produced a siderophore and reduced accumulated mortality in treatment tanks. Gram *et al* (2001) also used the above species against *A. salmonicida*. In vivo studies inhibited the growth of *A. salmonicida* while in vitro studies did not produce any probiotic effect.

Makridis *et al.* (2000a) obtained encouraging result with an unidentified gram negative strain PB-52 and 4:44. Both species colonized the gut of turbot larvae but there was no improvement in survival rate and no reduction in total aerobic flora. Makridis *et al.* (2000b) tried to enrich rotifer with these strains and *Artemia* nauplii with PB111 and PB61. Bacteria were effectively accumulated in both the live feeds within 20-30 minutes of feeding. Spanggaard *et al.* (2001) isolated 1018 bacterial strains and yeast from rainbow trout. The dominant antagonist among them was *Pseudomonas* and the strain was found to improve survival of rainbow trout against vibriosis. Sugita *et al.* (1996) tested the antibacterial ability of bacterial strains isolated from intestine of fresh water fish and

found that 7-12 target pathogenic strains were inhibited by strains of *Aeromonas caviae*, *A. hydrophila*, *Pseudomonas* spp. and *Clostridium* spp.

The yeast *Saccharomyces cerevisiae* has long been used for the culture of rotifers. Coutteau *et al.* (1990) suggested using chemically treated *S. cerevisiae* as a substitute for *Dunaliella*. A strain, *Saccharomyces cerevisiae* CBS 7764, isolated from rainbow trout intestine showed colonization potential (Andalid *et al.* 1999). The same effect was noticed by Ramirez *et al.* (2000) in European sea bass larvae. Yeast *Deharomyces hansenii* a polyamine producing yeast recovered from the digestive tract of fish improved the survival but reduced the growth rate of larval sea bass following incorporation into the diet (Ramirez *et al*, 2002). Gatesoupe (2002) used two commercial probiotic preparations, one containing *S. cerevisiae* I-1079 and the other *Pediococcus acidilacti* for encapsulating *Artemia* nauplii and later on feeding them to Pollock larvae. Growth rate was more with bacteria alone but it was better with a combination of both. Lara-Flores *et al.* (2003) reared tilapia fry using different probiotic treatments. One supplement contained *Streptococcus faecium* and *L. acidophilus* and the other yeast *S. cerevisiae*. Fry fed with probiotic showed a greater growth rate than control and those fed with yeast produced better feed efficiency, and growth performance. Li and Gatlin (2003) fed hybrid bass with diets containing different levels of *S. cerevisiae*. Enhanced weight gain and feed efficiency were observed in fishes fed with yeast. Exposure to the pathogen *Streptococcus iniae* resulted in no mortality compared to 20% mortality in control fishes.

In India, work regarding the application of probiotics in fishes are few. Mohanty *et al.* (1996) evaluated the potential of both *Lactobacillus coagulance* and *S. cerevisiae* for growing *Catla catla*. Naik *et al.* (1999) used a G-probiotic for growing Tilapia fry for 90 days. The fishes fed with the probiotic showed better specific growth rate, feed conversion rate and protein efficiency ratio in the group given 7.5 g G-probiotic/kg diet. The G-probiotic is known to contain yeast.

Report on application of probiotic organisms in the fish grow-out is rather scanty. Kennedy *et al.* (1998) used a strain of *Bacillus subtilis* (no.48) isolated from common snook, *Centropomus undecimalis*. The strain improved

survival rate of larvae, increased food absorption and enhanced growth. The same strain also produced uniform growth rate and survival rate when tested in *Mugil cephalus* and spotted sea trout. The number of potential pathogenic species was also decreased. Sugita *et al.* (1998) isolated a *Bacillus* species strain NM-12 from coastal fish dragonet and demonstrated its antibacterial activity against a wide range of intestinal bacteria of coastal fishes. The strain was found to produce a heat liable siderophore with molecular weight of 5 kDa. Boyd and Gross (1998) used a commercial preparation containing live Bacillus and observed a greater net production and higher survival in catfish production ponds. The compound couldn't produce any significant improvement in water quality.

Nikolskelainen *et al.* (2001a) administered a human probiotic *L. rhamnosus* ATCC 53101 to rainbow trout through feed. They noticed a decrease in mortality when challenged with *Aeromonas salmonicida*. Nikolskelainen *et al.* (2001b) investigated the potential probiotic properties of six LAB species identified for human use, *L. rhamnosus, L. casei, L. bulgaricus, L. rhamnosus* LC705, *Bifidobacterium lactis* Bb12, *L. johnsonii* and *Enterococcus faecium* for their effectiveness as fish probiotics. All the cultures showed adhesion to mucus but were not able to inhibit mucus binding *Aeromonas salmonicida*. But a co-culture showed inhibition on the growth of *A. salmonicida,* mediated by competition for nutrients rather than secretion of inhibitory substances. *L. rhamnosus* and *L. bulgaricus* were found to penetrate fish mucus better than other probiotic and based on bile resistance, mucus adhesion, mucus penetration and suppression of fish pathogens, they were recommended as candidate probiotic species in fish culture. Austin *et al.* (1995) tested the probiotic effect of *V. alginolyticus* against pathogenic activity of *A. salmonicida, V. anguillarum, V. ordalii* and Y. *ruckerii* in Atlantic salmon. The application of probionts led to a reduction in mortalities after a challenge with *A. salmonicida* and to a lesser extent to *V. anguillarum* and *V. ordalii* and not for Y. *rukerii.* The probionts sustained in the intestine even 21 days after the initial application.

2.5 Probiotic use in Crustaceans

Most of the scientific studies regarding the application of probiotics in crustacean aquaculture relate to shrimp hatchery and production. In India, large varieties of commercial preparations are available either as water additive or feed additive for shrimp farming (Anikumari *et al.* 2001). These preparations available for use in shrimp farms mostly contain *Bacillus* as the dominant species and about 15 *Bacillus* species were being used in these products (Jorry, 1998).

Moriarty (1998) was the first to try the effectiveness of these products for culturing *Penaeus monodon*. The treated ponds showed abundance of *Bacillus* species in sediment and water column, luminescent vibrios were reduced and the production was high with higher survival rate. The control ponds showed total loss after 80 days of culture due to *V. harveyii* infection. In another study Prabhu *et al.* (1999) used a commercial preparation and demonstrated an improvement in nutrient levels, average daily growth, production and reduction in NH_3 levels. McIntosh *et al.* (2000) used two commercial probiotic preparations containing Bacillus species for culturing *L. vannamei* with zero water exchange. There was no significant improvement in mean survival rate, final weight FCR and in water quality parameters. In another study Devaraja *et al.* (2002) applied two microbial products, product-1 containing *Bacillus* spp. and *Saccharomyces* spp., and product -2 *Bacillus* spp., *Nitrosomonas* spp. and *Nitrobacter* spp. in commercial *P. monodon* ponds for a period of 110 days. There was considerable increase on the mineralizing bacterial population and the average production was relatively high for pond which received product-1. *Bacillus* species predominated in all the ponds. Shariff *et al.* (2001) studied the effectiveness of a commercial product in *P. monodon* ponds with poorly prepared pond bottom. The product contained *Bacillus*, nitrite and sulphur oxidizing bacteria and yeast *Saccharomyces* species. The survival rate, FCR and final production in treated ponds were relatively higher than the control ponds. In ponds with well prepared pond bottom receiving the same product showed substantially higher production in a parallel study. Ravichandran and Jalaluddin (2001) used Environ-AC a water probiotic for the production of *P. monodon*. The total production was higher in treated ponds than the control ponds and there was reduction in total vibrio counts. Recently Menupol *et al.* (2003)

demonstrated the effects of ozone with or without probiotic treatments in *P. monodon* post larvae. The survival rate of farmed shrimp receiving probiotics coupled with ozonation was found to be significantly higher than control ponds.

Studies on the applicaton of probiotic species isolated from crustacean habitat are few. Rengpipat *et al.* (1998) isolated a *Bacillus* S11 bacterium from *P. monodon* habitats and fed to post larvae of *P. monodon* through feed in the grow-out ponds. The probiotic fed groups showed higher growth rate and survival when compared to control. The species dominated in the tank water and the shrimp showed 100% survival when challenged with *V. harveyii.* The species dominated the gut microflora of probiotic fed animals.

Lactic acid bacteria have not been reported as residential microflora of crustaceans. However attempts have been made to feed LAB species through encapsulated live feeds and commercial preparations. Uma *et al.* (1999) used Lacto-sacc containing *L. acidophilus*, *Streptococcus faecium* and yeast *Saccharomyces* spp. They observed a positive effect on the growth and survival of treatment ponds with *P. indicus.* Challenge experiment with *V. alginolyticus* resulted in low mortality rate in lactosacc fed animals than in control group. They attributed this to the immuno-potentiating effect of yeast and LAB.

2.6 Probiotic use in crustacean larviculture:

Earlier reports showing the influence of bacterial culture were mostly enrichment of rearing medium with nutrients for the growth of natural flora which in turn increases the productivity of water and feed availability for larvae. Maeda and Liao (1992) cultured *P. monodon* with and without soil extract in seawater. Isolated bacterial strains from prawn biotope were added to the treatment tanks. The bacterial strain promoted growth of larvae, especially a strain PM-4 gave higher survival rate, and moulting rate. In another study Maeda *et al.* (1992) added several nutrients to improve the growth of bacteria and yeast. Later this microbial culture was added to *Portunus trituberculosis* larvae culture. The bacterial numbers decreased rapidly as crab larvae fed on these microorganisms. A bacterial strain F3 seemed to promote the growth of larvae. The strain PM-4 was used again by Nogami and Maeda (1992) for culturing *P. trituberculosis.* The strain repressed the

growth of *V. anguillarum* in seawater, dominated the culture water and production rate of crab larvae significantly increased. In another experiment the strain PM-4 identified as *Thallasobacter utilis* was used as biocontrol agent for rearing *P. trituberculosis*. The larval survival was much higher when compared to control and the strain inhibited the growth of *V. anguillarum* and the fungus *Haliphthora* species.

Gil-Turnes *et al.* (1989) isolated an epibiotic *Alteromonas* strain from surface of *Palaemon macrodactylus* embryos. The strain inhibited the growth of pathogenic fungus *Laginedium calinectes* by the production of 2, 3-indolinedione (isatin). In another study Gil-Turnes and Fenical (1992) isolated Gram-negative epibiotic bacteria from embryo of *Homarus americanus*. The strain produced an antifungal substance 4-hydroxy phenethyl alcohol (tyrosol) that completely inhibited the growth of *L. calinectes*. Haryanti *et al.* (1998) used a strain BY-9 belonging to the genera *Alteromonas* for rearing *P. monodon* larvae from protozoea I to PL-10. The probiotic at a concentration of 10^6 CFU/ml inoculation daily in the water was effective in improving larval survival growth and suppressed the growth of pathogenic *V. harveyii*. In another study Abraham *et al.* (2001) isolated *Alteromonas* spp. TJP7 from *P. monodon* mysis 2. The strain inhibited pathogenic *Vibrios* especially *V .harveyii and V. splendidus* in-vitro as well as in-vivo in *P. indicus* larvae. The inhibitory activity of the strain was thought to be due to the production of inhibitory secondary metabolites released in to the environment. Tanasomwang *et al.* (1998) isolated 48 strains of bacteria showing inhibitory activity from *P. monodon* larvae, *Artemia* nauplii and rearing water and 27 strains isolated showed inhibitory activity against 32-94% of *Vibrios* tested and were identified as *Alteromonas*- like organisms. These strains were found to produce an antibiotic, a thermo labile protein with high molecular weight extracellularly.

Yasuda and Taga (1980) evaluated the effect of two *Pseudomonas* strains P-1 and P-7 for mass culture of rotifer. The strain P-7 was found to be superior as rotifer population multiplied four times after two days and 6.5 times after 3 days. Nutrient generation in tanks were improved which in turn might have helped multiplication of rotifers. *Pseudomonas* was found to be a good food supplement for the growth of *Artemia* (Gorosope *et al.* 1996). A combination of heat dried

Pseudomonas and rice bran resulted in better survival and growth of *Artemia* nauplii as source of protein and amino acid. Chythanya *et al.* (2002) isolated a *Pseudomonas* 1-2 strain identified as *P. aeruginosa* from estuarine water. The strain produced inhibitory compounds against *V. harveyii*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela* and *V. vulnificus* in invitro experiments. The strain produced an extra cellular low molecular weight non-proteinaceous heat stable anti-vibrio compound, which could be pyocyanine.

Griffith (1995) reported the use of *V. alginolyticus* in Equadorian shrimp hatcheries. The hatchery down time was reduced from approximately 21 days to 7 days annually and the production volume increased by 35% and the antibiotic use was reduced during the period 1991-1994. In another study Garriques and Arevalo (1995) used *V. alginolyticus* isolated from seawater for rearing *P. vannamei* larvae. Average survival and growth rate were higher in the treatment tanks compared to those receiving antibiotics and control. The occurrence of *V. parahaemolyticus* was totally suppressed in tanks receiving *V. alginolyticus*.

Although the yeast *Saccharomyces* spp. has been included in many of the commercial probiotic preparations, studies regarding their effect on shrimp production are few. There are many reports on the effectiveness of immuno-stimulants prepared from these species. Nates (1990) used different levels of Perkin Brewers Dried yeast for rearing the shrimp *Litopenaeus vannamei* for 35 days. The survival and growth rate was comparatively better in treatment tanks but not significant. The species have been used for feeding crustacean live feeds and their effect has been well documented.

Patra and Mohamed (2003) used *Saccharomyces boulardii* for enriching *Artemia* nauplii. The survival rate and resistance against pathogenic *V. harveyii* infection were significantly improved in treated nauplii than the control. Scholz *et al.* (1999) used cells and β-glucan of *S. cerevisiae*, an isolate of *S. exigouos* containing xeaxanthin (HPPRI) and *Paffia rhodozyma* for rearing juvenile *Penaeus vannamei*. The animals showed improved resistance to vibriosis infection and the diet containing *P. rhodozyma* lead to increased larval survival.

2.7 Probiotic use in Molluscs:

The microbial flora of rearing water serves as a source of food for molluscan larvae, but the prevalence of opportunistic forms results in poor production rates at times. Martin and Mengus (1977) inoculated a single beneficial strain to cultures of bivalve larvae but the beneficial effect of the added strain was not consistent in repeated experiments. Douillet and Langdon (1993) isolated 21 strains of marine bacteria from oyster and algal cultures and added them to bacteria free cultures of oyster larvae. Most were detrimental to larval survival and growth but the strain CA2 enhanced larval survival and growth. In another study Douillet and Langdon (1994) added the same strain to axenic larval cultures of oyster *Crassostra gigas*. Addition at 10^5 cells/ ml^{-1} to cultures of algae fed larvae increased larval growth, the proportion of larvae that set to produce spat and the subsequent size of spat. The bacteria might have provided essential nutrients not present in the algae or improved larval digestion by contributing enzymes.

Riquelme *et al.* (1996) isolated *Alteromonas haloplanktis* strain showing inhibitory activity against pathogens, *V. ordalii, V. parahaemolyticus, V. anguillarum* and *Aeromonas hydrophila* from gonads of *Argopecten purpuratus* brood stock. Later Riquelme *et al.* (1997) evaluated a total of 506 bacterial isolates from hatchery and laboratory sources for the production of inhibitory substances against *V. anguillarum*. Eleven strains were found to be effective and one of these strains (*Vibrio* spp.) protected the scallop larvae against *V. anguillarum* related pathogens. In another study Riquelme *et al.* (2000) carried out experiments using three selected strains of bacteria named as 11, C33 and 77 isolated in the previous experiment. The larvae ingested cells of strains 11 and 77 but not C33 and the strain 77 became the dominant bacteria of the larval microflora. Gibson *et al.* (1998) tested *Aeromonas* strain A199 showing bacteriocin like inhibitory activity against *Vibrio tubiashii* infection in *C. gigas* larvae during a challenge experiment. The strain could prevent the mortality where as in control all the larvae died in 5 days. Nakamura *et al.* (1999) isolated 51 strains from *C. gigas* cultures and tested their inhibitory activity against three *Vibrio* species. The strain S21 showing the highest vibriostatic activity was used as biocontrol agent for rearing *C. gigas* larvae. The larvae showed 78% survival when challenged with *V. alginolyticus* for 24 hours. Avendano and Riquelme (1999) used two bacterial strains, 11 and C33 for feeding *Argopecten purpuratus*. Significant ingestion of strain 11 when

directly added to the water and C33 when given in conjunction with micro algae was noticed, suggesting the use micro algal cultures as vectors for the introduction of probionts.

2.8. Probiotic use in Algae culture

Microalgae may have an impact on bacterial community of the larval tanks (Bell *et al.* 1974., Salvesen *et al.* 2000) and may promote or inhibit bacterial growth by production of organic exudates and toxic metabolites (Brock and Clyne, 1984; Duff *et al*, 1996). Rico-Mora and Voltolina (1995) isolated a strain SK-05 from *S. costatum* cultures and demonstrated the effectiveness of the strain in promoting *Artemia* nauplii growth. The same strain prevented the growth of *V. alginolyticus* in *S. costatum* culture by competitive exclusion (Rico-Mora *et al.*, 1998). Suminto and Hirayama (1997) tested the effect of marine bacterium, *Flavobacterium* sp. on the growth of three microalgae, *Chaetoceros calcitrans*, *Isochrysis galbana* and *Pavlova lutheri*. The bacterium was effective in keeping higher cell density during the stationary phase and the specific growth rates of *C. gracilis* in treated cultures were found to be higher than the controls. The strain dominated among the bacterial flora in the cultures. Avendano and Riquelme (1999) selected three bacteria strains based on their efficiency to grow in the extra cellular products of *I. galbana* and tested their effect on the growth of *I. galbana*. The effect on growth rate was not significant but the strain C33 was found to inhibit the pathogen *V. anguillarum*.

Salvesen *et al.* (2000) evaluated the impact of algal growth rates on the bacterial load of, *Skeletonema costatum*, *Chaetoceros mulleri, Nanochloropsis oculata*, *Isochrysis galbana, Pavlova lutheri* and *Tetraselmis* species. Higher bacterial load were found to be associated with Bacillariophyceae and bacterial levels were lower in *Tetraselmis* spp. and *P. lutheri. Tetraselmis* showed the low levels of opportunistic bacteria and *Vibrios* were low in *P. lutheri* in semi-continuous cultures Yousoff *et al.* (2002) applied a commercial probiotic product containing Bacillus and yeast to shrimp culture ponds to study the effect on phytoplankton communities. Diatoms and Dinoflagellates dominated the treatment ponds where as Cyanoflagellates dominated the control ponds by the end of production ponds. Rajeev Kumar (2003) reported improved algal growth rate with prolonged stationary period

with a single dose of *S. boulardii* (10^4 CFU/ml). Total aerobic count and *Vibrios* were considerably reduced when compared to the control.

Inhibitory activity of algae and its effectiveness in establishment of beneficial strains also have been reported (Bell *et al.* 1974). Kitto and Regunathan (1997) used an extract skelon made from *S. costatum* for feeding *P. monodon* larvae. The extract showed bacteriostatic activity and luminescent *Vibrio* was inhibited. Naviner *et al.* (1999) extracted active components from *Skeletonema costatum* and tested it against some pathogens of shell fish and fish. The growth of *V. anguillarum* and several species of *Vibrio* appeared to be inhibited. Olsen *et al.* (2000) used *Tetraselmis* for feeding *A. fransiscana* nauplii. The algae reduced total Vibrio counts, especially *V. alginolyticus* and relative diversity of associated bacterial flora increased. The effect may be possibly due to the removal of substrate for the opportunistic bacteria. When these *Artemia* were fed to Atlantic halibut similar effect was observed. The effect of another probiotic bacterium when cultured along with *C. mulleri* was demonstrated by Gomez-Gil *et al.* (2002). The bacterial density increased when cultured with algae but the algal density was unaffected when compared to the control. The strain growth was thought to be due to utilization of nutrients produced by algae or by the high organic material available when the algal cells die.

MATERIALS AND METHODS

3.0. MATERIALS AND METHODS

Experimental facility

The experiments were conducted using facilities in the laboratory and hatchery.

3.1. Laboratory unit

All microbiological work was carried out in I.F.S Project Laboratory Room in Molluscan Fisheries Division (MFD) of CMFRI Head Quarters, Cochin.

3.1.1. Microbiology

3.1.1.1. Sterilization

All glasswares and diluents used for bacteriological work were steam sterilized in an autoclave at 15 lbs/in for 20 minutes. All bacteriological media were steam sterilized at 10 lbs/in for 15 minutes. The sterilized glasswares were then transferred to a Laminar flow hood to avoid further contamination. All bacteriological media required for the experiment were procured from Hi Media Lab (Mumbai), and stored as per the instruction given by the manufacturer.

3.1.1.2. Bacteriological media and Chemicals

Following media were used for microbiology and Algal culture.

Bacteriological media

a. Sabouraud Dextrose Agar

Special peptone	10 g
Dextrose	20 g
Agar	15 g
Distilled water	1000 ml
Final pH (at 25°C)	6 – 6.3

b. *Vibrio harveyii*-media (Singh and Philip, 1993)

Prawn muscle extract	50 ml
Peptone	5 g
Yeast extract	1 g
Glycerol	30 ml
Seawater	1000 ml
Agar	15 g
Final pH (at 25°C)	7.2 ± 0.2

c. NCIM media for *Lactobacillus*

Glucose	5 g
Lactose	5 g
Sodium acetate	6 g
Yeast extract	5 g
Liver extract	10 g
Salt A	5 ml
Salt B	5 ml
Agar	15 g
Distilled water	1000 ml
Final pH	7.6

d. Thiosulphate Citrate Bile Sucrose (TCBS) Agar (M189, HiMedia, Mumbai)

Yeast extract	5 g
Protease Peptone	10 g
Sodium Thiosulphate	10 g
Sodium Citrate	10 g
Ox Bile	8 g
Sucrose	20 g
Sodium Chloride	10 g
Ferric Citrate	1 g

Bromo Thymol Blue	0.04 g
Agar	15 g
D.W.	1000 ml
Final pH (at 25°C)	8.6 ± 0.2

e. Seawater Agar

Yeast extract	5 g
Peptone	5 g
Beef Extract	3 g
Agar	15 g
Seawater	1000 ml
Final pH	8.6 ± 0.2

Phytoplankton culture media

Conway's or Walne's culture media (Walne, 1974)

Constituents	Quantity
Solution A	
Potassium Nitrate	100 g
Sodium orthophosphate	20 g
EDTA (Na)	45 g
Boric acid	33.4 g
Ferric Chloride	1.3 g
Manganese Chloride	0.36 g
Distilled water	1000 ml
Solution B	
Zinc Chloride	4.2 g
Cobalt Chloride	4.0 g
Copper Sulphate	4.0 g
Ammonium Molybdate	1.8 g
Distilled water	1000 ml

Solution C	
Vitamin B1 (Thiamin)	200 mg / 100 ml D.W
Vitamin B12 (Cyanocobalamine)	10 mg / 100 ml D.W
Solution E	
Sodium Silicate	1 ml / 1000 ml D.W

A, B and C (each) in different reagent bottles were prepared. 1ml of A, 0.5ml of B and 0.1 ml of C were added to 1000 ml of filtered and sterilized seawater.

3.1.2. Microbial strains

3.1.2.1. *Saccharomyces boulardii* (Plate.1)

Saccharomyces boulardii (SB) is commercial human probiotic yeast produced by Laboratories Biocodex, France and available in lyophilized powder form in gelatin capsules. *S. boulardii* was provided by Dr. F.J. Gatesoupe, IFREMER Centre de Brest, Plouzane, France and was stored at 4°C in a refrigerator. Characterization of SB was carried out by means of API 20C Aux test kit (Bio Merieux, France) comprising of 20 biochemical tests.

Preparation of SB broth

Contents of the capsule were transferred to Sabouraud Dextrose broth aseptically and incubated at room temperature under constant agitation. Inoculum (10%) from this culture was then used for further production of SB broth. After serial dilution, spread plates were made in duplicate on Sabouraud Dextrose Agar plates and incubated at 37°C for 48 hours. Total CFU/ml were enumerated as per Pothoulakis *et al.* (1993). Standardisation of the time for maximum CFU/ml production was also determined by counting the CFU/ml at 24, 48 and 72 hours.

3.1.2.2. *Lactobacillus plantarum* (Plate.2)

The strain was obtained from MTCC (Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India), MTCC code 1325 in powder form in sealed glass vials and stored in refrigerator at 4°C. Characterization of *L. plantarum* was carried out by means of API 20A test kit (Bio Merieux, France) comprising of 24 biochemical tests.

Preparation of *L. plantarum* broth

Growth of the strain was tested using, instruction given by MTCC, LMRS media supplied by HiMedia Labs and also in Lactobacillus media given by National Collection of Individual Microorganisms (NCIM), Pune. As the strain was found to grow better on the media composition given by NCIM, this media was selected for further production of the strain. The strain was transferred aseptically from the vial to culture broth and incubated at 37°C for 48 hours. The CFU/ml was determined by spread plate method on agar plates. The strain thus cultured was stored in agar slants and also in soft agar in test tubes at 4°C in refrigerator for subculture and use.

3.1.2.3. *Bacillus subtilis* (Plate.3)

The strain was isolated from NCIM media plates during the probiotic feeding experiment conducted on *Penaeus monodon* larvae using *L. plantarum.* Identification of the strain was done using fatty acid profile method TSBA 40. The strain was found to grow well on NCIM media for LAB. The strain was found to form dominant flora and showed *in vitro* antagonistic activity against *V. harveyii* and therefore it was selected for use as a candidate probiotic species for further enrichment experiments. The isolated strain was purified by subculture and stored in agar slants and tubes at 4°C in refrigerator for further production. The CFU/ml after 48 hours incubation at 37°C was determined on NCIM media plates.

3.1.2.4. *Vibrio harveyii* (Plate. 4)

Pure culture of luminescent *Vibrio*, *Vibrio harveyii* isolated from diseased shrimp was obtained from Centre for Fish Diagnosis and Management (CFDDM), CUSAT, Cochin and sub cultured as per the method given by Singh and Philip (1993). The culture was stored in agar slants and tubes at 4°C in refrigerator after 24 hours incubation at 37°C.

3.2. Hatchery

Hatchery included two sections, larval rearing unit and algal culture unit. The facility was set up in Marine Hatchery complex, CMFRI, Cochin.

3.2.1. Seawater

Seawater collected from sea off Kochi having 32 ppt salinity and 8.1 pH was stored in reservoir for hatchery use. After settling for 24 hours, seawater was pumped into 1 ton and 500 liter FRP tanks. The salinity was reduced to 30 ppt and chlorinated with sodium hypochlorite to a level of 30 ppm active chlorine. The water was mixed well and the aeration was removed. After 24 hours, vigorous aeration was provided and residual chlorine was removed with the application of Sodium thiosulphate as per the requirement and allowed to settle. Settled water was then pumped to 500 l overhead tank and filtered through a gravity sand filter and stored in 500 l black coloured tanks with cover.

3.2.2. Aeration

Aeration was given from air lines connected to a 5HP air blower. Air stones with air tubes were provided to the rearing containers for air supply.

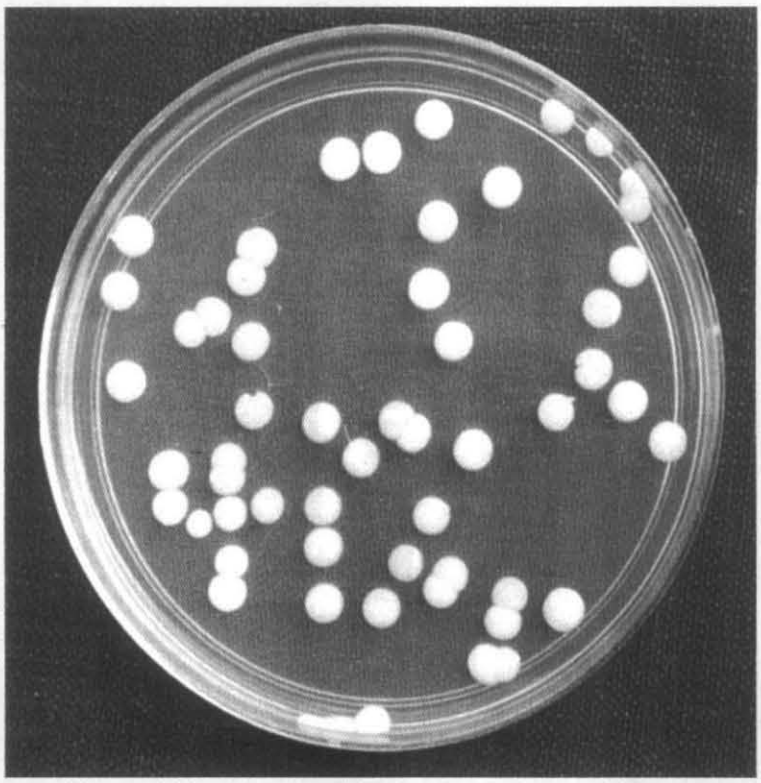

Plate 1. SB colonies on Sabouraud Agar plate

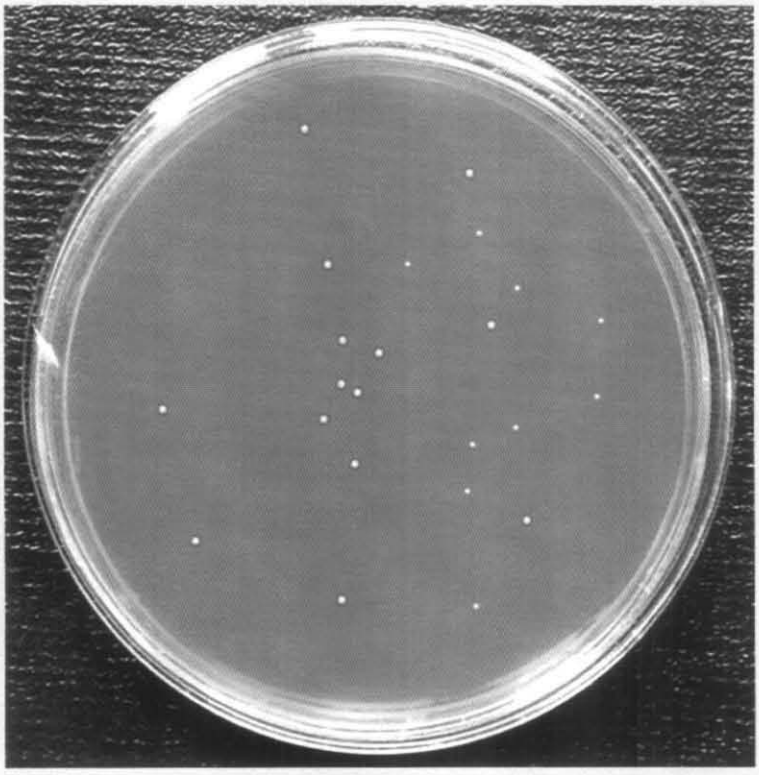

Plate 2. *L. plantarum* colonies on NCIM media plate

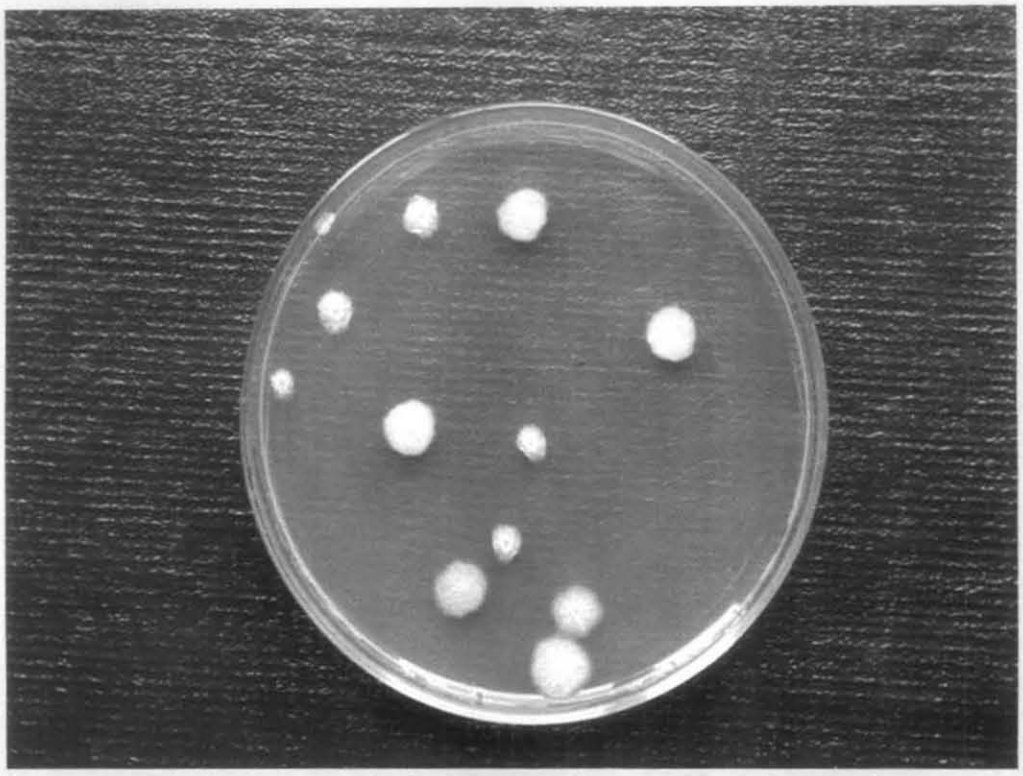

Plate 3. *B. subtilis* colonies on NCIM media plate

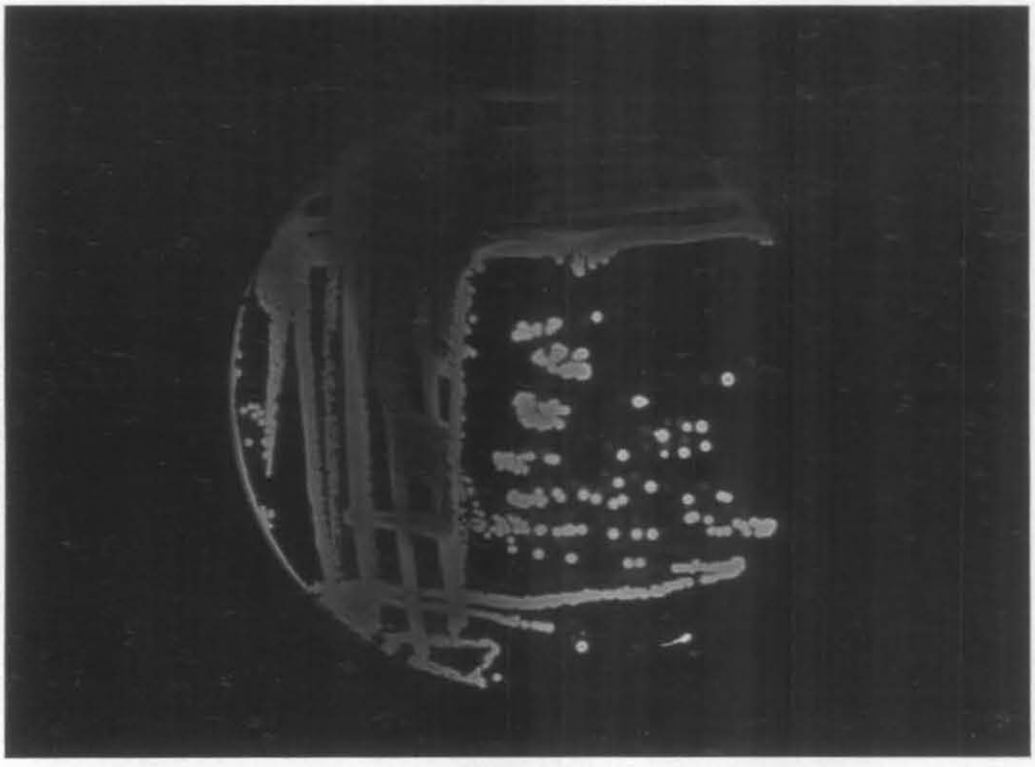

Plate 4. *Vibrio harveyii* colonies showing luminiscence

3.2.3. Filtering unit

A 60 l plastic bin was fitted with an outlet at the bottom and filled with oyster shells, activated charcoal and fine sand respectively from the bottom to make a good filtering unit. Sand layer was maintained at 60 cm thickness to provide efficient filtration. The filtering unit was connected to the overhead tank for water supply.

3.2.4. Larval rearing experiments

The experiments were conducted in two different steps. In both the steps, the following parameters were monitored.

a. Level of assimilation of probiotic microorganisms by larvae and post larvae.

b. Growth determined by finding out the weight gained after feeding probiotics.

c. Survival rate: determined every alternate day by counting the number of live larvae in each control and treatment units.

d. Evaluation of the success of probiotics feeding assessed by conducting a *Vibrio* challenge test using *Vibrio harveyii.*

e. Effect on other bacterial flora evaluated by counting total aerobic flora on Sea Water Agar plates and *Vibrios* on TCBS plates.

3.2.4.1. Experiment I

Effect of enrichment of probiotic organisms in zoea and mysis stage larvae of *Penaeus monodon.*

3.2.4.1.1. Larval rearing units (Plate.7)

The experiment was carried out in 3 l transparent flat bottom glass containers. Control and treatment units were kept in triplicates. Treatments were

allotted using random allotment method. The jars were filled with chlorinated and dechlorinated and filtered seawater with 30 ppt salinity.

3.2.4.1.2. Larval rearing

Healthy nauplii of *P. monodon* were procured from commercial hatcheries and acclimatized in the marine hatchery complex at CMFRI, Kochi. The larvae after reaching N_6 stage were collected using their positive photo tactic behavior, counted and stocked in glass jars at the rate of 100 nauplii/ litre. After moulting to zoea 1 the larvae were given both probiotics and algae. The larvae were reared up to PL. 1 stage in fifteen uniform size, 3 liter glass jars with 2.5 litre of treated and filtered sea water. The feeding was carried out daily in the morning after water exchange. Water exchange was carried out at the rate of 50%/day. Enumeration of larval density was carried out once in two days to determine survival rate. The larvae were observed everyday under microscope to assess the larval metamorphosis and health condition.

3.2.4.1.3. Feeding

a. Algae

The marine diatom *Chaetoceros calcitrans* was given with an initial concentration of one lakh cells/ml and later changed to *ad libitum*.

Algae culture unit (Plate. 5 and 6)

Pure culture of *Chaetoceros calcitrans* was obtained from FEMD algal laboratory (CMFRI) and used for subculture. Walne media (Walne, 1974), as mentioned in section 3.1.1.2. was used for the culture. Treated, filtered and boiled seawater with a final salinity of 32 ppt was used for pure culture. Ten percent inoculum was added from the stock culture to a volume of 3 litre, enriched seawater in transparent pearlpet jars and cultured indoors with sufficient aeration and light. After 48 hours when the culture reached its maximum exponential phase (3 to 4 million cells/ml), it was harvested and used for mass production in 15 l translucent plastic containers (buckets) with sufficient aeration and light. Harvesting was done after 48 hours. New cultures were made every day to get a minimum quantity of 10 l culture

Plate 5. Indoor algal culture

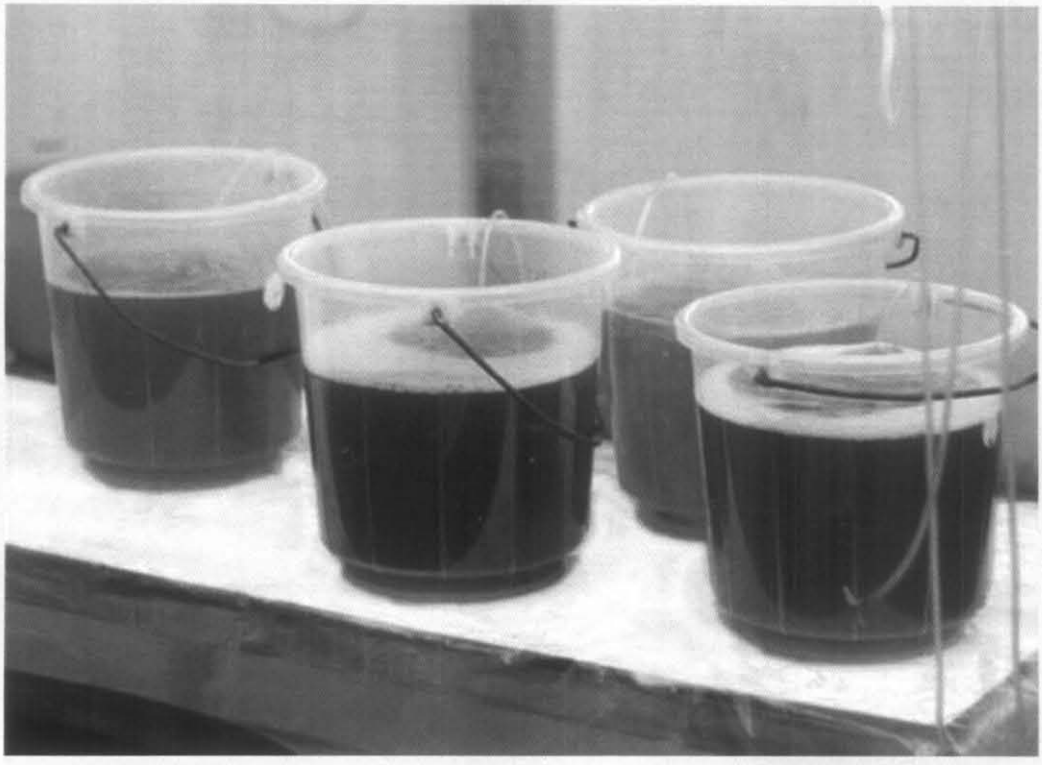

Plate 6. Out door algal culture

Plate 7. Larval rearing (Zoea to PL. 1)

Plate 8. Post larval rearing unit (PL. 3 to PL. 20)

(23 million cells/ ml) every day. The cultures were observed every day under microscope before feeding and inoculation. Impure cultures were discarded and the containers were disinfected when required. Every two weeks stock culture was renewed with supply of inoculum from the algal laboratory.

b. Probiotic

Three probiotics strains *Saccharomyces boulardii, Lactobacillus plantarum* and *Bacillus subtilis* were used for feeding experiments. The methods are summarized in Table 2.

Table 2. Experiment trials with *P. monodon* using different enrichment concentrations and treatments

Sl No	Trail No	Rearing stage	Enrichment with	Concentrations used	Treatment No.
1	TR-1	*P. monodon* Zoea –PL.1	SB	10^3 & 10^4	T_1 & T_2
2	TR-2	*P. monodon* Zoea –PL.1	SB	10^3, 10^4, 10^5, 10^6	T_1, T_2, T_3 & T_4
3	TR-3	*P. monodon* Zoea –PL.1	LAB	10^2, 10^3 & 10^4	T_1, T_2 & T_3
4	TR-4	*P. monodon* Zoea –PL.1	LAB	10^5, 10^6 & 10^7	T_1, T_2 & T_3
5	TR-5& TR-6	*P. monodon* Zoea –PL.1	*Bacillus subtilis*	10^4, 10^5 & 10^6	T_1, T_2 & T_3
6	TR-7& TR-8	*P. monodon* Zoea – PL.1-PL. 20	SB enriched *Artemia*	10^4	*T_1 & **T_2
7	TR-9	*P. monodon* Zoea –PL. 3 -PL.20	*Bacillus subtilis* enriched *Artemia*	10^5	*T_1 & ** T_2

- (fed with enriched dead *Artemia*); ** (live enriched *Artemia*)

1. ***S. boulardii***

Three trials of feeding experiments were carried out using *S. boulardii.* Broth culture containing live cells of *S. boulardii* were prepared as per methods given in section 3.1.1.2. In TR_1, broth was directly added to the culture medium at 10^3 for T_1, 10^4 for T_2 and 10^5 for T_3 treatments. As this method polluted the rearing medium and resulted in poor survival of larvae, the following method was used for harvesting pure colonies and preparing saline suspensions.

Preparation of saline suspensions

Pure cultures of probiotics were sub-cultured by overlaying sterile cellophane film on respective SB agar plates for *S. boulardii,* NCIM media agar plates for *Lactobacillus plantarum* and *Bacillus subtilis*. The cellophane sheets were previously cut into small circles as per the size of the plates and steam sterilized. One film each was overlaid on prepared agar plates and stored at 4°C. Pure cultures of probiotics were swabbed on these plates as and when required and incubated at 37°C for 48 hours. After incubation, cellophane films were aseptically transferred to sterile saline and agitated. The cell suspensions thus prepared was transferred to a sterile flask and stored at 4°C for further use. Total CFU/ml in this suspension was determined by using spread plate technique on respective agar plates after serial dilution.

1. *S. boulardii*

Feeding trial with cells suspended in saline: TR 1 cells suspended in saline were used. In both trials treatment T_1 represent 10^3, T_2-10^4, T_3 - 10^5 and T_4 -10^6 CFU/ml of *S. boulardii*. In T_4, except in one replicate, total mortality resulted in two days.

2. *Lactobacillus plantarum*

Pure culture of *L. plantarum* was isolated on NCIM Agar plates from stored agar tubes. Saline suspension of *L. plantarum* cells was prepared as per the method described for *S. boulardii* from NCIM Agar plates. Total CFU/ml in the

suspensions was counted on NCIM Agar plates after spread plate. Two trials were conducted using this probiotics. In TR 3 three treatments (T_1 -10^2, T_2 -10^3, and T_3 -10^4) with appropriate control were kept in triplicates. In TR 4 the concentrations used were T_1 - 10^5, T_2 - 10^6, and T_3 - 10^7.

3. *Bacillus subtilis*

Broth culture containing *B. subtilis* was prepared as per the methods given in section 3.1.1.2. Two trials were conducted using this probiotic organism. In both the trials cells suspended in saline were prepared as already mentioned for *S. boulardii* using NCIM media plates. In TR 5 and TR 6 the concentrations used were T_1 – 10^4, T_2 – 10^5, and T_3 – 10^6.

3.2.4.1.4. Bacteriology

Bacteriology of the larvae were conducted during ZIII and MIII stage. Ten larvae from each replicate were collected in sterile plastic vials and brought to the laboratory. The larvae were transferred aseptically to sterile sieves inside the laminar flow hood and washed with enough sterile saline to eliminate any external contamination. The larvae were then transferred to sterile homogenizers and homogenized thoroughly. The volume was made up to 5 ml and mixed thoroughly using a Vortex shaker and serial dilution of the homogenate was carried out. Total CFU/ml was determined by spread plating in duplicate for the following:

a. *S. boulardii*: Plated on SB Agar plates in duplicate at 10^{-1} and 10^{-2} concentration.

b. *L. plantarum*: Plated on NCIM media agar plates in duplicates at 10^1 and 10^2 concentration.

c. *B. subtilis*: Plated on NCIM media agar plates at10^{-2} and 10^{-3} concentrations.

d. *Vibrio*: Plated on TCBS Agar plates at 10^{-2} and 10^{-3} concentrations.

e. Total aerobic flora: Determined by plating on Seawater Agar plates at 10^2 and 10^3 concentrations.

Plate 9. *Vibrio* challenge experiment

The spread plates were incubated at 37°C for 48 hours for *S.boulardii*, *L. plantarum* and *B. subtilus* and 37°C for 24 hours for *Vibrio* and total aerobes. Enumerations of total CFU/ml were carried out after incubation.

3.2.4.1.5. *Vibrio* challenge test (Plate.9)

This test was carried out using pathogenic *V. harveyii* isolated from diseased *P. monodon* (section 3.1.1.2). As in the case of probiotics cultures subculture was carried out on Nutrient Agar plates overlaid with cellophane. The cells were harvested after 24 hours incubation at 37°C and suspended in saline. Fresh cultures were prepared before the start of each challenge experiment and CFU/ml was determined before storage at 4°C.

Challenge experiment was carried out in one liter white sterile polycarbonate bottles with 700ml treated and filtered seawater. Ten post larvae (PL.1) from each replicate were collected and transferred aseptically to these bottles. *Vibrio* cells in saline were added at 10^5 CFU/ml and mixed well. No aeration was given during the experiment. The bottles were covered with their respective caps to avoid contamination. Larval survival was enumerated after 24 and 48 hours. A negative control was also kept in triplicates without pathogen.

3.2.4.1.6. Weight gain

After the rearing experiments (PL.1 stage), 20 samples from each replicate were collected and preserved in 5% formalin. Larval weights were determined in an electronic balance with 0.1mg accuracy after one month to avoid any post-mortem distortion in weight (Lockwood and Daly, 1975).

3.2.4.2. Rearing *P. monodon* post larvae (PL. 3 to PL. 20) using probiotic enriched *Artemia* Instar-II nauplii

The experiments were conducted using enriched *Artemia* metanauplii. Two trials were conducted with *S. boulardii* enriched nauplii and one trial with *B. subtilis* enriched nauplii.

3.2.4.2.1. Experiment-II. *Artemia* enrichment experiments

a. Artemia cysts

Good quality *Artemia* cysts were procured from commercial aqua feed suppliers. Two brands, O.S.I (San Fransisco Bay) and Red Dragon were used for enrichment experiments. The sealed cans containing dry cysts were kept in a cool dry place away from light. The cysts were observed under microscope to check their morphological appearance and defects if any.

b. *Artemia* hatching and decapsulation (Plate. 10)

Cysts were weighed out and hydrated for one hour in fresh water with aeration. After hydration, the cysts were collected using a 120µ sieve. Decapsulation of the cysts was then done following the method described by Lavens and Soregeloos (1996).

Decapsulation

Hydrated cysts were collected using 120µ sieve and transferred to hypochlorite solution @ 1g cyst/ 14 ml hypochlorite solution with 0.5 g active product. The pH of the solution was raised to about 10 by adding 40% NaOH solution @ 0.33ml/1g cysts. Aeration was given vigorously to keep the cells in suspension. After 5 to 10 minutes, when the colour of the cysts changed from dark brown to pale orange and orange, the cysts were collected on a 120µ sieve and washed with fresh water to remove the chlorine. Later all traces of chlorine were removed by dipping the cysts in 0.1% $Na_2S_2O_3$ solution. The cysts were again washed with fresh water and kept for incubation and hatching.

Hatching

Decapsulated cysts were incubated for hatching in treated seawater @ 20 g cysts/l. Sufficient aeration and light were provided for hatching. The incubation was done up to 24 hours and hatched out Instar - I nauplii were harvested by exploiting the positive photo tactic behavior of the nauplii using 120µ sieve. Harvesting

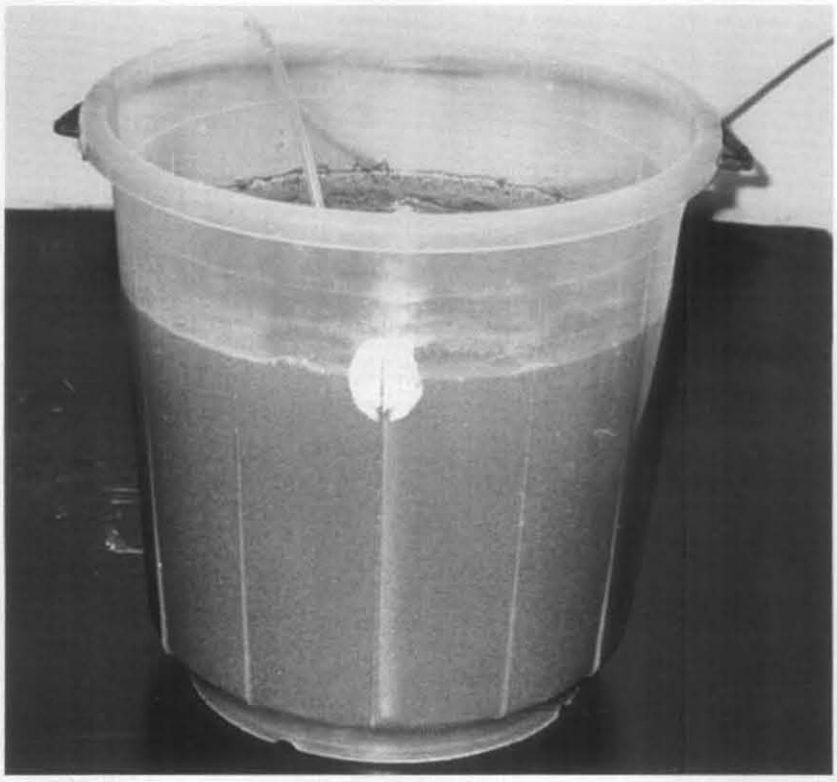

Plate 10. *Artemia* cyst incubation

Plate 11. *Artemia* metanauplii enrichment

was repeated twice with the addition of fresh seawater to remove shells and unhatched cysts completely.

c. *Artemia* enrichment unit (Plate. 11)

The experimental unit was set up in the microbiology laboratory of MFD, CMFRI, Kochi. The unit consisted of cylindrical flat bottom glass jars of 3 liter capacity each. Control and treatment jars were arranged in triplicates. The jars were filled with 2 liters of treated and filtered seawater. Sufficient aeration was provided using small air pumps. Clean and disinfected air tubes and stones were used for each jar. The jars were kept covered with clean black cloth. The harvested Instar - II nauplii after 28 hours were stocked in each jar at the rate of 20 nauplii/ml.

Probiotic enrichment

Three probiotic species *S. boulardii*, *L. plantarum* and *B. subtilus* were used for enrichment experiments. With *S. boulardii* the experiments were conducted using both the brands of *Artemia*. *L. plantarum* enrichment experiment was conducted using Red Dragon brand while OSI brand was used for *B. subtilus* enrichment experiments. The following parameters were monitored for all the probiotic species:-

a. Optimum enrichment duration for the three probiotic species.

b. Variations in optimum enrichment duration and concentrations between the two brands of for *S. boulardii*.

c. Effective concentrations at which enrichment can be carried out.

d. Enrichment

a. *S. boulardii*: Two treatments T_1 and T_2 were kept in triplicates. T_1 and T_2 correspond to 10^3 and 10^4 concentrations of *S. boulardii* broth. The control and treatment jars were made in triplicates. Experiment regarding the effective concentrations of *S. boulardii* was already carried out in another study (Patra and Mohamed, 2003). As survival and enrichment was better for 10^3 and 10^4

concentrations, these two concentrations were selected to find out the optimum enrichment duration for *S. boulardii*.

b. *L. plantarum*: NCIM media broth after 48 hours incubation containing 10^8 CFU/ml was used for enrichment experiments. Four treatment units T_1, T_2, T_3 and T_4 corresponding to 10^3, 10^4, 10^5, and 10^6 respectively, were used for the experiment.

c. *B. subtilis*: NCIM media broth containing 10^8 CFU/ml of *B. subtilis* was used for enrichment experiment. OSI brand *Artemia* was used for the experiments. Three treatments T_1, T_2, T_3 corresponding to 10^3, 10^4 and 10^5 concentrations were kept in triplicates. As higher concentrations resulted in low nauplii survival and poor rearing conditions, concentrations above 10^5 were avoided.

d. Bacteriology

Samples of 10 ml were drawn aseptically every hour after enrichment using 5 ml sterile tubes and closed tightly. One ml from each sample were transferred to a sterile 120 µ sieve and washed with sterile 18 ppt seawater. Later it was transferred to a sterile glass homogenizer and the larvae were homogenized thoroughly (the methodology followed is same as in section 3.2.4.1.4. for the bacteriology).

The plates were incubated in BOD incubator at 37°C for 48 hours. Hourly sampling was done up to 6 hours and then at 9^{th}, 12^{th}, and 24^{th} hours. Enumeration of total CFU/ml was done after 48 hrs.

3.2.4.2.2. Experiment – III: Post larvae rearing

The larvae rearing unit was set up in the marine hatchery complex, CMFRI, Kochi. The experiment was carried out for a period of eighteen days. Post larvae of *P. monodon* during PL. 2 stage were procured from commercial hatcheries and acclimatized in the marine hatchery complex. Stocking was done at the rate of 25/liter during PL. 3 stage. As consumption of enriched metanauplii by PL. 1 and PL. 2 were found to be less due to bigger size and faster movement of *Artermia* metanauplii,

feeding was started from PL. 3 and continued to PL. 20 stage. Cent percent water exchange was done daily and excess nauplii if any were removed. Dead larvae were removed during water exchange and larval density and survival enumerated on every alternate day. Conditions of larvae were observed under microscope after water exchange. Pure culture of algae *Chaetoceros calcitrans* was added daily to the rearing water to reduce light penetration and cannibalism and nitrogenous waste removal. The tanks were covered with clean black cotton cloth to avoid contamination and also to reduce light.

Rearing units (Plate. 8)

Rearing units consisted of cylindroconical gray colour epoxy coated fiber glass tanks of 40 liter capacity with central stand drain pipes. The tanks were filled with 30 liters of treated and filtered seawater of 30 ppt salinity. Sufficient aeration was provided from the bottom to keep the larvae and feed in suspension. Two treatments with control were kept in triplicates for all the larval rearing trials. T_1 received enriched dead nauplii kept in refrigerator for 24 hours and T_2 received fresh enriched nauplii. Control was given freshly hatched out nauplii. Since many hatcheries give killed *Artemia* nauplii to post larvae to minimize the energy spent on capturing them this procedure was adopted for T_1. *Artemia* concentration was adjusted every day after observing the consumption pattern.

3.2.4.2.2.1. Enriched *Artemia* nauplii feeding

Optimum enrichment duration was assessed in the *Artemia* enrichment experiments and the following concentrations were used for enrichment and subsequent feeding.

a. *S. boulardii* metanauplii were enriched for 4 hours using 10^4 concentration.

b. *B. subtilis* – were enriched for 4 h using 10^5 concentration

For feeding experiments Instar - I nauplii were harvested as per the requirement. 1/3 of the harvested portion were washed with fresh water and seawater using 120µ sieve and fed to control larvae. 2/3 of the portion after moulting to Instar - II

was enriched for four hours in 2 liter glass jar. After enrichment the nauplii were washed with fresh water and seawater using 120µ sieve and divided equally into two portions. One portion fed to T_2 treatment and the other portion was kept in refrigerator in concentrated form. T_1 treatment larvae were fed with the portion kept in refrigerator.

Bacteriology

Bacteriology of the larvae was conducted during PL. 8, PL. 14 and PL. 20 stage. Ten post larvae from each replicate were collected in plastic vials and brought to the laboratory. The larvae were transferred aseptically to sterile sieves (600µ) inside the laminar flow hood and washed with 50% alcohol to reduce their activity and to remove any external contamination. Afterwards they were washed with enough sterile 15 ppt seawater to remove alcohol. The larvae were then transferred to sterile homogenizers and homogenized thoroughly (the methodology followed is same as in section 3.2.4.1.4).

3.2.4.2.2.2*Vibrio* challenge test

This test was carried out using pathogenic *V. harveyii* suspended in saline. Fresh cultures were prepared before the start of each challenge experiment, CFU/ml determined and stored at 4°C. Challenge experiment was carried out during PL. 10 and PL. 20 stage (see section 3.2.4.1.5)

3.2.4.2.2.3 Weight gain

10 samples from each replicate during PL. 10 and PL. 20 stages were collected and preserved in 5% formalin (see section 3.2.4.1.6).

Experiment. IV. Level of colonization after stopping enrichment

3.3. Level of colonization:

Level of was checked only for SB and *B. subtilis* in larvae and for *B. subtilis* in post larvae. As isolation of LB in zoea and mysis were nil and SB in PL. 20 was poor, the level of colonization were not checked for these experiments.

3.3.1. *S. boulardii*

Larvae in T_2 (10^3 CFU/ml) were reared for 5 days after PL. 1. Post colonization was checked on SB agar plates during PL. 3 and PL. 5 (the methodology followed is same as in section 3.2.4.1.4).

3.3.2. *B. subtilis*

Larvae in T_2 (10^4 CFU/ml) were reared for 5 days after PL. 1. Post colonization was checked on NCIM media agar plates during PL. 3 and PL. 5 (the methodology followed is same as in section 3.2.4.1.4).

3.3.3. Post larval colonization of *B. subtilis*

Colonization after cessation of feeding enriched *Artemia* with *B. subtilis* was carried out for PL. 22 and PL. 25 on NCIM media agar plates (the methodology followed is same as in section 3.2.4.1.4).

3.4. Analyses

Analyses of the data to test for significant differences between treatment was conducted using SPSS 7.5 for Windows. Data were analysed with one way ANOVA. Level of significance was checked at 5% and at 1% level. Transformation of the data was done wherever necessary using $\sqrt{X+1}$ method. Level of significance was determined at 5% and 1% level. Post hoc test viz. Duncan's Multiple Range Test (DMRT) was conducted to test the homogeneity among treatments. Statistical analyses of post colonization studies were not conducted.

RESULTS

4.0 RESULTS

Growth of probiotic organisms

a *Saccharomyces boulardii*

Under continuous agitation, concentration of *S. boulardii* cells reached 10^6 CFU/ml within 48 h of incubation at 37°C. Total CFU/ ml was found to decline to 10^5 CFU/ml by 72 h. The colonies on SB agar were uniform, round and off white with strong fermenting smell. Saline suspension prepared from 32 plates (400ml media) contained 10^7CFU/ml *S. boulardii* cells. Microscopic observation revealed oval, budding, and single cells with nucleus inside. The viability of the cells was found to be same for a period of two weeks when stored at 4°C. Coding obtained with API 20 C AUX was 6000072 (Table 3).

b. *Lactobacillus plantarum*

A concentration of 10^8 CFU /ml was obtained under incubation at 37°C for 48 h. The concentration was found to decline after 48 h. The concentrations were $8x10^8$ CFU/ ml for 24 hours, $28x10^9$ CFU/ml for 48 hours and $160x10^7$ CFU/ml for 72 h. The colonies were small, uniform, round and pale white. Microscopic observation revealed Gram +ve rod shaped cells. Coding obtained with API 20 C AUX was 6000072 (Table 4).

c. *Bacillus subtilis*

The bacterial colonies developed well in NCIM media agar plates. The cells were Gram +ve and rod shaped under microscopic observation. On NCIM media agar plates the colonies were irregular, pale white and spreading. The concentrations observed were $23x10^7$ for 24 h, $1.8x\ 10^{10}$ for 48 hours and $2.4x10^{10}$ for 72 hours (Table 5).

Experiment I

4.1. 1. *Saccharomyces boulardii* (TR 1 and 2)

4.1.1.1 Larval survival

In TR 1 survival of zoea to PL.1 was poor when compared to TR 2 (Figs.1a and 1b). Total mortality was observed in T_2 after zoea stage in TR 1,

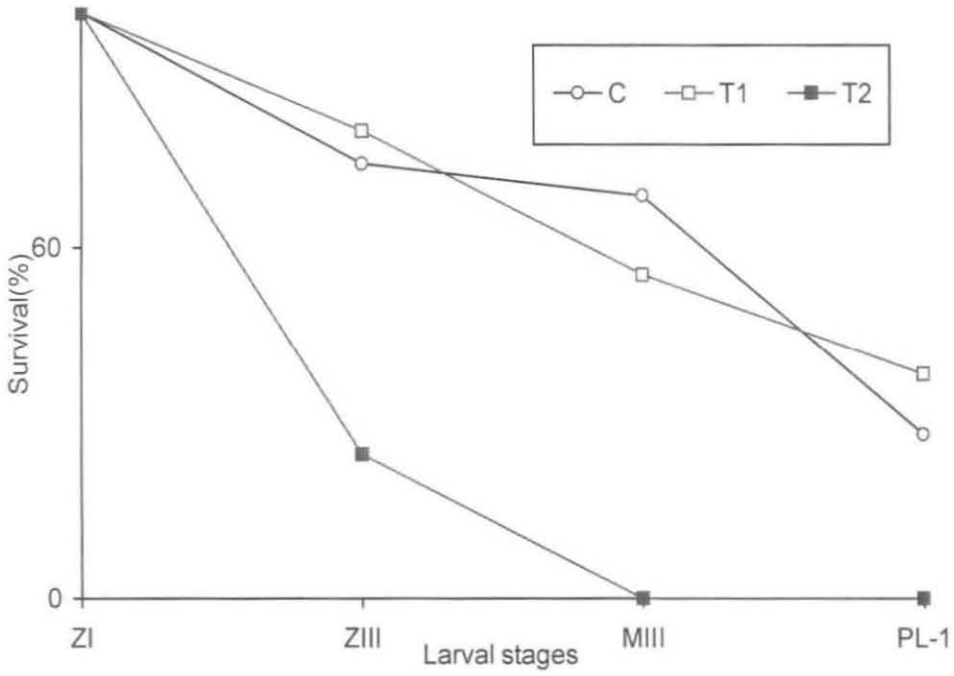

Fig.1a.TR.I. Mean survival of PL.1.*P.monodon* larvae after feeding SB

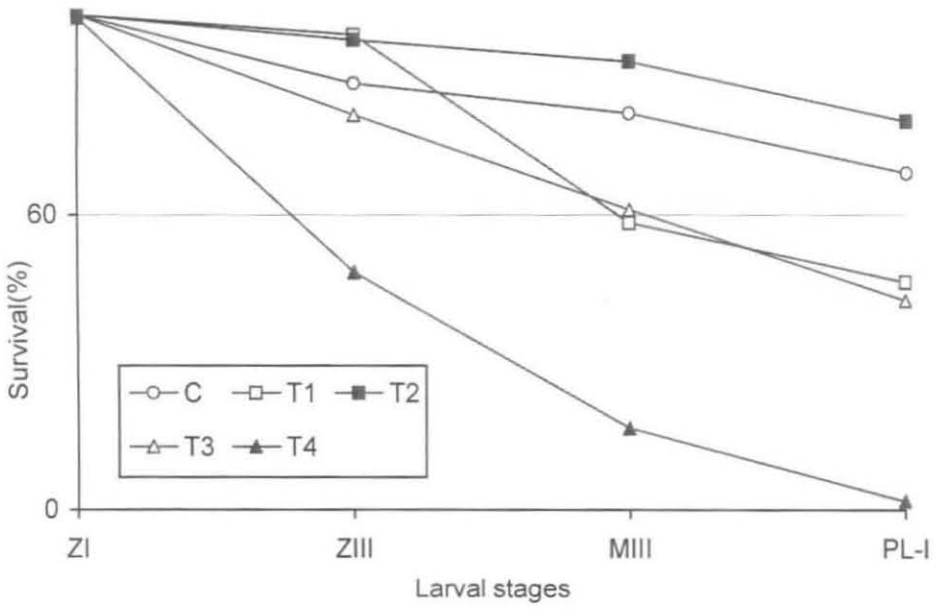

Fig.1b.TR .2.Mean survival of PL-I *P.monodon* after feeding SB

however treatment (T_1) showed higher survival compared to control and the difference was statistically significant (P = 0.000) (Table. 6). DMRT showed significant difference of T_1 over control and T_2 in TR 1. In TR 2 highest survival was observed in T_2 followed by control, T_1, T_3 and T_4 and the mean differences were not significant at 5% level. In T_4, total mortality was observed in two of the replicates after zoea stage.

Levels of enrichment

S. boulardii concentration showed an increasing trend in TR 1 and 2 zoea III with considerable variations among same treatments in both the trials. One way ANOVA showed no significant difference among treatments at 5% level for TR 1 and TR 2 for zoea III (Table. 7). Level of enrichment followed a linear trend corresponding to the concentrations added. Higher values were observed in TR 2 compared to TR 1(Figs. 2a and 2b). Mysis III showed higher levels of enrichment compared to zoea III. In TR 1 zoea III enrichment levels were higher for T_2 (13.6 CFU/larva) followed by T_1 (1.3 CFU/larva). Level of enrichment was the highest in T_4 in TR 2 during mysis III (1535 CFU/larva). In TR 2 around 4.5 CFU/larvae were ingested by zoea III in T_1 followed by 25 CFU/larvae for T_2, 85.17 CFU/larva for T_3, and 486.25 CFU/larvae for T_4. For mysis III the enrichment levels were 7 CFU/larvae for T_1, 14.8 CFU/larvae T_2 and 150.3 CFU/larvae for T_3 and the mean differences were highly significant among treatments (P = 0.000) (Table. 7).

4.1.1.2 Effect of enrichment on other bacterial flora

a. TCBS counts

Vibrio counts were low during initial stages and increased towards the end of culture period. In zoea III the counts were low in TR 1 compared to TR 2 and followed a linear fashion with probiotic concentrations. In control *Vibrio* counts were lower for zoea III and were higher for mysis III in both the trials. The mean differences (Table. 8) were not statistically significant in both the trials except for zoea in TR 2 (P = 0.000) with DMRT showing significant increase in T_4 over T_1, T_2, T_3 and control. In TR 1 and TR 2 total TCBS counts (*Vibrio*s) in mysis stage were low in T_2 and T_3 compared to control, except T_1 and T_4 where lower survival rates

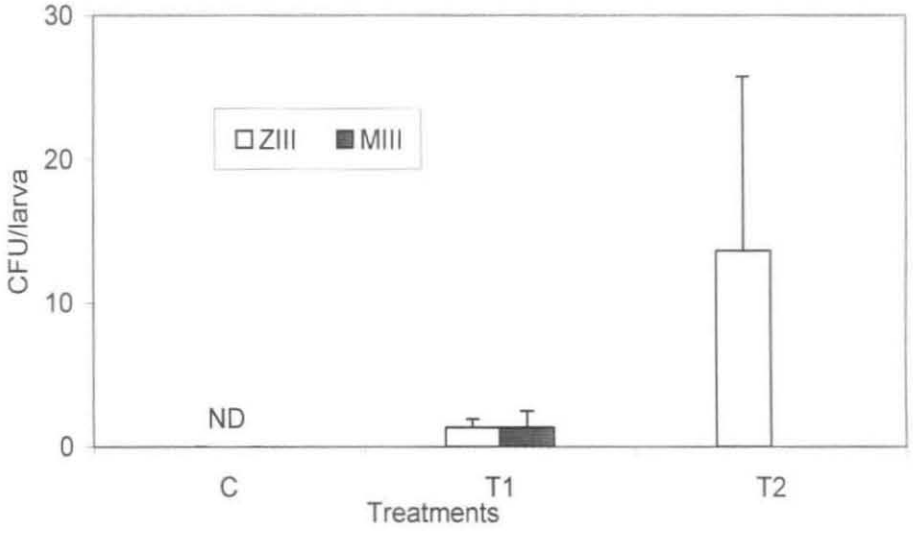

Fig.2a.TR.I. Enrichment level of SB in *P.monodon* zoea III and mysis III. ANOVA result not significant ND = Not Detected

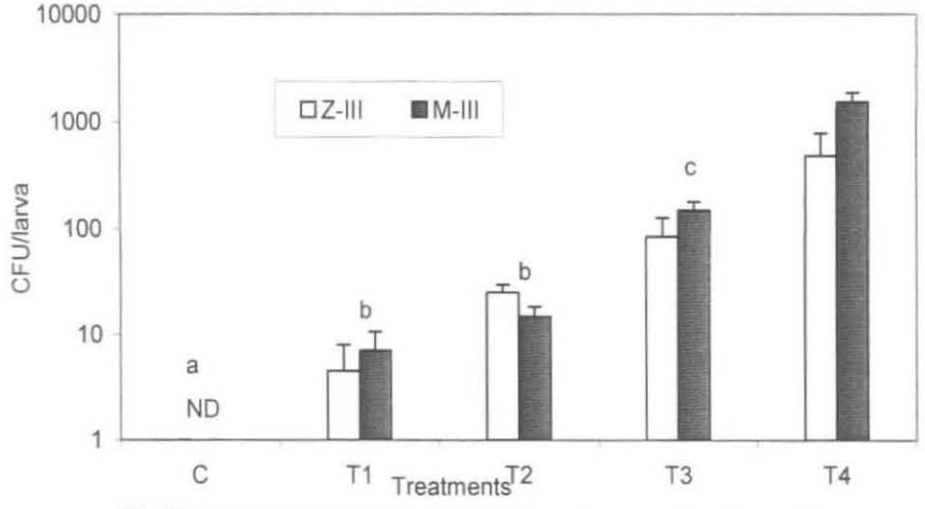

Fig.2b.TR.2. Enrichment level of SB in *P.monodon* zoea III and mysis III. ANOVA results of SB counts in Z-III are not significant.Non identical superscripts denote significant difference.T4 not used for statistical testing

were observed. Highest *Vibrio* counts ($4.85x10^4$ CFU /larva) were observed in mysis III in TR 2, T_4.

b. SWA counts

Results of the effects of SB enrichment on zoea III for TR 1 and 2 are given in Figs.3a and 3b and for mysis III in Figs. 4a and 4b. In TR 1 total aerobic counts were less in control compared to treatments for zoea III and mysis III. In TR 1 higher counts were observed in mysis III. One way ANOVA showed no significant difference among treatments at 5% level for zoea and mysis stage in TR 1(Table. 9) Total aerobic counts were high during initial phase of culture in TR 2 with higher counts in T_4 followed by T_3, control, T_2 and T_1 but the mean differences were not statistically significant. In TR 2 mysis III, control showed lower values compared to treatments and the mean differences were significant ($P = 0.028$) with DMRT showing significant by higher values in T_2 over control and T_1.

4.1.1.3. Pathogen challenge

In TR 1 after *Vibrio* challenge, survival rates were higher in T_1 compared to control. All larvae died in control after 48 hours (Figs.5a). One way ANOVA showed highly significant difference between control and T_1 ($P = 0.000$) after 48 hours, while after 24 h the mean differences were not significant (Table 5). In TR 2, T_1 and T_2 showed lower survival rates and T_4 showed 40% survival followed by control and T_3 after 48 hours (Figs. 5b). After 48 hours negative control showed higher values compared to treatments and control in both the trials. In TR 2 mean differences were significant after 48 hours ($P= 0.012$) (Table. 10) with DMRT showing significant difference in T_3 over T_2 and control and also between control and T_1. In TR 2 after 24 hours treatments showed higher survival rates compared to control but the results were not statistically significant.

4.1.1.4. Effect on weight gain

Among treatments, weight of PL. 1 ranged from 0.20 mg to 0.31 mg in TR 1 (Fig. 6) One way ANOVA showed highly significant difference among control and T_1 in TR.1 ($P = 0.008$) (Table 11). In TR 2, also T_1 showed highest weight gain (0.31 mg) followed by T_2, T_3 and control but the mean differences were not statistically significant.

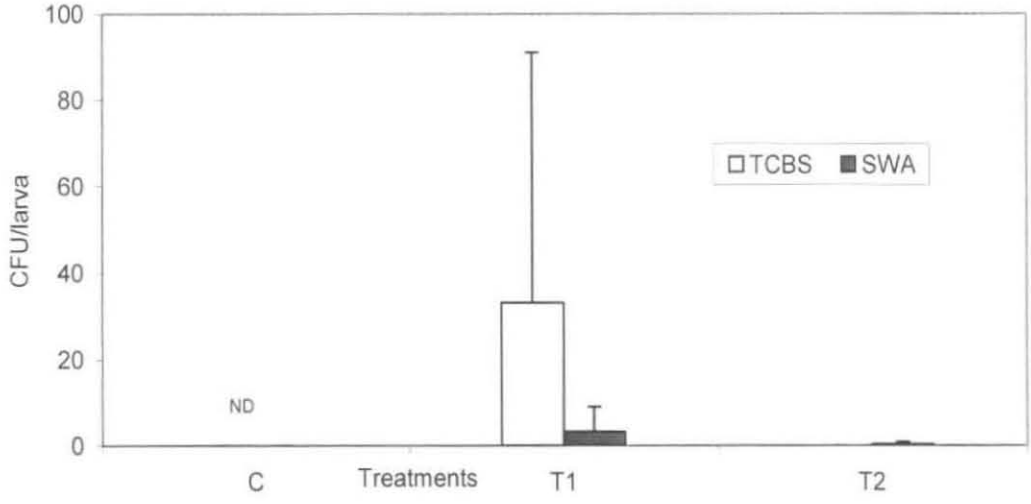

Fig.3a.TR.1. Effect of enrichment of SB on TCBS and SWA plate counts in *P.monodon* zoea-III. ANOVA result not significant

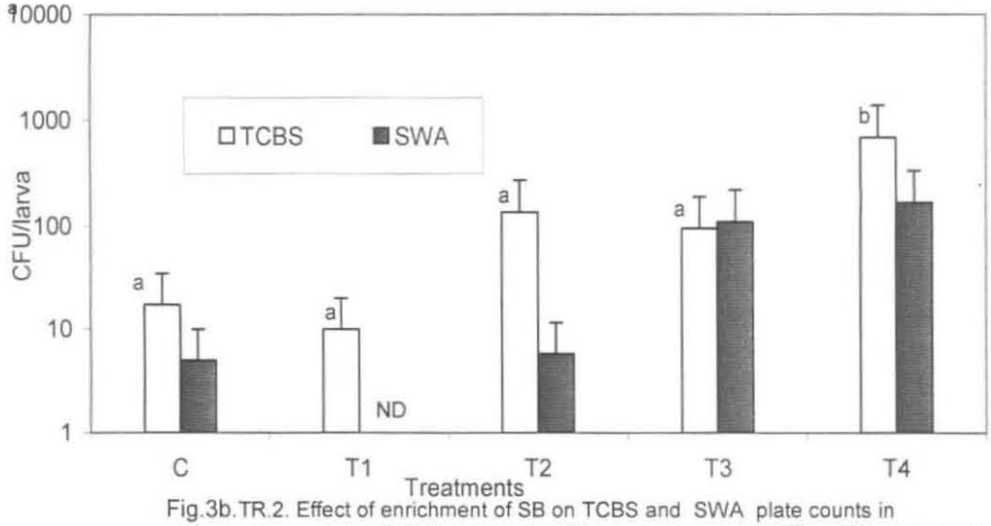

Fig.3b.TR.2. Effect of enrichment of SB on TCBS and SWA plate counts in *P.monodon* zoea-III. ANOVA results of SWA counts are not significant.Non-identical superscripts denote significant difference.ND = Not detected

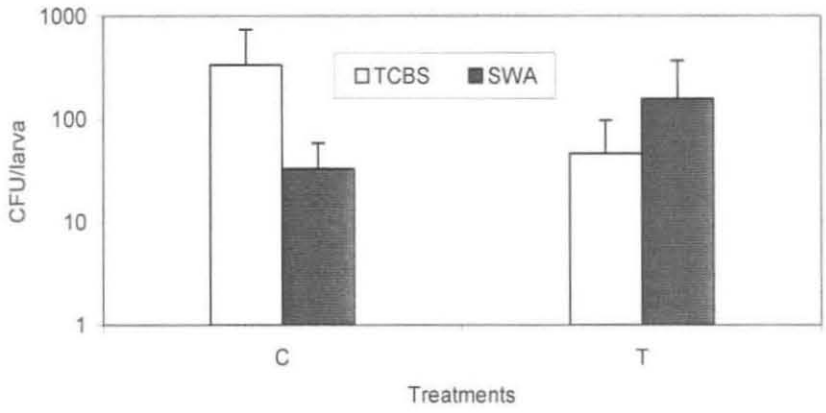

Fig.4a.TR.1. Effect of enrichment of SB on TCBS and SWA plate counts in *P.monodon* mysis-III. ANOVA result not significant

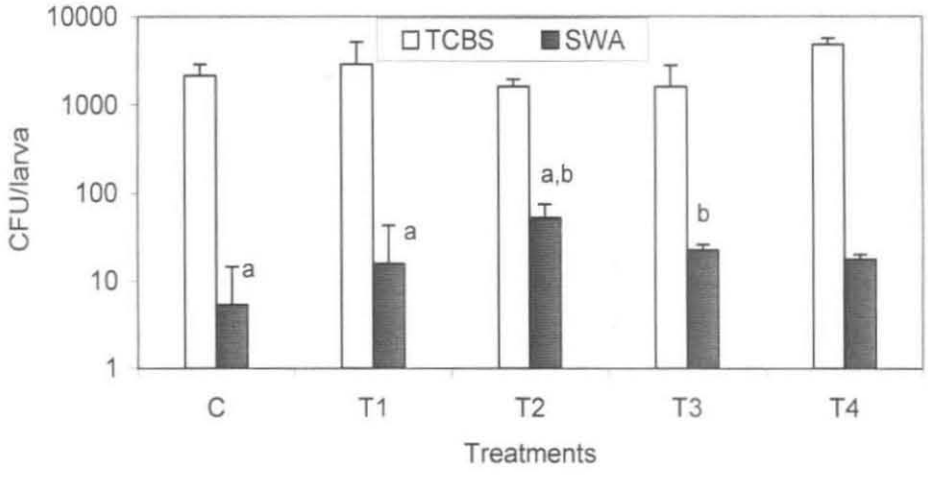

Fig.4b.TR.2. Effect of SB feeding on TCBS and SWA plate counts in *P.monodon* mysis-III. TCBS counts not significant . T4 not used for statistical analysis .Non identical superscripts are significantly different means

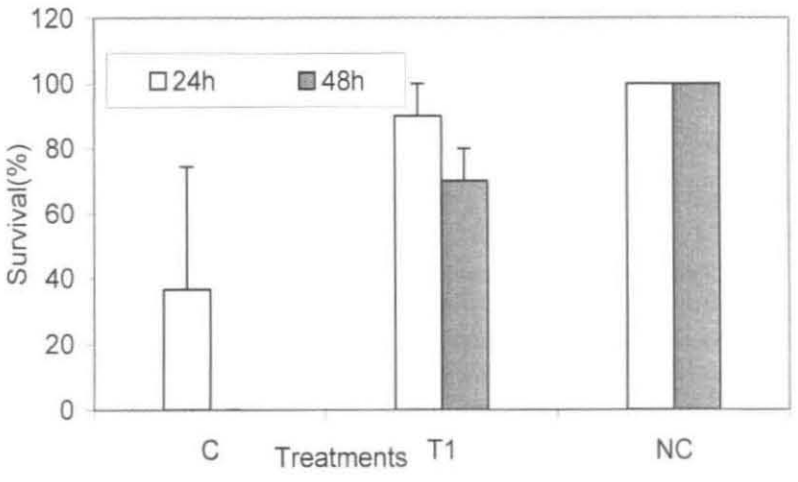

Fig.5a.TR.1. Mean survival of PL.1.*P.monodon* larvae on challenge with *V.harveyii* after feeding SB. Anova results not significant

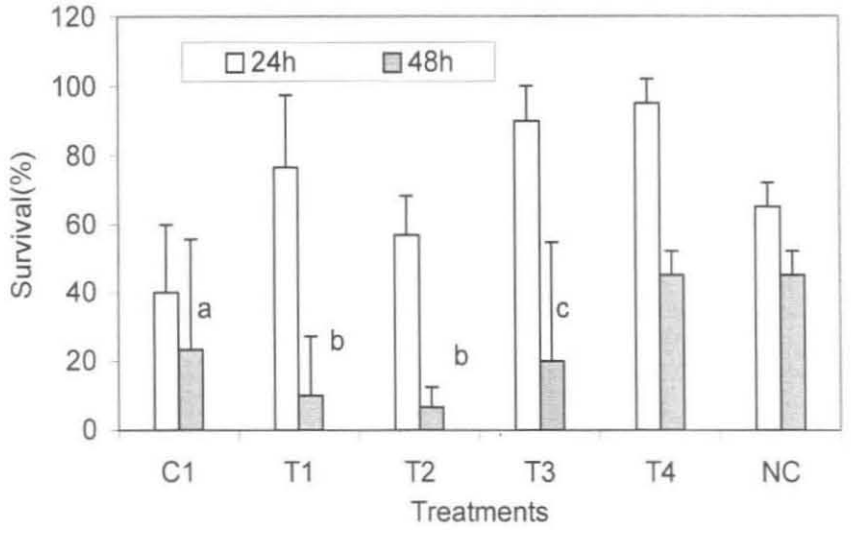

Fig.5b.TR.2. Mean survival of PL.1.*P.monodon* larvae on challenge with *V.harveyii* for 48 h after feeding SB. ANOVA results of 48h vibrio challenge not significant.Non-identical superscripts denote significant difference.

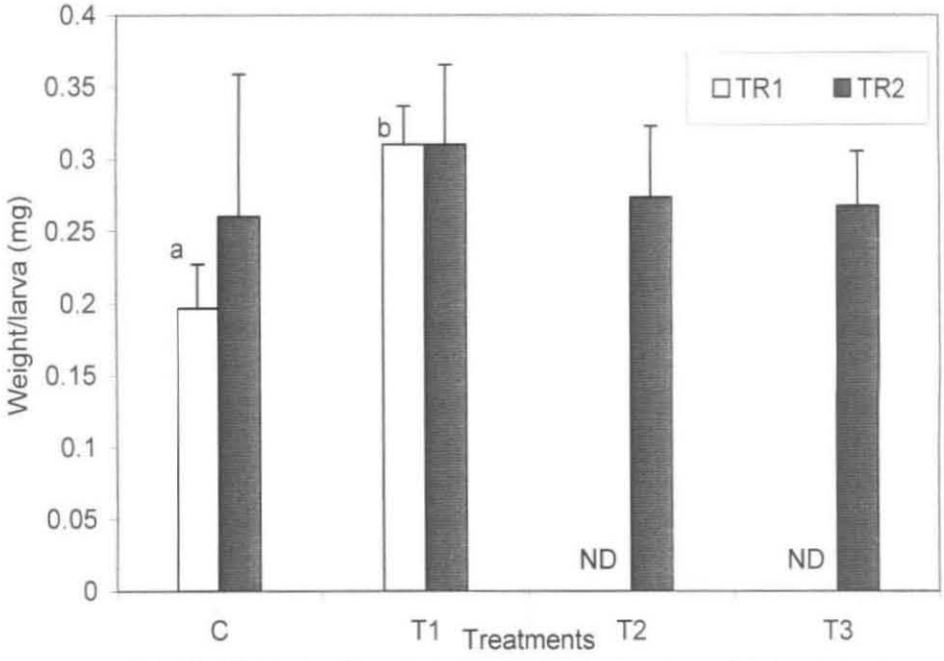

Fig. 6. Weight of PL- I *P.monodon* larvae in TR-1 after feeding SB. Weight gain in trial 2 not significant. Non-identical superscripts are significantly different. ND=Not determined

4.2 *Lactobacillus plantarum* (TR 3 and 4)

4.2.1 Larval survival

Larval survival was comparatively higher in TR 3 than TR 4 (Fig.7a and 7b). In TR 3 highest survival was observed in T_2 (76.5%) followed by T_1, T_3, control and T_4. One way ANOVA showed highly significant difference among treatments in TR 3 (P = 0.007) (Table. 6) with DMRT showing significant difference in mean survival in T_2 over control, T_3, and T_4 and also between T_1 and control. In TR 4 highest survival was in T_1 followed by control, T_3 and T_2. Mean survival

showed no significant difference among treatments at 5% level. Mortality was higher in TR 4 with total mortality after zoea in one of the replicates of T_2 and T_3.

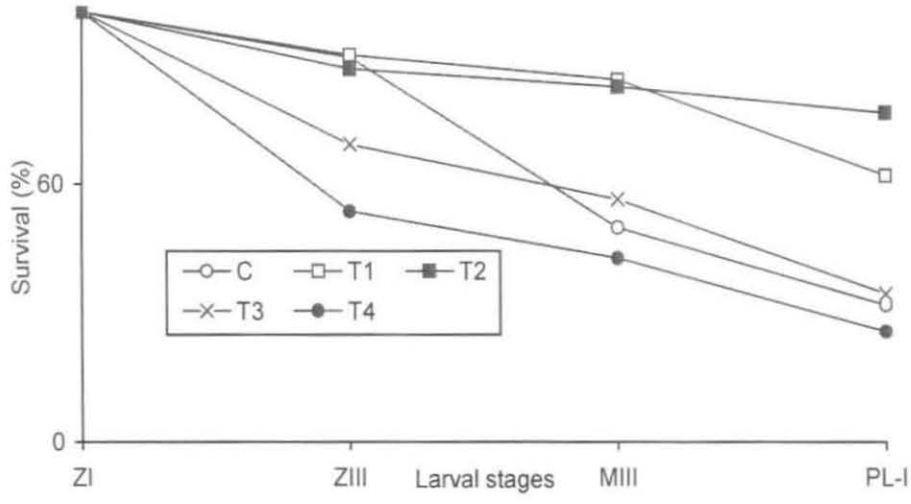

Fig. 7a. TR. 3. Mean survival of *P monodon* PL-I after feeding *Lactobacillus plantarum*

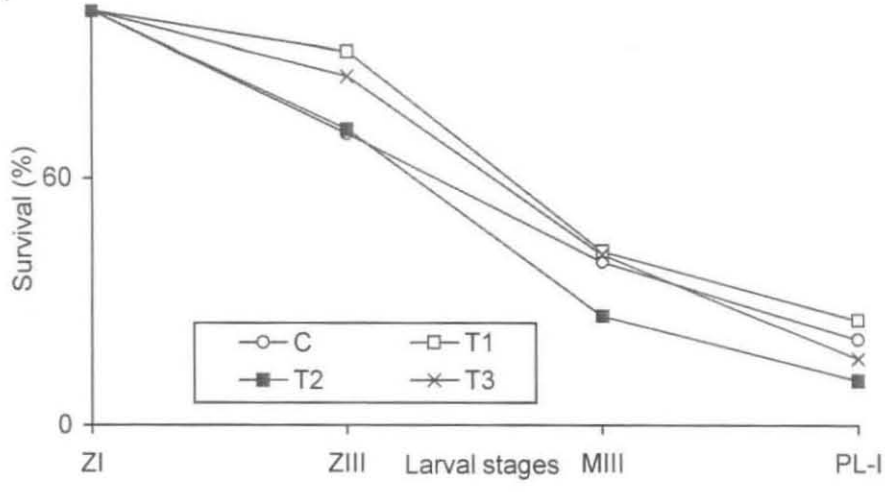

Fig. 7b. TR. 4. Mean survival of *P monodon* PL-I after feeding *Lactobacillus plantarum*

4.2.2. Level of enrichment

In both the trials bacterial sampling did not result in isolation of any LAB colonies from the larvae.

4.2.3. Effect on other bacterial flora

a. TCBS counts

Generally mysis III showed higher TCBS counts compared to zoea. In TR 3 and 4, *Vibrio* counts in zoea and mysis showed an increasing trend corresponding to the probiotic concentrations and were lowest for control compared to treatments in both the trials. In TR 3 zoea III, T_3 showed higher values compared to other groups. One way ANOVA for zoea III in TR 3 showed significant difference among treatments at 5% level (P = 0.023) (Table. 8) with DMRT showing significant difference in T_3 over control, T_1, T_2, and T_4, and not among other treatments. In TR 4 zoea, *Vibrio* counts were lower than control in T_2 and the mean differences were not statistically significant among treatments (Table. 8). In TR.3, mysis control showed comparatively lower values compared to treatments and the mean difference was significant at 5 % level (P = 0.013) with DMRT showing significant difference in control over T_1 and T_3. *Vibrio* values were higher for TR 3 compared to TR 4.

SWA counts

Results on the effects of LB enrichment in zoea III for TR 3 and 4 is given in Fig.8a and 8b and for mysis III in Fig. 9a and 9b.The aerobic counts were higher for mysis III compared to zoea III in both the trials. In TR 3 zoea SWA counts were highest for T_3 followed by control, T_2, T_1 and T_4. In TR 4 it was highest for control followed by T_3, T_2, and T_1. One way ANOVA showed no significant difference among treatments at 5% level for zoea III in both the trials. In TR 3 mysis III SWA counts were highest for T_4 followed by T_1, T_3, T_2, and control and the mean differences were highly significant among treatments (P = 0.005) (Table. 9). DMRT showed significant differences between control and all treatments and in T_2 over T_1 and T_4. In TR 4 mysis highest total aerobic count was observed for T_3 followed by control, T_2, and T1 and the mean differences were not statistically significant.

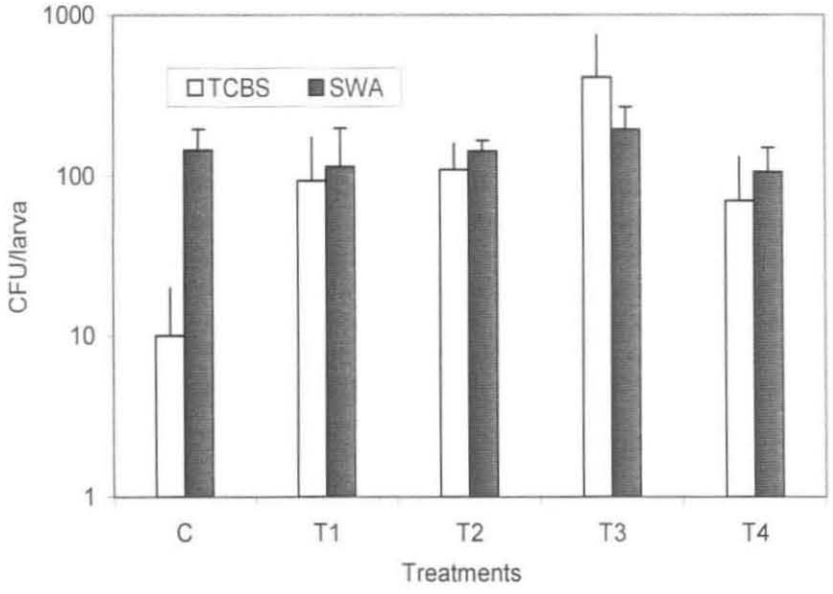

Fig. 8a. TR. 3. Effect of enrichment of *Lactobacillus plantarum* on TCBS and SWA plate counts in *P. monodon* zoea III. ANOVA result not significant

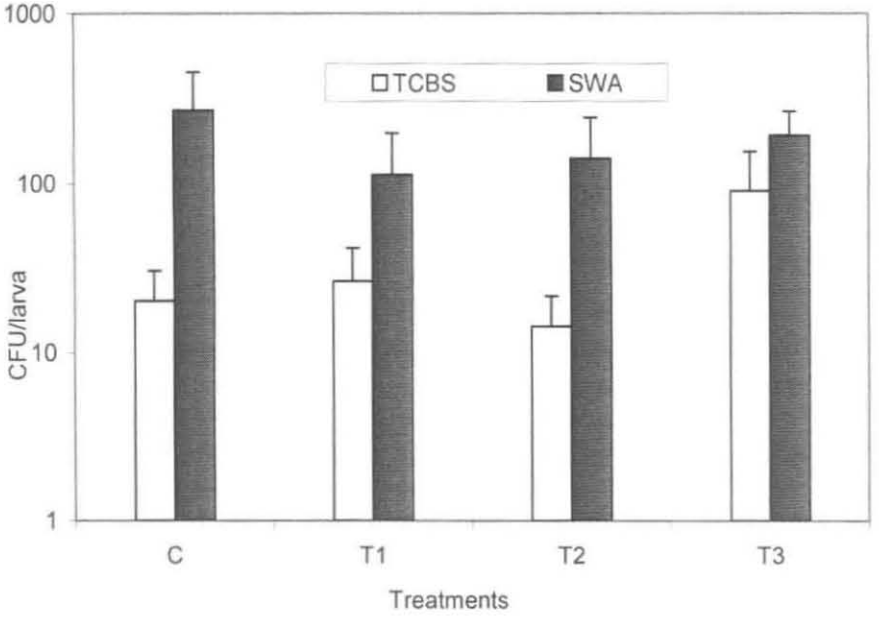

Fig. 8b. TR. 4. Effect of enrichment of *Lactobacillus plantarum* on TCBS and SWA plate counts in *P. monodon* zoea III . ANOVA result not significant.

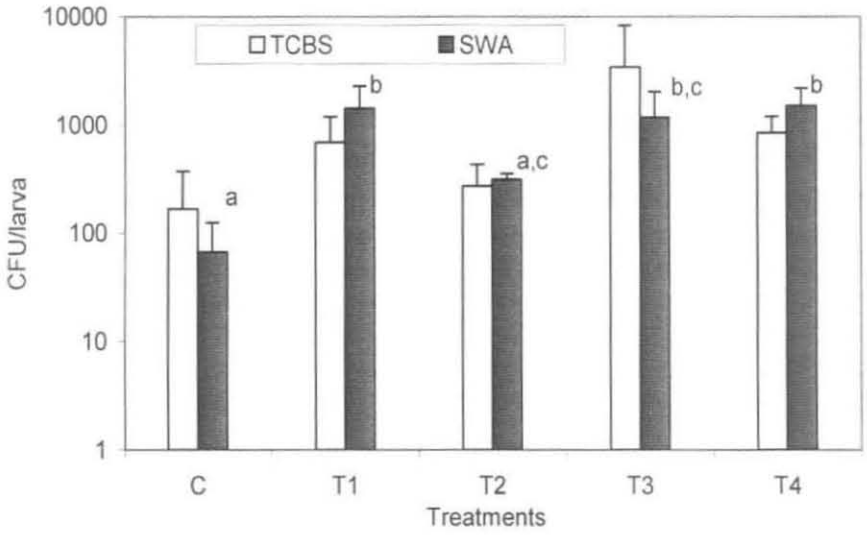

Fig. 9a. TR. 3. Effect of enrichment of LB on TCBS and SWA plate counts in *P.monodon* mysis III. ANOVA results of TCBS counts not significant .

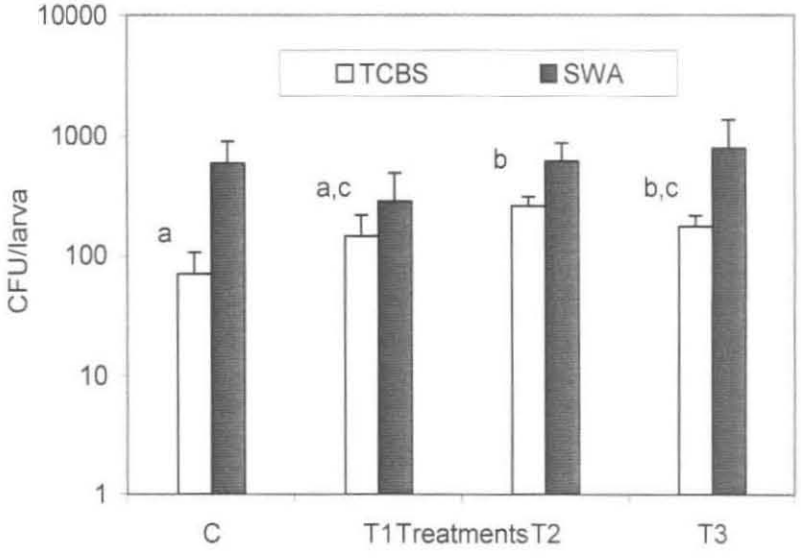

Fig. 9b. TR. 4. Effect of *Lactobacillus plantarum* feeding on TCBS and SWA plate counts in *P.monodon* mysis III. ANOVA results of SWA counts not significant.Non identical superscripts denote significant difference.

4.2.4. Pathogen challenge

In TR 3 and 4, mean survival after 48 h challenge was comparatively similar for treatments and control. In TR 3 highest survival was recorded in T_1, T_2 and control followed by T_3 and T_4 (Fig. 10a and 10b). After 24 h also the survival rate were similar for the experiment groups. In TR 4 highest survival was recorded in T_1 followed by T_3 with similar percentage for T_2 and control. In both trials negative control showed higher survival rates compared to treatments and control after 48 hours. One way ANOVA showed no significant difference among treatments in both the trials (Table. 10).

4.2.5. Effect on weight gain

Weight gain was observed only for TR 3 (Fig. 11). Highest weight was observed for T_2 (0.40 mg) followed by T_3 (0.37 mg), and T_1 (0.35 mg). For both control and T_4, the weight was similar (0.33 mg). One way ANOVA revealed significant difference among treatments at 5% level ($P = 0.049$) (Table 11). DMRT showed significant difference in T_2 over control and T_1.

4.3. *Bacillus subtilis* (TR. 5 and 6)

4.3.1. Larval survival

Higher survival rates were observed for TR 5 compared to TR 6 (Fig.12 a and 12 b). In TR. 5 higher survival was observed for T_3 followed by T_2, control and T_1. Mean differences in survival were statistically significant ($P = 0.007$) (Table. 6) with DMRT showing significant higher survival in T_3 over T_1 and control. In TR. 6 mean survival was highest for T_1 followed by control, T_2 and T_3. One way ANOVA showed significant difference among treatments at 1% level ($P = 0.010$) (Table. 6) with DMRT revealing significant difference between control and other treatments over T_3.

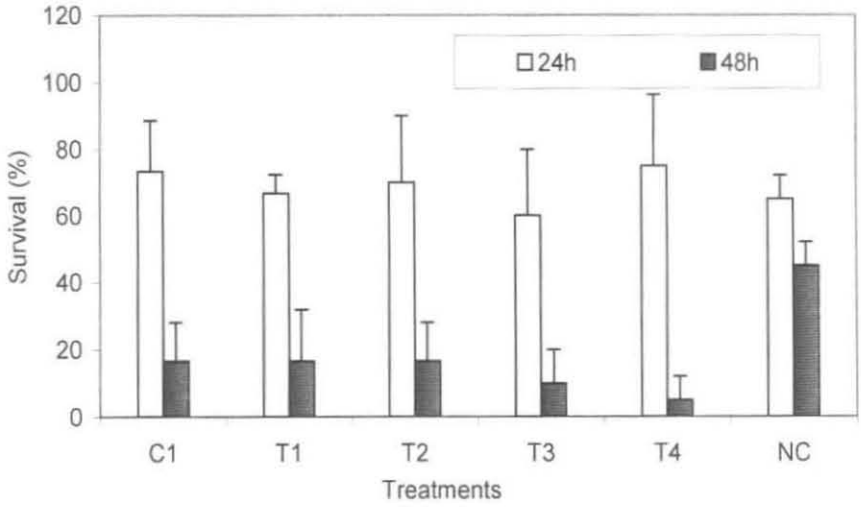

Fig. 10a. TR. 3. Survival of *P.monodon* PL-I after challenge with *V.harveyii* for 48 h after feeding Lactobacillus plantarum. ANOVA results of counts not significant

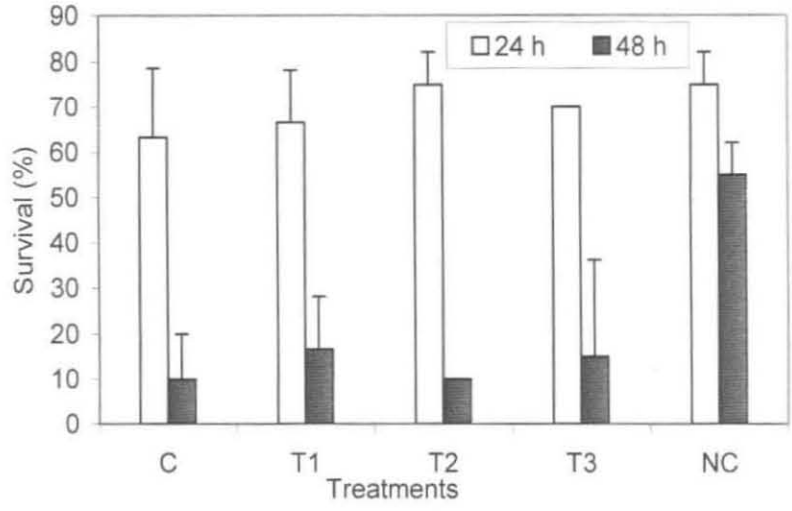

Fig. 10b. TR.4. Survival of *P.monodon* PL-I after challenge with *V.harveyii* for 48 h after feeding *Lactobacillus plantarum*. ANOVA results of counts not significant.

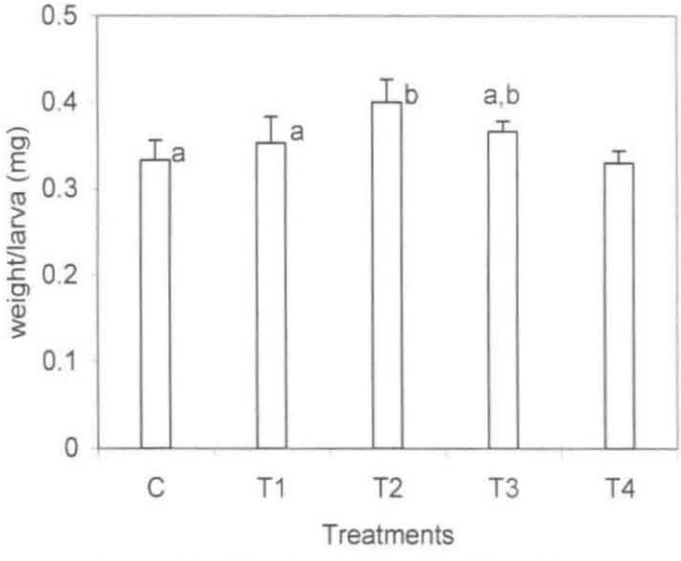

Fig. 11. Weight of *P. monodon* larvae after feeding *Lactobacillus plantarum*. T4 not used for statistical treatment.Non-identical superscripts denote significant differences

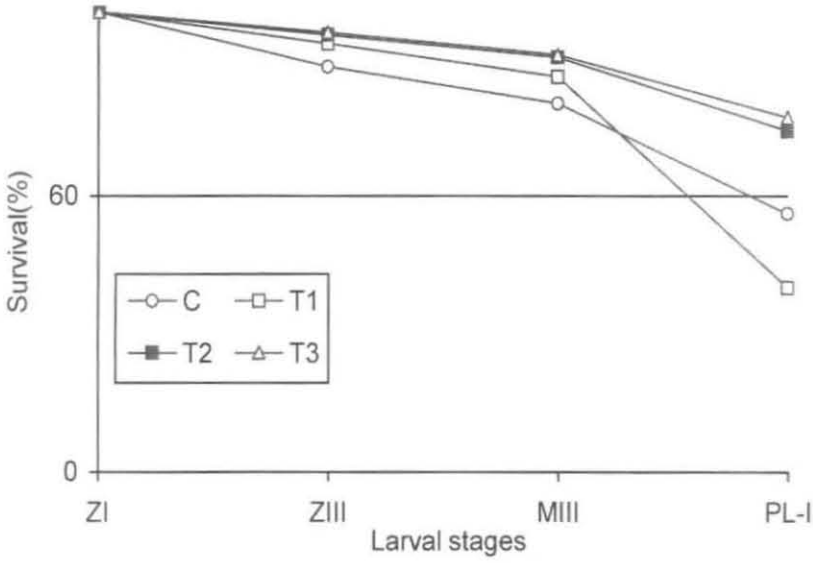

Fig. 12a. TR. 5. Mean survival of *P.monodon* larvae to PL-1 after feeding with *B.subtilis*

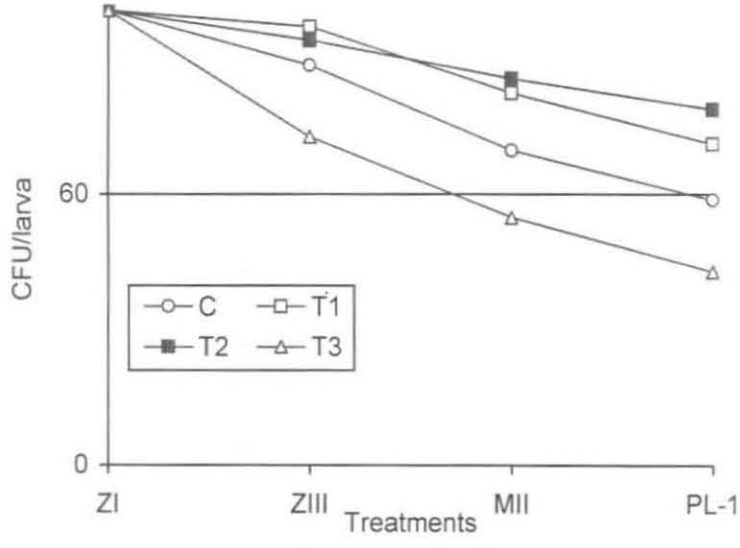

Fig. 12b. TR. 6. Mean survival of *P.monodon* larvae to PL-I after feeding *B.subtilis*

4.3.2. Level of enrichment

Higher levels of enrichment were noticed for mysis when compared to zoea in both TR 5 and 6 (Fig.13a and 13b). In TR 5 except for T_3, T_1 and T_2 showed enrichment levels increasing with increasing probiotic concentrations for both zoea and mysis. In zoea III the enrichment levels (CFU/larva) were 260 for T_1, 517 for T_2 and 170 for T_3. In mysis III the enrichment levels (CFU/larva) were 1500, 2133 and 857 for T_1, T_2 and T_3 respectively. Mean differences among treatments were highly significant for zoea and mysis (P = 0.000 for both) (Table. 7). In TR 6, zoea enrichment levels were higher in T_3 (180 CFU/larva) followed by T_1 (97 CFU/larva) and T_2 (32 CFU/larva) while in mysis the enrichment levels were higher for T_3 (3100 CFU/larva) followed by T_2 (1367 CFU/larva) and T_1 (213 CFU/larva). P = 0.000 for zoea and P = 0.002 for mysis respectively (Table. 7). DMRT showed highly significant difference in control over treatments and between treatments for both the trials.

4.3.3 Effect on other bacterial flora

a. TCBS counts

In TR.5 TCBS counts showed a decreasing trend in zoea III as the enrichment levels of *B. subtilis* increased, while for M III stage it showed an increasing trend (Fig. 14 a) for treatments. In TR 5, for both zoea and mysis, control showed higher counts compared to treatments. The mean differences were significant among treatments for both the stages (P = 0.000 and P = 0.015 respectively) (Table. 8) with DMRT showing significant by higher counts in control over treatments in zoea and mysis. In TR. 6 zoea *Vibrio* counts were highest for T_1 followed by T_3, T_2 and control with significant difference in T_1 over control and T_2 (P = 0.014) (Table. 3). Sampling of mysis III in TR 6 showed that TCBS counts were highest for T_3 followed by T_2, T_1 and control and the mean differences were not statistically significant (P = 0.177).

b. SWA counts

Results on the effects of *Bacillus* enrichment on other bacterial flora in zoea III for TR.5 and 6 are given in Fig.14a and 14b and for mysis III in Fig.15a and 15b. Total aerobic flora also showed a decreasing trend with increasing

concentrations of *Bacillus*. In TR. 5, higher values were observed during mysis III when compared to zoea III. Control showed marginally higher values compared to treatments. Mean counts for zoea III in TR. 5 was highest for T_2 followed by T_1, T_3 and control. For TR. 6 zoea III counts were highest for control followed by T_1, T_2 and T_3. During mysis III, in TR. 5 the counts were highest for T1 followed by control, T_2 and T_3. For TR 6 mysis III the values were highest for T_3 followed by control, T_1 and T_2. One way ANOVA did not show any significant difference among treatments for SWA counts in both the trials (Table. 9).

4.3.4. Pathogen challenge

Resistance to pathogen challenge was higher in treatments compared to control in both the trials (Fig.16a and 16b). TR 5 showed marginally higher levels of survival rate than TR 6 after 24 and 48 h. After 48 h, the larval survival in TR 5 was higher in T_2 (30%) followed by T_1, T_3 and control. In TR 6 survival after 48 hours was highest for T_1 and T_2 (6.7%) with total mortality in T_3 and control. One way ANOVA did not show significant differeces among treatments after 48 hours in both the trials (Table. 5). In TR 5 after 24 hours, T_2 showed higher rates of survival followed by T_3, T_1 and control. One way ANOVA showed highly significant difference among treatments after 24 h ($P = 0.002$) (Table. 10) with DMRT showing significant lower survival in control over various treatments. In TR 6 also the values were highest for T_2 followed by T_1, T_3 and control and the mean differences were not significant at 5% level.

4.3.5. Effect on weight gain

Among trials, TR 6 showed higher values when compared to TR 5 (Fig.17). T_2 showed highest value in both the trials (0.34mg). Mean value for weight gain was highest for T_2 in TR 5 (0.343mg) followed by control, T_1 and T_3, and the mean difference was highly significant among treatments ($P = 0.001$) (Table. 11) with DMRT showing significant difference in T_2 over control, T_1 and T_3. For TR 6, it was 0.342 mg for T_2 followed by T_1, control and T_3, and the mean differences were not statistically significant ($P = 0.084$).

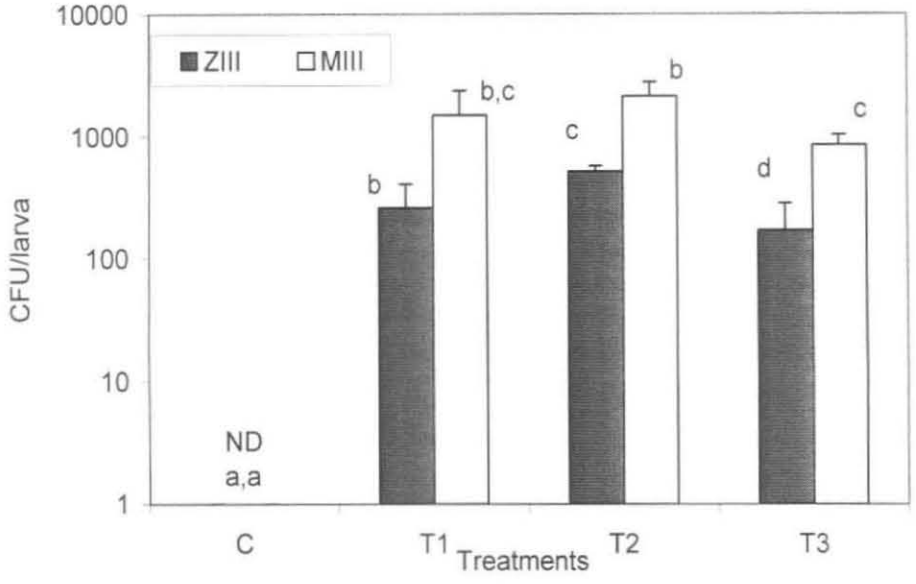

Fig. 13a. TR. 5. Enrichment level of *B.subtilis* in *P.monodon* larvae. zoea III and mysis III. Non-identical superscripts denote significant difference. ND = not detected

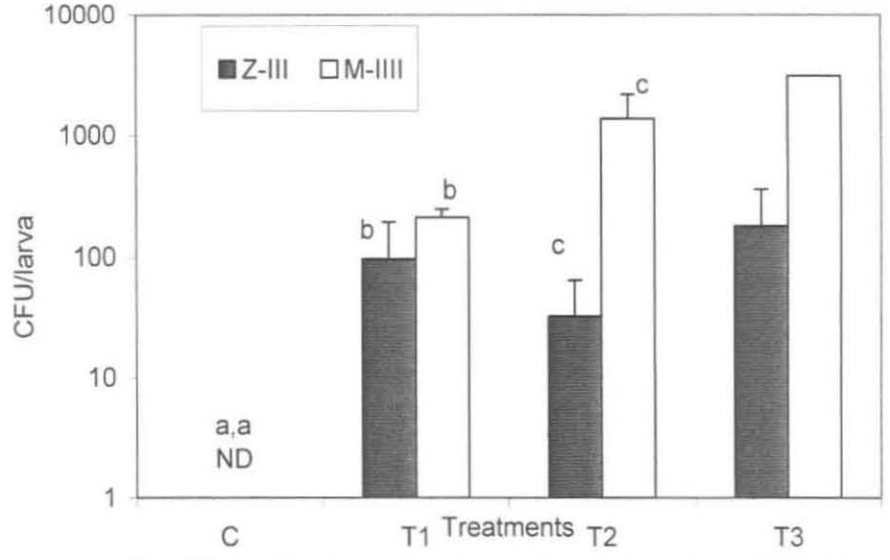

Fig. 13b. TR. 6. Enrichment level of *B.subtilis* in *P.monodon* larvae. zoea III and mysis III. T3 not used for statistical analysis .Non-identical superscripts denote significant differences.

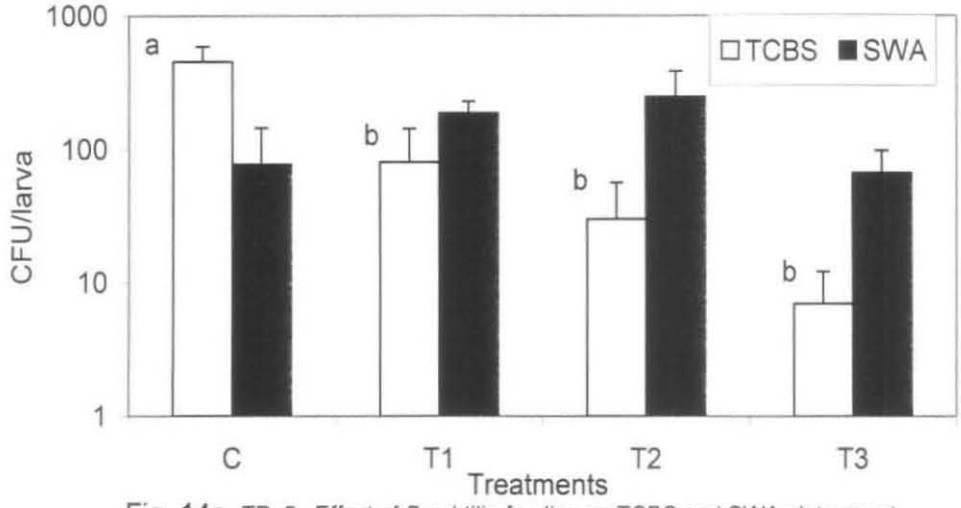

Fig. 14a. TR. 5. Effect of *B.subtilis* feeding on TCBS and SWA plate count *P.monodon* - zoea III. ANOVA results of SWA counts not significant. Non-identical superscripts denote significant differences.

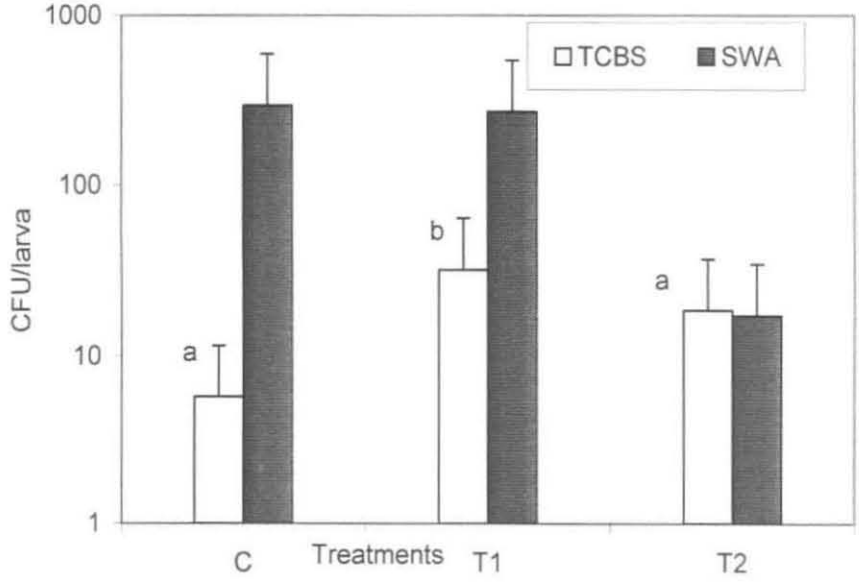

Fig.14b. TR. 6. Effect of *B.subtilis* feeding on TCBS and SWA plate counts in *P.monodon* zoea-III. ANOVA results of SWA counts not significant. Non-identical superscripts denote significant differences.

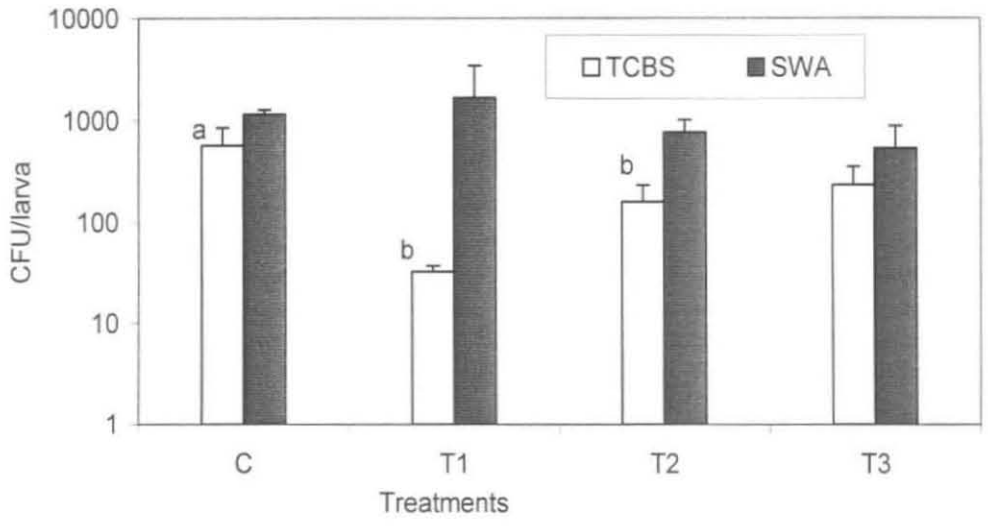

Fig.15a. TR. 5. Effect of *B.subtilis* feeding on TCBS and SWA plate counts in *P.monodon* - mysis III. T3 not used for statistical analysis. ANOVA results of SWA counts not significant .Non-identical superscripts denote significant differences.

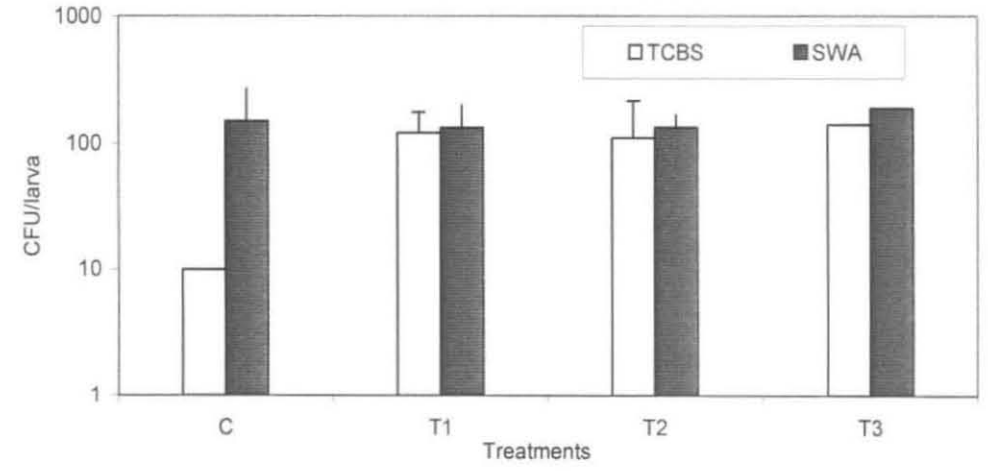

Fig. 15b. TR. 6. Effect of enrichment of *B.subtilis* on TCBS and SWA plate counts in *P.monodon* mysis III. ANOVA results not significant.

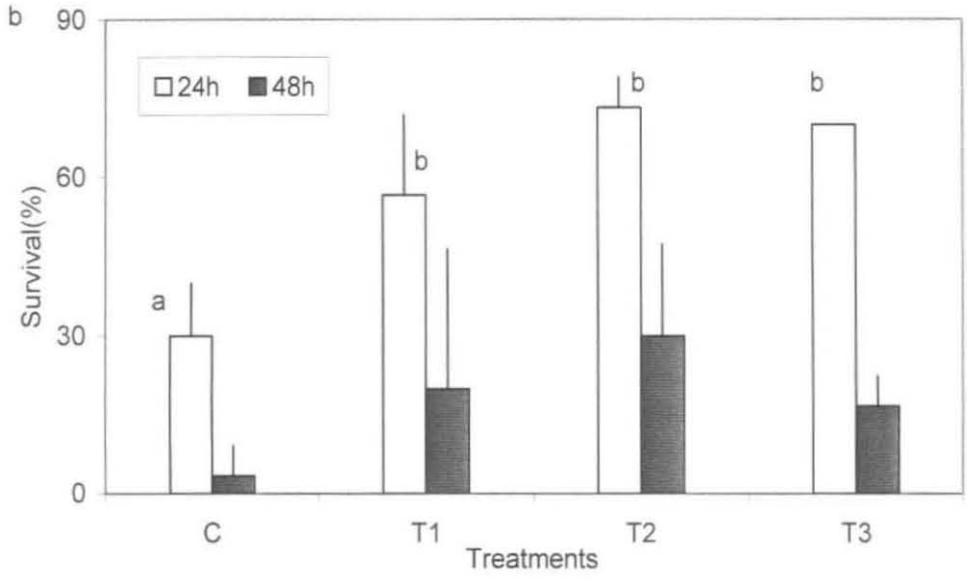

Fig. 16a. TR. 5. Survival of *P.monodon* larvae after challenge with *V.harveyii*. ANOVA results of 48h vibrio challenge not significant. Non-identical superscripts denote significant differences.

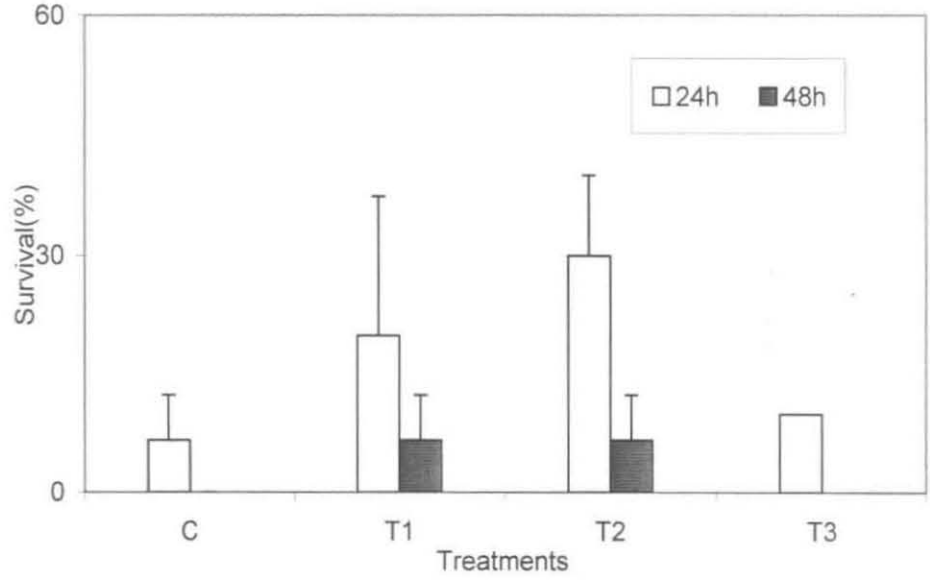

Fig. 16b. TR. 6. Survival of *P.monodon* larvae after challenge with *V.harveyii*. ANOVA results not significant

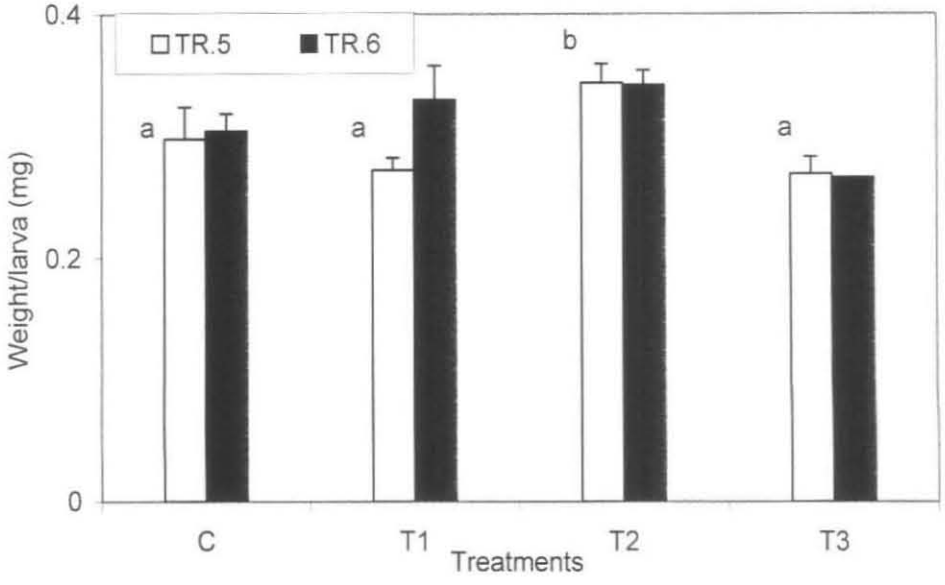

Fig.17. Weight of *P.monodon* PL-1 after feeding *B.subtilis*. Weight in TR-6 not significant. Non-identical superscripts denote significant differences

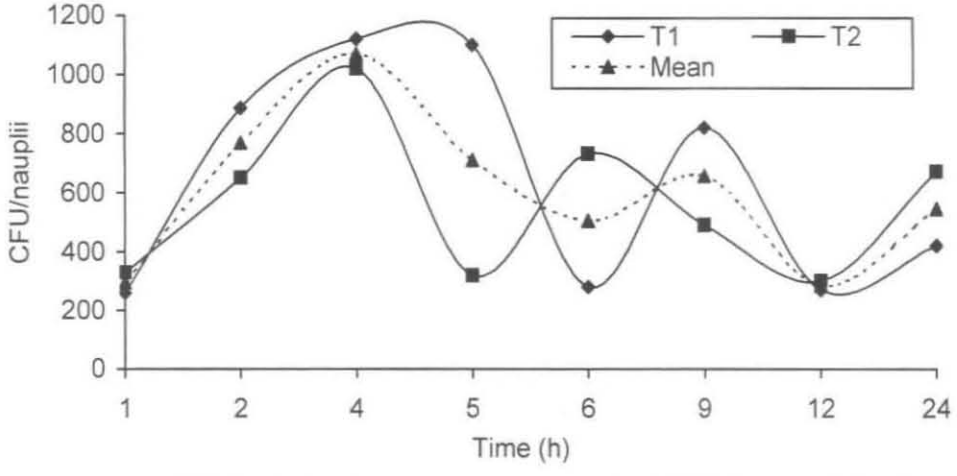

Fig.18.Artemia Red dragon brand optimum enrichment duration for SB

Experiment II

4.4.1. *Artemia* enrichment experiments

4.4.1.1. *Artemia* hatching rate

Hatching rate for both the brands were observed. Red Dragon brand showed lower hatching percentage (68 %) compared to OSI brand (89 %).

4.4.1.2. Optimum enrichment duration

4.4.1.2.1. *S. boulardii*

The optimum enrichment duration for *S. boulardii* was found to be 4 hours. In the initial phase, the level of enrichment followed linear fashion and after which fluctuation was observed for Red Dragon brand, in various treatments. For OSI brand fluctuations were similar for both the treatments.

4.4.1.2.1.1. Red Dragon brand

The enrichment counts for *S. boulardii* in *Artemia* metanauplii in T_1 and T_2 treatments showed an increasing trend (Fig.18). The enrichment levels showed a linear trend until 4 h, after which wide fluctuations were noticed. For T_1 with an initial concentration of 10^3 CFU/ml the average value for maximum enrichment concentration was 1.02×10^3 CFU/ larva and for T_2 (10^4CFU/ml) it was 1.12×10^3 CFU/larva. An average of 1.020×10^3 CFU/larva was incorporated into *Artemia* metanauplii by 4 h after which it came down to 3.0×10^2 CFU/larva by 12 h. Later, by 24 h the concentration was found to be slightly more (6.70×10^2 CFU/larva).

4. 4.1.2.1.2. O.S.I Brand

The enrichment concentration showed an increasing trend corresponding to the treatment concentrations (Fig. 19). The optimum enrichment duration was 4 h after which the enrichment level showed a decreasing trend by 12 hours. The maximum enrichment level was observed for T_2 treatment with a mean value of 3.41×10^3 CFU/larva. In T_1, the mean value for 4 hours was 2.00×10^2 CFU/larva.

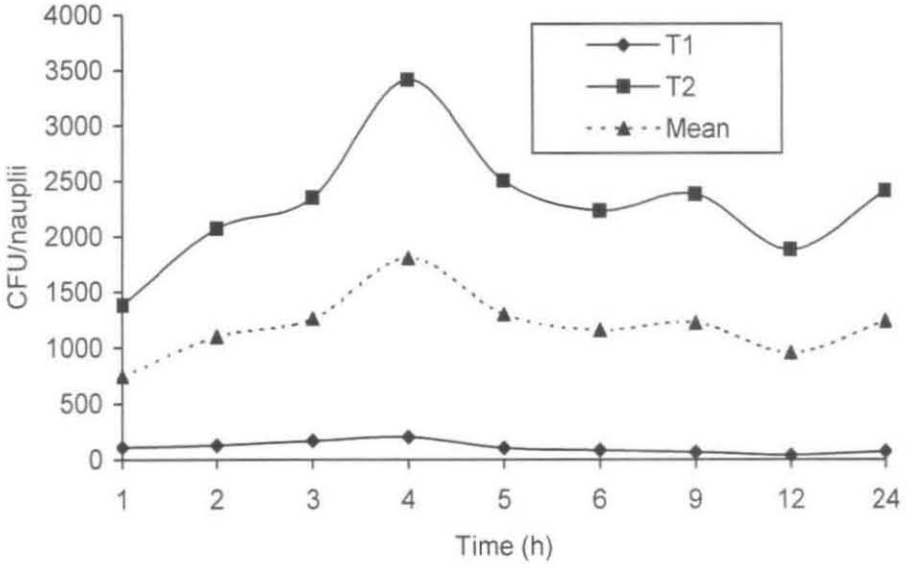

Fig.19. *Artemia* OSI brand optimum enrichment duration for SB

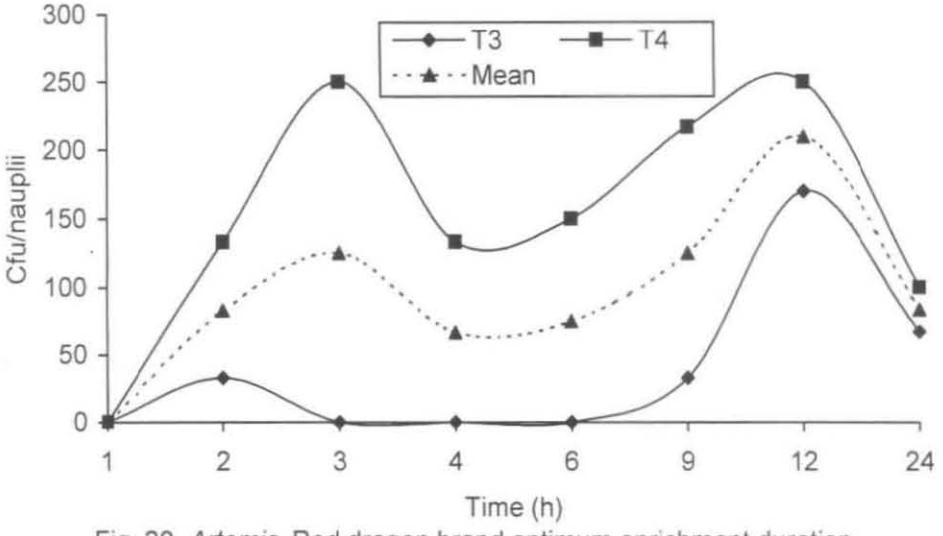

Fig. 20. *Artemia* Red dragon brand optimum enrichment duration for *Lactobacillus plantarum*

Even though the optimum enrichment duration was similar in both the treatments, metanauplii from OSI Brand showed higher levels of enrichment (3.4 x10^3 CFU/larva) when compared to Red Dragon (1.12 x 10^3 CFU/larva). In both the brands, enrichment levels were found to decline by 12 h and after which slight increase was noticed in both the treatments by 24 hours.

4. 4.1.2.2. *Lactobacillus plantarum*

Four concentrations were tried to find out the optimum enrichment duration using Red Dragon Brand (Fig.20). The enrichment levels were very low when compared with SB enrichment. Maximum enrichment took place by 12 h (2.5 x 10^1 CFU/larva) in T_4. The enrichment concentrations showed an increasing trend corresponding to the concentrations added. Very little enrichment was observed for 10^3 and 10^4 CFU/ml treatments (T_1 and T_2).

Mean enrichment level for T_3 and T_4 were highest at 3 hours and 12 hours (1.05 x 10^2 CFU/larva) and lowest during the initial 1 h period (1.7 x 10^1 CFU/larva).

4. 4.1.2.3. *Bacillus subtilis*

The enrichment experiment was carried out with only OSI Brand (Fig.21). The enrichment levels showed a linear trend corresponding to the treatment concentrations. The optimum enrichment duration was found to be 4 hours with *B. subtilis*. Highest enrichment level was recorded for T_3 treatment (2.093 x 10^3 CFU/larva) followed by T_2 and T_1 and lowest for T1 after 24 hours (4.0 x 10^1CFU/larva). The enrichment levels for all concentration showed a declining trend by 24 hours.

Enrichment levels followed a linear trend for all the three probiotics corresponding to the concentrations added. Except for *L. plantarum*, where the enrichment levels were low, the optimum enrichment duration was 4 h for all the probiotic organisms.

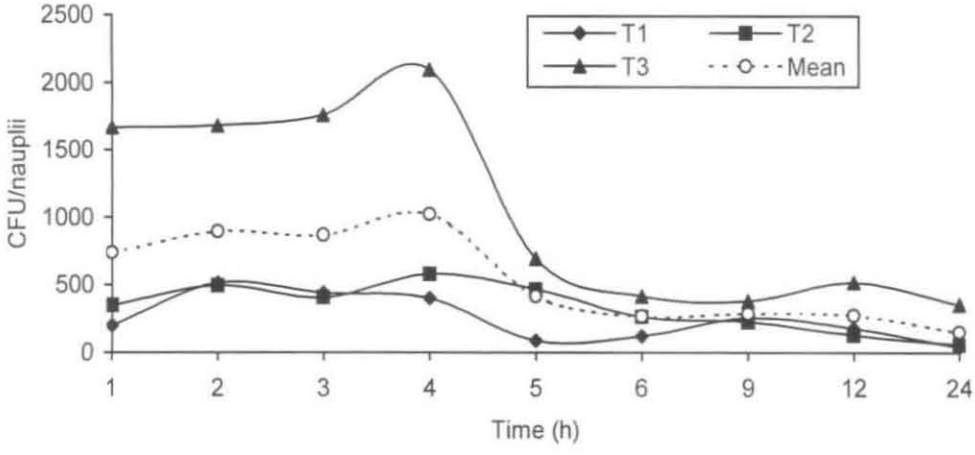

Fig. 21. *Artemia* OSI brand optimum enrichment duration for *B.subtilis*

4.5.0 Experiment-III: Post larvae rearing

4.5.1. *S. boulardii*

(TR 7 & TR 8). As the optimum enrichment duration was found to be 4 hours. The metanauplii were enriched for 4 h and then fed to post larvae after water exchange. The results of post larvae rearing with SB enriched *Artemia* metanauplii are given below.

4.5.1.1. Larval survival

TR 7 recorded higher survival rates compared to TR 8 for PL-20 (Fig.22a and 22b). Mean survival during TR 7 was highest for T_1 (81%) followed by control and T_2. In TR 8 the mean survival was highest for T_2 (74%) followed by T_1 and control. Mean differences among treatments were not statistically significant for both the trials at 5% level (Table. 6).

4.5.1.2. Level of enrichment:

SB could not be detected in during PL-8 and PL –14. SB was detected during PL 20 stage in T_1 and T_2 treatments for both the trials, with T_2 showing marginally higher values (Fig.23a and 23b). Mean SB counts for TR 7 was highest for T_2 followed by T_1. For TR 8 both the treatments showed similar values (1.3 CFU/larva). One way ANOVA for PL–20 SB counts showed significant difference among treatments for TR 7 and TR 8 ($P = 0.001$ for both the trials, Table. 7) with DMRT showing highly significant differences between treatments.

4.5.1.3. Effect of enrichment on other bacterial flora

a. TCBS counts

Vibrio counts on TCBS were higher in control compared to treatments, in all the samplings. In TR 7, except for PL-8, *Vibrio* counts were more for control compared to treatments. In PL-14 and PL-20 lower counts were observed for T_2 compared to T_1.Mean difference among treatments were significant for PL-14 and PL-20 ($P = 0.044$ and 0.007 respectively) (Table. 8) with DMRT showing significantly lower counts in T_2 over control and T_1. For PL-8 the mean

differences were not significant (P = 0.068). In TR 8, control showed higher values during the three sampling stages and T_2 showed comparatively higher values compared to T_1 and the mean differences were not statistically significant (P= 0.231 for PL 8, 0.083 for PL 14 and 0.126 for PL 20).

b. SWA counts

Results on the effects of SB enriched metanauplii feeding in TR 7 and TR 8 are given in Fig 24a and 24b.Total aerobic flora was high during initial phase of culture and decreased towards the end of culture period. In TR 7 control showed comparatively lower counts compared to treatments for PL-8, PL-14 and PL-20. In TR 8 higher values were recorded in control compared to treatments and the counts were lower for T_2 than T_1 in PL-14 and PL-20. One way ANOVA did not show any significant differences among treatments for PL-8 and PL-14 in TR 7and TR 8 while for PL-20 the difference was highly significant for TR 7 (P = 0.004) and not for TR 8 (P= 0.506) (Table 9).

4.5.1.4. Pathogen challenge

PL. 20 showed better survival compared to PL.10 after *Vibrio* challenge in both the trials (Fig.25a and 25b). In TR 7, PL. 10 after 48 hours and 24 hours showed higher rates of survival in treatments compared to control. However, ANOVA results showed no significant difference among treatments after 48 hours as well as 24 hours. PL. 20 also showed marginally higher survival rates in treatments compared to control after 24 hours and 48 hours but the mean differences were not statistically significant (P=. 055 and 0.236 respectively; Table. 8). In TR 8, PL. 10 the larvae showed higher survival rates in T_2 followed by T_1 and control after 48 hours and 24 hours but the mean differences were not significant. In PL. 20 after 48 hours and 24 hours the rate of survival was significantly high in treatments compare to control (P= 0.004 and 0.000 respectively) (Table. 10). DMRT showed highly significant differences between treatments and control.

4.5.1.5. Effect on weight gain

Weight gain of PL was more in TR 7 compared to TR 8 (Fig. 26a and 26 b). In both trials T_2 showed higher weight gain compared to T_1 and control. In TR 7,

PL. 8 weight gain was more for T_2 followed by control and T_1 while in PL. 20 higher values were observed for T_2 followed by T_1 and control. Mean difference was statistically significant among treatments for PL. 10 (P = 0.003) (Table. 11) with DMRT showing significantly higher values in T_2 over Control and T1. In PL. 20, mean differences were not significant among treatments.

In TR 8, PL. 10 weight gain showed similar trend for control and T_2 with T_1 showing lower value. ANOVA results showed highly significant difference for T_2 and control over T_1 (P = 0.000). In PL. 20 the value was higher in T_2 followed by control and T_1 and the mean differences were highly significant (P = 0.004) (Table. 11) with T_2 showing significantly higher mean weight over control and T_1.

4.5.2. *Bacillus subtilis*

(TR 9). Only one trial was conducted for post larvae rearing with *B. subtilis* enriched *Artemia* metanauplii.

4.5.2.1. Larval survival

Larvae showed comparatively higher survival in T_2 followed by control and T_1 (Fig. 27). One way ANOVA revealed highly significant difference among treatments (P = 0.008) (Table. 6) with DMRT showing significantly higher survival in T_2 over control and T_1.

4.5.2.2. Level of enrichment

Bacillus was detected from all the three sampling stages (Fig.28). In PL. 8 the level of enrichment was more for T_2 (7.5 CFU/larva) followed by T_1 (22 CFU/larva) while in PL. 14 it was higher for T_1 (66CFU/larva) followed by T_2 (17.8 CFU/larva). In PL. 20 enrichment levels were lower compared to PL. 8 and PL. 14 with T_2 showing higher levels compared to T_1. ANOVA showed highly significant difference among treatments for PL. 8, PL. 14 and PL. 20 (P = 0.010, 0.008 and 0.006 respectively, Table. 7) while between control and treatments the differences were significant. Between T_1 and T_2 the differences were significant in PL. 20.

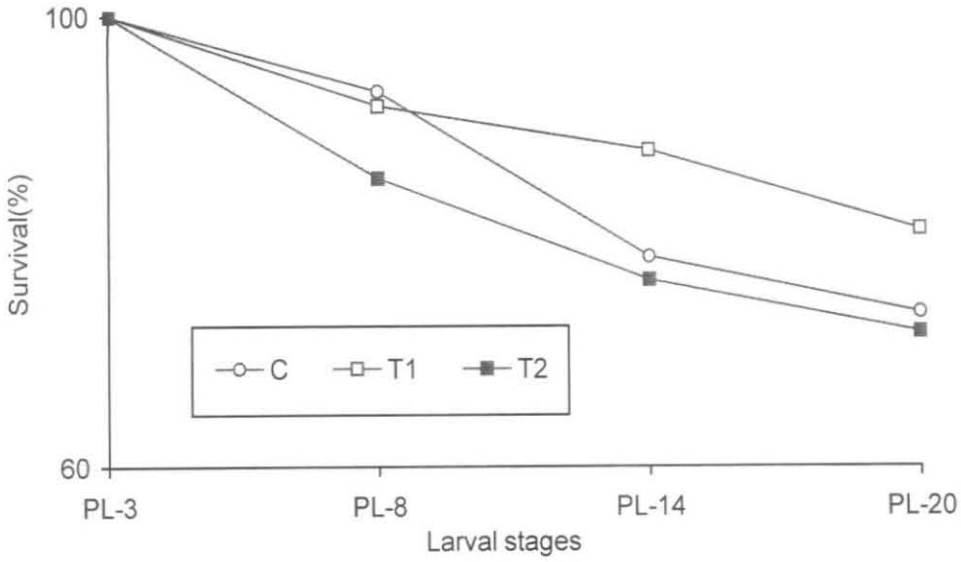

Fig.22.a.TR.7. Mean survival of *P.monodon* PL-3 to PL-20 fed with SB enriched *Artemia*

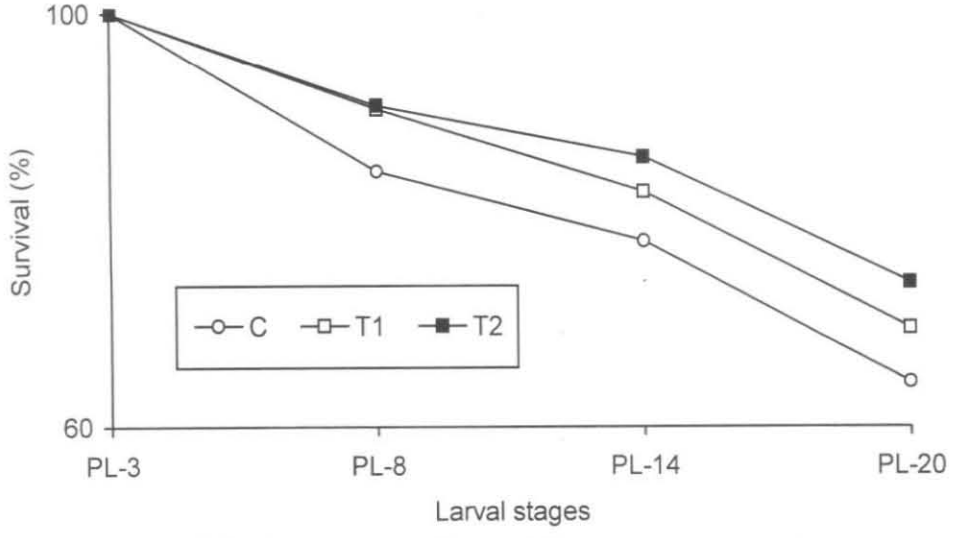

Fig.22. b. TR.8. Mean survival of PL - 20 *P. monodon* larvae after feeding SB enriched *Artemia*

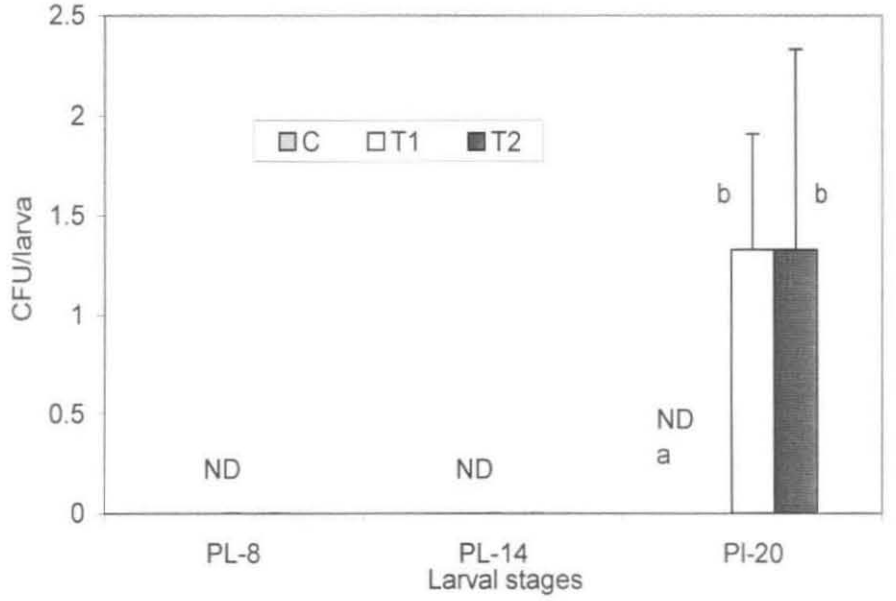

Fig.23. a. TR. 7. Enrichment level of SB in *P. monodon* post larvae fed with enriched *Artemia*. Non-identical superscripts denote significant differences.ND = not determined.

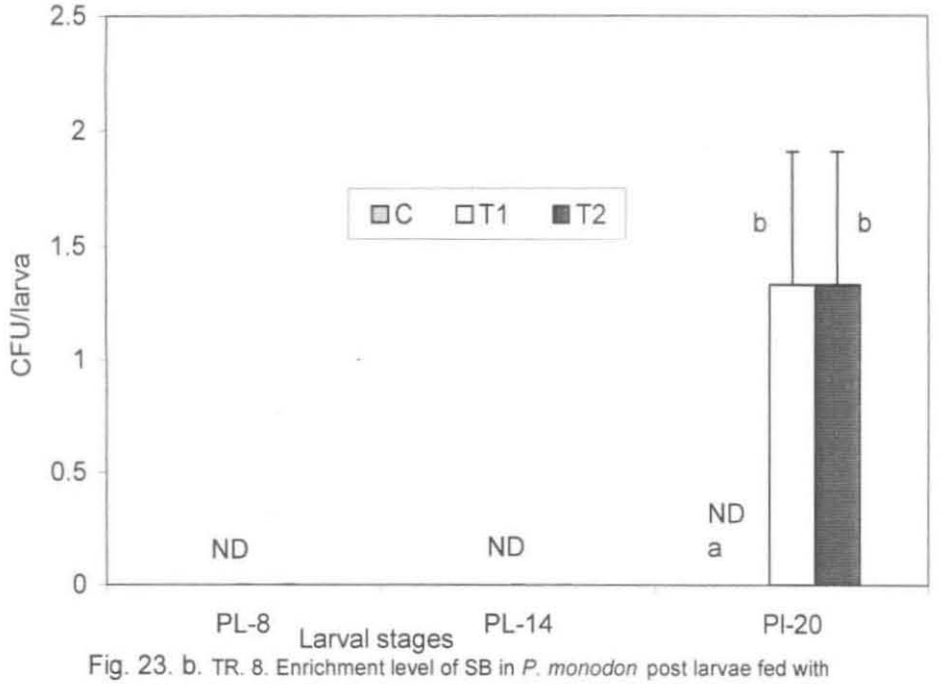

Fig. 23. b. TR. 8. Enrichment level of SB in *P. monodon* post larvae fed with enriched *Artemia* Non-identical superscripts denote significant differences.ND = not determined.

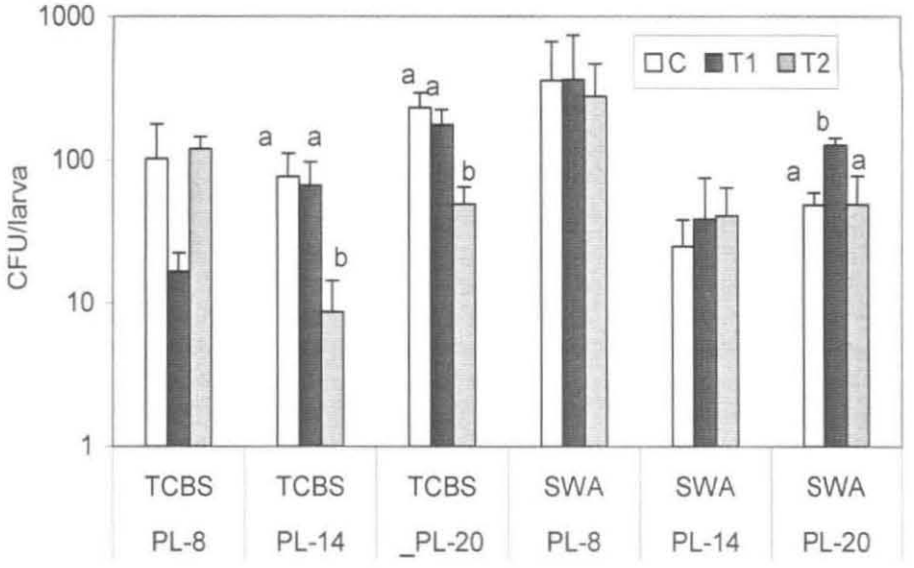

Fig.24. a.TR. 7. Effect of SB enriched *Artemia* on TCBS and SWA plate counts in *P.monodon* post larvae ANOVA results of TCBS counts in PL-8 and SWA counts in PL-8 and PL-14 not significant

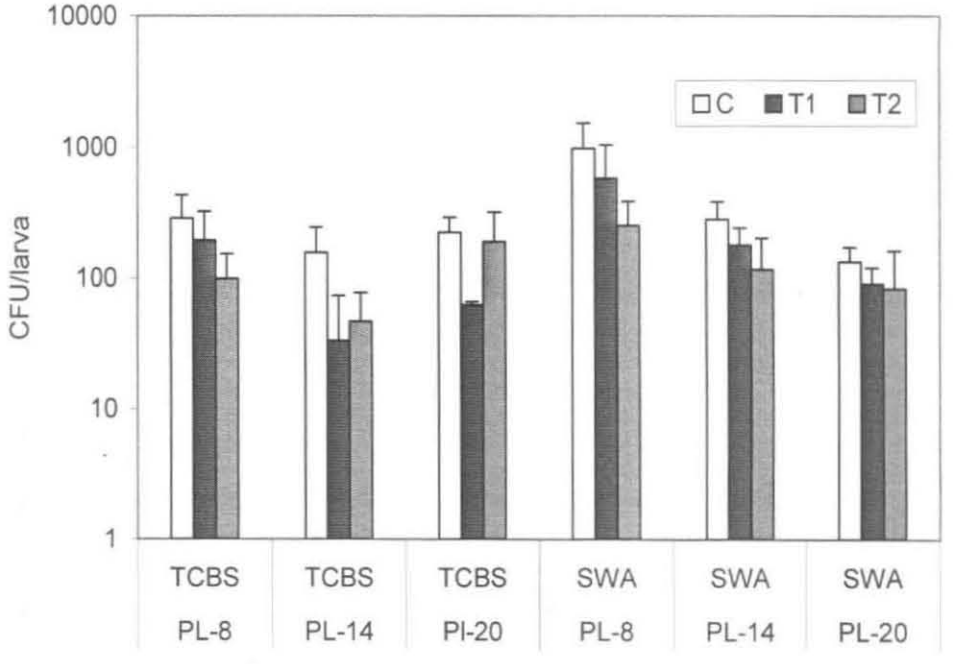

Fig.24. b.TR.8. Effect of SB enriched *Artemia* on TCBS and SWA plate counts in *P.monodon* post larvae. ANOVA results not significant.

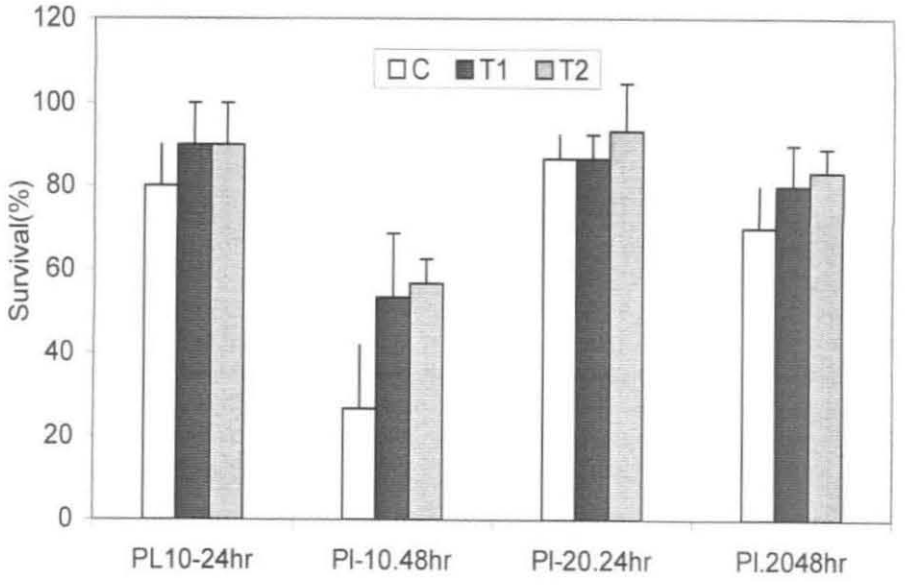

Fig.25.a.TR.7.Survival after challenge with *V.harveyii* for 48 h. ANOVA results not significant

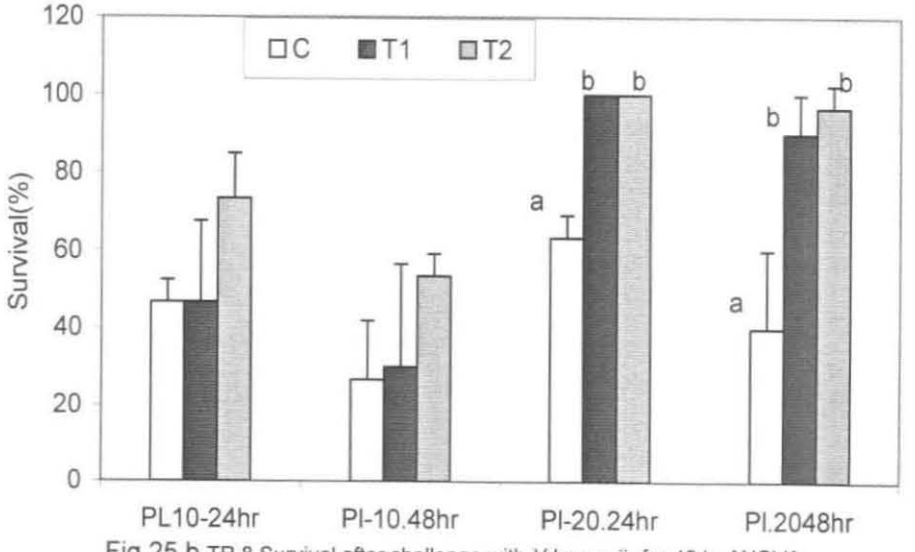

Fig.25.b.TR.8.Survival after challenge with *V.harveyii* for 48 h ANOVA results in PL-10 not significant.Non-identical superscripts denote significant difference

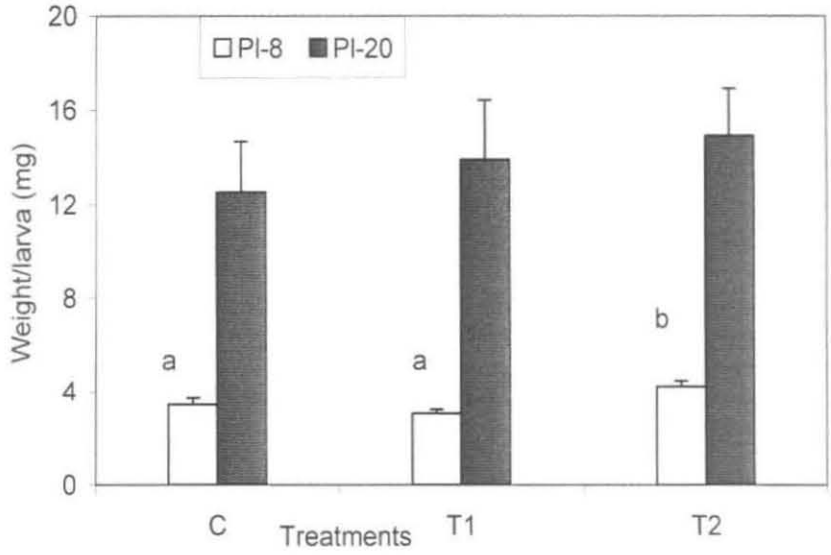

Fig. 26. a. TR.7. Weight after feeding SB enriched *Artemia* in *P.monodon* post larvae. ANOVA results in PL-20 not significant.Non-identical superscripts denote significant differences.

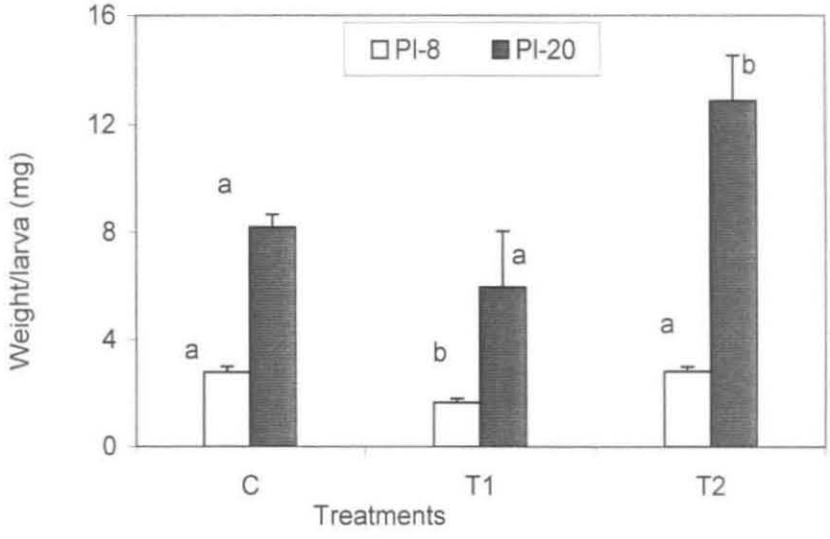

Fig.26. b.TR.8. Weight after feeding SB enriched *Artemia* in *P.monodon* post larvae.Non-identical superscripts denote significant differences.

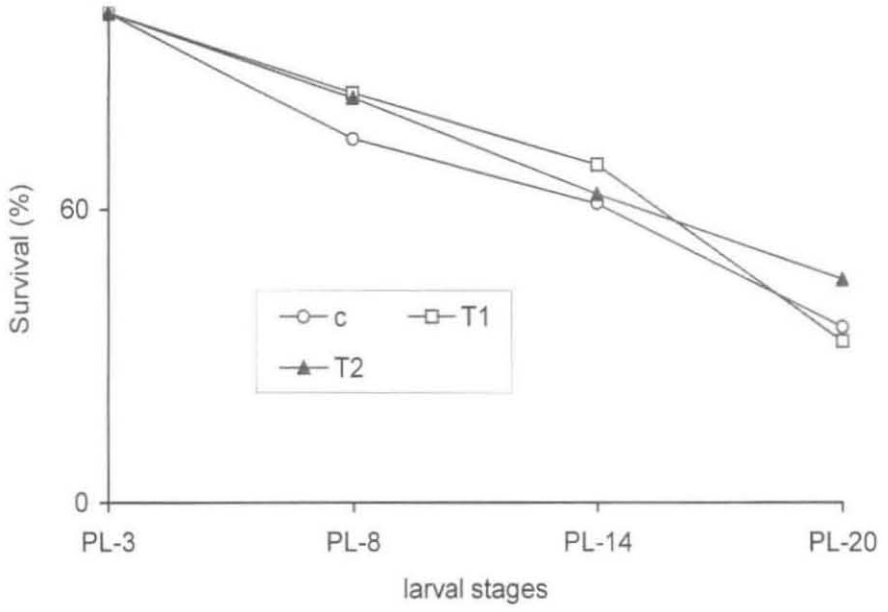

Fig. 27. TR-9. Mean survival of *P.monodon* post larvae after feeding with *B.subtilis* enriched *Artemia*

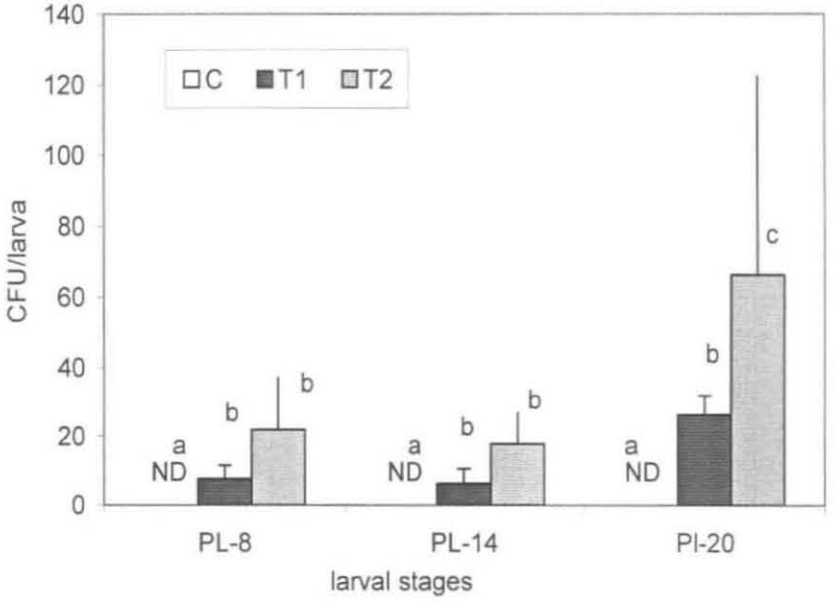

Fig.28. TR. 9. Enrichment level of *B. subtilis* in *P. monodon* post larvae fed with enriched *Artemia*. Non-identical superscripts denote significant differences.

4.5.2.3. Effect on other bacterial flora

a. TCBS counts

In PL. 8 *Vibrio* counts in TCBS were higher for T_2 followed by control and T_1, while for PL. 14 and PL. 20, control showed lower counts compared to treatments, with T_2 showing higher counts than T_1. Mean differences were significant among treatments for PL. 8 and PL. 20 (P = 0.001 and 0.003 respectively) (Table. 8) with DMRT showing significantly higher counts in T_2 over control and T_1. In PL. 8, the mean differences were not significant among treatments

b. SWA counts

Results on the effects of *Bacillus* enriched metanauplii feeding on other bacterial flora is given in Fig. 29. Total aerobic flora showed decreasing trend towards the end of culture period. SWA counts were higher in T_2 followed by control and T_1 for PL. 8 and PL. 20 while for PL. 14 control showed higher counts followed by T_2 and T_1. Mean differences were not significant among treatments for all the three samplings (Table 9).

4.5.2.4. Pathogen challenge

After 24 hours and 48 hours, PL. 10 showed higher rates of survival in treatments compared to control (Fig. 30). PL. 20 also showed the same trend after 24 hours as well as 48 hours. One way ANOVA show significant difference among treatments for PL. 20 after 24 hours (P = 0.016) and 48 hours (P= 0.05) while for PL. 10 results were not significant after 24 hours and 48 hours (Table. 10).

4.5.2.5. Effect on weight gain

In PL. 10 and PL. 20, T_2 showed higher values (1.88 mg and 7.86 mg respectively) followed by T_1 and control. (Fig. 31). One way ANOVA showed significant difference among treatments for PL. 10 and PL. 20 (P = 0.008 and 0.007 respectively) (Table. 11) with DMRT showing the values to be significantly high in T_2 over control and T_1.

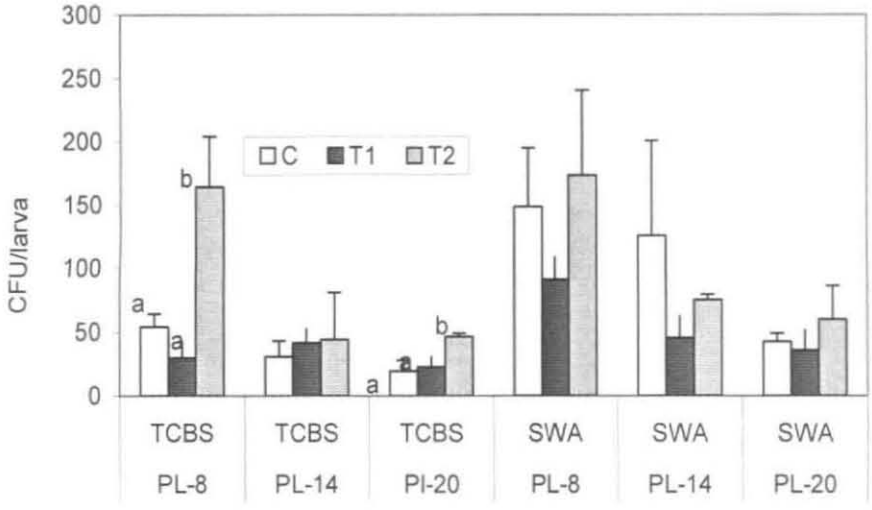

Fig.29. TR. 9. Effect of *B. subtilis* enriched *Artemia* on TCBS and SWA Plate counts in *P.monodon* Post larvae. ANOVA results of TCBS in PL-14 and SWA are not significant.

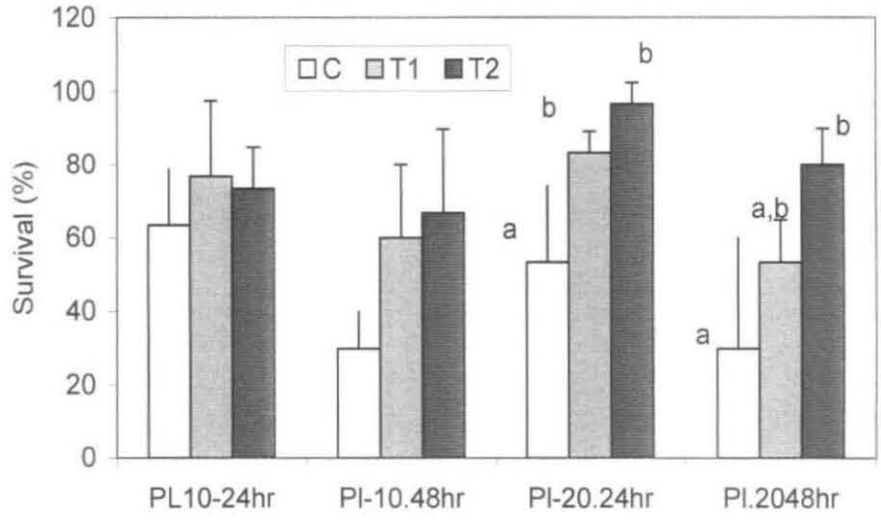

Fig. 30. TR.9. Survival after challenge with *V.harveyii* for 48 h (PL-10 & 20). ANOVA results of PL-10 at 24 and 48 h are not significant.Non-identical superscripts denote significant differences.

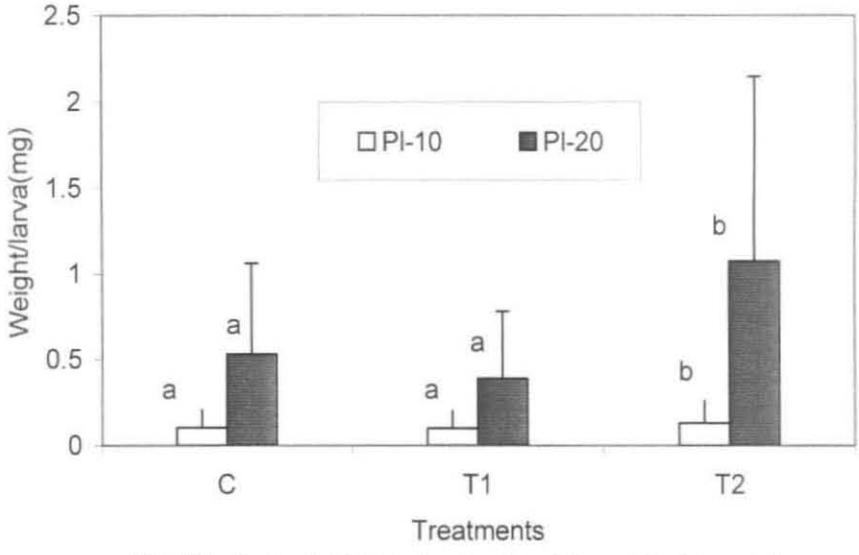

Fig. 31. TR. 9. Weight after feeding *B. subtilis* enriched *Artemia* in *P.monodon* post larvae. Non-identical superscripts denote significant differences.

4.6.0 Experiment-IV: Level of colonization after stopping enrichment

The level of colonization was checked only for SB and *B. subtilis* in larvae and for *B.subtilis* in post larvae after PL. 20. As isolation of *L. plantarum* in zoea and mysis were nil and SB in PL. 20 was poor, the level of colonization were not checked for these experiments.

4.6.1. *S. boulardii*

Level of colonization was found to decline as the days progressed. Only T_2 treatment (10^4) was kept for checking the colonization. The colonization levels were1.95x 10^3 for PL-1 followed by 7.5x 10^1 for PL-3 and 2x10^1 for PL-5 (Fig. 32).

4.6.2. *B. subtilis*

In *B. subtilis* also level of colonization after mysis III was found to decline after the feeding stopped. The effect was checked with T_2 treatment (10^5 CFU/ml) during PL. 1, PL. 3 and PL. 5. The colonization levels were found to be 2.67x10^2 for PL. 1, 5.33x10^1 for PL. 3 and 2.0x10^1 for PL. 5 (Fig. 32)

4.6.3. *B. subtilis* in post larvae after PL- 20

Level of colonization was found to be more in T_2 compared to T_1. The colonization levels were found to be 4.60x 10^2 CFU/larva in T_1 and 5.8x 10^1CFU/larva in T_2 for PL. 22, and 2.13x 10^1 CFU/larva in T_1 and 2.28x10^2 CFU/larva for T_2 in PL. 24 (Fig. 33)

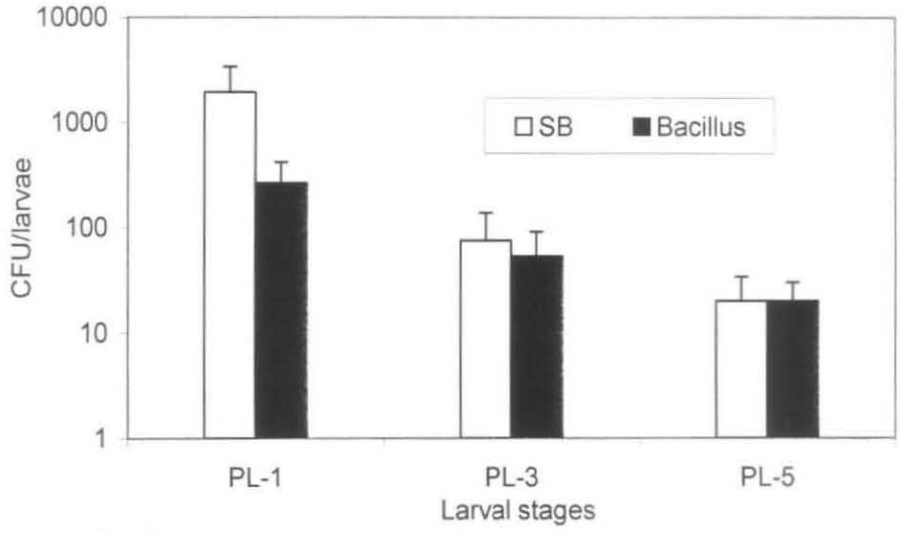

Fig. 32. Level of colonization after 1,3 and 5 days of SB and *B.subtilis* enrichment in mysis III

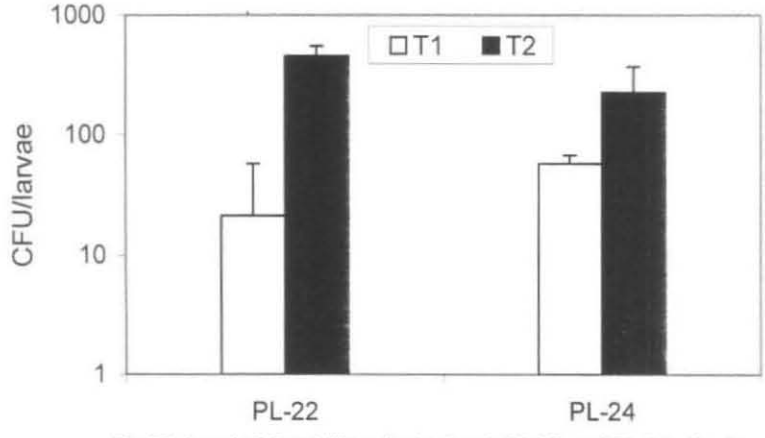

Fig.33. Level of *B.subtilis* colonization in PL-22 and PL-24 after 2 and 4 days post feeding.

Table 3. Biochemical reactions of *Saccharomyces boulardii* using Api -20 C AUX Test Code

Api -20 C AUX Coding	Code number - 6000072
Glucose	+
Glycerol	+
2-Keto –D-gluconate	-
L- Arabinose	-
D - Xylose	-
Adonitol	-
Xylitol	-
Galactose	-
Inositol	-
Sorbitol	-
A-methyl-D-glucoside	-
N-acetyl –D-glucosamine	-
Cellobiose	-
Lactose	-
Maltose	+
Saccharose/sucrose	+
Trehalose	+
Melezitose	-
Raffinose	+
Hyphae/pseudohyphae	-
Identification	*Saccharomyces boulardii*

Table 4. Biochemical reactions of *Lactobacillus plantarum* using Api -20 A Test Code

Api -20 A Test Coding	Code number - 47356732
Indole production	-
Urea	-
Glucose	+
Mannitol	+
Lactose	+
Saccharose	+
Maltose	+
Salicin	+
Xylose	-
Arabinose	+
Gelatin	-
Esculin	+
Glycerol	-
Cellobiose	+
Mannose	+
Melezitose	+
Raffinose	+
Sorbitol	+
Rhamnose	+
Trehalose	+
Catalase	-
Spore	-
Gram stain	+
Cocci	-
Identification	*Lactobacillus plantarum*

Table 5. Fatty acid profile for *B. subtilis*

RT	Response	Ar/Ht	R Fact	ECL	Peak Name	Percent	Comment 1	Comment 2
1.607	4.49E+08	0.026		7.028	SOLVENT PEAK		< min rt	
1.712	38416	0.021		7.238			< min rt	
1.874	477	0.022		7.563			< min rt	
1.992	521	0.035		7.779			< min rt	
2.208	3474	0.025		8.231			< min rt	
2.261	658	0.021		8.338			< min rt	
2.502	834	0.026		8.82			< min rt	
3.598	392	0.02		10.737				
4.313	2361	0.024		11.585				
4.692	263	0.024	1.051	11.999	12:00	0.07	ECL deviates 0.001	Reference 0.005
5.397	1298	0.03	1.026	12.615	13:0 ISO	0.36	ECL deviates 0.001	Reference 0.002
5.532	1416	0.044		12.733				
6.294	4004	0.031		13.334				
6.682	5949	0.033	0.993	13.619	14:0 ISO	1.58	ECL deviates 0.000	Reference 0.002
7.201	2443	0.034	0.982	13.999	14:00	0.64	ECL deviates 0.001	Reference 0.002
8.162	142452	0.036	0.968	14.625	15:0 ISO	36.91	ECL deviates 0.002	Reference 0.001
8.3	130225	0.038	0.966	14.714	15:0 ANTEISO	33.68	ECL deviates 0.001	Reference 0.000
8.691	5282	0.041	0.962	14.969	unknown 14.959	1.36	ECL deviates 0.010	
9.779	12143	0.039	0.951	15.626	16:0 ISO	3.09	ECL deviates 0.001	
10.398	16054	0.04	0.946	15.999	16:00	4.06	ECL deviates 0.001	Reference 0.002
11.074	634	0.044	0.941	16.389	ISO 17:1w10c	0.16	ECL deviates 0.001	Reference 0.002
11.299	3806	0.039	0.94	16.52	ANTEISO 17:1w9c	0.96	ECL deviates 0.004	
11.489	45454	0.04	0.939	16.63	17:0 ISO	11.42	ECL deviates 0.000	
11.649	21501	0.041	0.938	16.722	17:0 ANTEISO	5.4	ECL deviates 0.001	Reference 0.001
13.888	708	0.037	0.928	18.001	18:00	0.18	ECL deviates 0.001	Reference 0.001
13.958	3658	0.043		18.041				Reference 0.000
15.161	538	0.067	0.924	18.729	19:0 ANTEISO	0.13	ECL deviates 0.002	
15.766	584	0.045		19.076				
16.515	3672	0.036		19.505				

ECL Deviation: 0.003 Reference ECL Shift: 0.002 Number Reference Peaks: 11Total respnse: 404838 Total named: 388750
Percent named: 96.03% Total amount: 373615
Sim Index: 0.518 Matches: TSBA40 4.10 Entry name ***Bacillus subtilis***

Table 6. Result of one way ANOVA for Survival

Trial	F value	P value
TR.1	183.861	0.000*
TR.2	1.848	0.217
TR.3	6.6333	0.007*
TR.4	0.945	0.463
TR.5	8.773	0.007*
TR.6	7.634	0.010*
TR.7	1.671	0.265
TR.8	2.522	0.160
TR.9	12.188	0.008*

* The mean difference is significant at 1% level

Table 7. Result of one way ANOVA for Level of enrichment

Trial	Larval stage	F value	Significance
TR.1	Z. 3	4.501	0.064
	MIII	4.000	0.116
TR.2	Z. 3	2.822	0.083
	MIII	152.204	0.000*
TR.5	Z. 3	57.583	0.000*
	M III	27.504	0.000*
TR.6	Z. 3	76.939	0.000*
	M III	22.992	0.002*
TR.7	PL. 20	25.515	0.001*
TR.8	PL. 20	33.971	0.001*
TR.9	PL. 8	10.876	0.010*
	PL. 14	11.858	0.008*
	PL. 20	13.774	0.006*

* The mean difference is significant at 1% level

Table 8. Result of one way ANOVA for *Vibrio* counts.

Trial	Larval stage	F value	Significance
TR.1	Z. 3	1.000	0.422
	MIII	2.485	0.190
TR.2	Z. 3	121.757	0.000*
	MIII	0.758	0.549
TR.3	Z. 3	4.591	0.023*
	MIII	1.649	0.237
TR.4	Z. 3	3.414	0.073
	MIII	6.898	0.013*
TR.5	Z. 3	22.773	0.000*
	M III	6.620	0.015*
TR.6	Z. 3	9.528	0.014*
	M III	2.345	0.177
TR.7	PL. 8	4.351	0.068
	PL. 14	5.517	0.044*
	PL. 20	16.146	0.004*
TR.8	PL. 8	1.889	0.231
	PL. 14	3.89	0.083
	PL. 20	2.987	0.126
TR.9	PL. 8	24.673	0.001*
	PL. 14	0.273	0.770
	PL. 20	17.105	0.003*

* The mean difference is significant at 5% level

Table 9. Result of one way ANOVA for SWA counts.

Trial	Larval stage	F value	Significance
TR.1	Z .3	0.901	0.456
	MIII	1.029	0.368
TR.2	Z. 3	0.141	0.963
	MIII	5.812	0.028*
TR.3	Z. 3	0.633	0.65
	MIII	7.53	0.005*
TR.4	Z. 3	1.01	0.437
	MIII	0.949	0.462
TR.5	Z. 3	3.549	0.067
	M III	0.892	0.486
TR.6	Z.3	1.454	0.306
	M. III	0.048	0.953
TR.7	PL. 8	0.076	0.928
	PL. 14	0.325	0.734
	PL. 20		0.004
TR.8	PL. 8	2.225	0.189
	PL. 14	2.833	0.136
	PL. 20	0.765	0.506
TR.9	PL. 8	0.541	0.608
	PL.14	2.491	0.163
	PL. 20	1.455	0.305

* The mean difference is significant at 5% level.

Table 10. Result of one way ANOVA for *Vibrio* challenge.

Trial	Time	F value	Significance
TR.1	24 h	5.565	0.078
	48 h	147.000	0.000*
TR.2	24 h	5.448	0.025*
	48 h	0.294	0.829
TR.3	24 h	5.565	0.078
	48 h	147	0.000*
TR.4	24 h	5.448	0.025*
	48 h	0.294	0.829
TR.5	24 h	12.697	0.002*
	48 h	1.365	0.321
TR.6	24 h	2.846	0.135
	48 h	2.000	0.216
TR.7	24 h (PL. 10)	1.000	0.422
	48 h (PL. 10)	4.867	0.055
	24 h (PL. 20)	0.667	0.548
	48 h (PL .20)	1.857	0.236
TR.8	24 h (PL.10)	3.556	0.096
	48 h (PL. 10)	1.966	0.221
	24 h (PL. 20)	1.966	0.221
	48 h (PL. 20)	16.188	0.004*
TR.9	24 h (PL.10)	0.542	0.608
	48 h (PL.10)	3.323	0.107
	24 h (PL. 20)	8.867	0.016*
	48 h PL. 20)	4.971	0.053

*The mean difference is significant at 5% level

Table 11. Result of one way ANOVA for Weight gain

Trial	Larval stage	F value	Significance
TR.1	PL. 1	23.592	0.008*
TR.2	PL. 1	0.359	0.784
TR.3	PL. 1	4.097	0.049*
TR.5	PL. 1	16.691	0.001*
TR.6	PL. 1	3.862	0.084
TR.7	PL. 10	18.002	0.003*
	PL. 20	0.884	0.461
TR.8	PL. 10	38.510	0.000*
	PL. 20	15.234	0.004*
TR.9	PL. 10	11.747	0.008*
	PL. 20	12.749	0.007*

*The mean difference is significant at 5% level

DISCUSSION

5.0 DISCUSSION

Bacteria and yeast are the most common microorganisms used as probiotics, but they differ fundamentally in their mechanism of action, metabolism and resistance to pathogens. Four bacterial genera and one yeast genus (*Enterococcus*, *Bifidobacterium*, *Escherichia*, *Lactobacillus* and *Saccharomyces* respectively) are the basis for most preparations. In farmed animals and human clinical applications their beneficial effects are proven as growth promoters and immunostimulants (Fuller, 1989., Vanbelle, 1989.,and Czerucka *et al.* 1994).

In aquaculture, microorganisms are important for health of the animal cultured and also for environmental conservation. In nature microbial flora in the gut of aquatic organisms mostly reflects that of aquatic environment. However, in intensive larval production systems, many bacterial diseases originate in the gut and in such cases the normal flora is out balanced by pathogens. Antibiotic use has a temporary effect on the flora, with the increasing emergence of drug resistant strains. Use of probiotics helps in preventing the prevalence of drug resistant strains and other pathogenic bacteria. Application of probiotics in aquaculture is a recent development. *Bacilli, Lactobacilli* and *Saccharomyces* are the major groups of microorganisms used in commercial probiotic preparations meant for aquaculture.

Artemia nauplii have been reported as one of the best foods for most larval organisms under culture. Bio-encapsulation of *Artemia* nauplii has been practiced as a technique to improve the nutritional characteristic and also for safe delivery of drugs to fish and shrimp larvae. Bio-encapsulation with probiotic organisms was documented for rotifers (Gatesoupe, 1989 and 1991., Gracia de la Banda *et al.*, 1992), and *Artemia* metanauplii (Gatesoupe, 2002., Makridis *et al.* 2000a). The duration of enrichment required to effectively incorporate probiotic organisms in bio-carriers like *Artemia* nauplii is critical to ensure the process safety. *Artemia* metanauplii are continuous feeders and once the maximum ingestion rate is achieved there is a

chance for other opportunistic bacteria to flare up, and getting carried over to the predator organism. *Artemia* nauplii if not carefully handled can contaminate the larval culture system with bacteria (more than 10^7 CFU/g), mostly *Vibrios* which are potentially pathogenic or stress larvae to a point so that they become susceptible to viral infections (Lavens and Sorgeloos, 2000). Standardization of optimum duration was necessary to avoid contamination of larvae as well as to save the energy content of the nauplii.

In the present study *Artemia* enrichment experiments showed higher levels of enrichment for *S. boulardii* compared to *B. subtilis* and *L. plantarum*. The enrichment levels were 3410 for *S. boulardii*, 2093 for *B. subtilis* and 105 CFU/nauplii for *L. plantarum*. Similarly, Gatesoupe (2002) observed higher levels of enrichment of *Artemia* metanauplii with *S. cerevisiae* (200 CFU/nauplii) compared to *Pediococcus acidilacti* (69 CFU/nauplii) adopting a two step enrichment process. In the first step the *Artemia* metanauplii were enriched with yeast and in the second step enrichment was carried out with *S. cerevisiae*, and *P. acidilacti* in separate treatments and also with both in another treatment. He used 20 hours old *Artemia* nauplii, where as in the present study 4 hours old metanaupli were used. The higher yeast as well as bacterial count observed in the present study might be related to the nauplii size, since in smaller metanauplii filtration rate is high compared to large ones.

The *S. boulardii* enrichment levels (3500 CFU/nauplii) reported by Patra and Mohamed (2003) were comparable and similar to the enrichment results obtained in the present study. They enriched *Artemia* metanauplii with varying concentration of *S. boulardii* (10^2 to 10^4 CFU/ml) and observed increase in enrichment levels corresponding to the *S. boulardii* concentrations added. Enrichment levels also varied between *Artemia* strains for *S. boulardii* enrichment. Enrichment of OSI brand resulted in 3410 CFU/nauplii while for Red Dragon it was only 1120 CFU/ nauplii.

The enrichment levels in *Artemia* nauplii followed a linear trend corresponding to the concentration of probiotics added as reported by many authors

(Patra and Mohamed, 2003., Gelabert, 2003 and Makridis *et al.*, 2000a). The enrichment levels of *L. plantarum* obtained in the present study (10^2 CFU/nauplii) are substantially lower (10^4 CFU/nauplii) than the levels reported for LAB species by Villamil (2003) and Gatesoupe (1994). However the enrichment levels of *B. subtilis* did not vary from that of the above concentration. The difference in the enrichment levels of *L. plantarum* might be due to the differences in the *Artemia* strains used as observed by Makridis *et al.* (2000 a).

The optimum enrichment duration for *S. boulardii* as well as *B. subtilis* was found to be 4 hours, while for *L. plantarum* it was 12 hours. Gomez-Gil *et al.* (2001) also found the best enrichment duration to be 4 hours when metanauplii were enriched with drugs such as enrofloxacan and oxytetracycline. They had attributed this to the ability of *Artemia* metanauplii to bio-encapsulate the maximum concentration of particles in a period ranging from less than 1 hour to around 8 hours depending on the substance ingested, after which the organism reach equilibrium between ingestion of the substance and excretion of waste products. Gelabert (2003) observed higher filtration efficiency for smaller metanauplii when compared to larger ones and attributed this to the higher metabolic activity of the smaller individuals.

In the present study application of probiotic organisms to the shrimp larval rearing system was performed in two different approaches. For rearing zoea to post larvae the selected probiotics were directly added to the culture medium since the larvae are filter feeders during this period. In the second set of experiments from post larvae 3 to 20 probiotic organisms were enriched in to *Artemia* metanauplii and were then fed to the larvae, as raptorial feeding becomes the dominant mode of feeding during this stage (Loya-Javellana, 1989).

Generally all the three-probiotic organisms were found to improve the survival rate of larvae from zoea to PL. 1 (76-78%). The effective concentrations, which resulted in higher survival rates compared to the control were, 10^3 CFU/ml for *L. plantarum*, 10^4 CFU/ml for *S. boulardii* and 10^5 CFU/ml for *B. subtilis*. Haryanti *et al.*

(1998) observed an increase in survival from zoea to PL. 10 with the addition of a probiotic bacterial strain, BY-9 culture broth at 10^6 CFU/ml. The treatment tank resulted in higher survival rate (70%) compared to control (35%) in PL. 1. Garriques and Arrevalo (1995) also reported higher survival rate for *P. vannamei* larvae with the addition of *V. alginolyticus* culture broth to the treatment tank. They observed 90% survival rate in treatment tanks compared to control (70%) for PL. 10. In both the cases the survival rates of post larvae in treatments were higher than the result obtained in the present study, when broth was added to culture medium. Variations in the result obtained might be related to the type of medium used. Besides the culture volume used was higher (18 to 25 tonnes) than in the present study.

Studies on the use of *Bacillus subtilis* and *S. boulardii* in penaeid larval rearing from zoea to post larvae have not been reported so far. Uma (1995) had observed increase in survival rate corresponding to the concentration *L. plantarum* added to the larval rearing medium of *P. indicus* larvae from PL. 15 to PL. 30. The concentrations in the treatment ranged from 10^3 to 10^6 CFU/ml and the corresponding survival rates ranged from 72±4 to 94±2%. However she observed a survival rate (72%), at 10^3 CFU/ml application of *L. plantarum* as in the present study. The lower survival rate obtained in the present study may be due to the sensitive nature of zoea and mysis stage to the changes in quality of rearing medium compared to the post larvae from PL. 15 to PL. 30. Gildberg *et al.* (1995) reported higher cumulative mortality than the control in Atlantic salmon fry given *Carnobacterium* sp. They have attributed this to the reduction in intestinal pH which could have helped in the colonization by *Aeromonas salmonicida* and also to the reduced lytic activity of the proteolytic enzymes in lower pH conditions. Contrary to the present study Gatesoupe (1991) noticed lower survival rates in turbot larvae fed with LAB enriched *Artemia* metanauplii.

Survival was poor in treatments where higher concentrations of probiotics were added. Similar results were reported by Azad *et al.* (2002) who found lower percentage survival for *P. monodon* larvae in treatments where higher

concentration of photobacteria, *Rhodovulum sulfidophilum*, was used along with the alga *Skeletonema*. They used varying proportions of bacteria 1%, 2%, 3%, and 5% along with *Skeletonema* for protozoea, while mysis and post larvae were fed the above diet supplemented with *Artemia* nauplii. They observed higher survival rate with 2 and 1% *R. sulphidophilum* in the diet followed by 3 and 5%. They had attributed this to the deterioration in water quality with higher levels of *R. sulphidophilum* inclusion. In the present study the trials using more than 10^5 CFU/ml of probiotics resulted in poor survival and the mortality occurred from mysis I stage onwards. In zoea and mysis the increased concentrations of probiotics might have resulted in accumulation of cell aggregates on protozoeal cephalic appendages, which might have inhibited effective feeding and respiration leading to mortality. The deposition of excess solid particles or organic matter in the incubation tanks increases the possibility of fouling and colonization by pathogens and other microorganisms resulting in poor survival or even death (Simon, 1978).

In experimental trials where post larvae were reared from PL. 3 to PL. 20 with enriched metanauplii, treatments fed with *B. subtilis* resulted in significantly higher survival rates than control. Post larvae given *S. boulardii* enriched nauplii gave higher survival rates than *B. subtilis* fed groups but the results were not significantly different from control. Gatesoupe (2002) also noticed higher survival rates in pollack larvae fed *Artemia* nauplii enriched with yeast compared to *P. acidilacti* in a two step enrichment process. In the first step the *Artemia* metanauplii were enriched with yeast and in the second step enrichment was carried out with *S. cerevisiae*, and *P. acidilacti* in separate treatments and also with both in the same treatment. He observed 33% survival in *S. cerevisiae* enriched treatments compared to 15% in treatment with *P. acidilacti* and 24% in combination of both but the results were not significant. Lara-Flores *et al.* (2003) used diets containing bacterial and yeast supplements for rearing *Tilapia* fry. The bacterial supplement contained *Streptococcus faecium* and *L. acidophilus* and the yeast diet contained *S. cerevisiae*. They observed higher survival rates in yeast fed tanks compared to the treatment tanks given bacteria. Similarly when yeast *S. cerivisiae* is used, Nates *et al.* (1994) and Devaraja *et al.* (2002) noticed

higher survival rates in treatment ponds compared to control in *P. monodon* and *Liptopenaeus vannamei* grow outs respectively.

Enrichment of *B. subtilis* in larvae as well as post larvae resulted in significant improvement in survival rate. Use of *Bacillus* sp as a probiotic in shrimp larval rearing has not been reported so far. In shrimp grow-out ponds also when *P. monodon* were fed with probiotics containing *Bacillus* sp higher survival rates were reported (Moriarty, 1998., Devaraje *et al.*, 2002 and Rengpipat *et al.*, 1998). Rengpipat *et al.* (1998) used *Bacillus* S11 strain isolated from *P. monodon* habitats and fed it to *P. monodon* PL. 30 by incorporating in to the formulated feed for 100 days. With the supplementation of 10^{10} CFU/g feed the survival rate observed in treatments (33%) were higher than control (15.8%). Moriarty (1998) also observed similar results in *P. monodon* grow outs when *Bacillus* is used at an abundance of about 10^4 to 10^5 CFU/ml. The survival rate obtained with *Bacillus* (73%) was found to be highly significant compared to control. Even though the culture methods adopted in the present study are different from that of pond conditions the result obtained in the present study is similar to that obtained by the above authors. *Bacillus* species were reported to have the capacity to produce antibiotics and also, enzymes that can break down polysaccharides, nucleic acids and lipids. These properties might have helped the larvae in improving food conversion and resistance to pathogens which inturn might have reflected in the overall survival of the larvae.

Application of *S. boulardii* and *B. subtilis* resulted in high enrichment levels during mysis III compared to zoea. In zoea and mysis the enrichment levels increased as the concentration of probiotic organisms increased. In *P. monodon* ingestion rate is reported to peak at M III (Loya-Javellana, 1989). Ingestion rate inturn is the function of animal size and feeding efficiency. *B. subtilis* gave higher levels of enrichment (2133 CFU/larva) compared to *S. boulardii* (1535 CFU/larva). It was not possible to re-isolate *L. plantarum* could not be re-isolate from zoea and mysis in both experimental trials, indicating that either the sampling method was inadequate to capture the organism in the selective media used, or *L. plantarum* was quickly

eliminated from the gut. However Strom and Ringo (1993) observed an enrichment level of 70% of total CFU in cod larvae fed with *L. plantarum*. *Lactobacilli* are not commonly found in shrimp gut (Coloroni, 1985., Hameed, 1993., Singh *et al.* 1998). On the other hand in fish guts they form 10% of the bacterial flora. In fish larval rearing Lactobacilli have been widely used with considerable degree of success (Gatesoupe, 1994., Garcia de la Banda *et al.* 1992). The present study shows that *Lactobacilli* are not an ideal probiotic species for shrimp larvae.

In post larvae it was possible to re-isolate *B. subtilis* from PL. 8, PL. 14 and PL. 20 stages where as re-isolation of *S. boulardii* was possible only from PL. 20. The enrichment level of *B. subtilis* varied from 18 to 22 CFU/larva fed with live nauplii whereas it ranged from 6 to 26 CFU/larva in treatment fed with enriched dead nauplii. Similarly low levels of enrichment (4 CFU/ larva) were noticed in turbot larvae fed with *Bacillus* spores enriched rotifers (Gatesoupe, 1991). Sridhar and Raj (2001) isolated *Bacillus* and *Micrococcus* strains from the gut of wild *P. indicus*. *Bacillus* was coated on to compounded feeds as emulsion containing $5x10^7$ cells/g feed. At the end of growth trial it was possible to re-isolate *Bacillus* at $6.3x10^6$ CFU/shrimp. Similar results were observed by Rengpipat *et al.* (1998) in *P. monodon* fed with *Bacillus* S11. They had observed an enrichment level of 10^7 to 10^8 CFU/g in *P. monodon* PL-30 when fed with *Bacillus* S11 incorporated diet containing10^{10} CFU/g for 100 days. These enrichment levels were higher than the enrichment results obtained in the present study. Incorporating *Bacillus* along with compounded feed may be a better option for enrichment in late post larval stages when compounded feeds are used. Enriched *Artemia* nauplii could be an effective live feed for early post larval stages from PL. 3 onwards since preying on fast moving and larger metanauplii is difficult for larval stages from mysis to PL. 2 as observed during the present study.

Post larvae showed enrichment level of only 1 CFU/larva when fed with *S. boulardii* enriched metanauplii in both trials. Even though enriched metanauplii contained approximately 3400 CFU of *S. boulardii* it was not reflected in post larvae, as the feeding levels were approximately 18 metanauplii/ larva from PL. 12 to PL. 15

and 20- 25 metanauplii/larva from PL. 15 to PL. 20. This result suggests that to achieve sufficient colonization of *S. boulardii* feeding levels should be increased substantially to above 20 enriched metanauplii/day. The lower levels encountered during the post larval stage could be due to the lysis of *S. boulardii* cells by enzymes in the larval gut. Lyses of *S. boulardii* cells by digestive enzymes inside the gut of human volunteers were suggested by Klein *et al.* (1993). Results obtained with live as well as dead *Artemia* were the same for both trials pointing to the viability of yeast cells inside dead *Artemia* nauplii and the possibility of cell lysis inside the post larval gut.

In the enrichment experiments with all the three probiotics from zoea to PL. 1, TCBS counts as well as SWA counts were low during initial phase of culture and increased towards the end of culture period. Lower bacterial counts noticed in the initial phase can be related to the culture medium being chlorinated and filtered seawater being used for the culture. The subsequent increase in bacterial counts might be due to the multiplication of opportunistic bacteria in the rearing medium triggered by the addition of microalgae and the organic waste from the larvae. Combination of high larval densities, debris from dead larvae and high loads of organic matter due to the addition of intensively produced live feed stimulates selection and growth of opportunistic bacteria in the larval rearing tanks (Garriques and Arevalo, 1995). Opportunistic bacteria will take advantage of ecological changes introduced, when seawater is used in aquaculture (Skjermo and Vadstein, 1999). Moreover rearing technology for intensive production of larvae creates a highly artificial environment, which promotes bacterial growth and affects bacterial selection negatively. Higher counts of *Vibrios* observed in treatment with 10^6 concentrations could have resulted from higher concentrations of probiotic leading to the clumping of particles, which could have triggered the growth of opportunistic bacteria.

Enrichment of *S. boulardii* and *B. subtilis* significantly reduced the *Vibrio* counts in mysis stage while the effect of *L. plantarum* was not significant. Hariyanti *et al.* (1998) noticed significant reduction in *Vibrio* counts in the rearing water after enriching *P. monodon* larvae, zoea to PL.10 with bacterial strain BY-9. Garriques and

Arrevalo (1995) also noticed reduction in potentially pathogenic *Vibrios* in the larval rearing tanks of *P. vannamei* when treated with *V. alginolyticus*. The *Vibrio* counts in the above studies were low (10^2 CFU/ml with BY-9 and 10^2-10^3 CFU/larva with *V. alginolyticus*). The higher *Vibrio* counts observed in the present study were from the larval gut whereas with BY-9 it was from rearing water. *Vibrios* are dominant component of gut micro flora and will always be at higher concentration compared to the rearing water. The lower counts obtained in Garriques and Arrevalo's studies might be due to the effectiveness of *V. alginolyticus* against another *Vibrio* since antagonistic effect is higher between two Gram negative organisms (Irianto and Austin, 2002). Competitive exclusion of pathogenic *Vibrios* has been suggested by Garriques and Arrevalo (1995). The present study clearly shows the competitive exclusion of *Vibrio* species by *S. boulardii* and *B. subtilis*. *S. boulardii* may also have some antagonistic effect on *Vibrio* species. Czerucka *et al.* (1994) observed reduction in *Vibrio* induced cholera toxin levels in rat epithelial cells incubated with *S. boulardii*. Similarly a reduction in dominant *Vibrio* spp was noticed in rotifers enriched with *Bacillus* spores (Gatesoupe, 1991).

In spite of higher *Vibrio* counts observed during mysis III the survival rate of larvae in the treatment tanks were significantly higher than control. This could be due to the fact that the *Vibrios* observed might be opportunistic rather than pathogenic, however clear incidence is lacking. Anderson *et al.* (1989) observed *Vibrios* as the dominant microflora in the larvae of hatchery reared *M. rosenbergii* and concluded that even though the members of the genus are pathogenic they are not primary pathogens and exist in and around crustaceans in marine and brackish water environment as part of their normal micro flora. Similarly, Singh *et al.* (1998) reported *Vibrio* as the dominant micro flora in the gut of pond reared *P. indicus*. He postulated the beneficial as well as harmful effect of the species in the life of animal, being capable of producing various hydrolytic enzymes that enhances digestive process.

Addition of the three probiotics did not result in any significant variation in SWA counts. Hariyanti *et al.* (1998) also reported no significant variation in SWA

counts in *P. monodon*, zoea to PL.10 on being fed with strain BY-9. Gildberg *et al.* (1995) observed no significant difference in total aerobic flora among control and treatments in Atlantic salmon fed with *Carnobacterium* species. On the other hand Strom and Ringo (1993) observed increase in total aerobes in control from 10^2 to 10^4 CFU/larva as the culture progressed, while in the tanks treated with *L. plantarum* the bacterial count remained same. Gatesoupe (1991) also observed a significant reduction in total aerobic counts in rotifers fed with *L. plantarum*. The insignificant effect of *L. plantarum* observed in the present study might have resulted from the absence of *L. plantarum* cells inside the gut of larvae (as shown in the enrichment studies) to compete for space and nutrients.

In post larvae, TCBS as well as SWA counts decreased towards the end of culture period with *S. boulardii* as well as *B. subtilis* enriched *Artemia*. Reduction in SWA counts was 60-70% while it was around 50% for *Vibrio*. As mentioned in the case of larvae the reduction in *Vibrio* counts might have been due to the antagonistic effect of *S. boulardii* against *Vibrio* species. Rengpipat *et al.* (1998) had observed significant reduction in *Vibrio* counts (10^2 CFU/larvae) in treatment tanks after feeding *P. monodon* with *Bacillus* S11 incorporated in diets for 100 days while variation in SWA counts were not significant from control. The *Vibrio* counts are comparable to that of the present study (10^2 CFU/larva. In another study Moriarty (1998) also observed low *Vibrio* counts in pond water and sediment treated with *Bacillus* in *P. monodon* grow outs. When DMS series containing *Bacillus* were applied *V. harveyii* counts reduced from $3x10^3$ CFU/ml to less than $1x10^2$ CFU/ml in pond water while it was absent in sediment. In both the studies the reason attributed was competitive exclusion of other bacterial flora by *Bacillus*.

Resistance to a pathogenic *V. harveyii* infection in PL. 1 stage was higher for *S. boulardii* fed groups than *B. subtilis*. *L. plantarum* did not produce any significant response. Enrichment of *S. boulardii* significantly improved the survival rate of PL.1 during a 48 hours pathogen challenge. Addition of *S. boulardii* broth gave highly significant effect ($P< 0.01$) compared to the addition of cells alone. When *S.*

boulardii broth was added it required only 10^3 CFU/ml of *S. boulardii* where as when cells alone were added 10^6 CFU/ml was required to surmount the pathogen infection. Patra and Mohamed (2003) also reported a similar effect in *Artemia* metanauplii enriched with *S. boulardii* broth at 10^4 CFU/ml concentration. They observed 91% survival in the treatment given *S. boulardii* broth. In the present experiment 70% survival was observed in treatment given 10^3 CFU/ml of *S. boulardii* broth. The lower survival rate observed might be because of the highly sensitive nature of PL. 1 larvae compared to *Artemia* metanauplii. The higher survival rate observed with the addition of broth compared to cells alone might be due to availability of nutrients in the culture broth.

Post larvae fed with *S. boulardii* enriched *Artemia* resulted in higher survival rates after challenge compared to control after 48 as well as 24 hours. Yeast cells are known to contain β 1-3 glucans in their cell walls that can act as immuno-stimulating agents. β 1-3 glucans in *S. cerevisiae* was found to enhance the immune functions of hybrid striped bass (Li and Gatlin, 2003) and *P. monodon* (Song *et al.*, 1997). Sakai *et al.* (2001) reported that nucleotides from *S. cerevisiae* could enhance the activity of phagocytic cells in common carp. β 1-3 Glucans enhanced the non-specific immune response of shrimps and in turn reduced shrimp mortality caused by opportunistic pathogens (Song *et al.* 1997). *S. boulardii* is reported to have some kind of antagonistic activity against *Vibrio* species. Czerucka *et al.* (1994) observed 40% reduction in cholera toxin induced cAMP levels in rat IEC- 6 cells. *S. boulardii* cells were found secrete a protein of 20 kilodaltrons that inhibit the activity of cholera toxin in their experiment. This protein factor might have induced resistance in the larvae against the pathogenic *V. harveyii*.

Addition of *Bacillus* at the rate of 10^4 to 10^5 CFU/ml resulted in higher survival in challenge test with *V. harveyii*. In post larval experiments also treatments fed with *B. subtilis* enriched metanauplii resulted in significantly higher survival rates compared to control. Rengpipat *et al.* (1998) had observed a similar results when they challenged *P. mondon* post larvae with *V. harveyii*, and observed 100% survival in

treatments compared to 26% in control and the dominance of *Bacillus* S11 in the treatment tanks. Menupol *et al.* (2003) fed *P. monodon* post larvae for a month with *Bacillus* S11 incorporated in feed and observed 80% survival after 48 hours and 43% survival after 144 hours challenge with *V. harveyii*. Both the studies mentioned above suggested competitive exclusion by non-pathogenic *Bacillus* S11 as the main reason for this beneficial effect. These results are comparable to that of the present study. Moriarty (1998) suggested the efficiency of *Bacillus* genus to secrete many enzymes that degrade slime and bio-films, which help *Bacillus* and their antibiotics to penetrate slime layers around Gram-negative bacteria. Competitive exclusion by *Bacillus* species was also suggested as cause for their probiotic effect. Another possibility is the production of siderophores, an anti-bacterial substance as suggested by Sugita *et al.* (1998).

Enrichment of *L. plantarum* in zoea and mysis in the present study did not result in any significant improvement in resistance to *V. harveyii* infection. Similar effect was reported in *P. indicus* post larvae (PL. 30) by Uma (1995) when *L. plantarum* was directly added to the rearing medium containing PL. 15. The survival ranged from 50 to 65% and the highest was noticed in control. In another study Uma *et al.* (1999) had reported increase in resistance to *V. harveyii* chllenge when LAB species were given along with yeast in *P. indicus* grow out. They have attributed this to the immuno-potentiating effect of yeast rather than bacteria. Gatesoupe (1994) and Nikolskelainen *et al.* (2001a) had also noticed an increase in resistance in larval turbot when challenged with pathogenic *Vibrio*.

Application of the three probiotic organisms to zoea and mysis resulted in significant increase in weight at the end of the experiments. Both the bacterial species promoted higher weight gain than *S. boulardii*. Among bacterial species *L. plantarum* resulted in higher weight gain compared to *B. subtilis*. All the treatments resulted in growth higher than the control diets suggesting that the addition of probiotics mitigated the effect of stress factors.

In *S. boulardii* enrichment experiments, growth performance of PL. 1 was significantly different from control. When cells alone or culture media was added 10^3 CFU/ml of *S. boulardii* enrichment resulted in better performance of larvae compared to higher enrichment levels. Similarly Li and Gatlin (2003) observed better growth performance in hybrid sea bass fed with 1% dietary supplementation of yeast compared to 2% and 4% level inclusions. In another study Lara-Flores *et al.* (2003) had observed higher individual weight gain and specific growth rate with yeast compared to treatments fed with probiotic bacteria and control. They have recommended 0.1% supplement of yeast in *Tilapia* dry feeds to stimulate productive performance. It can be concluded from the present study and those described above that lower concentration of yeast inclusion is beneficial to host compared to higher concentrations.

Ramirez *et al.* (2002) also reported increase in weight gain in groups fed with yeast compared to control in sea bass larvae. They noticed increased amylase and trypsin secretion in yeast fed groups and also increased secretion of polyamines from yeast cell. Polyamines improve cell metabolism and proliferation by stimulating RNA, DNA and protein synthesis. The above properties of yeast cells might have induced increase in growth in *S. boulardii* enriched larva and post larva in the present study.

Application of *L. plantarum* resulted in significantly higher weight gain for treatments given 10^2 CFU/ml and 10^3 CFU/ml of cells at PL. 1 stage. Uma (1995) observed increase in weight gain corresponding to the concentrations of *L. plantarum* added in *P. indicus* PL. 30. Contrary to the present observation she noticed lowest weight gain for treatments given 10^3 CFU/ml. Uma *et al.* (1999) used Lacto-sacc containing *L. acidophilus*, *Streptococcus* and *Saccharomyces* species and observed a positive effect on growth and survival rates in *P. indicus*. Contradictory to the present study, Gatesoupe (1991) observed lower weight gain in turbot larvae fed with *L. plantarum* enriched *Artemia* nauplii. But he had observed significant increase in mean weight by day 20 in groups fed with *L. plantarum* enriched rotifers. Gildberg *et al.*

(1998) also reported insignificant difference in *Carnobacterium* fed groups compared to control.

Enrichment of *B. subtilis* in larval as well as post larval experiments resulted in significant improvement in weight gain. Post larvae given *Bacillus* enriched live nauplii performed better than those given dead enriched nauplii. Rengpipat *et al.* (1998) observed significant increase in weight gain in *P. monodon* post larvae reared for 100 days with *Bacillus* S11. The treatment groups resulted in higher weight gain (7.06 g) compared to control. However Moriarty (1998) observed 39 g in control compared to 28 g in treatment ponds treated with DMS series containing *Bacillus*. This could be related to reduction in number of animals /area due to higher mortality in the control. *Bacilli are* known to produce special compounds that can break down polysaccharides, nucleic acids and lipids. This property might have nutritionally benefited the larvae resulting in increased weight gain.

Post larvae fed with live enriched nauplii showed comparatively higher performance for all the parameters observed compared to control. Post larvae fed with dead live enriched *Artemia* appeared healthy with increased black pigmentation compared to those given dead enriched *Artemia*. *S. boulardii* and *B. subtilis* were found to be viable in the gut, as significant difference in the enrichment could not be observed between both groups. This technique can be adopted for feeding younger larval stages from mysis to post larvae as larvae can capture dead metanauplii more efficiently than live ones.

When probiotic bacteria are used in aquaculture to protect fish against disease caused by bacterial pathogens, the strains to be administered should have the antagonistic property and capacity to colonize the host. Post feeding colonization has not been reported in shrimps so far. Colonization after discontinuing the probiotic supplementation was checked for *S. boulardii* and *B. subtilis*. Since *L. plantarum* could not be re-isolated from larvae, this species was not used for checking post feeding colonization. In both *S. boulardii* and *B. subtilis* the number of CFU/larva decreased

gradually to 10^2 CFU/larva in five days. In *S. boulardii* enriched larvae *S. boulardii* counts decreased from 1950 to 20 CFU/larva. Klein *et al.* (1993) noticed a reduction in *S. boulardii* counts in five days once the oral administration was stopped. In their study healthy human volunteers were given single oral dose of 1 g *S. boulardii*. The time to achieve maximum concentration in the stool was 36-60 hours, after which *S. boulardii* counts reduced to below detectable level in 2-5 days. They have suggested possible lysis of *S. boulardii* cells inside the intestine by the digestive enzymes of the host. In the present study it is evident that *S. boulardii* can be used as an effective probiotic for rearing shrimp larvae. Possible lysis of the cells in filter feeding stages of zoea and mysis may not be a factor for the reduction in *S. boulardii* counts as there will be continuous evacuation of the gut contents. The later process might have resulted in reduction of *S. boulardii* counts once the feeding stopped.

In fishes, Rameriz *et al.* (2002) reported the capacity of *Debaryomyces hansenii* and *S. cerevisiae* to adhere to the intestinal walls of sea bass after 41 days post hatch, when compounded feed incorporated with the above yeast species was given, the fluorescent labeled cells remained on the intestinal segments even after three rinsings. Both the species were recovered at 10^2 CFU/larva, which is similar to the present observation. They have attributed the ability of yeast cells to adhere to fish mucus mediated by both specific adhesion and cell surface hydrophobicity.

B. subtilis also showed a reduction to 10^2 CFU/larva 5 days after cessation of feeding in PL. 5. In the present study post larvae also showed colonization of *B. subtilis* in the larvae suggesting that when compared to *S. boulardii* and *L. plantarum*. *B. subtilis* is effective in colonizing digestive tract as well as in producing significant probiotic effect. Joborn *et al.* (1997) fed rainbow trout with *Carnobacterium* divergance strain K1 at 4 $x10^7$ CFU/g through feed for 6 days. Post feeding colonization was checked after 4 days. They observed 10^5 CFU/ml in faecal pellets even 4 days after post feeding suggest that the cells were able to multiply and colonize in the digestive tract. However in the present study concentration of *B. subtilis* in faecal pellets was not determined.

Robertson *et al.* (2000) used *Carnobacterium divergens* strain K1 for feeding rainbow trout fry and fingerlings and Atlantic salmon fingerlings by incorporating in to feed at 10^7cells/ g. The experiment showed constant increase in *C. divergence* population up to 7.4 x 10^6 CFU/g of intestine after 28 days of feeding. However levels declined gradually for 6 days after cessation of feeding such that *Carnobacterium* species could not be detected in the intestine 6 days later, while in rainbow trout fry it could be detected in the intestine for 10 days. In post larvae reduction in *B. subtilis* counts was only 9% after 5 days post feeding, which could be due to the effectiveness of the species to colonize the gut. Duration of colonization after cessation of feeding in the above experiment is similar to that observed in the present study.

In larval rearing from zoea to PL. 1 true colonization was absent, but a sustained transient state was maintained artificially by the regular re-introduction of fresh bacteria through diet. In post larvae colonization levels were not checked after 6^{th} day to find out whether there is permanent colonization in the gut. Except in the case of true colonization *S. boulardii* and *B. subtilis* satisfy the major requirement of being effective probiotics, as isolates that remain viable in the intestinal tract and benefits the host by way of increasing the survival rate, increasing the resistance of the larvae to a pathogenic *Vibrio* and by imparting improved growth and weight gain and, thus overall improving the health and nutritional status of the larva. The lack of consistency in the results obtained in the repeated trials is a biological truism due to the inherent variability of biological systems and individual batches of larvae and rearing conditions. From the experiments described above it can be concluded that *S. boulardii* and *B. subtilis* can be used as candidate probiotic species for shrimp larviculture while *L. plantarum* is not a suitable species. Further research is needed to find out the effect on the use of consortium of the species used in the present study since performance of each species was at varying levels for the parameters checked.

SUMMARY AND CONCLUSIONS

SUMMARY

- The present study was undertaken to evaluate effectiveness of three probiotic organisms in *P. monodon* larval rearing system. The probiotic organisms used were, *Saccharomyces boulardii*, *Lactobacillus plantarum* and *Bacillus subtilis*. The experiments were conducted in two different sections. In the first section where the larvae were reared from zoea to post larvae the probiotic organisms were directly added to the rearing medium. In the second set of experiments post larvae were reared from PL. 3 to PL. 20 with probiotic enriched *Artemia* metanauplii.

- The parameters studied were level of enrichment of probiotic organisms in larvae and post larvae, survival and weight gain, effect on other bacterial flora and resistance to a pathogenic *V. harveyii* infection. Attempts were made to find out the optimum enrichment duration and maximum enrichment levels of the three probiotic organisms in *Artemia* metanauplii. During the study post feeding colonization levels were checked for both *S. boulardii* and *B. subtilis* from PL 1 to Pl 5. In post larvae, colonization was checked only for *B. subtilis* after PL. 20.

- In *Artemia* enrichment experiments *S. boulardii* and *B. subtilis* gave comparatively higher enrichment levels compared to *L. plantarum*. Enrichment levels followed a linear trend for all the three probiotics corresponding to the concentration added.

- Optimum enrichment duration for *S. boulardii* as well as *B. subtilis* was found to be 4 h while for *L. plantarum* it was 12 hours. The maximum enrichment levels were 3410 CFU/nauplii for *S. boulardii,* 2093 CFU/nauplii for *B. subtilis* and 105 CFU/nauplii for *L. plantarum*. Enrichment levels also varied between *Artemia* brands for *S. boulardii* enrichment. Enrichment of OSI brand resulted in 3410 CFU/nauplii while for Red Dragon it was 1120 CFU/ nauplii.

- Generally all three probiotic organisms were found to improve the survival rate of *P. monodon* larvae from zoea to PL.1. Higher survival was observed for *B. subtilis* followed by *L. plantarum* and *S. boulardii.* The effective concentration, which resulted in higher survival rates, was 10^3 CFU/ml for *L. plantarum,* 10^4 CFU/ml for *S. boulardii* and 10^5 CFU/ml for *B. subtilis.* In all experiments, higher levels of probiotics application resulted in lower survival rates. Addition of culture media directly to rearing system resulted in poor survival rates compared to addition of cells alone.

- In experimental trials where post larvae were reared from PL.3 to PL. 20 with enriched metanauplii, Treatments fed with *B. subtilis* resulted in significantly higher survival rates. Post larvae given *S. boulardii* enriched nauplii gave higher survival rates than *B. subtilis* fed groups but the results were not significantly different from control. The lower survival rates observed in *B. subtilis* fed group might have resulted from the variation in the quality of larvae among the batches used.

- Application of *S. boulardii* and *B. subtilis* showed peak enrichment levels during mysis III. In zoea and mysis the enrichment levels increased as the concentration of probiotics organisms increased. *B. subtilis* showed higher levels of enrichment compared to *S. boulardii* . Beyond 10^5 CFU/ml applications the enrichment level remained at 10^4 CFU/larva only. It was not possible to re-isolate *L. plantarum* from zoea and mysis in both the experiments trials, indicating that either the sampling method was inadequate to capture the organism in the selective media used, or *L. plantarum* was quickly eliminated from the gut.

- It was possible to re-isolate *B. subtilis* from PL. 8, PL.14 and PL. 20 stages after feeding with enriched metanauplii where as reisolation of *S. boulardii* was possible only from PL. 20. The enrichment levels of *B. subtilis* in treatments fed with live enriched nauplii were high compared to the treatment given dead enriched nauplii. The enrichment levels for treatment fed with live enriched nauplii were 22 CFU/ larvae for PL. 8, 18 CFU/larvae for PL.14 and 66

CFU/larvae for PL. 20 and in treatment fed with dead enriched nauplii the enrichment levels were 8 CFU/larvae for PL. 8, 6 CFU/larvae for PL-14 and 26 CFU/larvae for PL. 20. *S. boulardii* enrichment levels in post larvae were same for both the treatment groups (1 CFU/larvae).

- In experiments where probiotics organisms were directly added to zoea and mysis *Vibrio* counts were observed to be low during initial phase of culture and increased towards the end of culture period while in post larvae fed with enriched nauplii an opposite trend was observed.

- Enrichment of *S. boulardii* as cells significantly reduced the *Vibrio* counts in mysis stage. Addition of *L. plantarum* resulted in no significant variation in *Vibrio* counts during mysis stage (range 10^2 -10^4 CFU/larva). Addition of *Bacillus subtilis* significantly reduced *Vibrio* counts in zoea and mysis in the first trial. While the effect was not apparent in the second trial.

- Post larvae given *S. boulardii* and *B. subtilis* showed significantly higher levels of *Vibrio* counts in treatments fed with live enriched metanauplii in PL. 8 and the counts decreased towards the end of culture period

- Generally M III showed higher SWA counts compared to zoea with all the three probiotics organisms. Addition of *S. boulardii, L. plantarum* and *B. subtilis* did not result in any significant variation in SWA counts.

- In the post larval rearing experiment feeding *S. boulardii* and *B. subtilis* enriched *Artemia* resulted in lower SWA counts in PL 14 and PL 20.

- Resistance of probiotic fed larvae to pathogenic *V. harveyii* infection in PL1 stage was higher for *S. boulardii* fed group than *B. subtilis*. *L. plantarum* did not produce any significant response.

- Addition of *S. boulardii* significantly improved the survival rate of PL.1 during a 48 hours pathogen challenge. Addition of *S. boulardii* broth gave highly

significant effect compared to the addition of cells alone. When *S. boulardii* broth was added it required only 10^3 CFU/ml of *S. boulardii* where as when cells alone were added 10^6 CFU/ml was required to surmount the pathogen infection. In post larval experiments on challenge with *V. harveyii* both PL. 10 and PL. 20 showed higher survival rates in *S. boulardii* enriched *Artemia* fed groups. The effect was more significant in PL. 20 for groups fed with live enriched metanauplii compared to the treatments fed with dead enriched nauplii. This clearly establishes that 10^3 CFU/ml of *S. boulardii* broth and 10^6 CFU/ml of *S. boulardii* cells are effective as probiotic for *P. monodon* in zoea and mysis stage.

- In PL.1, addition of *B. subtilis* enhanced disease resistance in treatment groups. About 10^4 to 10^5 CFU/ml of *B. subtilis* were required to impart resistance to the pathogen infection. In the post larval experiments, *B. subtilis* enriched *Artemia* metanauplii resulted in significant improvement in resistance to pathogenic *V. harveyii*. Treatment fed with live *Artemia* meta nauplii resulted in higher survival rates compared to dead enriched meta nauplii.

- Application of the three probiotics organisms to zoea and mysis resulted in significant increase in weight at the end of the experiment. Both the bacterial species produced higher weight gain than the yeast *S. boulardii*. Among the bacterial species *L. plantarum* resulted in higher weight gain compared to *B. subtilis* enriched metanauplii.

- Enrichment of *S. boulardii* in zoea and mysis resulted in significant improvement in weight. Among treatments, 10^3 CFU/ml application produced significantly higher growth. When cells alone or culture media is applied the weight gain was highest for 10^3 CFU/ml. Application of *L. plantarum* resulted in significantly higher weight gain for treatments given 10^2 CFU/ml and 10^3 CFU/ml of cells at PL. 1 stage. Enrichment with *B. subtilis* also resulted in significant increase in weight gain.

- Post larvae (PL. 3 to PL. 20) fed with *B. subtilis* enriched *Artemia* metanauplii resulted in significant weight gain how ever the survival rate were lower than *S. boulardii* fed groups. In *S. boulardii* fed groups the increase in weight gain was not significant. In post larval experiments treatments given live enriched metanauplii gave significantly higher weight gain where as the group given dead enriched metanauplii produced lower growth.

- Colonization levels after discontinuing the probiotic supplementation was checked for *S. boulardii* and *B. subtilis* from PL. 1 stage. Both the species showed a decline in number when the supplementation was discontinued. Colonization levels of *S. boulardii* declined from 1950 to 20 CFU/larva in five days where as for *B. subtilis* it was from 267 to 20 CFU/larva.

- In post larvae, level of post feeding colonization was checked only for *B. subtilis*. The colonization levels did not show much reduction in four days. There was only 7% and 9% reduction in colony counts in treatments fed with live nauplii and dead nauplii, respectively. This clearly indicates that *B. subtilis* can effectively colonize the digestive tract of post larvae.

REFERENCES

6.0 REFERENCES

Abraham, T. J., Shanmugam, S. A., Uma, A., Palaniyappan, R., and Dhevendaran, K., 2001. Biocontrol of shrimp bacterial pathogens using penaeid larva associated bacterium, *Aeromonas* sp. *J. Aqua. Trop.* 16(1): 11-22.

Anderson, I. G., Shamsudin, M. N. and Nash, G., 1989. a preliminary study on the aerobic heterotrophic bacterial flora in giant freshwater prawn, *Macrobrachium rosenbergii*, hatcheries in Malayasia. *Aquaculture.*, 81:213-223.

Andlid, T., Vazquez –Juarez, R. and Gustafsson, L., 1998. Yeast isolated from the intestine of rainbow trout adhere to and grow inintestinal mucus . *Mol. Mar. Biol.biotechnol.*,7:115-116

Anikumari, N. P., Pandi Rajan, Balram Krishna M. and Mohamed, K. S. 2001. A preliminary survey on the use of probiotics by shrimp farmers in Nellore District, Andhra Pradesh. *In*: Singh, I. S. B., Pai, S. S., Philip, R. , Mohandas, A. (Eds). Aquaculture medicine Fish Diagonosis and Management, CUSAT, Kochi, India., pp 123-128.

Austin, B., Stuckey, L. F., Robertson, P. A. W., Effendi, I., 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.

Avendano, R. and Riquelme, C. 1999.Establishment of mixed-culture probiotics and microalgae as food for bivalve larvae. *Aquacult. Res.*, 30: 893-900.

Axelsson L T, Chung T C, Dobrogosz W J, Lindgren L E.1987 . discovery of a new antimicrobial substance produced by *Lactobacillus reuteri. FEMS microbiology reviews* 46: 65

Azad, S. A., Chong, V. C. and Vlkineswary, S. 2002. Phototrophic bacteria as feed supplement for rearing *Peneaus monodon* larvae *J. World. Aqua .Soc.*, 33:158-168.

Baticados, M. C. L., Lavilla-Pitogo, C. R., Cruz-Lacierda, E. R., dela Pena, L.D., Sunaz, N. A., 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyii and V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. *Dis. Aquat. Org.*, 9: 13-133.

Bell, W. H., Lang, J. M. and Mitchell, R., 1974. Selective stimulation of marine bacteria by algal extracellular products. *Limnol. Oceanogr.*, 19 : 833-839.

Bergh, O., 1995. Bacteria associated with early life stages of halibut, *Hippoglossus hippoglossus* L., inhibit growth of a pathogenic *Vibrio* sp. *J. Fish Dis.,* 18, 31-40.

Boyd, C. E., Gross, A., 1998. Use of probiotics for improving soil and water quality in aquaculture ponds. *In*: Book of Abstracts. The Fifth Asian Fisheries Forum, November 11-14, 1998: Cheng Mai, Thailand. International Conference of Fisheries and Food Security beyond the year 2000. pp-33.

Brock, T. D. and Clyne, J., 1984. Significance of algal excretory products for growth of epilimnetic bacteria. *Appl. Envt. Microbiol.*, 47: 731-734.

Brock, T. D., 1974. Biology of Microorganisms, 2nd edn., Prentice- Hall Englewood Cliffs, NJ, 852pp.

Buts, J. P., Bernasconi, P., Vaerman, J. P., Dive, C., 1990. Stimulation of secretory IgA and secretory component of immunoglobulins in small intestine of rats treated with Saccharomyces boulardii. Digestive diseases and sciences, 35: 251-256.

Buts, J. P., Ge'rard, C. and Delmee, M. 1993. *Saccharomyces boulardii* for *Clostridium difficile* – associated enteropathies in infants. *J. of Pediatric Gastroenterology and Nutri.*, 16, 419-425.

Chang, C.I. and Liu, W.Y., 2002. An evaluation of two probiotic bacterial strains, *Enterococcus faecium* SF68 and *Bacillus toyoi*, for reducing edwardsiellosis in cultured European eel, *Anguilla anguilla* L. *J. Fish Dis.*,25 :311 -315

Chythanya, R., Karunasagar, I. and Karunasagar, I., 2002. Inhibition of shrimp pathogenic *Vibrios* by a marine *Pseudomonas* 1-2 strain. *Aquaculture.*, 208: 1-10.

Coloroni, A., 1985. A study on the bacterial flora of giant prawn, *Macrobrachium rosenbergii* larvae fed with *Artemia salina* nauplii. *Aquaculture,* 49:1-10.

Coutteau, P., Lavens, P. and Sorgeloos, P., 1990 Baker's yeast as a potential substitute for live algae in aquaculture diets: *Artemia* as a case study *J. World Aquacult. Soc.*21:1-9

Czerucka, D., Roux, I. and Rampal, P. 1994. *Saccharomyces boulardii* inhibits secretagogue-mediated adenosinee 3' – 5' –cyclic monophosphate induction intestinal cells. *Gasteroenterology*, 106, 65-72.

Davidson, P. M. and Hoover, D. G., 1993. Antimicrobial components from Lactic Acid Bacteria. In: Lactic Acid Bacteria (ed. Salminen, S. and von Wright, A.). Marcel Dekker,Inc. New York.

Devaraja, T. N., Yusoff, F. M. and Shariff, M., 2002. Changes in bacterial populations and shrimp production in ponds treated with commercial microbial products. *Aquaculture,* 206: 245-256.

Douillet, P. and Langdon, C. J., 1993. Effects of marine bacteria on the Culture of Axenic Oyster *Crassostrea gigas* (Thunberg) larvae. *Biol. Bull.* 184: 36-51.

Douillet, P. A., Langdon, C. J., 1994. Use of a probiotic for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunberg). *Aquaculture*, 119: 25-40.

Duff, D. C. B., Bruce, D. L. and Anita, N. J., 1996. The antibacterial activivy of marine planktonic algae. *Can. J. Microbiol.*, 12: 877-884.

Earnshaw, R. G., 1992. The antimicrobial action of lactic acid bacteria: Natural food preservation system. In: :Lactic acid bacteria in health and disease (Ed.Wood, B. J), pp-484

FAO, 2003 & 2001. Food and Agriculture organization of the United Nations (FAO).FISHSTAT.Online.http//www.fao.org/fi/statist/FiSOFT/FISHPLUS.asp

Fukusho, K. 1980. Mass production of a copepod, *Tigris japonicus* in combination culture with rotifer *Brachionus plicatilis* fed omega yeast as a food source. *Bull. Jap. Soc. Sci. Fish.*, 46: 625-629.

Fuller, R., 1989. Probiotics in man and animals. *J. Appli. Bacteriol.*, 66: 365-378.

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 *P. vannamei* post larvae in Ecuador. In: Swimming through troubled waters. Proceedings of the special session on shrimp farming. (ed. Browd, C. L., and Hopkins, J. S) World aquaculture society. Baton Rouge LA, USA, pp. 53-59.

Gatesoupe, F. J., 1991. The effect of three strains of lactic bacteria on the production rate of rotifers, *Brachionus plicatilis*, and their dietary value for larval turbot, *Scophthalmus maximus*. *Aquaculture.*, 96:335-342.

Gatesoupe, F. J., 1993. *Bacillus* sp. spores as food additive for the rotifer *Brachionus plicatilis*: improvement of their bacterial environment and their dietary value for larval turbot, *Scophthalmus maximus* L. *In*: Kaushik, S. J., Luquet, P. (Eds.), Fish Nutrition in Practice. Institut National de la Recherche Agronomique, Paris, France, Les Colloques, Vol. 61, pp. 561-568.

Gatesoupe, F. J., 1994. Lactic acid bacteria increase resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic *Vibrio*. *Aquatic Living resour.*, 7: 277-282.

Gatesoupe, F. J., 1997. Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. *Aquat. Living Resour.*,12: 239-246.

Gatesoupe, F. J., 1999. The use of probiotics in aquaculture. *Aquaculture.*, 180: 147-165.

Gatesoupe, F. J., Arakawa, T. and Watanabe, T. 1989. The effect of bacterial additives on the production rate and dietary value of rotifers as food for Japanese flounder, *Paralichthys olivaceus*. *Aquaculture.*, 83, 39-44.

Gatesoupe, F.J., 1989. Further advances in the nutritional and antibacterial treatments of rotifers as food for turbot larvae, *Scophthalmus maximus* L. In: Aquaculture – A Biotechnology in Progress. (Eds. De Pauw, N., jaspers, E., Asckefors, H., Wilkins,N.) European Aquaculture society, Bredene, Belgium, pp. 721-730.

Gatesoupe, F.J., 2002. Probiotic and formaldehyde treatments of *Artemia* nauplii as food for larval pollack, *Pollachius pollachius*. *Aquaculture.*, 212 : 347-360.

Gelabert, R. F. 2003. Bioencapsulation in *Artemia*. II. Influences of the particle concentration in the enrichment process. *Aquacult.*, 200, 147-159

Gibson, L. F., Woodworth, J. and George, A. M., 1998. Probiotic activity of *Aeromonas* media on the Pacific oyster, *Crassostrea gigas*, when challenged with Vibrio tubiashii. *Aquaculture,* 169: 111-120.

Gildberg, A. and Mikklsen, H., 1998. Effects of supplementing the feed to Atlantic cod (*Gadus morhua*) fry with lactic acid bacteria and immuno-stimulating peptides during a challenge trial with *Vibrio anguillarum*. *Aquaculture.*, 167:103-113.

Gildberg, A., Johansen, A. and Bogwald, J. 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.*, 138:23-24.

Gildberg, A., Mikkelsen. H., Sandaker, E. and 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.

Gil-Turnes, M. S. and Fenical, W., 1992. Embryos of Homarus americanus are protected by epibiotic bacteria. *Biol. Bull.*, 182: 105-108.

Gil-Turnes, M. S., Hay, M. E. and Fenical, W., 1989. Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science*, 246: 116-118.

Goemez-Gil, B., Cabanillas-Ramos, J., Paez-Brambila, S., Roque, A., 2001. Standardisation of the bioencapsulation of enrofloxacin and oxytetracycline in *Artemia franciscana* Kellogg, 1906. *Aquaculture*, 196: 1-12.

Gomez-Gil, Roque B., A. and Velasco-Blanco , 2002. Culture of *Vibrio alginolyticus* C7b, potential probioticbacteria with the microalgae *Chaetoceros mulleri* . *Aquaculture.*,211:43-48.

Gorosope, J. N., Nakamura, K. Abe, M. and Higashi , S.,1996. Nutritional contribution of *Pseudomonas* species in *Artemia* culture . *Fish. Sci.*, 62:914-918.

Gracia-de-la-Banda, I., Chereguini, O. and Rasines, I. 1992. Influence of lactic bacterial additives on turbot (*Scophthalmus maximus* L.) larvae culture. *Bol. Inst. Esp. Oceanogr.* 8, 247-254.

Gram, L., Lovold, T., Melchiorsen, J., Spanggaard, B., Huber, I. and Nielsen, T.F., 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Appl. Environ. Microbiol.*, 65:969-973.

Gram,L., Lovold,T., Nielsen,J., Melchiorsen,J., Spanggaard, B. 2001. In vitro antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. *Aquaculture.*, 199: 1-11.

Griffith, D.R.W., 1995. Microbiology and role of probiotics in Ecuadorian shrimp hatcheries. In: Lavens, P., Jaspers, E., Roelants, I., (Eds.), Larvi' 95-Fish and Shelfish larviculture Symposium. European Aquaculture society, Special publication, Vol.24, Gent, Belgium, p. 478.

Hall, R. H., 1966. Nisin and food production, *Proc. Biochem.*, 1:461.

Hameed, A. S. S. 1993. A study of aerobic heterotrophicbacterial flora of hatchey reared eggs, larvae and post larvae of *Penaeus indicus* . *Aquaculture.*, 117:195-204.

Hariyanti, Sugama, K., Tsumura. 1998. Use of BY-9 as a probiotic agent in the larval rearing of *Penaeus monodon*. *In*: Book of Abstracts. The Fifth Asian Fisheries Forum, November 11-14, 1998: Cheng Mai, Thailand. International Conference of Fisheries and Food Security beyond the year 2000. pp. 74.

Harris, L. J., Daeschel, M. A., Stiles, M. E., and Klaenhammer, T. R., 1989. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. *J. Food Prot.*, 52: 384-387.

Harzevilli, A. R. S., Duffel, H. V., Dhert, Ph., Swings, J. and Sorgeloos, P., 1998. Use of a potential probiotic *Lactococcus lactis* AR21 strain for the enhancement of growth in the rotifer *Brachionus plicatilis* (Muller). *Aquacult. Res.*, 29: 411-417.

Havennar, R. and Huis in't Veld, J. H. J., 1992. Probiotics: a general view. In: Lactic Acid Bacteria in health and disease, Vol.I (ed. Wood J. B. J). Elsevier, Amsterdam, pp. 477.

Horne, M. T., Richards, R. H., Roberts, R. J., Smith, P. C., 1977. Peracute vibriosis in juvenile turbot *Scophthalmus maximus*. *J. Fish. Biol.*, 11: 355-361.

Huys, L., Dhert, P., Robles, R., Ollevier, F., Sorgeloos, P. and Swings, J., 2001. Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. *Aquaculture.*,191: 25-37

Inglis, V., Abdullah, S. Z., Angka, S.L., Chinabut, S., Chowdhary, M. B. R., Leano, E. M., MacRae, I. H., Sasongko, A., Somsiri,T., Yambot, A.V., 1997. Survey of resistance to antibacterial agents used in aquaculture in five South East Asian countries. In: Diseases in Asian aquaculture III. (Eds. Flagel, T. W., MacRae, I. H.), Fish health sect., Asian Fish. Soc., Manila, Philippines. pp. 331-337

Irianto, B. and Austin, B., 2002. Probiotics in aquaculture. *J. Fish Dis.*, 25: 633-642.

Jöborn A., Olsson J. C., Westerdahl, A., Conway P.L. and Kjelleberg S. 1997. Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. *J. Fish Dis.*, 20: 383–392.

Jorry, D. E. 1998. Use of probiotics in penaeid shrimp growout. *Aquaculture magazine*, 1:62-77.

Kennedy,S.B., Tucker, J.W., Neidig, C.L., Vermeer, G.K., Cooper, V.R., Jarrel, J.L., Sennet, D.G., 1998. Bacterial management strategies for stock enhancement of warm water marine fish: a case study with common snook. (*Centropomus undecimalis*). *Bull.Mar.Sci.*, 62, 573-588.

Kitto, M. R. and Regunathan, C., 1997. *Skeletonema* can kill luminosis in shrimp hatcheries. *Fish farmer (Nov/Dec)* pp: 37.

Klein, S. M., Elmer, G. W., Mc Farland, L. V., Surawicz, C. M. and Levy, R. H. 1993. Recovery and elimination of the biotherapeutic agent, *Saccharomyces boulardii*, in healthy human volunteers. *Pharmaeutical Res.* 10 (11), 1615-1619.

Kozasa, M., 1986. Toyocerin (*Bacillus toyoi*) as growth promoter for animal feeding. *Microbiol. Aliment. Nutr.* 4:121-135.

Lara-Flores, M., Olvera-Novoa, M. A., Guzman-Mendez, B. E. and Lopez-Madrid, W., 2003. Use of bacteria *Streptococcus faecium* and *Lactobacillus acidophilum* and the yeas *Saccharomyces cerevisea* as growth promoters in Nile tilapia (*Oreochromis niloticus*). *Aquacult.*, 216: 193-201.

Lavens, P. and Sorgeloos, P. 2000. Experiences on importance of diet for shrimp postlarval quality. *Aquaculture.*, 191: 169-176.

Lavens, P., Sorgeloos, P., (Eds.) Manual on the production and use of live food for aquaculture. *FAO Fisheries Technical Paper.* No. 361, Rome, FAO. p-295.

Lavens, P., Sorgeloos, P., 2000. Experience on importance of diet for shrimp postlarval quality. *Aquaculture*, 191: 169-176.

Lewus, C. B. and Montville, T. J., 1991. Detection of bacteriocins produced by lactic acid bacteria. *J. Microbiol. Methods.*, 13: 145.

Li, P. and Gatlin, D. M., 2003. Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops* X *M. saxatilis*). *Aquaculture.*, 219: 681-692.

Lightner, D. V., 1983. Diseases of cultured penaeid shrimp. In: CRC handbook of mariculture. Volume 1. Crustacean aquaculture. (Ed. McVey, J. P.), CRC Press, Boca Raton, Florida, USA. pp. 289-320.

Lilly, D. M. and Stillwell, R. H., 1965. Probiotics: growth promoting factors produced by microorganisms. *Science*, 147: 747-748.

Lockwood, S. J. and Daly, C. D. B., 1975. Further observations on the effect of preservation in 4% neutral formalin on the length weight of 0 group flat fish. *J. Cons. Int. Explor. Mer.* 36(2) :170-175.

Loya-Javellana, G. N. 1989. Ingestion, saturation and growth response of *Penaeus monodon* larvae to food density. *Aquaculture.*, 81, 329-336.

Maeda, M. and Liao, I. C., 1992. Effect of bacterial population on the growth of prawn larva, *Penaeus monodon. Bull. Natl. Res. Inst. Aquaculture.*, 21:25-29.

Maeda, M., Nogami, K., Ishibashi, N., 1992. Utility of microbial food assemblage for culturing a crab, *Portunus trituberculatus. Bull. Natl. Res. Inst. Aquaculture*, 21: 31-38.

Maeda, M., Nogami, K., Kanematsu, M. and Hirayama, K., 1997. The concept of biological control methods in aquaculture. *Hydrobiol.*, 358:285-290.

Makridis P., Fjellheim A.J., Skjermo J. and Vadstein O. 2000a. Control of bacterial flora of *Branchionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions. *Aquacult.*ure, 185: 207–218.

Makridis P., Fjellheim A.J., Skjermo J. and Vadstein O. 2000b. Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulation in rotifers. *Aquacult. International* 8, 267-280.

Martin, Y. P. and Mengus, B. M., 1977. Utilization de souches bacteriennes selectionees dans l'alimentation des larves de larves de Mytilus galoproveincialis (LMK) (Mollusque bivalve) en clevages experimentaux. *Aquaculture.*, 10: 253-262.

Matty, A. J. and Smith, D., 1978. Evaluation of a yeast, a bacterium and an alga as a protein source for Rainbow trout. I. Effect of protein level on growth, Gross conversion efficiency and protein conversion efficiency. *Aquaculture.*, 14: 235-246

McFarland, L. V. and Bernasconi, P. 1993. *Saccharomyces boulardii*: A review of an innovative biotherapeutic agent. *Microbial Ecol. In Health Dise.*, 6, 157-171.

McIntosh, D., Samocha, T. M., Jones, E. R., Lawrence, A. L., McKee, D. A., Horowitz, S. and Horowitz, A., 2000. The effect of a commercial bacterial supplement on the high-density culturing of *Litopenaeus vannamei* with a low- protein diet in an out door tank system and no water exchange. *Aquacult. Eng.*, 21: 215-227.

Metchnikoff, E. 1908. The nature of man. Studies in Optimistic Philosophy. William Heinemann, London.

Meunpol, O., Lopinyosiri, K. and Menasveta, P., 2003. The effects of ozone and probiotics on the survival of black tiger shrimp (*Penaeus monodon*). *Aquaculture.*, 220: 437-448.

Mohanty, S.N., Swain, S.K., Tripathi, S.D., 1996. Rearing of catla (*Catla catla* Ham.) spawn on formulated diets. *J. Aqua. Trop.* 28: 253-258

Montes, A. J. and Pugh, D. G., 1993. The use of probiotics in food- animal practice. *Vet. Med.*, 88: 282-288.

Moriarty, D. J. W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture.*, 164: 351-358.

Muroga, K., 2001. Viral and Bacterial diseasesof marine fish and shell fish in Japanese hatcheries. *Aquaculture.*, 202: 23-44.

Naik, A. T. R., Murthy, H. S. and Ramesha , T. J. 1999. Effect of graded levels of gut probiotive on growth, survival and feed conversion of tilapia *Oreochromis mossambicus* . *Fishery.teschnol.* 36:63-66.

Nakamura, A., Takahashi, K. G. and Mori, K., 1999. Vibriostatic bacteria isolated from rearing seawater of oyster brood stock: potentiality as biocontrol agents for *Vlbriosis* in oyster larvae. *Fish Path.*, 34(3):139-144.

Nates, F. S. 1990. Effect of brewer's yeast on growth and survival of white shrimp *L.eptopenaeus vannamei.* snates@ zeiglerfeed.com

Naviner, M., Berge, J. P., Durand, P. and Bris, H. L., 1999. Antibacterial activity of the marine diatom *Skeletonema costatum* against aquacultural pathogens. *Aquaculture.*, 174: 15-24.

Nicolas, J. l., Ansquer, D. and Besse, B. 1990. Influence of bacterial flora on performances of larval rearings in marine aquaculture. In: Microbiology in poecilotherms (ed Léser, R.) Elsevier Science, Publishers B.V., Paris France pp.177-185.

Nikoskelainen, S., Salminen, S. Bylund, G. and Ouwehand, A., C 2001a. charachterisation of the properties of human and diary derived probiotics for prevention of infectious diseases in fish . *Appl. Environ. Microbia*l.,67:2430-2435

Nikoskelainen, S., Ouwehand, A., Salminen, S. and Bylund, G., 2001 b. Protection of rainbow trout (*Onchorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus. Aquaculture.*,198:229-236.

Nogami, K. and Maeda, M., 1992. Bactria as biocontrol agents for rearing larvae of crab *Portunus trituberculatus. Can. J. Fish. Aquat. Sci.*, 49: 2373-2376.

Olsen, A .I., Olsen, Y., Attramadal, Y., Christie, K. Birkbeck, T. H. Skjermo, J. and Vadstein, O., 2000. Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. *Aquaculture.*, 190 :11-25

Onarheim, A., M. and Raa, J. 1990. Characteristics and possible biological significance of an autochtonous flora in the intestinal mucosa of sea water fish. In: Microbiology in poecilotherms (Ed. Lesel R.) Elsevier Science Publishers, Amsterdam pp.197-201.

Park, J., Park, W. J., Jeong, H. D., 2001. Immunological efficacy of *Vibrio vulnificus* bacterins given as an oral vaccine in the flounder *Paralichthys olivaceous*. *Aquaculture*, 201: 187-197

Parker, R. B., 1974. Probiotics. The other half of the antibiotic story. *Anim. Nutr. Health*, 29:4-8.

Parker, R.B., 1974. Probiotics. The other half of antibiotic story. Anim. Nutr. Health 29, 4-8.

Patra, S. K. and Mohamed, K. S. 2003. Enrichment of *Artemia* nauplii with the probiotic yeast *Saccharomyces boulardii* and its resistance against a pathogenic *Vibrio*. *Aquacult. Internat.*, 11: 505-514.

Pothoulakis, C., Kelly, C. P., Joshi, M. A., Gao, N., O'Keane, C. J., Castagliuolo, I. and Lamont,m J. T. 1993. *Saccharomyces boulardii* inhibits *Clostridium difficile* toxin a binding and enterotoxicity in rat ileum. *Gastroentrology*, 104, 1108-1115.

Prabhu, N. M., Nazar, A. R., Rajagopal, S. and Khan, S.A., 1999. Use of probiotics in water quality management during shrimp culture. *J. Aqua. Trop.*,14 (3): 227-236.

Price, R. J. and Lee, J. S., 1970. Inhibition of *Pseudomonas* species by hydrogen peroxide producing *Lactobacilli*. *J. Milk Food Technol*, 33: 13.

Pybus, V., Loutit, M. W., Lamont, I. L. and Tagg, J. R., 1994. Growth inhibition of the salmon pathogen *Vibrio ordalii* by a siderophore produced by *Vibrio anguillarum* strain VL4355. *J. Fish Dis.*,17:311-324.

Rajeev Kumar, 2003. Effect of bacterial load and probiotics on the sustainability of microalgal cultures. M.F.Sc. Dissertation, CIFE, Mumbai. 52pp.

Ramirez Tovar, D., Zambonino Infante, J.L., Gatesoupe, F .J., Vazquez Juarez, R., Flos, R.; Creswell, L., 2000, Responsible Aquaculture in the New Millenium, Abstracts and Contributions of the International Conference Aqua 2000, 2–6 May, Nice, France. European Aquaculture Society Special Publication No. 28, EAS, Oostende, Belgium. p. 16

Ramirez Tovar, D ., Zambonino, J., Cahu, C., Gatesoupe, F.J., Vazquez Juarez, R. and Lésel, R., 2002. Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae" *Aquaculture.*, 204: 113-123

Ravichandran, R. and Jalaluddin, R. S., 2001. Stress management strategy with probiotics for preventing shrimp diseases. *Appl. Fisheries and Aquaculture.*, 1(1): 73-74.

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

Rico-Mora, R. and Voltolina, D., 1995. Bacterial interactions in *S. costatum* culture on the survival of *Artemia franciscana* nauplii. *J. of Invert. Pathol.*, 66: 203-204.

Rico-Mora, R., Voltolina, D. and Villaescussa-Celaya, J. A., 1998. Biological control of *V. alginolyticus* in *S. costatum* (Bacillariophyceae) and cultures. *Aquacult. Eng.*, 150: 45-62.

Ringo, E and Strom, E., 1994. Microflora of Arctic charr, Salvelinus alpines (L.). I. The gastrointestinal microflora of free living fish, and the effect of diet and salinity on intestinal microflora. Aquaculture and fisheries Management. 25, 623 – 629

Ringo, E. and Birkbeck, T.H., 1999. Intestinal microflora of fish larvae and fry. *Aquacult. Res.*12: 73-93

Ringo, E. and Gatesoupe, F. J., 1998. Lactic acid bacteria in fish: a review. *Aquaculture.*, 160, 177-203.

Ringo, E. and Vadstein, O., 1998. Colonization of *Vibrio pelagicus* and *Aeromonas caviae* in early developing turbot (*Scophthalmus maximus* L.) larvae. *J. Appl. Microbiol.*, 84:227-233.

Ringo, E., Birkbeck, T. H., Munro, P. D., Vadstein, O. and Hjelmeland, K., 1996. The effect of early exposure to *Vibrio pelagicus* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L.) larvae. *J. Appl. Bacteriol.* 81, 207-211.

Ringo, E., Olsen, R. E., Overli, O and Lovik, F. 1997b. Effect of dominance hierarchy formation on aerobic microbia associated with the epithelial mucosa of subordinate and dominant individuals of Artic charr, *Salvelinus alpinus* (L.). *Aquacult. Res.*, 28, 901-904

Riquelme, C., Arya, 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. *Aquacult.*, 181: 25-36.

Riquelme, C., Arya, R., Vergara, N., Rojas, A., Guaita, M. and Candia, M., 1997. Potential probiotic strains in the culture of Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). *Aquaculture.*, 154: 17-26.

Riquelme, C., Hayashida , G., Arya, R., Uchida, A., Satomi, M., and Ishida, Y., 1996 a. Isolation of a native bacterial strain from the scallop *Argopecten purpuratus* with inhibitory effects against pathogenic *Vibrios. J. Shellfish Res.*,15(2):369-374.

Robertson P. A. W., O'Dowd, C., Burrells, C., Williams, P. and Austin, B. 2000. Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture.* 185, 235-243.

Sakai, M., Taniguchi, K., Mamoto, K., Ogawa, H., Tabata, M., 2001. Immunostimulant effects of nucleotide isolated from yeast RNA on carp. *Cyprinus carpio L. J. Fish Dis.*, 24: 433-438.

Salminen, S., Ouwehand, A., Benno, Y. and Lee, Y. K., 1999. Probiotics : How they should be defined? *Trends in Food Sci and Tech.*,10: 107-110.

Salvesen, I., Reitan, K. I., Skjermo, J. and Oie, G., 2000. Microbial environments in marine larviculture: Impacts of algal growth rates on the bacterial load in six microalgae. *Aqua. Intl.*, 8: 275-287

Savage, D. C., 1987. Microorganisms associated with epithelial surface and stability of the indigenous microflora. Die Nahrung., 31(5) : 383.

Scholz, U., Garcia Diaz, G., Ricque, D.,Cruz Suarez, I. E., Vargas Albotes, F. and Latchford, J., 1999. Enhancement of Vibriosis resistance in Juvenile *P. vannamei* by supplementation of diets with different yeast products. *Aquaculture.*, 176: 271-283.

Schroder, K., Clausen, E., Sandberg, A. M. and Raa, J., 1980. Psychrotrophic *L. plantarum* from fish and its ability to produce antibiotic substances. In: Advances in Fish science and Technology (ed. Connel, J. J.). Fishing News Books, Farnham, Surrey, England, pp. 480-483.

Shariff, M., Yusoff, F. M., Devaraja, T. N. and Rao, S., 2001. The effectiveness of a commercial microbial product in poorly prepared tiger shrimp, *Penaeus monodon* (Fabricus) ponds. *Aquacult. Res.*, 32:181-187.

Simon , C. M. 1978. the culture of the diatom *Chaetoceros gracilis* and its use as a food for penaeid protozoeal larvae . *Aquacult.*,14:105-113.

Singh, I. S. B., Lakshamana Perumal Swamy , P. and Chrandra Mohan , D. 1998. Bacterial flora of pond reared *Penaeus indicus* (Milne Edwards) *J. Aqua. Trop.*, 13:133-142.

Singh, I. S. B., Philip, R., 1993. Comparative efficiency of media for isolating heterotrophic bacteria from larvae and larval rearing water of *M.rosenbergii. Indian. J. Microbiol.*, 33(1): 67-69.

Skjermo J. and Vadstein O. 1999. Techniques for the microbial control in the intensive rearing of marine larvae. *Aquaculture,* 177: 333–343

Smith, P. and Davey, S., 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress inducible furunculosis by a fluorescent pseudomonad. *J. Fish Dis.* 16, 521-524.

Song, Y. L., Lie, J. J., Chan, I. C and Sung, H. H., 1997. Glucan indiced disease resistance in Tiger shrimp (*Penaeus monodon*). In: Fish Vaccinology (eds. Gudding, R., Lillehaung, A., Mdtlyng, P. J. and Brown, F.) *Dev. Biol. Stand. Karger.*, :431-421.

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K. F., Gram, L., 2000. The microflora of rainbow trout intestine. A comparison of traditional and molecular identification. *Aquaculture*, 182: 1-15.

Spanggaard, B., Huber, I., Nielsen, J., Sick, E. B., Bressen Pipper, C., Martinussen, T., Slierendrecht, W. J. and Gram, L. 2001. The probiotic potential against *Vibriosis* of the indigenous microflora of rainbow trout. *Env. Microbiol.*, 3:755-765

Sridhar, M and Raj, R. 2001. Efficacy of gut probiotics in enhancing growth in *Peanaeus indicusin* post larvae. *In*: Aquaculture medicine (Eds. Singh, B., Rosamma, S., Das, A. M.,). FDDM, CUSAT, Cochin (in press)

Strom, E. 1988. Melkesyrebakterier I Fisketarm. Isolasjon, Karakterisering og egenskaper. *M.Sci. Thesis*. The Norwegian College of Fishery Science (In Norwegian), 88 pp.

Strom, E. and Ringo, E., 1993. Changes in bacterial flora in developing cod, Gadus morhua (L.), larvae after inoculation of *Lactobacillus plantarum* in the water. In: Physiological and Biochemical Aspects of Fish Larval Development. (Eds. Walther, B., Fyhn, H.J.), University of Bergen, Norway, pp 226-228.

Sugita, H., Matsuo, N., Shibuya, K. and Deguchi, Y., 1996. Production of antibacterial substances by intestinal bacteria isolated from coastal crab and fish species. *J. Mar. Biotechnol.*, 4:220-223.

Sugita, H., Tanaami, H., Kobashi, T. and Deguchi, Y., 1981. Bacterial flora of coastal bivalves. *Bull. Jpn. Soc. Sci. Fish.*, 47:655-661.

Sujita, H., Hirose, Y., Matsuo, N. and Deguchi, Y., 1998. Production of antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. *Aquaculture.*, 165:269-280.

Suminto, K. and Hirayama, K., 1997. Application of a growth promoting bacterium for stable mass culture of three marine microalgae. *Hydrobiologia*, 358: 223-230.

Tanasomwang, V., Nakai, T., Nishimura, Y. and Muroga, K., 1998. *Vibrio* inhibiting marine bacteria isolated from black tiger shrimp hatchery. *Fish Pathol.*, 33, 459-466.

Tannock, G. W., 1997. Modification of the normal microbiota by diet, stress, antimicrobial agents and probiotics. *In*: Mackie, R. I., With, B. A., Isaacson, R.E. (Eds.), Gastrointestinal Microbiology, Vol. 2, Gastrointestinal Microbes and Host interactions. Chapman and Hall Microbiology Series, International Thomson Publishing, New York, pp. 434-465.

Uma, A. 1995. Influence of probionts on the growth and survival of penaid shrimp in nursery phase. .*M.Sc. Thesis.*, Department of Aquaculture, F.C. and R. I., TANUVAS, Tuticorin.

Uma, A., Abraham, T. J., Jeyaseelan, M. J. P. and Sundararaj, V., 1999. Effect of probiotic feed supplement on performance and disease reistance of Indian White Shrimp *Penaeus indicus* (H. Milne Edwards). *J. Aqua. Trop.*, 14(2): 159-164.

Vanbelle, M., Teller, E. and Focant, M. 1989. Probiotics in animal nutrition: a review. Publ. l'Unite de Biochimie de la Nutrition, 1348 Louvain-la-Neuve, Belgique.

Villamil, L., Figueras, A., Planas, M. and Novoa, B. 2003. Control of *Vibrio alginolyticus* in Artemia culture by treatment with bacterial probiotics" *Aquaculture.*, 43-56

Walbanks, S., Martinez-Murcia, A. J., Philips, B. A. and collins, M. D. 1990. 16 S rRNA sequence determination for members of the genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus salmoninarum* sp. nov. Int. *J. Syst. Bacteriol.* 40, 224-230.

Walne, P. R. 1974. Culture of bivalve molluscs. 50 years experience at Conway. Fishing New (Books) Ltd., Surrey, pp. 1- 173.

Yasuda, K. and Taga, N., 1980. Culture of *Brachionus plicatilis* Muller using bacteria as food. *Bull. Jap. Soci. Sci. Fish.*, 46(8): 933-939.

Yousoff, F. M., Zubaida, M. S., Matias, H. B. and Kwan, t. S. 2002. Phytoplankton succession in intensive marine shrimp culture ponds treated with commercial bacterial product. *Aquacult. Res.*, 33: 269-278.