EFFECT OF BACTERIAL LOAD AND PROBIOTICS ON THE SUSTAINABILITY OF MICROALGAL CULTURES

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CERTIFICATE

Certified that the dissertation entitled "**EFFECT OF BACTERIAL LOAD AND PROBIOTICS ON THE SUSTAINABILITY OF MICROALGAL CULTURES**", is a record of independent bonafide research work carried out by **Mr. Rajeev Kumar** during the period of study from September 2001 to August 2003 under our supervision and guidance for the degree of **Master of Fisheries Science (Mariculture)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title.

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I hereby declare that the dissertation entitled "**EFFECT OF BACTERIAL LOAD AND PROBIOTICS ON SUSTAINABILITY OF MICROALGAL CULTURES**" 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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सारांश

"सूक्ष्मशैवालों के पालन की निरन्तरता में जीवाणु-लदान और प्रोबायोटिक का प्रभाव" नामक अध्ययन (1) सूक्ष्मशैवाल पालन में जीवाणु वैविद्यता के जाँच करने (2) सूक्ष्मशैवाल खाद्यों के माध्यम से समुद्री डिम्भकीय पालन पर परिवहित जीवाणु लदान ज़ान लेने और (3)सूक्ष्मशैवाल संवर्धन में एक प्रोबायोटिक जोडने से इसके जीवाणु वैविद्यता, बढती और पुष्टि जानने के लिए किया था ।

इन लक्ष्यों को पूरा करने के लिए दो परीक्षणों की अभिकल्पना की और उनका निष्पादन किया । प्रथम परीक्षण में दो सूक्ष्मशैवाल जातियाँ कशाभी *आइसोक्राइसिस गाल्बाना* और डयाटम *कीटोसिरोस* को 250 मि ली धारिता के संवर्धन फ्लास्क में रखकर उनकी बढती और जीव वैविद्यता का माप किया । दूसरे परीक्षण में *कीटोसिरोस* जाति के संवर्धन में *साक्रोमाइसेस बोलारडी* यीस्ट को प्रोबायोटिक के रूप में 10^4CFU/ml की सघनता में एक एकल जोड (एकल जोड उपचार) या एक दैनिक जोड (दैनिक जोड उपचार) के रूप में जोड दिया । प्रोबायोटिक जोड के बिना एक परीक्षण भी चलाया था । संवर्धन फ्लास्कों की शैवाल कोशिकाओं की सघनता रोज़ देख लिया और कुल वायुजीवी वनस्पति-जात (SWA पर), कुल विब्रियो (TCBS पर) और कुल यीस्ट (सबोर्ड ऐगर पर) का मूल्यांकन कुछ विशेष दिनों पर किया । परिणाम यह सूचित किया कि परीक्षणात्मक पालन सूक्ष्मशैवाल बढती की सभी अभिलक्षक अवस्था याने कि प्रारंभिक अवस्था (लाग), बढती (लॉग), स्थिरता (स्टेशनरी) घटती (डिक्लाइनिंग) और मर जाने की अवस्था दिखायी । अध्ययन यह दिखाता है कि सूक्ष्मशैवाल संवर्धकों को यदि घटती की अपेक्षा बढती और स्थिरता की अवस्थाओं में उपयोग किये जाए तो डिम्भकीय संवर्धन में जोडनेवाले जीवाणुओं की मात्रा 3-4 के क्रम में कम कर दिया जा सकता है । *कीटोसिरोस* और *आइसोक्राइसिक गाल्बाना* दोनों के पालन में जाति प्रचुरता को छोडकर जीवाणु वैविद्यता के सभी हिस्सा वर्धमान शैवाल सघनता के साथ वृद्धि की प्रवणता दिखायी । परीक्षित सूक्ष्मशैवाल पालन में प्रारंभ, बढती की प्रारंभिक अवस्था और बढती के श्रृंगकाल और मर जाने की अवस्था में जीवाणु टाक्सा का गुच्छ देखे गये थे। प्रोबयोटिक यीस्ट *एस.बोलारडी* के साथ एकल जोड उपचार में शैवाल की बढती नियन्त्रित और दैनिक उपचार की तुलना में उच्च दिखायी पडी थी ।

औसत कुल वायुजीवी वनस्पतिजात नियन्त्रित और दैनिक उपचार में बढती की प्रवणता दिखायी जबकि एकल जोड उपचार में इसकी बढती कम थी । पदानुक्रमिक गुच्छ विश्लेषण उपचारों के बीच गणनीय विभिन्नता दिखायी पडी थी । नियन्त्रित पालन की अपेक्षा एकल जोड उपचार में जीवाणु टाक्सा में विचारणीय विभेदन सूचित करते हुए जीवाणु गुच्छों की समरूप प्रतिशतता में गणनीय बढती देखी गयी थी ।

ABSTRACT

This study entitled "Effect of bacterial load and probiotics on sustainability of microalgal cultures" was carried out with the objective of (1) investigating the bacterial diversity in microalgal cultures (2) to find out the bacterial load transferred to marine larval rearing systems via microalgal feeds and also (3) to study the effect of addition of a probiotic to microalgal cultures on its bacterial diversity, growth and sustenance.

For fulfilling the objectives two experiments were devised and executed. In the first experiment two microalgal species, the flagellate *Isochrysis galbana* and the diatom *Chaetoceros* sp. were maintained as 250 ml cultures and their growth and bacterial diversity was measured. In the second experiment, the probiotic yeast *Saccharomyces boulardii* was added in 10^4 CFU/ml concentrations either as a single addition (Treatment SA) or as a daily addition (Treatment DA) to *Chaetoceros* species culture. All treatments were made in triplicate and a control without addition of probiotic was maintained. The algal cell density in the culture flasks was taken daily and the total aerobic flora (on SWA), total vibrios (on TCBS) and total yeasts (on Sabouraud agar) were assessed on specific days. Results indicated that the experimental cultures showed all the typical phases of microalgal growth ie, lag, log, declining, stationary and death phases. The study shows that if microalgal cultures are used when they are in the log and stationary phase rather than in the declining phase, the amount of bacteria added to the larval culture medium can reduced by 3-4 orders of magnitude. All the measures of bacterial diversity except species richness showed and an increasing trend with increasing algal density in both *Chaetoceros* and *Isochrysis galbana* cultures. There was marked clustering of bacterial taxa during lag, early log, peak log and death phases in the microalgal cultures tested. The addition of the probiotic yeast *S. boulardii* in treatment SA resulted in significantly ($P<0.01$) improved algal growth rates with prolonged log and stationary period, when compared to control and treatment DA

The mean total aerobic flora showed a steeply increasing trend in control and DA treatments, while the trend in SA treatment was that of slow increase. The hierarchical cluster analysis showed remarkable differences between treatments. There was a marked increase in the similarity percentage of bacterial clusters indicating a much better discrimation of bacterial taxa in treatment SA as compared to control.

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INTRODUCTION

1.0 INTRODUCTION

Algal aquaculture worldwide is estimated to be a US$ 5 billion per year industry. The largest portion of the industry is represented by macroalgal production for human food in Asia, South America and Africa. Microalgal aquaculture is much smaller in economic impact but it is the subject of much research. Microalgae are cultured for direct human consumption and for extractable chemicals but current use and development of cultured microalgae is increasingly related to their use as feeds in marine animal aquaculture (Wikfors and Ohno, 2001).

Unicellular marine algae are widely used as food in the hatchery production of commercially valuable fish and shellfish. Oysters and clams feed by filtering them from seawater. Rotifers and brine shrimps also ingest algae and are then themselves used as food for larval fish and prawns. In some systems algae are added to the water containing fish or prawns to improve its quality. Hence, the culture of microalgae is an essential prerequisite for the rearing operations of economically important cultivable organisms in a hatchery system.

Marine microalgae are single celled plants and like all plants, contain chlorophyll, which traps the energy from light and uses it to convert nutrients and carbon dioxide in the seawater into organic growth. In the laboratory, or hatchery, a collection of algal cells, which are growing and dividing, is known as culture (Laing, 1991).

In microalgal culture the most important parameters that regulate its growth, are 1) *Nutrient quality and quantity* 2) *Light*, 3) *pH*, 4) *Turbulence*, 5) *Salinity* and 6) Temperature. The ranges of optimal environmental conditions for culturing marine microalgae are temperature- 18-24^{0}C, salinity- 20-24 ppt, light intensity- 2500-5000 lux, photoperiod- 16L: 8D and pH- 8.2-8.7.

Several methods have been developed for production of algae for use as food for various marine animals and their larvae. The terminology used to describe the type of algal culture includes the following.

1) Indoor/outdoor - Indoor culture allows control over illumination, temperature, nutrient level, contamination with predators and competing algae, whereas outdoor algal systems make it very difficult to grow specific algal cultures for extended periods.

2) Open/closed - Open cultures such as uncovered ponds and tanks (indoors or outdoors) are more readily contaminated than closed culture vessels, such as flask, carbouys, bags etc., which are always kept closed either by plugging with cotton or by any other means.

3) Axenic (=sterile)/xenic - Axenic cultures are free of any foreign organisms such as bacteria and require strict sterilization of glass wares, culture media and vessels to avoid contamination. The latter makes it impractical for commercial operations.

Five phases have been recognized during the growth of an algal culture (Gopinathan, 1996). They are:

I. Lag or Induction phase: The cells taken from the stock culture room and inoculated to a new flask have to acclimatize to the surroundings or to the new medium. Hence there will be no cell division for a few hours and this stage is known as lag or induction phase

II. Exponential (Log) phase: Once the cells are acclimatized to the surroundings, they start multiplication and grow rapidly. It is assumed that within 8-16 hrs, the cell will divide into 2 and further these cells carry on the growth till the culture reached its maximum concentration. This growing phase is known as exponential phase.

III. Declining phase: Once the cells reach its maximum concentration, the growth and multiplication of the cells will be arrested and slowly the cultures show the symptom of decline. This arrested growth of the cells on the culture is known as declining phase.

IV. Stationary phase: After the arrested growth the culture will be stationary without any further cell division for a few days. Actually, stationary phase is a prolonged one in the case of flagellates. For this they may develop some

cover or cyst or matrix around its body for thriving in the unfavourable conditions. In the stationary phase, if the cells get a new environment they start further growth and reproduction.

V. Death phase: After a long period in the stationary phase, the cells lose its viability and start to die and thus the culture becomes useless, either for reculturing or for feeding.

In mass culture, the main problems of microalgal production are cost, growth and harvesting and predator infestation. In laboratory culture growth of microalgae is highly inconsistent with frequent collapse of cultures due to ciliate infestation, bacterial load and lack of appropriate controlled condition like temperature, pH, light intensity etc. The economic loss in hatcheries due to collapse of microalgal cultures is difficult to quantify but is known to be substantial.

Improvement in the growth of some microalgae has been demonstrated when bacteria are present. Enhanced growth of the microalga *Tetraselmis chuii* was obtained when cultured with bacteria commonly found in its culture (Canizares-Villanueva et al., 1993). The same effect was observed for *Chaetoceros gracilis* then grown with *Flavobacterium* sp., wherein significant improvement in the specific growth rate of the microalga was obtained and the stationary growth phase lasted longer (Suminto and Hirayama, 1997). However, these authors did not find the same improvement for *Isochrysis galbana* or *Pavlova lutheri*, which point to a species – specific relationship.

In livestock nutrition, growing public disquiet over the use of antibiotics in feed additives has encouraged commercial interest in probiotic as an alternate therapy. So in a wide sense, probiotics is often used as an opposite of antibiotics i.e. as a promoter of life. Probiotics are defined as "A live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989).

The sole purpose of probiotics is to promote colonization of desirable bacteria in the gut by application of live microorganisms from either, indigenous or exogenous sources. In otherword, probionts are organisms, which contribute to intestinal microbial balance.

It is possible for probionts, through oral administration, in effective doses, to establish and eventually colonize the digestive tract, increase the natural flora of digestive tract, and to prevent colonization of pathogenic organisms, thus promoting optimal utilization of the feed. Most commonly used probiotics in animal nutrition are LAB or lactic acid bacteria (e.g. *Lactobacillus acidophilus* and *Streptococcus faecium* etc.) and some strains of *Bacillus* sp. The search for new probionts with additional benefits is continuing in many laboratories around the world. These probionts have numerous mechanisms or mode of action. Few of them are listed below.

1. Probiotics inhibit the proliferation of pathogenic bacteria by producing organic acids and antibiotics substances or by reducing the pH.

2. Probiotics prevent the adhesion of pathogenic bacteria by producing Hydrogen peroxide (H_2O_2)

3. Probionts produce certain metabolites, which are able to neutralize bacterial toxins in situ.

4. Probionts help to prevent pathogenicity and by their own enzymes, increase the digestive utilization of feeds or detoxify injurious metabolites from the flora.

Therefore probionts not only inhibit the proliferation of pathogenic bacteria but also improve nutritional value of food by increasing feed conversion efficiency (FCE) and live weight gain as well as stimulation of non-specific immune response.

Microalgal cultures are a virtual storehouse of various microorganisms and therefore by feeding microalgae to marine larvae we inadvertently transfer these potential pathogenic microorganisms to the culture medium. The consequences are well known, with low survival and poor quality larvae, besides failure of microalgal cultures due to overgrowth of microorganisms.

The main objectives of the study are:

1. Investigations on bacterial biodiversity in microalgal cultures and study of their role in sustaining of microalgal cultures.

2. To find out under standard culture condition, the bacterial load that is transferred to larval rearing systems through microalgal cultures.

3. Study the effect of addition (singly and daily) of probiotic yeast to microalgal cultures on its bacterial biodiversity and sustenance.

The two microalgae species used for the culture experiments are *Isochrysis galbana* and *Chaetoceros* sp. while the former is a flagellate, the latter is a diatom. *Isochrysis* is used as the main food for the larvae of bivalves in molluscan hatcheries. In shrimp hatcheries *Chaetoceros* is mainly used for feeding the developing stages of shrimp larvae. The probiotic experiment was carried out only with *Chaetoceros* sp.

In the present experiment the probiotic yeast *Saccharomyces boulardii* (SB) was used to study its effect on other microorganisms in the algal culture medium and also to study its overall effect on growth and sustenance of the microalgal culture. SB is nonpathogenic yeast, isolated from lychee fruit, used to treat intestinal illnesses due to *Clostridium difficile*-induced pseudomembranous colitis and antibiotic associated diarrhoea in humans. This yeast is well suited as a treatment agent because it is able to achieve high concentrations in the colon quickly, maintain constant levels, does not permanently colonize the colon, and does not translocate easily out of the intestinal tract compared to other colonic flora. It is also effective and safe for oral ingestion in case of adults as well as children infected with acute diarrhoea. Although, it is used as both preventive and therapeutic agent for the treatment of diarrhoea caused by *Vibrio cholera* in human beings and farm animals, its use in aquatic organisms has been reported only in the live feed organism *Artemia* against *V. harveyi* (Patra and Mohamed, 2003).

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

Microalgae are indeed the biological starting point for energy flow through most aquatic ecosystems and as such are the basis of food chain in many aquaculture operations (Bardach et al., 1972). Hence, maintaining algal cultures is an integral part of aquaculture. Marine microalgae are the main food source in production of larvae, juveniles and adults of bivalves and combination of several algal species are often used. Algal cells must be within an acceptable size, be non-toxic and have a digestible cell wall to be ingested and digested (DePauw and Pruder 1981). Cells must furthermore have a proper chemical composition to be a high nutritional value; microalgae are also supplemented to larviculture of marine fishes and comprise part of the diet of many freshwater and brackish water fishes. In addition to their nutritional support, supplementation of microalgae also has an impact on the composition of the bacterial community in the larval tanks (Tubiash et al., 1965; Bell et al., 1974; Kellam and Walker, 1989 and Reitan et al., 1994). Variation in these factors may also have an effect on the growth of bacteria associated with the microalgae.

The algae–bacteria interactions are complex and can be recognized, as competition, commensalism or parasitism. Microalgae may promote and /or inhibit bacterial growth by production of organic exudates and toxic metabolites (Duff et al., 1966; Kogure et al., 1979; Cole, 1982; Brock and Clyne, 1984). Bacteria on the other hand, may have a stimulating effect on the algae through decomposition of organic metabolites or through the production of stimulative substances for algal growth (Delucca and McCracken, 1978; Riquelme et al., 1988). During the initial growth phase of microalgae, the bacterial colony biomass increases exponentially at a rate comparable to the maximum specific growth rate (Pirt, 1967; Hattori, 1985; Salvesen and Vadstein, 2000). This relationship was used to obtain a rough estimate of the proportion of opportunistic fast growing bacteria by Salvesen and Vadstein (2000). The method relates only to cultivable bacteria and is based on the assumption that lag time is negligible to the time required to form a visible colony, which is not true for all species.

Marine microalgae are routinely monocultured in Japanese hatcheries, however, their population growth is often unstable, with their lag phases sometimes too long and their stationary phases sometimes too short (Okauchi, 1991). These phenomena may be related to the propagation of unfavourable bacterial organisms in the cultures (Suminto and Hirayama, 1997). Several reports show that some bacterial strains isolated from the marine environment have suppressive or lytic effects on the growth of several marine species of microalgae (Sakata, 1990; Sakata et al., 1991; Imai et al., 1991; Mitsutani et al., 1992).

In contrast, other bacterial strains that promote the growth of marine microalgae were reported from laboratory investigations (Riquelme et al., 1988; Fukami et al., 1991; Suminto and Hirayama, 1996). However, cultures promoting bacterial strains have not been fully utilized in the practical mass production of marine microalgae. Suminto and Hirayama (1993) reported a direct relationship between diatom growth and bacterial population, in semi – mass culture tanks of diatoms in an outdoor culture system.

Suminto and Hirayama (1993) isolated twelve bacterial strains from the semi – mass culture tanks, and cultured in axenic condition, the diatom *Chaetoceros gracilis* together with each one of the isolated bacterial strains, to test their promotive and suppressive effects in diatom growth. They found only one bacterial strain that had a significant promotive effect in diatom growth. Suminto and Hirayama (1993) further tested the practical use of the growth promoting bacterial strain, *Flavobacterium* (strain DN-10) for stabilizing mass culture of three marine microalgae in an indoor culture room.

Microalgae have been shown to have positive effects on fish larvae during first feeding, and this has been attributed to improvement in the nutritional quality of the live feed (Reitan et al., 1993) and to microbial factors (Skjermo and Vadstein 1993; Stottrup et al., 1995). Addition of algae to the water in fish tanks alters the composition of the bacterial flora associated with larvae (Skjermo and Vadstein 1993; Bergh et al., 1994) and bacterial growth and composition of the flora in the water depends on both the algal species and state of growth of the algae (Salvesen et al., 2000).

Many workers (Duff et al., 1966; Kellum and Walker, 1989; Austin and Day, 1990; Austin et al., 1992) have reported that the effect of microalgae on bacterial flora of live feeds has so far not been studied well. One hypothesis is that microalgae contribute to a change in the bacterial composition of live feed organisms and also reduce its number by expelling the gut contents, which act as a substrate for bacterial proliferation. Algae can possibly also affect the bacterial community in the live feed by production of antibacterial substances, as reported for *Tetraselmis*.

Antibacterial activity is present in all algal classes and algal extracts have been shown to inhibit growth of several opportunistic pathogens of fish and shellfish; many of them being assigned to the genera *Vibrio* (Visco et al., 1987; Austin and Day, 1990; Pesando, 1990; Austin et al., 1992; Naviner et al., 1999). Antibacterial substances are in general most abundant in slow growing cultures; as for example, in the early period of the stationary growth phase when the competition for nutrients is high due to high levels of both algae and bacteria (Borowitzka, 1995).

There are numerous reports relating to the stimulation of bacterial growth by extra cellular products of microalgae (Hellebust 1974; Servais and Billen 1993; Munro et al., 1995; Rico-Mora and Voltolina 1995). In the case of Vibrio sp., it has been mentioned that the growth of the species is stimulated after the occurrence of microalgal blooms (Colwell 1984). It has also been reported that the bacterial flora associated with the microalgae *Isochrysis tahitiana*, a species that is used in aquaculture, does not include Vibrionaceae (Lodeiros, et al., 1991). Species of the microalgae *Chaetoceros* have been used widely to feed larval stages of penaeid shrimp, specially the protozoa stage. Commonly the algal density fed to the shrimp larvae is around 10^5 cells, ml^{-1} (Wyban and Sweeney, 1991) but varies with the larval stage. Microalgae used in shrimp hatcheries usually have a natural bacterial load between 10^4 and 10^7 CFU ml^{-1} of heterotrophic bacteria but Vibrios are rare. (Lizarraga-partida et al., 1997).

Whittaker and Feeny (1971) have suggested that marine bacteria are capable of utilizing algal extra cellular products and such a relationship between bacteria and planktonic algae may be deduced from the ability of bacterial contaminants to grow in algal culture (Vela and Guerra, 1966; Berland et al., 1969) or from nutritional studies of such bacteria (Berland et al., 1970) which can then be

related to specific algal products (Belly et al., 1973). Suminto and Hirayama (1996) reported that in a culture tank for mass production of food diatoms, the actively growing population of bacteria or diatom exhibits a mutually suppressive effect on the growth of other population.

Two principal systems have been developed for rearing of larval turbot (Pillay, 1993). In intensive rearing conditions, larvae are stocked at high density (> 10 larvae/l.) usually with regular addition of rotifers enriched with fatty acid supplements as a food source. For extensive rearing conditions 1 larvae/l are maintained at a lower concentration such that rotifers and unicellular algae can be added initially at concentrations appropriate to form a balanced 3 – component food chain in which the rotifers graze on algae and thus maintain a nutritionally – beneficial food source for the larvae. The incorporation of algae is also beneficial in reducing ammonia level in water and larval survival rates in extensive rearing are generally higher and more reproducible than in the intensive rearing system. The principal cause of losses in larval rearing is considered to be due to bacteria but recognized pathogens were not prevalent amongst bacterial isolates from a range of trials involving different food sources locations and rearing conditions (Munro et al., 1994).

To counteract the harmful activities of some of these, as yet undefined, bacteria the addition of beneficial (probiotic) or harmless bacteria has been considered by several workers (Gatesoupe et al., 1999; Gatesoupe 1990). It is important that the added bacteria should not adversely affect the rotifers or algae, as well as the larvae.

Munro et al., (1994) studied the effect of bacteria, isolated from larval turbot, on growth of *Pavlova lutheri.* Of the 41 bacteria tested, 23 inhibited growth in various degrees, 8 had no effect and 10 were weak growth stimulants. Four bacteria, identified as a *Flavobacterium*, *Vibrio fluvialis*, *Vibrio natrigens* and *Vibrio* sp. were strongly inhibitory and the *Flavobacterium* inhibited growth of *Pavlova lutheri.* Inhibition was due to a heat – labile factor released by the *Flavobacterium* into the culture medium. The *Flavobacterium* also produced bacteriocin, which inhibited the growth of a range of Vibrios. Bacteria antagonistic towards algae would be undesirable in larval rearing and if bacteria are to be selected which are beneficial

(probiotics) in larval rearing systems their possible interaction with algae must be considered.

Salvesen et al., (1999) carried out a first feeding experiment with turbot as a two factorial design with filtered vs. microbially matured water as one factor, and with vs. without microalgae added to the tanks as the other. Application of microbial matured water had a highly positive effect in the growth of the larvae from day 5 after hatching and onwards, algal addition gave the greatest effect in size from day 12, although both factors were highly significant. A positive interaction effect of combining matured water with the addition of microalgae was also observed. The positive effects may be related to the observed daily in colonization of the larvae in the early stages of first feeding. Evaluation of the water microflora by criteria based on the degree of maturation showed a lower proportion of opportunistic (i.e. r – selected) bacteria in tanks with matured water containing microalgae. It was suggested that rearing of turbot larvae in microbially matured water to which microalgae are added will lower the proliferation of opportunistic bacteria on the mucosal surfaces of the larvae, with more viable and fast – growing larvae as the result.

Fabregas et al., (1993) studied the use of tris [tris (hydroxymethyl) amino methane], a compound often used as a buffer in microalgal culture media, which sustains active bacterial growth in non–axenic microalgal cultures when sodium phosphate is present. The low pH levels caused by bacterial growth and probably the depletion of phosphorus in the medium caused the collapse of *Phaeodactylum tricornutum* cultures resulted in a reduction of microalgal growth from 32×10^6 to 1.1×10^6 cells /ml. This emphasizes the need for care when interpreting the results of non – axenic microalgae cultures in which tris or other organic buffer is added.

Probiotics

The word probiotic is often used as an opposite of antibiotics i.e. is promoter of life (Gatesoupe, 1991) the research on probiotic for aquatic animals is increasing with the demand for environmentally friendly aquaculture. The probiotic concept is pertinent only when the administered microbes survive in the

gastrointestinal tract, otherwise more general terms are suggested like biocontrol when the treatment is antagonistic to pathogens or bioremediation when water quality is improved (Gatesoupe, 1999). Use of probiotic in larviculture of fish, shrimp and molluscs, has been progressing and the results have been encouraging.

Artificial production of scallop seed has been seriously affected by the occurrence of massive larval mortalities of which one probable case has been bacterial infection of the algal culture, especially by Vibrio sp., (Robert et al., 1996). The success of scallop seed production in hatcheries was thus affected (Disalvo 1991; Navarro et al., 1991; Riquelme, et al., 1995). One of the usual methods of controlling proliferation of pathogens in hatcheries has been by using antibiotics. However, although larval survival is often significantly increased by the use of such compounds, this technique often results in the selection of antibiotic – resistant bacterial strains (Sarti and Giorgetti, 1988; Fitt et al., 1992).

An alternative to the use of chemical therapeutics is biological control by means of probiotic strains of bacteria. The use of probiotics has been studied extensively in terrestrial organisms (Conway, 1989). However, these methods have only recently been studied in marine environments, as antagonists to diseases in the culture of fish (Westerdahl, et al., 1991; Olsson, et al., 1992; Bergh, 1995) bivalve molluscs (Ruiz, et al., 1995; Riquelme, et al., 1996) and crustaceans.

In selecting a probiotic agent, it is desirable to obtain microorganisms autochthonous to the site of application, thus avoiding the introduction of exotic bacterial agents to the system (Hansen, 1993). The method of isolating pathogens antagonistic to bacteria from bivalve larval cultures and then introducing them en masse into new larval cultures may provide a key factor for the control of pathogens in these cultures (Riquelme, et al., 1997).

Probiotic microorganisms have been used to enhance the resistance to disease of aquatic animals with variable results (Griffith, 1995; Riquelme et al., 1997; Gibson et al., 1998; Gomez Gil et al., 2000). *Vibrio alginolyticus* has been tested as a probiotic in *Litopenaeus vannamei* larvae with promising results and it was found to give some protection against the disease (Austin et al., 1995; Garriques and Arevalo. 1995). *V. alginolyticus* was also detected in healthy rotifers and a positive relation

between the survival of turbot larvae and the presence of this Vibrio in the rearing environment was established (Gatesoupe, 1990).

A potential probiotic strain coded C7b and identified as *V. alginolyticus*, prove to be able to outgrow several other bacterial strains isolated from seawater then a bioassay was alone employing a technique based on the disk difficusion method (Gomez Gil, 1998). This stain also demonstrated to be effective in overgrowing potential pathogenic Vibrios isolated from the haemolymph of diseased shrimps and had no detrimental effect to the shrimp larvae.

Although the use of probiotic bacterial strains in microalgal cultures does not come within the strict definition of probiotic usage, recent work by Avendano and Riquelme (1999) and Gomez-Gil et. al., (2002) has shown the significance of such probiotic addition in marine larviculture. One of the obvious advantages of such treatments is that microalgal cultures can be used as vectors for the delivery of bacterial antagonists to bacterial pathogens in marine larviculture.

Avendano and Riquelme (1999) established the feasibility of incorporating bacteria with the ability to produce inhibitory substances (BPI) into axenic cultures of *Isochrysis galbana* with the object of using this microalga as a vector for transmitting BPI into cultures of larval bivalves as antagonists of pathogenic bacteria in these cultures. As a first step, the ability of seven strains of BPI to grow in extracellular products of *I. galbana* was evaluated, with positive results with four of these (334, C33, 11, and 77). Subsequently, the effect of the addition of these strains on the growth of *I. galbana* was evaluated. Comparison of growth rates of I. *galbana* with and without the addition of BPI showed no significant differences ($p<0.05$). A stable and persistent inhibitory capacity of strain C33 on the pathogen *Vibrio anguillarum* was also observed. Finally studies were made on the ingestion of BPI by larvae of *Argopecten purpuratus*. Results demonstrated significant ingestion of strain 11 ($p<0.05$), when it was inoculated directly into the water, and bacterium C33, when delivered in conjunction with the microalga. Upon evaluating incorporation and maintenance of BPI strains 11 and C33 after 5 days of larval culture, they observed the major presence of strain C33 (3×10^2 CFU/larva) compared with strain 11 (90 CFU/larva). The results obtained suggested that it was

feasible to use microalgal cultures as vectors for the introduction of bacterial antagonists to bacterial pathogens in molluscan larval culture.

Gomez-Gil et. al., (2002) studies made to evaluate the performance of the microalga *Chaetoceros muelleri* then cultured with a potential probiotic bacterium *Vibrio alginolyticus* strain C7b as compared when both are cultured alone in medium f/2. Strain C7b grew significantly better and lasted longer when grown with the microalga than when grown alone. The microalgal density was not affected by the presence of the bacteria compared when grown alone. *C. muelleri* and the bacterial strain C7b can be cultured together for up to 9 days to achieve a high density (5.15 x 10^6 and 6.63 x 10^4 cell/ml, respectively) and then fed to the protozoeal and mysis stages of penaeid shrimp.

Saccharomyces boulardii

Apart from *Lactobacillus* sp. and *Bacillus* sp. there are many other new probionts with additional benefits and research is continuing in many laboratories around the world. Among them, the yeast *Saccharomyces boulardii* is an emerging one.

McFarland and Bernasconi (1993) reviewed the use of *S. boulardii* as a non-pathogenic yeast, which has been used as both a preventive and therapeutic agent for the treatment of variety of diarrhoeal diseases. They also confirmed its safety for oral administration. According to their observation *S. boulardii* maintains a high stable level as long as the yeast is taken daily and once the agent is discontinued, *S. boulardii* is quickly eliminated from the colon. Klein et al., (1993) found that as the SB dose increased, the mean steady state concentration of SB increased significantly. They also investigated that percentage recovery was not dependent on dose provided.

Buts et al., (1993) proved that oral administration of *S. boulardii* is effective in reducing morbidity and mortality due to *Clostridium difficile* induced pseudomembranous colitis in a selected group of infants and children with persistent intestinal symptoms related to toxigenic *C. difficile* over growth.

Pothoulakis et al., (1999) studied the mechanism of SB's protective effects by studying the binding of (3H) toxin A to its brush border receptor. The effect of toxin A on secretion, epithelial permeability and morphology in rat ileal loops in vivo was also examined in rats pretreated with *S. boulardii*. As a result *S. boulardii* reduced (3H) toxin A-receptor binding in a dose-dependent fashion. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of ileal brush border exposed to *S. boulardii*-conditioned medium revealed a diminution of all brush border proteins. Treatment of rats with *S. boulardii* suspension reduced fluid secretion and mannitol permeability caused by toxin A.

Czerucka et al., (1994) studied inhibition of the secretion induced by cholera toxin (CT) in rat jejunum by the yeast *S. boulardii*. They studied the mechanism by which *S. boulardii* protects intestinal cells against cholera toxin (CT). They observed that SB -conditioned medium significantly reduced CT - induced cAMP level in IEC-6 cells. Although well known, and available commercially as a human and farm animal probiotic, the use of *S. boulardii* for aquatic animals has been reported only in *Artemia* sp., against *V. harveyi* (Patra and Mohamed, 2003).

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

For fulfilling the objectives the following two experiments were devised and executed.

3.1 EXPERIMENT 1

Two microalgal species, the flagellate *Isochrysis galbana* and the diatom *Chaetoceros* sp. were maintained in triplicate as 250 ml cultures and their total floral counts were made on specific days to assess the bacterial biodiversity.

3.1.1 Seawater

3.1.1.1 Source

Seawater of salinity 30-35 ppt was brought from Mannassery near Fort Kochi and stored in 50 tonne cement tanks.

3.1.1.2 Seawater Treatment

After settling, the seawater were pumped into 500 liter FRP tank and treated with sodium hypochlorite (30 ppm active chlorine). After 24 hours the residual chlorine was checked with O-Toloudine and removed with Sodium Thiosulphate. This was followed by filtration of seawater through gravity sand filter and then stored in clean plastic bins. Salinity was adjusted to 30 ppt with aged tap water. The filtered seawater was then boiled and cooled. Appropriate quantity of nutrient media was added and microalgal culture was done under laboratory condition.

3.1.2 Rearing Containers

Pre-sterilized conical flasks of 500 ml capacity were used for this purpose. Cultures of *Isochrysis galbana* and *Chaetoceros* sp. were made in triplicate. Flasks were filled with filtered and boiled seawater after addition of appropriate concentration of nutrient media. Each flask was filled up to 250 ml with addition of 10% [25 ml] pure inoculum of *Isochrysis galbana* and *Chaetoceros* sp. respectively.

3.1.3 Enrichment Media

For the successful culturing of microalgae (diatoms or nannoplanktors), various chemical culture media have been used depending on the type of organism cultured and their growth phase. Microalgae in any water body require nutrients such as nitrate, phosphate (in an approximate ratio of 6:1) and silicate. Silicate is especially used for the growth of diatoms, which utilize this compound for production of their outer shells. Micronutrient consists of various trace metals, vitamins like thiamin (B_1), cyanocobalamine (B_{12}) and amino acids. The composition of Conway's & Walne's culture media (Walne, 1974) is given below.

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	1 lit.
Solution B	
Zinc chloride	4.2 g
Cobalt chloride	4.0 g
Copper sulphate	4.0 g
Ammonium molybdate	1.8 g
Distilled water	1 litre
Solution C	
Vitamin B_1 (Thiamin)	200 mg in 100 ml distilled water
Solution D	
Vitamin B_{12} (Cyanocobalamine)	10 mg in 100 ml distilled water
Solution E	
Sodium silicate	1 ml dissolved in 1000 ml

Solution	**Quantity in ml (for 2 l of filtered sea water)**
A	2.0
B	1.0
C	0.2
D	0.1
E	2.0

3.1.4 Rearing System

Culture flasks were maintained in indoor conditions at 28^0C with 12-hour light and 12- hour dark condition. Lighting was provided with fluorescent lights and the incident light was measured as 500-lux/ flasks

3.1.5 Duration of Culture

All cultures were maintained until they showed signs of decline and collapse

3.1.6 Sampling

3.1.6.1 Determination of Algal Cell Concentration

Among the several procedures available for determining the algal cell concentration, haemocytometric counting is the simplest method. Other counters used for determining the cell density of microalgae are Coulter counters, Turbidometer, Spectrophotometer, Video endoscopy etc.

The apparatus used for counting the algal cells in the present study was a haemocytometer with an improved Neubaeur ruling. The device is quite suitable for counting algal cells less than even 3μ size. Before counting, both the cover slip and the chamber were rinsed with clean distilled water and dried with blotting paper. Occasional cleaning with alcohol ensures the free flow of algae over the counting area. The face of counting chamber is composed of two gridded surfaces separated by canals. The cover slip was placed on the support bars along the canals and a drop of homogenously mixed algal suspension was delivered from a Pasteur pipette by touching the pipette tip on the edge of the cover slip where it hangs over the V-shaped loading part. Slight pressure will cause the algal suspension to flow evenly across the surface, but not into the canals or on the top of the cover slip.

Both sides of chamber were loaded to seal the cover slip properly. Each half of the haemocytometer surface contains nine large grids. Only those algal cells, which fall with the four large corner grids, were counted. Each large corner grid is further sub-divided into 16 small squares. Cells, which fall on a border were counted if at least half the cell is within the square, but only two borders were

acknowledged (either top or bottom and either left or right) so that cells were not counted twice.

To determine the algal cell density (number of algal cells per ml of sample), the number of algal cells in the four corner grid areas were counted and the mean was taken. The mean value when multiplied by 10^4 gave the actual cell concentration per ml of the sample. If the algal suspension is very thick suitable dilution with seawater was made. Similarly, three to four samples were taken from each algal suspension and the average value was taken as the final cell concentration.

The average number of cells in 1 ml was calculated as

Average count per chamber x 10^4 = Total number of cells/ ml

3.1.6.2 Bacteriological Sampling

Microbiological sampling was done once in 4 days for total aerobic flora and total vibrios from all replicate flasks. Microbial sampling involves procedures like serial dilution, spread plating of serially diluted sample as well as spread plating of particular dilution and was followed sequentially.

(a) Serial dilution technique: 4 or 5 sterilized test tubes of 4.5 ml were arranged serially in the test tube stand inside the laminar flow chamber. 0.5 ml of sample was transferred to first test tube and mixed well with the help of a vortex mixer. This was 10^{-1} dilution. Similar procedure was followed to prepare the other dilutions up to 10^{-6}.

(b) Spread plate procedure: Appropriate dilutions were decided for spread plating in different media (Table 1) like Seawater agar (SWA) plates for enumerating total aerobic flora and thiosulphate citrate bile sucrose (TCBS) plates for enumerating vibrios. 0.1 ml of chosen dilution were poured on to the different media plates and spread uniformly with sterilized glass rods by rotating the lower part of Petri dishes clockwise and anti-clockwise randomly.

3.1.6.3 Bacterial Enumeration

After 48-hour incubation of Petriplates at 37^{0}C colonies found on the agar were counted for all dilutions. Plates with colonies between 30 and 300 were recorded and expressed as colony forming units per ml (CFU/ml).

3.1.7 Bacterial Identification

3.1.7.1 Isolation

About 5 – 6 dominant cultures from each sample giving preferably a count of 3 -30 well separated colonies on SWA were randomly selected and purified by streak dilution method using preset and dried seawater agar plates. All well isolated colonies were picked on to SWA slants labeled and incubated for 18 - 24 h at 37°C. The pure cultures thus obtained were maintained at room temperature and used for identification.

3.1.7.2 Identification Protocol

The cultures were identified up to the genus level by following the identification scheme proposed by Surendran and Gopakumar (1981). The identification scheme included Grams reaction and microscopy, penicillin sensitivity, catalase and cytochrome oxidase tests, oxidation/ fermentation of glucose. and pigmentation.

3.1.7.3 Tests used for Identification

3.1.7.3.1 Grams Reaction and Microscopy

A thin smear of the test culture was prepared by emulsifying a speck of the culture using a drop of sterile DW on the middle of a dirt and grease free glass slide. The smear was allowed to air dry and heat fixed by passing through the blue flame of Bunsen burner (3-4 times). The heat fixed smear was flooded with crystal violet for 1 min. followed by water washing, flooded again with Grams iodine for another minute followed by washing with water. Destaining was done by drop wise addition of alcohol over the smear held in inclined position till the washings were free

of violet colour and thoroughly washed with water. The smear was counter stained with Saffranine for 1 minute and washed with water.

The air-dried smears were examined under oil immersion objective (100x) for the colour and shape of cells, and for the presence of spores if any. The cells with violet, bluish purple or bluish violet was taken as Gram positive and those, which had red colour as Gram negative.

3.1.7.3.2 Penicillin Sensitivity Test

Pre-set antibiotic agar plates were prepared and dried in laminar flow chamber for 30 minutes. The plates were removed from the chamber, divided into 4 quarters and each quarter was marked appropriately. A little of the test culture was smeared over 4-cm^2 area in each quarter. A filter paper disc impregnated with penicillin (2.5 IU Penicillin) was then placed on the surface of each smear. Plates were incubated at 37°C for 18-24 hours without inverting. The cultures showing zones of clearance around the discs were considered sensitive to 2.5 IU penicillin.

3.1.7.3.3 Catalase Test

A speck of test culture was emulsified with two drops of Hydrogen Peroxide (30%) on a clean glass slide. The effervescence i.e. evolution of gas bubbles within few seconds indicated positive reaction for the test.

3.1.7.3.4 Oxidase Test

1% N N N N Tetramethyl-p-phenylene diamine dihydrochloride was freshly prepared and used for the test. A smear of the test culture was prepared on a filter paper impregnated with Kovac's cytochrome oxidase reagent using a sterile platinum loop. Development of blue colour within 10-15 seconds was taken as positive for the test.

Table 1. Composition of media used in the study

Media	Composition	Quantity (grams)	Procedure
Seawater Agar (SWA)	Peptone	5	Final pH at 25°c 7.2 ± 0.2 sterilized at 15 lbs for 15 min.
	Yeast	1	
	Fe PO_4	0.1	
	Agar	15	
	DW	1000 ml	
Thiosulphate citrate bile sucrose (TCBS)	Yeast extract	5	Final pH at 25°c 8.6 ± 0.2. Boil to dissolve the medium completely.
	Protease peptone	10	
	Sodium thiosulphate	10	
	Sodium citrate	10	
	Ox bile	8	
	Sucrose	20	
	Sodium chloride	10	
	Ferric citrate	1	
	Bromo thymol blue	0.04	
	Thymol blue	0.04	
	Agar	15	
	DW	1000 ml	
Sabouraud Broth	Special peptone	10	Final pH at 25°c 5.6 ± 0.2
	Dextrose	20	
	DW	1000 ml	
Sabouraud Agar	Special peptone	10	Final pH at 25°c 6-6.3
	Dextrose	20	
	Agar	15	
	DW	1000 ml	
Antibiotic agar	Peptone	10	Sterilized at 15 lbs for 15 min. Final pH 7.2
	Nacl	10	
	Agar	15	
	DW	1000 ml	
H and L Glucose O/F medium	Peptone	1	Dissolve and adjust pH to 7.1 and 1 ml of 0.1% phenol red solution is added. Dispensed in 8ml quantities in 15cm x 12 mm tubes and sterilized at 10 lbs for 20 min.
	Nacl	0.5	
	K_2HPO_4	0.4	
	Dextrose	1	
	Agar	0.3	
	DW	100 ml	
Tryptone Glucose Agar (TGA)	Tryptone	0.5	After adjusting pH to 7.2, sterilized at 15 lbs for 15 min.
	Beef extract	0.3	
	Nacl	0.5	
	D- Glucose	0.1	
	Agar	1.5	
	DW	100 ml	

3.1.7.3.5 Hugh and Leifson's Glucose oxidative/fermentative Reaction Test

Using a sterile platinum needle, a little of the test culture was stab inoculated into the H&L glucose oxidative/fermentative medium in such a way that at least 2 cm long column of the medium at the bottom of the tube was left un-inoculated. The tubes were incubated at 37°C for 18-24 h and observed for the change in colour of the medium.

Growth of bacteria along the line of inoculation and a yellow colour throughout the medium was taken as positive for fermentative reaction i.e., fermentative with acid but no gas (FANG). The gas bubbles trapped in the medium indicated the fermentation with acid and gas (FAG) reaction. Deep pink colour only at the top portion of the medium was considered as alkaline top reaction, and a yellow colour at the top part of the medium was taken as oxidative reaction. Both alkaline top and oxidative reactions were considered as Non-fermentative (NF).

3.1.7.3.6 Pigmentation

The colour of the cultures on SWA slants was noted after 72-96 hours of incubation at room temperature.

The results of above tests were used to identify the cultures based on the identification scheme of Surendran and Gopakumar (1981).

3.2 EXPERIMENT 2

In this experiment the effect of the addition of a probiotic yeast *Saccharomyces boulardii* (SB) on 250 ml *Chaetoceros* sp. cultures was studied. All experimental procedures followed were similar to Experiment 1 and as mentioned in Section 3.1.

3.2.1 Culture of Probiotic Organism

3.2.1.1 Source of Yeast

The pure culture of *S. boulardii* as lyophilized powder was reisolated on to Sabouraud Agar plates from gelatin capsules obtained from LABORATORIES BIOCODEX, France

3.2.1.2 Storage

Pure culture of SB in Sabouraud broth was kept in a test tube of 10 ml capacity and its mouth was plugged with a cap and further wrapped with Parafilm to prevent entry of moisture and other microorganisms. Such samples were stored in a refrigerator at 4°C.

3.2.1.3 Standardization of Culture of SB in Broth

To know the exact concentration of yeast in broth, the following steps were followed:

(i) 50 ml of Sabouraud broth was sterilized in 100 ml conical flask at 15 psi (121°C) for 15 minutes.

(ii) 0.5 ml of inoculum of SB was added to 50 ml of sterilized broth.

(iii) Then the conical flask was plugged well with cotton and was agitated continuously in a shaker at low RPM at room temperature for 48 h.

(iv) Concentration of yeast in broth and its colour was checked at periodic interval at 24, 36, 48, 72 and 90 h to confirm the peak concentration by counting total CFU on Sabouraud agar plates incubated at 37°C in BOD incubator. Required concentration of yeast in broth was achieved in 48 – 72 h.

3.2.2 Experimental Treatments

Three treatments were made in triplicate for *Chaetoceros* sp. culture. In the first, which was the control, 250 ml of enriched Walne media was inoculated with 10% *Chaetoceros* culture. In the second treatment (treatment SA - single addition) 2.5 ml of SB culture of 10^6 CFU/ml concentration was added such that the concentration of SB in the 250 ml *Chaetoceros* culture was 10^4 CFU/ml. In the third treatment (DA - daily addition) 2.5 ml of 10^6 CFU/ml SB culture was added daily to *Chaetoceros* culture.

3.2.3 Sampling

3.2.3.1 Determination of Algal Cell Concentration

The *Chaetoceros* cell density in each experimental flask was counted daily as described in section 3.1.6.1.

3.2.3.2 Bacteriological Sampling

Microbiological sampling was done once in 4 days for total aerobic flora (on SWA plates), total vibrios (on TCBS plates) and total SB (on Sabouraud Agar plates) from all replicate flasks. Microbial sampling involves procedures like serial dilution, spread plating of serially diluted sample as well as spread plating of particular dilution and was followed sequentially as described in section 3.1.6.2.

3.2.4 Bacterial Enumeration and Identification

Procedures outlined in section 3.1.6.3 and 3.1.7 were followed.

3.3. Analyses of Data

All algal count and microbial count data were averaged and expressed as mean ± standard deviation (SD). The data were graphed for analysis of trends. Statistical comparisons of mean algal cell counts in different treatments were done with one-way ANOVA using SPSS software.

3.3.1 Analysis of Bacterial Diversity

Conventional indices of community diversity were used to measure the biodiversity of bacterial taxa in culture flasks. To measure diversity the Shannon-Weiner Index was calculated by applying the formula –

$$H' = -\sum p_i \bullet \log(p_i)$$

Where, p_i is the fraction of individuals in a species.

The magnitude of H' is affected not only by the distribution of the data but also by the number of categories. The diversity may be expressed as a

proportion of the maximum possible diversity (H'_{max} = log k, where k is the number of categories), called the relative diversity or species evenness of the data:

$$J' = H' / H'_{max}$$

The Simpson measure of diversity expresses the dominance of or concentration of abundance into one or two commonest species of the community. If two individuals are drawn at random from a population of *N* individuals, the probability that both individuals belong to the same species is:

$$C = \sum_{i=1}^{s} \frac{n_i(n_i - 1)}{N(N-1)}$$

where, n_i is the number of individuals of the i^{th} species. Simpson's index is most appropriate when we are looking into the relative degree of dominance of a few species in the community, rather than the overall evenness of the abundance of the species.

Species richness, Margalef's `d', is a measure of the number of species present in the sample, making some allowance for the number of individuals.

$$d = (S-1) / Log(N)$$

Where, S is the number of species in each sample and N is the number of individuals in each sample.

The above analyses were carried out with the help of the software PRIMER™ (Plymouth Routines in Multivariate Ecological Research) version 5.2.8. Based on a similarity matrix generated by the software using the Bray-Curtis measure, hierarchical clustering of species groups in different days of culture was done. The resulting dendrogram indicated the similarity of bacterial diversity in different days of culture, relating specifically to the lag, log, stationary and death phase of the cultures.

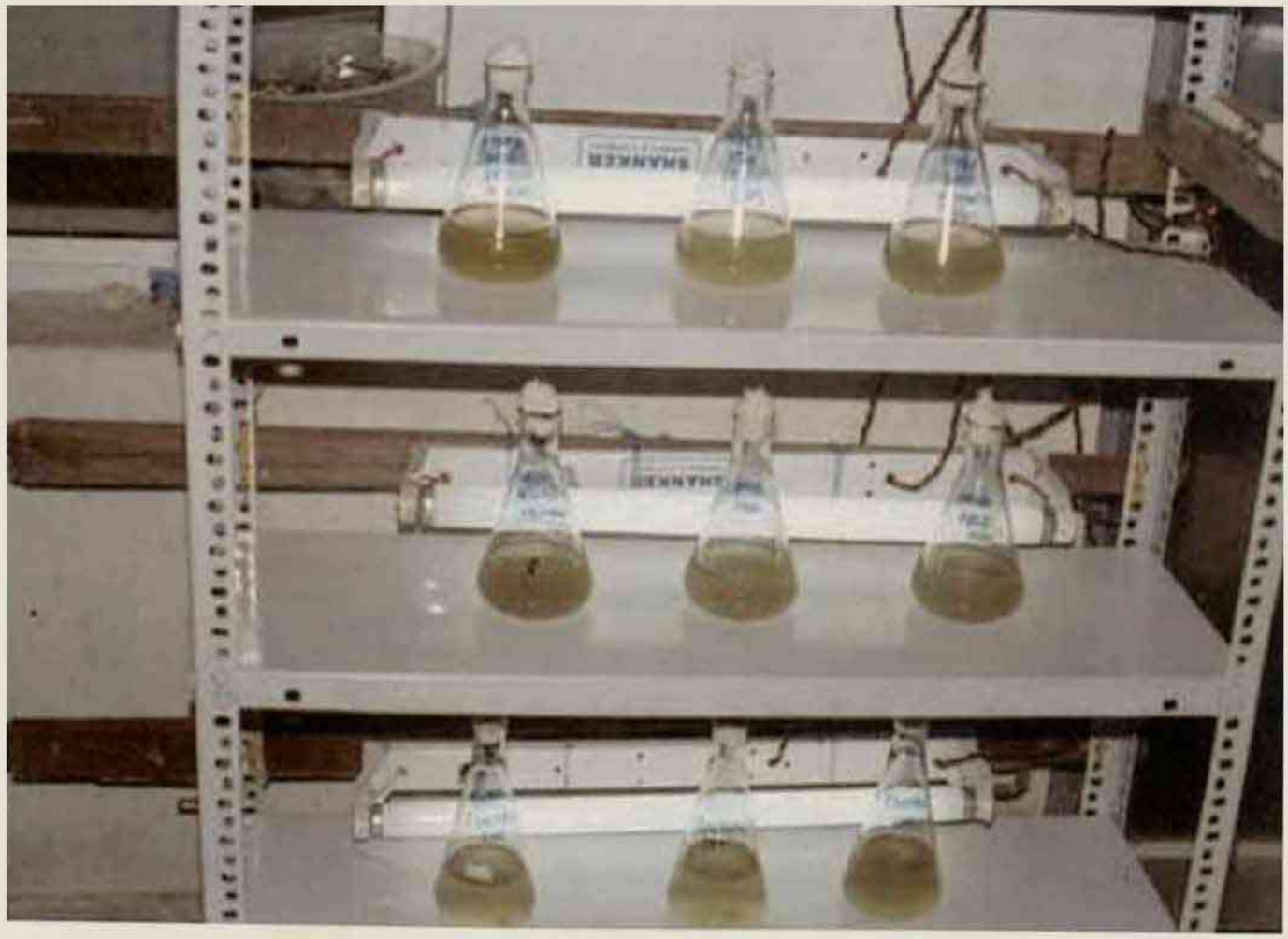


Plate No. 1 Experimental setup for culture of *Chaetoceros* sp. together with the probiotic *S. boulardii*

RESULTS

4.0 RESULTS

4.1 *Chaetoceros* Culture

Mean *Chaetoceros* cell counts in culture flask against time is shown in Fig.1. A steady increase in cell counts of *Chaetoceros* was observed from first day of culture and maximum concentration (2.9 million cells/ml) was attainted on Day 22 after which it gradually started declining and finally it crashed on Day 37. The pattern of culture was a typical one and very similar to microalgal growth of any kind showing lag, log, stationary, declining and death phases.

4.1.1 Bacterial Diversity in *Chaetoceros* Culture

The biodiversity indices during *Chaetoceros* sp. culture are given in Table 2. Only 2 bacterial species were identified in the culture from Day 1 to 22, and on Day 32, 3 species were observed. The bacterial taxa identified upto genus levels were *Micrococcus sp.*, *Acenetobacter* sp., and Enterobacteriaceae. The total aerobic flora as CFU/ml in the culture showed an increasing trend with age of the culture, with the exception of Day 22 (peak log phase) when it declined to very low levels. The species evenness, Shannon's index and Simpson diversity showed an increasing trend until Day 22, after which they declined on Day 32. The species richness index (Margalef d) showed a decreasing trend until Day 17 and then increased. The Simpson diversity index and species richness index were observed to be inversely proportional (Fig.2).

The results of the bacterial taxa cluster analysis are shown in the dendrogram (Fig.3). The analysis indicates that the composition of the bacterial taxa during Day 1 and 6 (Lag phase) show a similarity of nearly 70%. The bacterial taxa on the other sampling days are very dissimilar to each other. On Day 22, which is close to the peak Log phase, the bacterial taxa were very dissimilar to Day 32, which is close to the death phase of the culture. Day 22 bacterial taxa were also very dissimilar to Day 17 taxa.

4.2 *Isochrysis galbana* Culture

Mean cell counts of *I. galbana* in the culture flask is shown in Fig.4. The growth curve observed was a typical one with all phases of microalgal growth. Maximum cell concentration (9.1 million cells/ml) was observed on Day 26 of the culture, after which the culture entered the declining phase. The culture crashed on Day 39 at a concentration of 0.18 million cells/ml.

4.2.1 Bacterial Diversity in *Isochrysis galbana* Culture

The biodiversity indices of *I. galbana* culture with respect to total aerobic flora counts are shown in Table 3. More number of bacterial taxa was observed in *I. galbana* cultures as compared to *Chaetoceros* cultures. The number of taxa fluctuated between 2 and 3 during the culture duration. Three taxa were consistently observed from Day 18 to 28, which was the peak Log phase of the culture. The bacterial taxa identified upto genus levels were *Staphylococcus* sp., *Micrococcus* sp., *Pseudomonas* sp. and *Arthrobacter* sp. The total aerobic flora as CFU/ml showed a progressive increase from Day 1 reaching 47 million/ml on Day 38. The Shannon's index and the Simpson diversity showed an increasing trend up to Day 23 (peak log phase) and then subsequently declined. The species evenness index however, continued to show an increasing trend until the crash of the culture. The species richness index and the Simpson diversity showed an inverse proportionality only until Day 13 (Fig. 5), after which they were proportional.

The bacterial taxa hierarchical cluster analysis clearly showed the similarity in taxa based on the different phases of the microalgal culture (Fig.6). The bacterial taxa during Day 18 and 23 (log phase) showed more than 80% similarity. The taxa during these days were dissimilar to the taxa present during the lag phase of culture (Day 1, 4 and 8). The taxa on Day 33 and 38 also showed 50% similarity and pertained to the period when the culture was about to crash.

4.3. Effect of Probiotic *S. boulardii* addition on *Chaetoceros* Culture

Mean *Chaetoceros* counts in control flasks showed a normal trend and the culture lasted for 23 days (Fig.7). Maximum cell concentration of 0.9 million-cells/ ml was observed on Day 13 and thereafter the declining and death phase was seen.

In the case of treatment daily addition (DA), the culture lasted for a very short duration of 8 days and the maximum concentration was less than 0.5 million-cells/ ml. The treatment single addition (SA) was the longest lasting (up to 31 days). Until Day 3 poor growth was observed and after Day 6 the mean cell concentration crossed 1-million cells/ ml. The mean maximum cell concentration observed (2.3 million cells/ ml) in this treatment was on Day 22. This treatment proved to be significantly more long lasting and attained higher cell concentration than the other treatment and control. One-way ANOVA (Table 4) indicated that the algal cell counts in peak log phase in different treatments were significantly ($P < 0.01$) different.

Table. 4 One–way ANOVA of algal cell counts in different treatments in Experiment 2.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ALGCNT	Between Groups	6.3E+12	2	3.1E+12	17.097	003
	Within Groups	1.1E+12	6	1.8E+11		
	Total	7.4E+12	8			

4.3.1 Bacterial Diversity in *Chaetoceros* Culture Treatments

The biodiversity indices of *Chaetoceros* culture under different treatments are shown in Table 5. The number of species in control varied from 1 to 2, in treatment DA from 2 to 3 and treatment SA from 1 to 3. The mean total aerobic flora showed a steeply increasing trend in control and DA treatments, while the trend in SA treatment was that of slow increase. Highest CFU/ml was observed on the last day of culture for all the treatments (31.9 million in control; 79.9 million in DA and 0.9 million in SA). The total CFU/ml was remarkably low in the treatment SA as compared to control and DA treatments. Since the control and SA treatments showed only one colony type on some sampling days, the diversity indices for those days could not be calculated. As such, clear trends were not discernible form the diversity indices except in DA treatment, which showed an inverse relationship between Species richness and Simpson diversity. In general, the Simpson diversity index was at maximum at the time when the cultures were in peak log phase in treatment SA and control.

The results of the hierarchical cluster analysis of bacterial taxa for all treatments are shown in Fig.8. In the control, Day 1 and 6 (Lag phase) formed a single cluster with 80% similarity in bacterial taxa. Day 11 (Log phase) taxa formed a separate cluster and Day 16 and 21 (Death phase) formed a separate cluster with low similarity. In treatment DA, there was no similarity between bacterial taxa on Day 1, 6 and 11. In treatment SA, the similarity in bacterial taxa in initial Log phase (Day 6 and 11) and peak Log phase (Day 16 and 21) was more than 90%. The clustering of these groups was markedly higher than in the control. The bacterial taxa on Day 28, when the culture was in the death phase formed a separate cluster.

4.3.2 Qualitative and Quantitative Bacterial Counts in Treatments

The mean aerobic flora counts on SWA in control DA and SA treatments are shown in Fig.9. Progressive increase in the counts over time was observed. However, the rate of increase in counts was very high in DA treatment and control, where from 10^4 CFU/ml the count increased to 10^7 CFU/ml in DA and control in 11 and 21 days respectively. On the other hand, in treatment SA, the rate of increase in counts was much lower from 10^4 to 10^6 CFU/ml in 28 days. The mean TCBS *Vibrio* counts (Fig.10) showed an increasing trend in treatment DA, while in SA treatment, the trend was that of decrease, and it was finally absent on Day 28. In control, vibrios were observed only from Day 16, at very low levels, and it increased steeply to 10^4 CFU/ml on Day 21. The mean yeast count on SB agar in the different treatments is shown in Fig.11. From an initial concentration of 10^5 CFU/ml, the counts were maintained at between 10^5 and 10^6 CFU/ml in treatment DA until collapse of the culture. While in treatment SA, from an initial count of 10^5 CFU/ml it decreased to 10^4 CFU/ml on Day 6 and was absent from Day 11 onwards.

Fig.1. Mean *Chaetoceros* Cell Counts in Culture Flask. Vertical lines indicate standard deviation

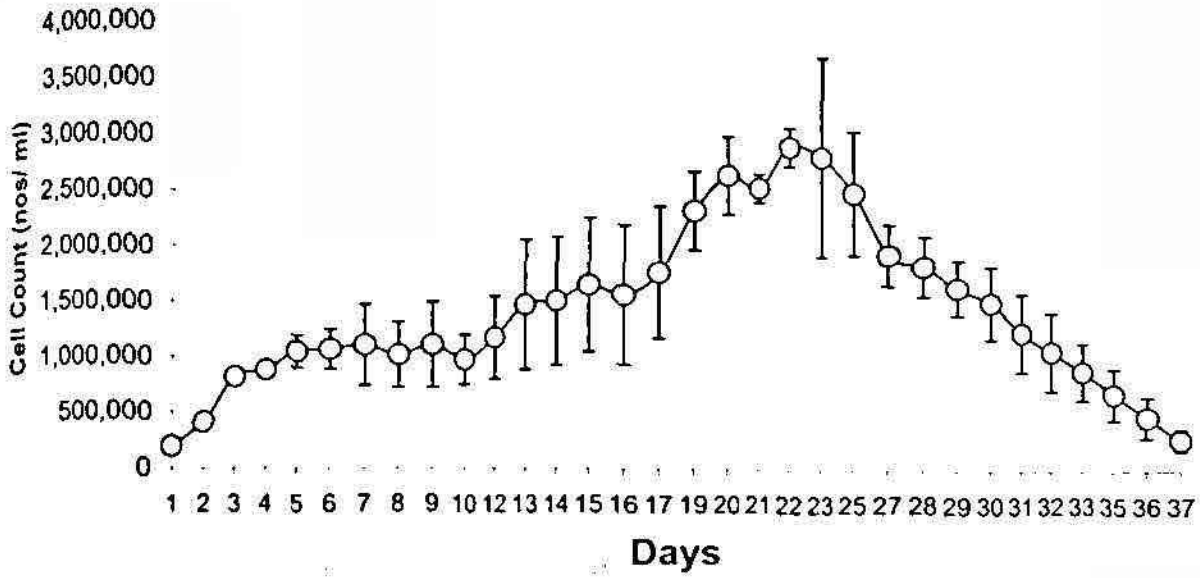


Fig.2. Bacterial Taxa Biodiversity in *Chaetoceros* culture

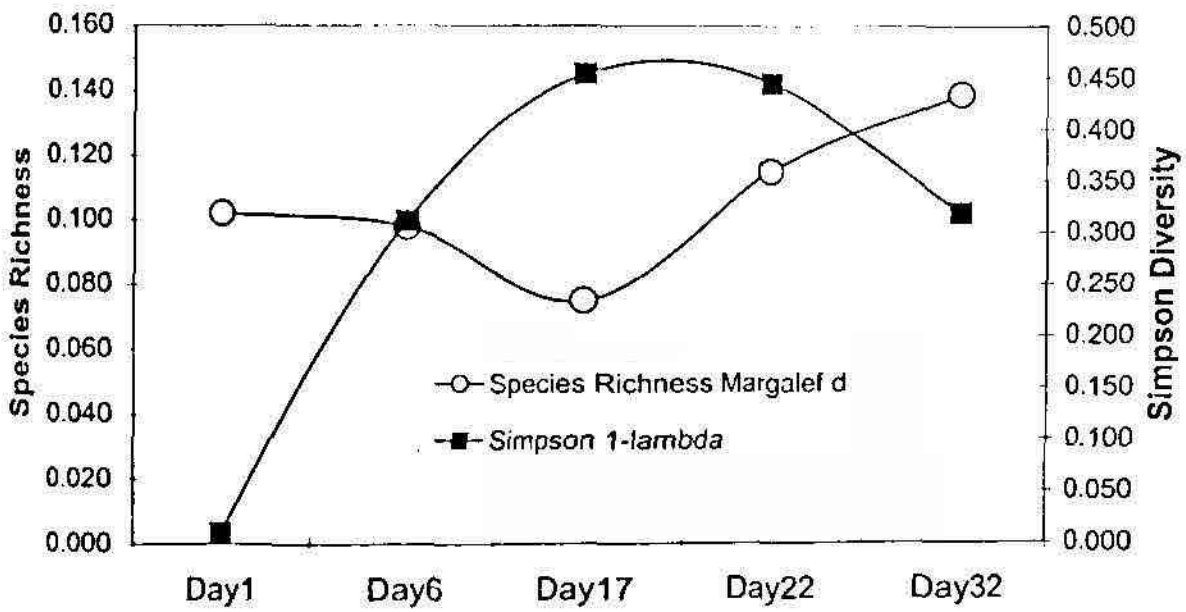
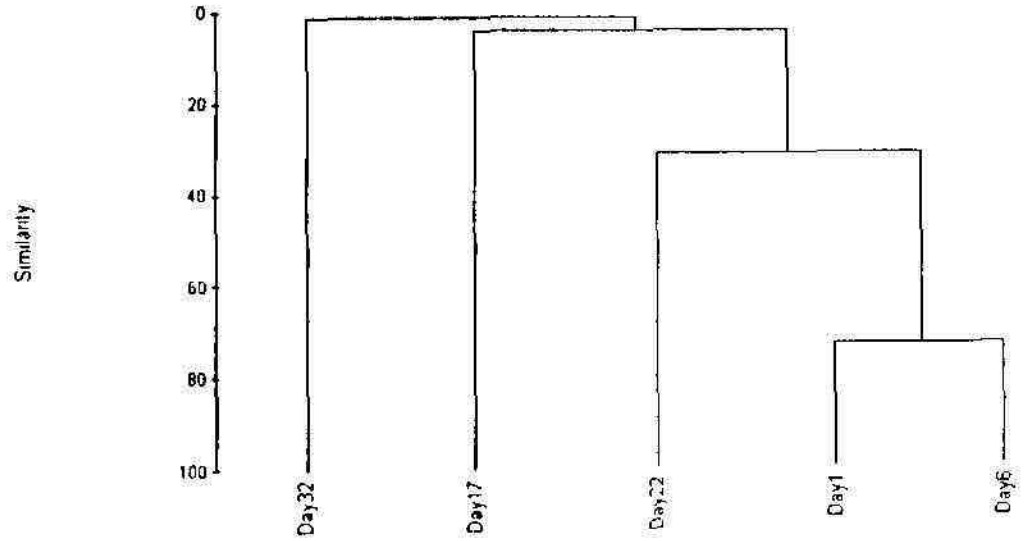


Table 2. Biodiversity indices during *Chaetoceros* sp. culture

	Total Species	Total CFU	Species Richness Margalef d	Species Evenness J'	Shannon-Index H'	Simpson Diversity 1-lambda
Day1	2	18067	0.102	0.049	0.034	0.011
Day6	2	27666	0.098	0.707	0.490	0.311
Day17	2	613333	0.075	0.932	0.646	0.454
Day22	2	6000	0.115	0.918	0.637	0.445
Day32	3	1800000	0.139	0.552	0.607	0.319

Fig 3. *Chaetoceros* sp culture bacterial taxa cluster analysis



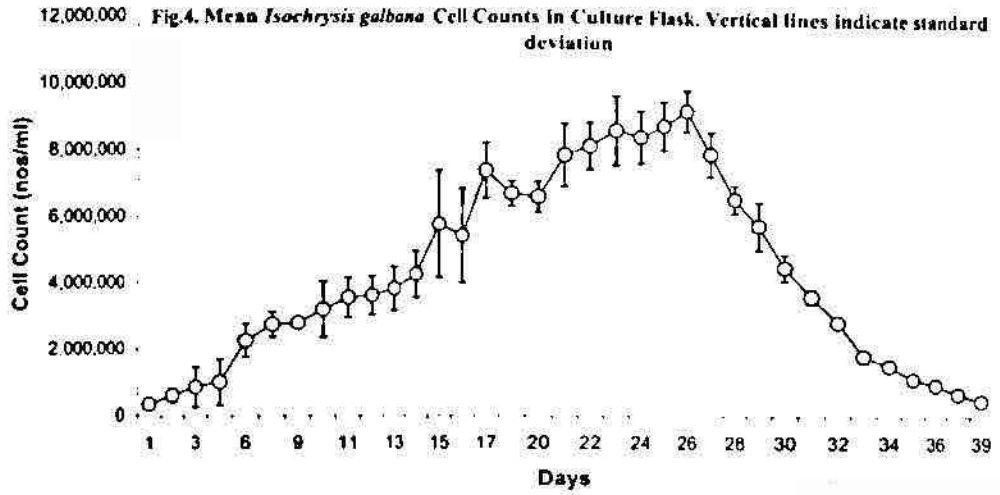


Fig.4. Mean *Isochrysis galbana* Cell Counts in Culture Flask. Vertical lines indicate standard deviation

Fig.5. Variation in biodiversity indices during *Isochrysis galbana* culture

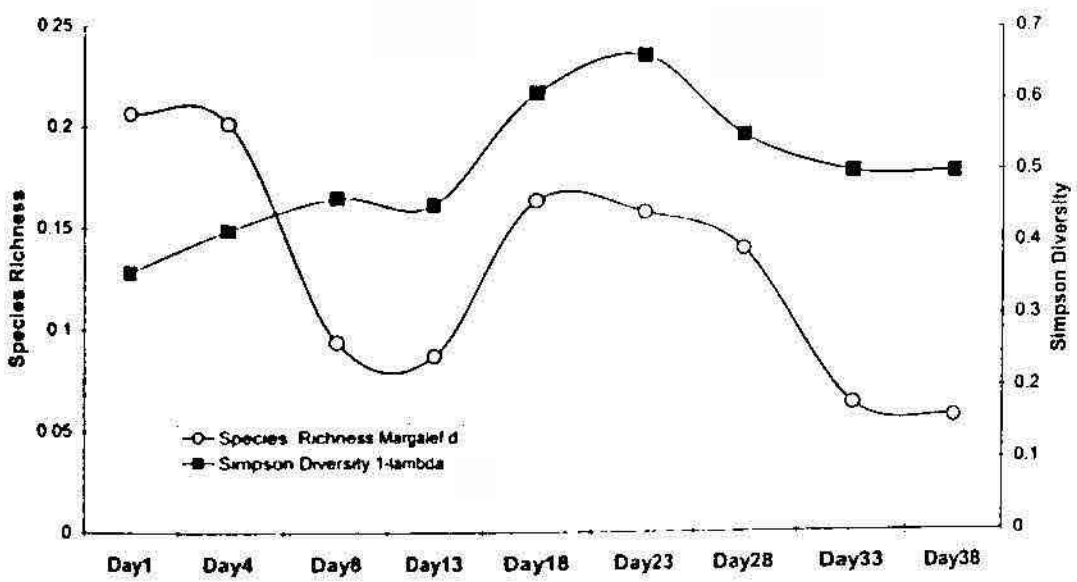


Table 3. Biodiversity indices during *Isochrysis galbana* culture

	Total Species	Total CFU	Species Richness Margalef d	Species Evenness J'	Shannon-Weiner Diversity H'	Simpson Diversity 1-lambda
Day1	3	16166	0.206	0.593	0.652	0.358
Day4	3	20666	0.201	0.681	0.748	0.416
Day8	2	41666	0.094	0.943	0.653	0.461
Day13	2	96666	0.087	0.929	0.644	0.452
Day18	3	205000	0.164	0.910	0.999	0.607
Day23	3	316666	0.158	0.990	1.088	0.660
Day28	3	1633333	0.140	0.852	0.936	0.550
Day33	2	6999999	0.063	0.998	0.692	0.499
Day38	2	47010000	0.057	0.997	0.691	0.498

Fig 6. *I. galbana* culture bacterial taxa cluster analysis

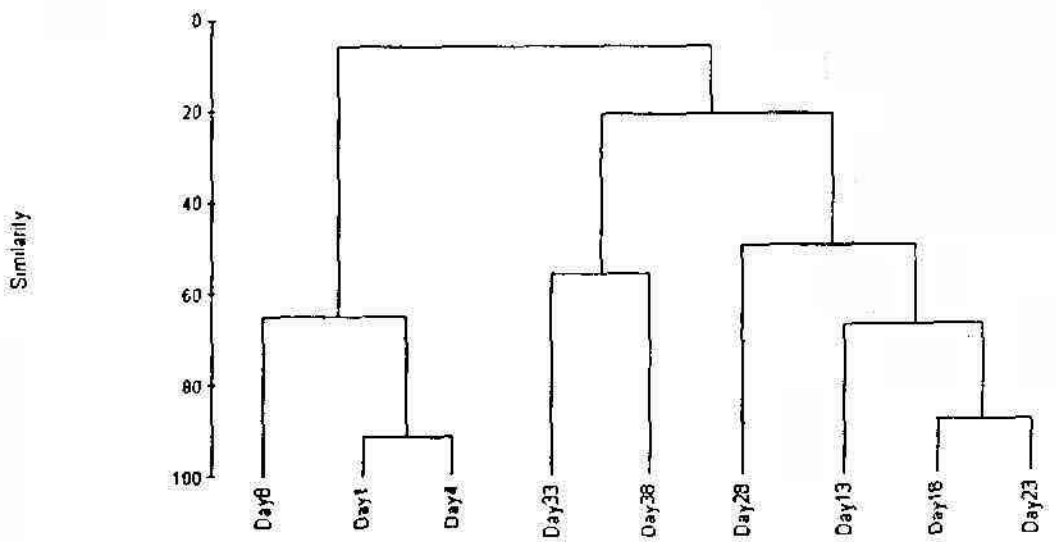


Fig.7. Effect of probiotic *Saccharomyces boulardii* addition on *Chaetoceros* Culture. Vertical lines indicate standard deviation

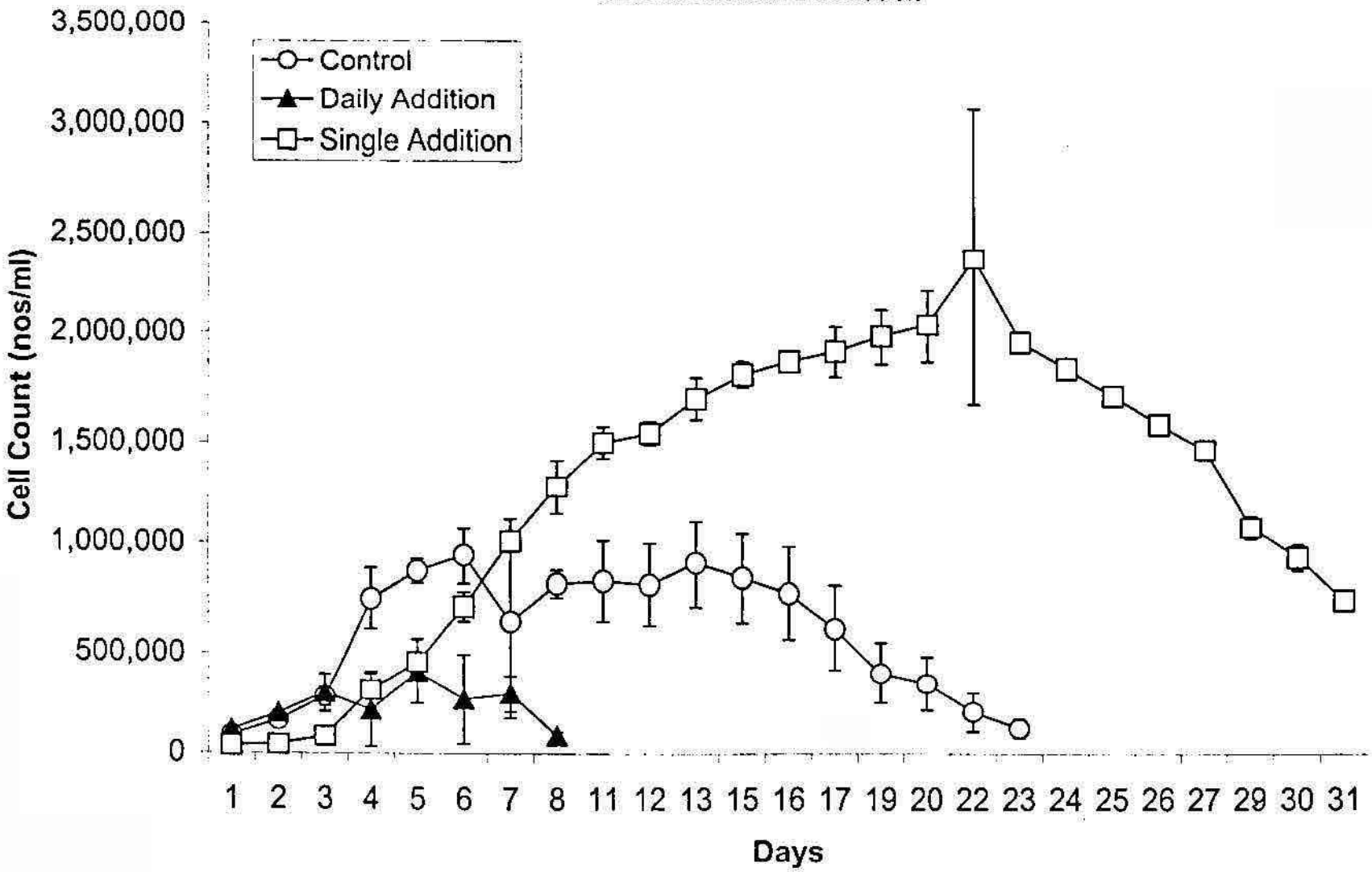


Table 5. Biodiversity indices in *Chaetoceros* culture under different treatments, daily addition, single addition and control

CONTROL	Total Species	Total CFU	Species Richness Margalef d	Species Evenness J'	Shannon-Index H'	Simpson Diversity 1-lambda
Day1	1	13000	0.000	0.000	0.000	0.000
Day6	2	19667	0.101	0.474	0.329	0.183
Day11	1	1033333	0.000	0.000	0.000	0.000
Day16	2	7333333	0.063	0.845	0.586	0.397
Day21	2	31999999	0.058	0.777	0.538	0.353

DAILY ADDITION	Total Species	Total CFU	Species Richness Margalef d	Species Evenness J'	Shannon-Index H'	Simpson Diversity 1-lambda
Day1	2	13334	0.105	0.469	0.325	0.180
Day6	2	1500000	0.070	0.802	0.556	0.369
Day11	3	79999999	0.110	0.790	0.868	0.500

SINGLE ADDITION	Total Species	Total CFU	Species Richness Margalef d	Species Evenness J'	Shannon-Index H'	Simpson Diversity 1-lambda
Day1	2	13334	0.105	0.469	0.325	0.180
Day6	1	21667	0.000	0.000	0.000	0.000
Day11	1	19667	0.000	0.000	0.000	0.000
Day16	3	270001	0.160	0.480	0.527	0.279
Day21	2	250000	0.080	0.353	0.245	0.124
Day28	2	920000	0.073	0.450	0.312	0.171

Fig.8

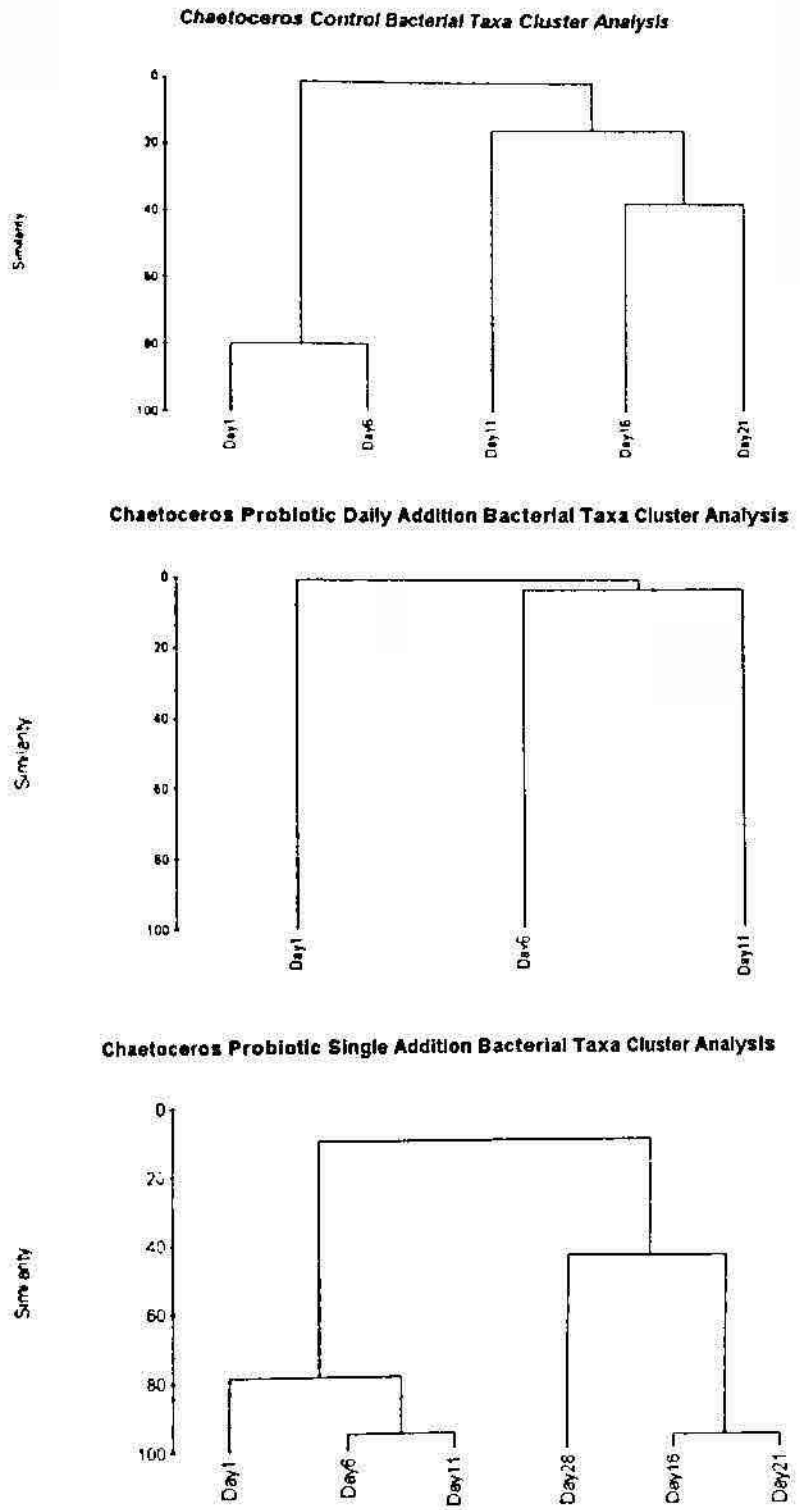

Chaetoceros Control Bacterial Taxa Cluster Analysis
Similarity
0
20
40
60
80
100
Day1
Day6
Day11
Day16
Day21
Chaetoceros Probiotic Daily Addition Bacterial Taxa Cluster Analysis
Similarity
0
20
40
60
80
100
Day1
Day6
Day11
Chaetoceros Probiotic Single Addition Bacterial Taxa Cluster Analysis
Similarity
0
20
40
60
80
100
Day1
Day6
Day11
Day28
Day16
Day21

Fig.9. Mean Bacterial Counts on SWA after *S. boulardii* addition. Vertical lines indicate standard deviation

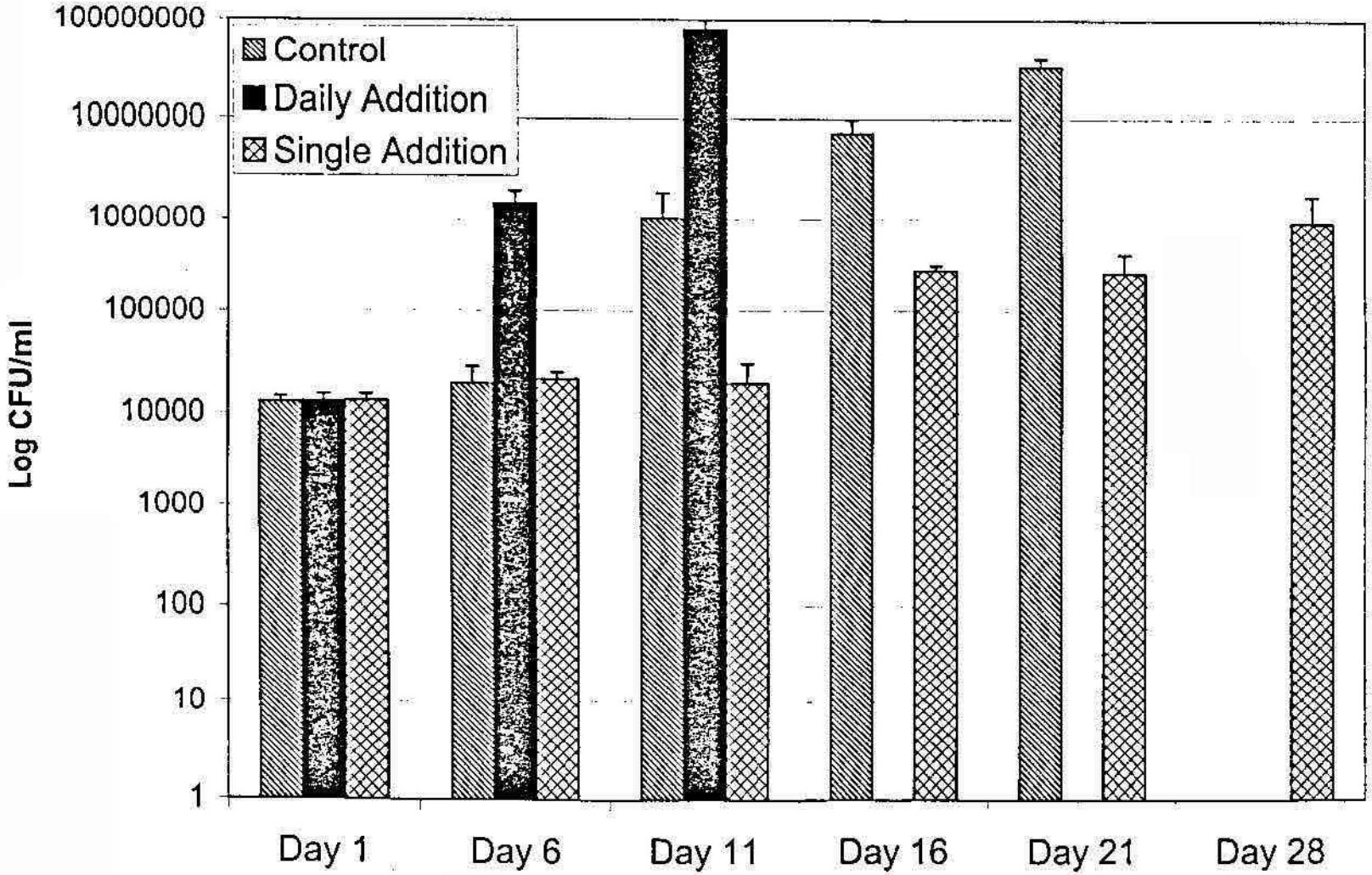


Fig.10. Mean Vibrio Counts on TCBS after addition of *S. boulardii*. Vertical lines indicate standard deviation

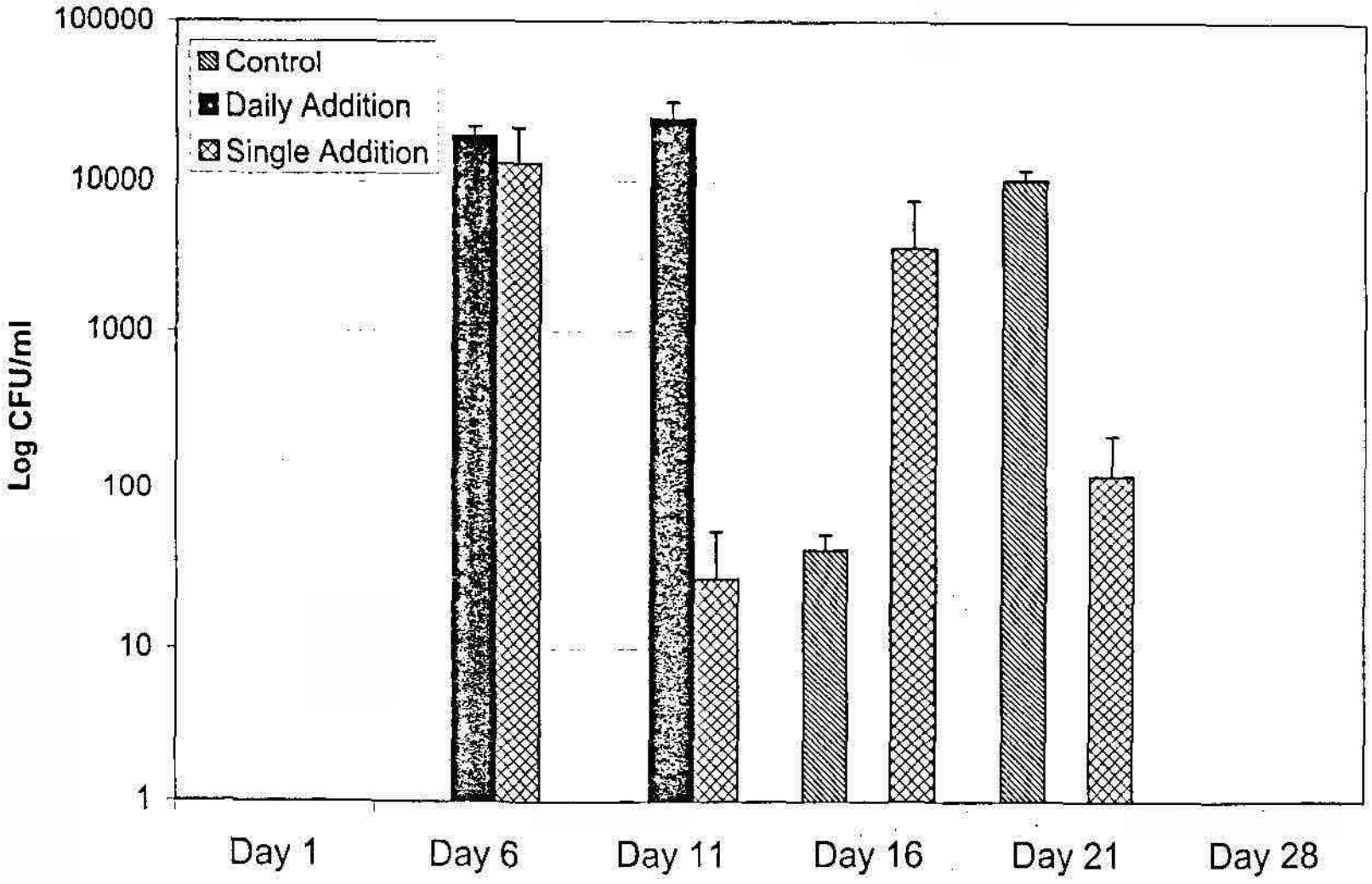
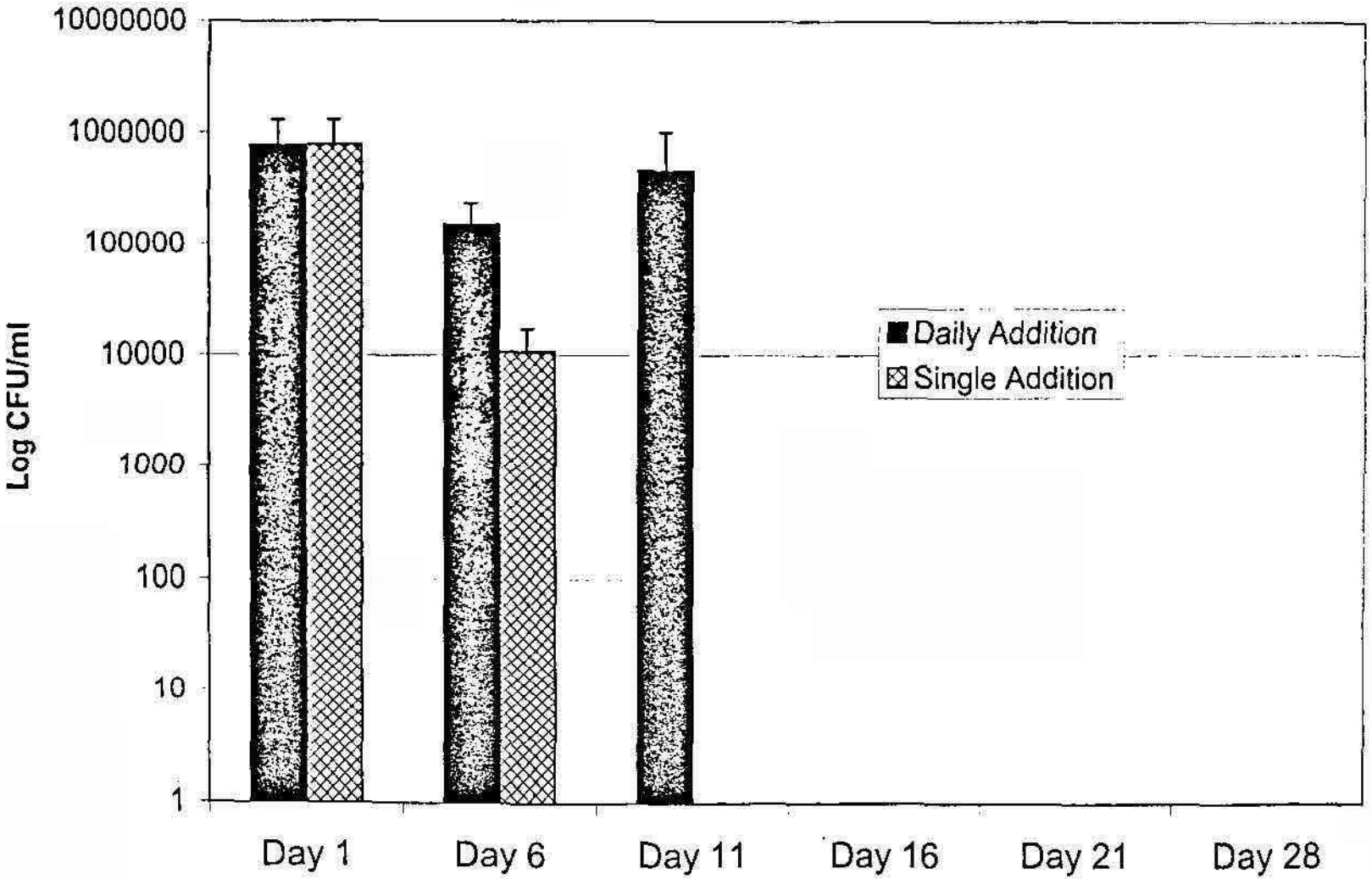


Fig.11. Mean Yeast counts on Sabouraud Agar after addition of *S. boulardii.* Vertical lines indicate standard deviation



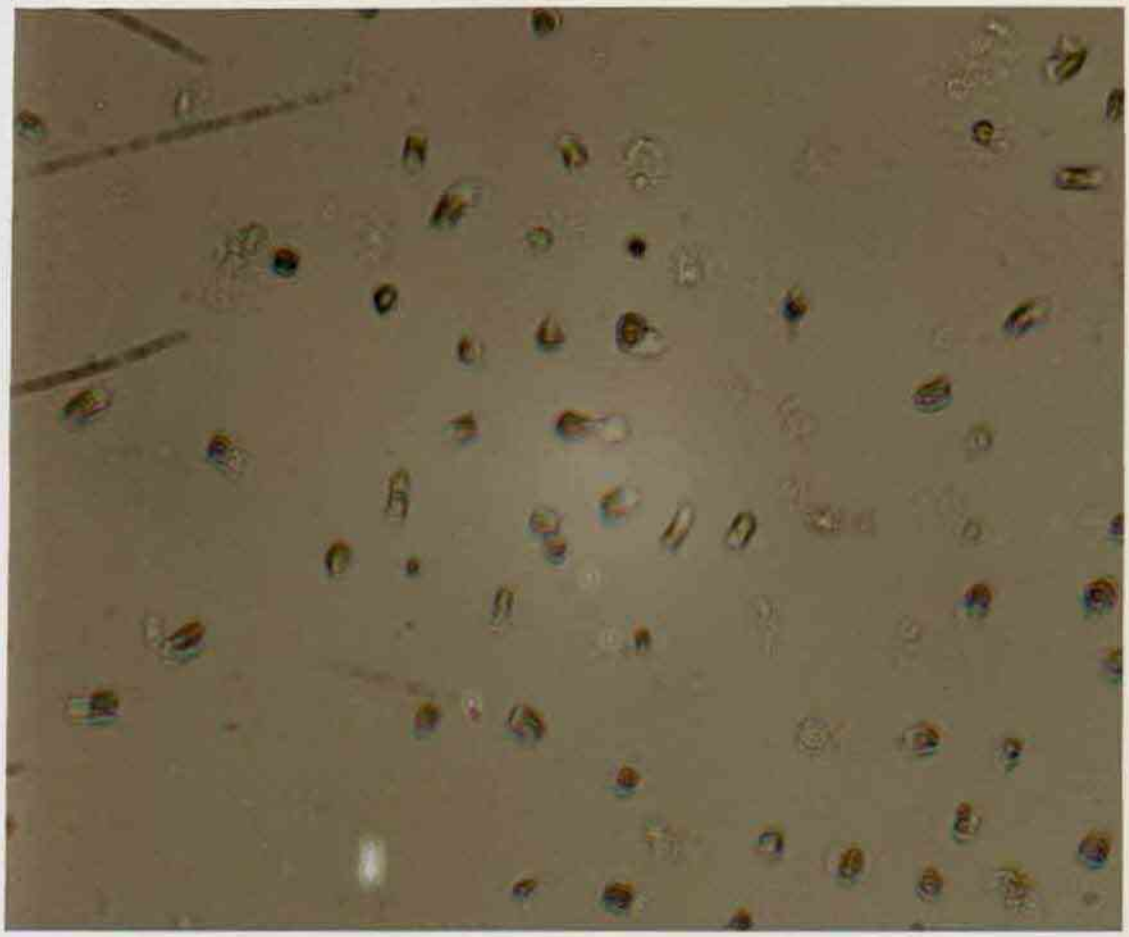


Plate No. 2. *Chaetoceros* sp. cells in Walne medium (mag. 10X40x)

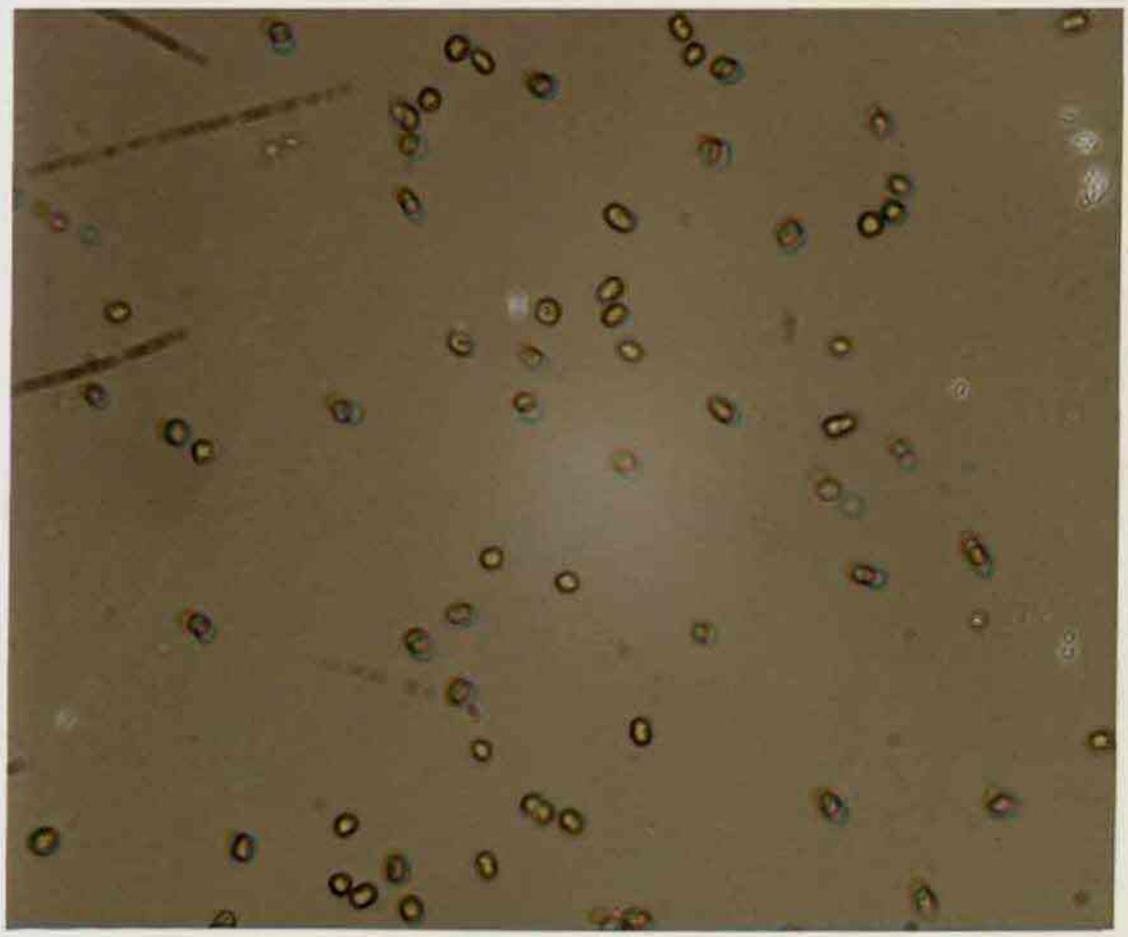


Plate No. 3. *Isochrysis galbana* cells in Walne medium (mag.10X40x)

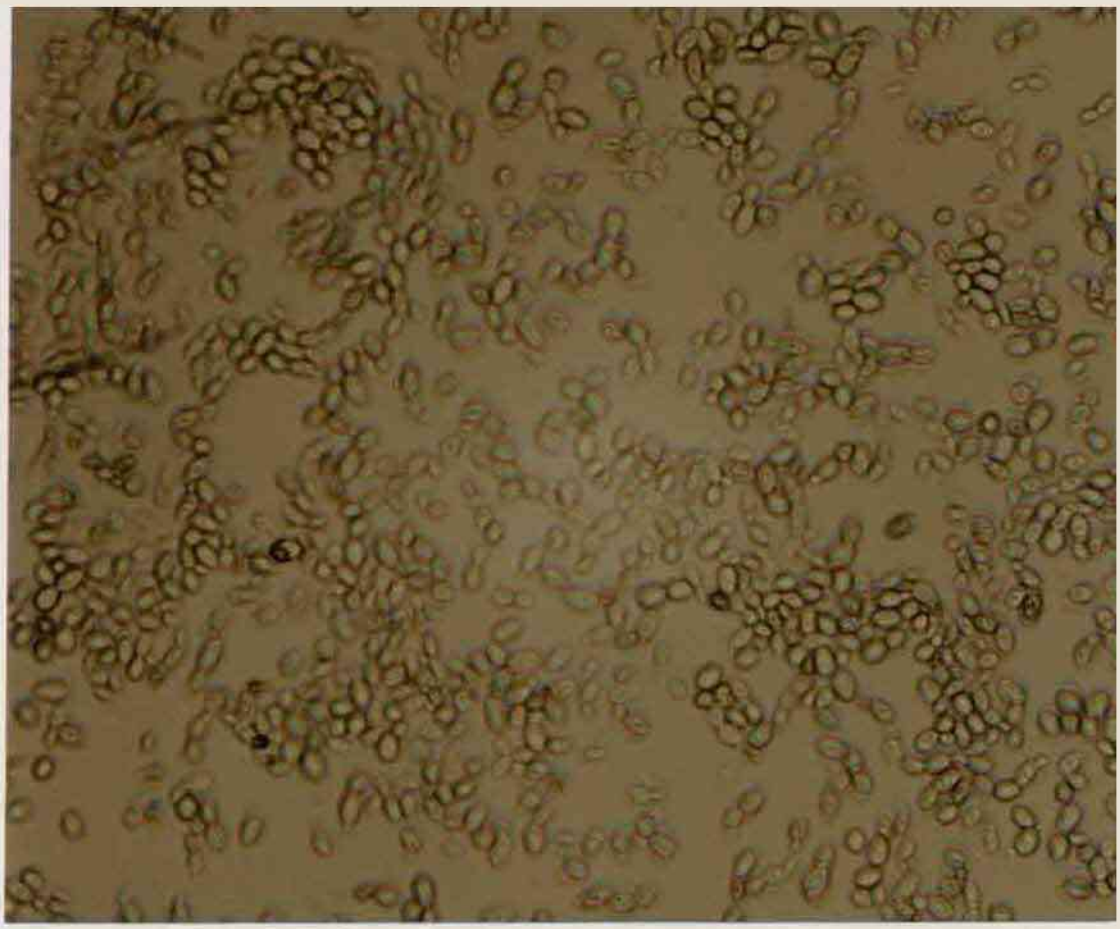


Plate No. 4. *Saccharomyces boulardii* cells in sabouraud culture (mag. 10X100x)

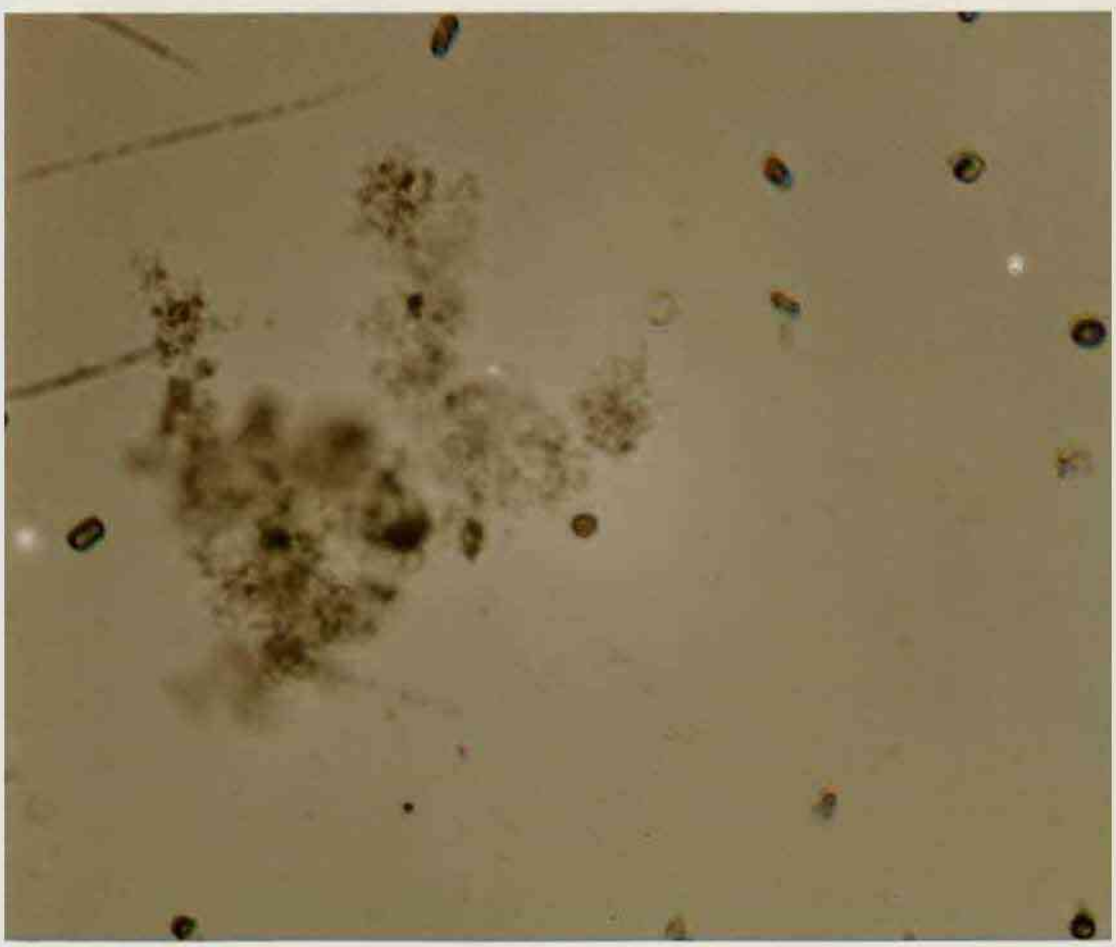


Plate No. 5 Clumping of *Chaetoceros* sp. and *S. boulardii* cells in daily addition(DA) treatment (mag. 10X40x)

DISCUSSION

5.0 DISCUSSION

The present study shows that the microbial environments in algal cultures systems are complex with high degree of variability. The number of dominant bacterial species recorded in the experimental trials varied from 1 and 3. Algal cultures are known to produce antibacterial exudates (Duff et al., 1996; Kogure et al., 1979), and it is possible that the number of species recorded is low due the presence of these exudates. Generally, more number of bacterial colonies was observed in *Isochrysis galbana* than in *Chaetoceros* cultures. In both these cultures, the mean total aerobic count was less (10^3-10^5 CFU/ml) during the exponential and stationary phases than in the declining phase (10^6-10^7 CFU/ml) when the cultures were either senescent or dying. Salvesen et al., (2000) observed that higher bacterial levels were associated with slow growing microalgae or when the growth of the microalgae decreased. They attributed the reason to an effect of higher excretion of organic products from senescent algae together with the decomposition of dead cells, which provided an ideal substrate for bacterial growth.

Thus, microalgal cultures represent a substantial source of bacteria in marine larviculture systems. This study shows that if microalgal cultures are used when they are in the log and stationary phase rather than in the declining phase, the amount of bacteria added to the larval culture medium can be reduced by 3-4 orders of magnitude.

Bacterial diversity is dependent on not only the number of species in the community, but also on the relative abundance of each species. All the measures of bacterial diversity except species richness used in the present study showed an increasing trend with increase in algal density in both *Chaetoceros* and *I. galbana* cultures. Soon after the peak log phase these indices declined. Salvesen et al., (2000) reported for *Chaetoceros muelleri*, *I. galbana* and *Nanochloropsis occulata*, the diversity was lowered when algal growth rates were reduced. The opposite was true for *Tetraselmis* sp. and *Pavlova lutheri*, in which a more diverse bacterial community developed when there was reduction in algal growth rates.

The Simpson measure of diversity expresses the dominance of one or two component species of the community. In both the algal species tested this index was at the maximum at peak log phase, indicating that one or two species were dominating the bacterial community in the algal culture medium when the growth rate was high. Interestingly, the Margalef's species richness index showed an inverse proportion with Simpson diversity in *Chaetoceros* culture. Such a relationship could be due to an effect of inter-specific competition between bacteria and also may be due to a species-specific antibacterial activity from the algae. This relationship was not evident in *I. galbana* culture.

The hierarchical cluster analysis clearly established the dissimilarity in bacterial taxa occurring during the different phases of growth of *Chaetoceros* and *I. galbana*. There was marked clustering of bacterial taxa during the initial lag phase, early log phase and peak log phase and death phase.

The addition of the probiotic yeast *S. boulardii* as a single addition to *Chaetoceros* culture resulted in significantly improved algal growth rates with prolonged stationary period when compared to the control. The daily addition of the same yeast yielded very poor algal growth. Gomez-Gil et al. (2002) reported that *Vibrio alginolyticus* (strain C7b) could be cultured in the same system (F/2 media) as the microalgae *C. muelleri* without affecting the yield of either species. Other microalgal species (*Tetraselmis chuii*) have been observed to perform better when cultured with bacteria than when in axenic cultures (Canizares and Ontiveros, 1993; Suminto and Hirayama, 1997). Avendano and Riquelme (1999) compared the growth rates of *I. galbana* with and without the addition of bacterial inhibitory substances (BPI) and they found no significant differences.

The treatment DA in the present study had a culture media, which was very rich in aerobic bacteria (up to 10^7 CFU/ml) and vibrios on TCBS (up to 10^4 CFU/ml). The daily addition of Sabouraud media along with the yeast resulted in the formation of conjugates of media particles and algal cells, and this probably resulted in poor algal growth. In treatment SA where algal growth was significantly better, *S. boulardii* could not be detected in the medium beyond Day 6. The earlier initiation of death phase in control culture may be due to the absence of certain organic compounds or growth stimulators produced by the yeast *S. boulardii*. However it did

help in keeping low the total aerobic bacterial count in the medium to between 10^4 and 10^5 CFU/ml as compared to control, which had counts of $10^7$and 10^8 CFU/ml. *S. boulardii* treatment as a single addition also helped to keep the vibrios in TCBS at lower level than control (10^2 vs 10^4 CFU/ml on Day 21 and nil on Day 28). How exactly *S. boulardii* could improve the *Chaetoceros* growth rate remains to be investigated. Suminto and Hirayama (1997) also observed that the total bacterial population in *C. gracilis*, *I. galbana* and *P. lutheri* cultures was lower in the exponential phase when treated with the bacterial strain Flavobacterium (DN-10 strain) than in the stationary and death phases.

Treatment SA showed 162% increase in maximum algal density over that of control and delay in initiation of death phase. This result could have relevance in the mass culture of *Chaetoceros* in hatcheries where cultures are prone to frequent crashes. A similar, but much smaller (46%), increase in algal densities was observed by Avendano and Riquelme (1999) in *I. galbana* when grown with a strain of *Vibrio* (C33). Suminto and Hirayama (1997) also observed much improved growth of *C. gracilis* by the addition of a growth promoting bacterium *Flavobacterium* sp. However the same bacterial species did not promote the growth of *I. galbana* and *P. lutheri*.

A comparison of the diversity indices in different treatment did not reveal any marked changes. However, the hierarchical cluster analysis showed remarkable difference between treatments. There was a marked increase in the similarity percentage of clusters indicating a much better discrimination of bacterial taxa in treatment SA as compared to control. It is likely that such heightened discrimination helped in prolonging the culture in SA treatment.

Algae-bacteria interactions are complex and our understanding of the same is limited, but they are very important in maintaining a stable environment in algal culture systems. This study shows that the probiotic yeast *S. boulardii* can positively affect the growth and sustenance of *Chaetoceros* cultures. Besides the finding that one or two species of bacteria dominate the algal cultures during their log phase is an indication of the manipulations possible in algal culture systems to improve their sustenance.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Unicellular marine microalgae are cultured widely as food for larvae of commercially valuable fish and shellfish. The culture of microalgae requires control over parameters, which regulate its growth, like nutrients, light, pH, salinity and temperature. The laboratory and mass culture of microalgae is highly inconsistent with frequent collapse of culture due to protozoan infection, bacterial load and lack of appropriate environmental conditions.

Microalgal cultures are a virtual storehouse of various microorganisms, and therefore, by feeding marine microalgae to marine larvae, we transfer many potentially pathogenic microorganisms to the culture medium. The consequences are low survival and poor quality larvae, besides failure of microalgal culture due to over-growth of microorganisms.

The objective of the present study was to investigate bacterial diversity in microalgal cultures, and to find out bacterial load that is transferred to marine larviculture systems. Further the effect of addition of probiotic yeast to *Chaetoceros* culture on its bacterial diversity, growth and sustainability was also studied.

In the present study the flagellate *Isochrysis galbana* and the marine diatom *Chaetoceros* sp. were used. The probiotic yeast *Saccharomyces boulardii* was used as a probiont for this study. Standard procedures were followed for microalgal culture, bacterial counts and culture of probiotics. Conventional indices of community diversity were used to measure the diversity of bacterial taxa in culture flasks. Hierarchical cluster analysis was carried out to plot similarities in bacterial taxa occurring during different phases of microalgal culture.

The salient findings of the investigation are given below

1. Generally, more number of bacterial colonies was observed in *Isochrysis galbana* than in *Chaetoceros* cultures. In both these cultures, the mean total aerobic count was less (10^3-10^5 CFU/ml) during the exponential and stationary phases than in the declining phase (10^6-10^7 CFU/ml) when the cultures were either senescent or dying.

2. This study therefore shows that if microalgal cultures are used when they are in the log and stationary phase rather than in the declining phase, the amount of bacterial added to the larval culture medium can be reduced by 3-4 orders of magnitude.

3. All the measures of bacterial diversity except species richness used in the present study showed an increasing trend with increase in algal density in both *Chaetoceros* and *I. galbana* cultures. Soon after the peak log phase these indices declined.

4. In both the algal species tested the Simpson Diversity index was at the maximum at peak log phase, indicating that one or two species were dominating the bacterial community in the algal culture medium when the growth rate was high. The Margalef's species richness index showed an inverse proportionality with Simpson diversity in *Chaetoceros* culture. This relationship was not evident in *I. galbana* culture.

5. The hierarchical cluster analysis clearly established the dissimilarity in bacterial taxa occurring during the different phases of growth of *Chaetoceros* and *I. galbana*. There was marked clustering of bacterial taxa during the initial lag phase, early log phase and peak log phase and death phase.

6. The addition of the probiotic yeast *S. boulardii* as a single addition to *Chaetoceros* culture resulted in significantly ($P < 0.01$) improved (162% increase in maximum algal density) algal growth rates with prolonged stationary period when compared to the control. The daily addition of the same yeast yielded very poor algal density.

7. The addition of the probiotic yeast *S. boulardii* as a single addition helped in keeping low the total aerobic bacterial count in the medium to between 10^4 and 10^5 CFU/ml as compared to control, which had counts of 10^7 and 10^8 CFU/ml. *S. boulardii* treatment as a single addition also helped to keep the vibrios in TCBS at lower level than control (10^2 vs 10^4 CFU/ml on Day 21 and nil on Day 28). The mean total aerobic flora showed a steeply increasing trend in control and DA treatments, while the trend in SA treatment was that of slow increase.

8. In hierarchical cluster analysis there was a marked increase in the similarity percentage of clusters indicating a much better discrimination of bacterial taxa in treatment SA as compared to control. It is likely that such heightened discrimination helped in prolonging the culture in SA treatment.

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