5. DISCUSSION

In recent years there is an increasing interest to understand the relevance of microbes in aquaculture systems and their importance for prawn productivity. *Bacillus* and *Lactobacillus* are used in Culture and Production System to manipulate the microbial communities in order to reduce or eliminate, selected pathogenic microorganism like Vibrio and to improve the growth of cultured organisms.

As there is a paucity of information studies on indigenous flora like *Bacillus* and *Lactobacillus* in the perennial and pokkali aquaculture System of Cochin backwaters is un explored area, a study on the "Role of *Bacillus* and *Lactobacillus* from the marine environment for sustainable aquaculture Practices" was initiated and regular collection of water, sediment and shrimp intestine was made from two fixed station for a period of one year (2001 April – 2002 March). The result of the study show that:

1. Totally 248 strains of *Bacillus* and 166 strains of *Lactobacillus* were isolated from both Cherai and Valappu.

2. *Bacillus Subtilis* and *Lactobacillus acidophilus* were the most prominent species of Bacillus isolated. The biochemical potential
(proteolytic, amylolytic, caeseno lytic, Ureolytic, saccharolytic etc) was found very high.

3. 14 Species of Bacillus (B.subtilis, B.sphaericus, B.pumilus, B.megataruim, B.firmus, B.circulane, B.alvei, B.polymixa, B.brevis,B.coagulans, B.stearothermophilus, B.licheniformis, B.macurance and B.cereus(mycoides) and 8 species of lactobactobacillus(L.acidophilus, L.plantarum, L.fermentum, L.brevis, L.curvatus, L.casei, L.butchnerii, L.cosynifosmil and L.coprophihis) were isolated from Cherai and Valappu.

4. Statistical inference on the influence of environmental parameters on Bacillus and Lactobacillus, showed that significant correlation existed between environmental parameters such as salinity, dissolved oxygen, temperature, Phosphate, Nitrite, Nitrate and Ammonia.

5. Selected strains of 4 Bacillus species identified by biochemical potential was subjected to Microbial Identification System (MIS) and the identification was found correct based on the fatty acid profile.
Through out the period of study at Cherai and Valappu showed almost identical physico-chemical parameters such as salinity, temperature, Dissolved oxygen and pH, even though slight variation existed between the two stations which can be attributed to the tidal influence and under water currents to change in the Cochin back water system as the culture ponds are extensions of these estuary.

The temperature of the study area showed considerable seasonal fluctuation and recorded maximum of 34°C in Cherai while Valappu recorded 36°C and a minimum of 30.5-31°C was recorded from both the stations. The changes in temperature is mainly due to monsoonal influences in these aquaculture ponds: Lakshmanan et. al., (1982) reported sharp variation in temperature during onset and withdrawal of South West monsoon. Rheinheimer (1985) also reported that fluctuation in the seasonal temperature causes a change in the distribution of actinomycetes during monsoon which may be due to environmental parameters other than temperature as temperature was recorded low during monsoon. (Fig 2)

Lakshmanan et. al., (1982) also reported that salinity gradient in the northern side of the estuary is lower than the southern side. The present study revealed a gradual increase in the salinity from December to April in Cherai. Maximum of 28ppt was recorded in March a minimum during June.
July and August (3-5 ppt). The degree of salinity determines to a particularly large extent the living microbial communities in water. Majority of marine bacteria are halophilic. It is the Na$^+$ which is the vital necessity for most marine microbes and for some in additional Cl$^-$. (Macleod, 1965, 1968). Salinity which deviates to some degree from the optimum prolongs the generation time in all bacteria (Rheinheimer, 1985). (Fig 4). Minimum salinity at Cherai was recorded during July (4 ppt) while at Valappu during June (3 ppt). This is due to the monsoon and post monsoon rains, which in turn brought down the salinity to low range and thereby the bacterial counts.

According to Mayer-Reil (1972) adaptation is largely restricted to within certain salinity ranges characteristic for the particular ecological group ie that is the bacteria with the greatest salt tolerance are as a rule, also the most adaptable. Sridharan and Mohammed Salih (1985) reported wide variation in salinity which may be due to combined action of water movement, tidal variation and mixing. A maximum of 28 ppt was recorded in Cherai in March, April while Valappu showed only 24 ppt.

Maximum dissolved oxygen at Cherai was encountered during March (6.5 mg/l) while Valappu showed maximum during October (6.5 mg/l) (fig 4) the range of dissolved oxygen observed by Venkitesan et. al., (2001) was from 7-8.4 mg/l whereas in the presence study dissolved
oxygen ranged between (3.5-6.5mg/l). The aquaculture pond at Cherai had a maximum pH of 8.4 during march while Valappu recorded a maximum pH of 9.2 during June. Sing (1986) has observed similar results during his study where he reported that 35.7% of isolates from cultured *P indicus* gave good growth at PH 9. Nellan (1967) has reported that the schelei fjord (Baltic) at a time of vigorous plankton bloom had a pH of 9.5. The large fluctuation of pH affects the composition of bacterial flora. At Valappu and Cherai minimum pH recorded were 7.1 and 7.4 the comparatively higher pH due to higher photo synthetic activity the aquatic vegetation which removed the CO₂ from water column low pH may be due to decomposition of organic matter. (fig 5)

Nitrite Nitrogen values at Cherai ranged between 0.37 µg atm/l – 2.34 µgatm/l and Valappu recorded Nitrite values range in between 0.48mgatm/l – 3.96 µgatm/l. the reports of Venkitesan et. Al., (2001) and Lekhsmanan et. al., (1987) was found consistent to the presence study. Satpathy and Nair (1996) reported a range of 0.19-3.27 reaching almost parallel to the present study. (fig 7)

Nitrate ranged between 0.97. µgatm/l to 7.92 µgatm/l from Cherai station while Valappu recorded a minimum of 0.096 µgatm/l in November and maximum of 2.16 µgatm/l in September. In the present study the
highest nutrient value were noticed during the pre monsoon season while low values were recorded during post monsoon season coinciding with the influx of rainwater. At Cherai a maximum of 9.8 $\mu$gatm/l of NH$_3$ was recorded in June while Valappu recorded a maximum of 17.6mgatm/l in January the lowest values of NH$_3$ from Cherai and Valappu were recorded in January (0.45mgatm/l) and December (0.09$\mu$gatm/l) (fig 8)

Ammonia and Nitrate play an important role in the supply of energy for nitrifying bacteria and the oxygen bound in Nitrate can be used by the numerous flora capable of denitrification under anaerobic condition for the oxidation of organic material. Optimum concentration of nitrate and ammonia were found to enhance bacterial activity were as sub acute or chronic exposure to 1 ppm unionised ammonia or 10 ppm nitrite may result in gill damage, black gills and low level mortalities (Chen et. al., 1990) (fig 6)

At Cherai maximum phosphate values were recorded in September 2001 (7.92$\mu$gatm/l) and a minimum in April 2001 (0.04$\mu$gatm.l) while Valappu recorded a maximum of 8.9$\mu$gatm/l in April and a minimum of 0.4$\mu$gatm/l in July the high phosphate contain observed in April at Valappu may be due to the pre monsoon effects as recorded by Lakshmanan et. al., (1987). The activity of heterotrophic microorganisms
causes enrichment of nitrate and phosphate consequently regions where water from the deeper parts which are rich in nutrients well up to the surface show high productivity and a more abundant growth (Rheinheimer, 1985). (fig 9).

In the present study 3 different selective medias were used. Apart from Zobell’s marine agar as mentioned in the results for enumeration of TPC, Bacillus, Lactobacillus flora. It is always better to employ more than one medium to assess the antagonistic strain of bacillus and lactobacillus spp than by employing a single media. (Grey and Meyer; 1968).

The annual variation of TPC in water showed a maximum total heterotrophs in the month of September at Cherai (252x10³/ml) and August (24.8x10⁴/ml) at valappu. (Fig 12 and Fig9). Sediment sample at Valappu recorded a maximum during the month of March 2001 (18.0x10⁴/gm) while Cherai recorded a maximum in October 2001 (220 x 10³/gm). The correlation matrix between TPC of water and environmental parameter at Cherai showed that TPC was significantly positively correlated with Dissolved Oxygen and phosphate at 5% level and TPC was significantly correlated with temperature at 1% 0 level. In Valappu TPC was correlated with dissolved oxygen and nitrate at 1% level, while a negative correlation at 5% existed with salinity. The primary environmental factors influencing the sediment bacteria include moisture, temperature,
acidity, organic matter and inorganic ingredient supply. The influence of season occurs only from the combination of primary determinants. The number and types of *Bacillus and Lactobacillus* is determined by sediment type also. Valappu sediment was of clayey nature rich in organic matter which influenced the highest count of *Bacillus and Lactobacillus*. Alexander (1978) reported that bacterial density is influenced to a large extent by the organic matter content in the habitat. (Fig10 and 13) (Table1)

Valappu recorded the maximum no: of Bacillus and Lactobacillus during the study period which may be due to the tidal effect. Whenever maximum no: of Bacillus and Lactobacillus occurred, variety decreased. (Table 8 and 9). The minimum TPC was observed in monsoon and maximum TPC in post monsoon during the present study was found to be in accordance with the observation of Chandrika (1983). The heterotrophic bacteria of sandy beaches of Goa showed a maximum count of $134.46 \times 10^3 / \text{gm}$ and minimum of $0.47 \times 10^3 / \text{gm}$ in July and October. (Nair and Lokabharathi, 1980). This may be due to the sandy nature of the study area. Venketeswaran et. al., (1989) reported <2-80 cfu/gm in the sediment of the Seto island. The mean total viable counts ranged from $1.85 \times 10^5$ to $6.18 \times 10^6 / \text{gm}$ in shrimp; $1.80 \times 10^3 - 4.50 \times 10^3 / \text{gm}$ in rearing water and $1.82 \times 10^6 - 4.72 \times 10^6 / \text{gm}$ in sediment. Sharmila et. al., 1996 studied the bacterial flora of penaeid shrimp, *P.indicus*, pond water and sediment in a
semi-intensive surface water collected in September and October (1999) from fresh water marsh habitat showed bacterial density ranging from $9.7 \times 10^5$ to $1.3 \times 10^7$ cells/ml. The majority of 888 isolates were gram positive, of which 35% were bacillus spp. (Smith, T. W; Walker, E. D; Kaufman, M. G., 1998). Matrix correlation of TPC sediment showed negative correlation at 5% level with nitrate in Cherai, while in Valappu TPC showed no significant correlation with any of the environmental parameters in sediment. (fig 14and15)

TPC of shrimp intestine recorded a maximum during January 2002 ($160 \times 10^3$/gm) while a minimum of $98 \times 10^3$ was recorded during March. While Valappu station showed a maximum in March $26.8 \times 10^4$/gm while January recorded minimum $72.8 \times 10^3$/gm. The temporal and seasonal variations in the different environmental parameters are reflected in the quantitative and qualitative distribution of TPC. (Fig 13)

Correlation matrix of TPC at Cherai showed no significant correlation with any of the environmental parameters. At Valappu a significantly positive correlation existed between oxygen at 1% level and nitrate at 5% while salinity showed a negative correlation at 5% level. (Table 7and13).
This may be probably due to wide fluctuation in the water quality and the nature of pond substrate and organic enrichment in the sediment are the important factors restricting the abundance of TPC.

Singh (1986) observe that an increasing order of TPC from the alimentary canal of P.indicus. He has also observed that gram negative bacterial count was higher in intestine than body surface. Similar results were observed by Palaniappa (1982). Chandrasekharan (1985) has found that the bacterial flora varied from $10^6$-$10^7$/cm² in the body surface, $10^6$-$10^8$ gm in gills and $10^6$-$10^8$ in the intestinal content of P.indicus.

The low number of flora is present contrary to this low no: of flora in the present study indicates stress condition and wherever optimal conditions are obtained because of the dilution, low count has been observed.

Total Bacillus Count (TBC) from Cherai station recorded a maximum ranging between $22.2\times10^3$-$50\times10^3$/gm while a minimum of $1-2\times10^3$/gm was recorded. Valappu recorded a maximum of $79\times10^3$-$98\times10^3$/gm, while a minimum value recorded as $1-2\times10^3$/gm.

The bacillus counts were higher in Valappu when compared to Cherai and that the highest counts were recorded during monsoon season.
as it is found that matrix correlation of Cherai showed that significant correlation existed between total bacillus count and Ammonia at 5% level while at Valappu station 5% correlation existed with temperature, phosphate and nitrate.

This may be the reason for increased count in Valappu when compared to Cherai. Studies of Ostenvick et al., (2004) revealed that bacillus species from 8 different rivers were 15-1400 cfu/100ml while Binimol (2004) isolated $6.3-24 \times 10^9$/gm of bacillus from mangrove sediments. Variations in different environmental parameters and composition of sediments and substrate availability are reflected in the quantitative and qualitative distribution of bacillus. The flora composition exhibited a different picture in the Cherai and Valappu culture ponds.

Total lactobacillus count was recorded maximum in May ($210 \times 10^3$/gm) at Cherai while at Valappu showed maximum in July ($170 \times 10^3$/gm), rest of the months showed low count in both the station. Matrix correlation of Total bacillus Count at Cherai showed negative correlation at 5% level with pH of water while no correlation existed with other parameters. While in Valappu Total Lactobacillus exhibited 5% significance with phosphate and dissolve oxygen each. (Table 10 and 4)
Studies of Nair et. al., (1997) reported that LAB counts were always 1-2 log cycles in the case fishes from internal trade. Lactobacillus were present in fresh and brackish water fishes. But LAB was not present in ocean fresh fish. Reports of Ringo et. al., (1998) and Halami et. al., (1991) also revealed that lactic acid bacteria dominated in the intestine of fish and fowl.

8 species of lactobacillus were isolated from both Cherai and Valappu. *Lactobacillus acidophilus* dominated in both the station total of (22.28%), the other species *L. plantarum* (20.48%) *L. fermentum* (16.86%), *L. brevis* (11.44%), *L. curvatus* (10.24%), *L. casei* (6.62%), *L. butchenerii* (6.02%), *L. coryniformis* (3.61%) and *L. coprophilus* (1.20%). A total of 166 strains were isolated and identified using Bergy’s manual of determinative bacteriology (1974) and Cowan and Steel, (1977) from the two stations. (Table 30)

Forty seven strains of homofermentative rod shaped and 5 heterofermentative sphere shaped lactic acid bacteria were isolated by Tanasupawat et. al., (1998) from 4 kinds of fermented fish. Four strains were identified as lactobacillus pentoses and one strain as *L. plantarum*. About 90% of lactobacillus strains were isolated and characterised from fresh and frozen fish by Nair et. al., (1999).
All these studies in parallel to the present study shows that lactic acid bacteria are candidate species of the gut. They colonise in the gut and produce bacteriocin which may act antagonistic to gram negative fish pathogen. So the need to use antibiotics in future aquaculture can be reduced by periodically administering the flora. Seventy-eight strains of lactobacillus were isolated from fillets of vacuum-packed smoked and salted herring by Gancel et al. (1997).

Binimol (2004) isolated 4 species of Bacillus – B. subtilis, B. licheniformis, B. pumilus and B. oleronicus from Mangalavanam mangrove sediments. 86 Bacillus strains were totally isolated out of which bacillus species such as B. cereus and Bacillus subtilis representing from all the samples. Nirmala Thampuran recorded 30% B. subtilis, 40% B. coagulans, 10% B. alvei, 5% B. brevis and 5% B. pumilus from dried, barred and cured fish from canned fish. B. subtilis, B. pumilus occurred 20% each, B. alvei occurred 40% where as pickled fish harboured 44% B. subtilis, 28% B. coagulans, 8% B. pumilus and 4% B. alvei, B. brevis and B. megaterum each.

In the present study about 14 species of bacillus strains i.e., B. subtilis (44), B. sphaericus (24), B. pumilus (27), B. megaterium (39), B. firmus (22), B. circulans (15), B. alvei (n=8), B. polymyxa (n=10), B. brevis (11), B. coagulans (9), B. steareothermophilus (7), B. licheniformis (8),
B. maceran (3) and B. cereus (mycoides) (n=1) were isolated from the two stations Cherai and Valappu. (Table 25 and 26)

Ivanova et. al., (1999) isolated 20 aerobic sporeforming Bacillus of which only species of Bacillus, B. subtilis, B. cereus, B. licheniformis, B. firmus, B. pumilus, B. mycoides and B. lentus from the marine environment and in relation to the present study. B. subtilis and B. pumilus were most abundant species associated with marine environment. This may be so because the strains were able to utilise a wide range of organic compounds were halotolerant and alkali tolerant and reflect their great metabolic flexibility.

Quantitative variations in the different environment parameters and composition of sediments, substrate availability are reflected in the quantitative and qualitative distribution of Bacillus in the present observation. The flora composition exhibited a different picture in Cherai and Valappu aquaculture ponds. B. subtilis was dominant species in both the aquaculture pond which suggest that B. subtilis has got enzyme potential to degrade and refractory organic compounds received by high organic load through terrestrial inputs and anthropogenic influence in this environment. (Table 37)

The predominance of stress tolerant species getting mutated with adaptive enzymes existed with low microbial diversity wherever optimal
conditions are not met with. The enzyme potential shows an impact on environmental parameters which control the distribution of Bacillacea at the same time indicate the quantum of endurance warranted by the flora to tide over by the range of environmental stress. High microbial diversity of Bacillus species is to stabilise the ecosystem as the ecosystem and the flora is highly fragile.

Several taxonomic studies of bacillus and related species have been conducted by various workers. Ivanova et. al., (1999) isolated 16 representatives of the genus bacillus from seawater samples. Ivanova and Mikhail (1999) isolated 20 aerobic endospore forming bacillus. Binimol (2004) isolated 4 species from mangrove ecosystems. Further problems that arise in making comparisons are that different set of characters may have been used and the same test may not give the same results in different laboratories explaining the variations that occur in the certain tests for the classification of bacterial isolates (Bryant et. al., 1986). The present study helped to establish whether data collected from different times by employing standard techniques were reliable and could be combined to provide a useful taxonomic result.

The procedure for the identification of bacillus was exemplified by Bergy’s Manual of determinative bacteriology (1986) and Cowan and Steel’s (1977) Manual for identification of medical bacteria. The
comprehensive and practical description of conventional test media and methods set out in Bergy's Manual as appropriate and widely used for the characterisation of most of the bacillus species. All the strains of bacillus from Cherai and Valappu were gram positive rods which were highly motile.

The spore shapes were oval for most of the species except for *B. sphaericus* with round spores and the spores position were either central, terminal or subterminal. In most cases the spores were central in position. In relation to the present study Alexander, 1977 reported that 60-100% of soil bacilli population exist in the inactive spore state. Claus and Berkeley (1986) reported that genus bacillus count of a large no: of diverse rod shaped gram positive bacteria that are motile by peritrichous flagella and are aerobic. The numbers are capable of producing endospores that are highly resistant to unfavourable environmental condition.

79.54% of bacillus from Valappu and 96.9% of bacillus strains from Cherai showed a high tolerance at 45°C while temperature tolerance at 65°C was exhibited only by strain *B. stearothermophilus* from both stations. (Table 25 and 26) Alkali tolerance of 5% was exhibited by 77.2% of strain from Cherai and 71.95% of strains in Valappu station. Halotolerance at 7% NaCl was exhibited by 69% of strains from Cherai and 73.3% of strains from Valappu. The study shows that bacillus species exhibited a high
salinity, temperature and alkali tolerance which make them suitable as pond probiotics.

All the strains from both Cherai and Valappu produced the enzyme catalase by cytochrome oxidase was produced by 88.4% of *B. subtilis* only. Indole production and Arginine dihydrolysis was weak in all the strains of bacillus. Nitrate was reduced to nitrite by 81.06% of bacillus from Valappu and 67.6% of strains from Cherai station. This shows that Bacillus species exhibited a high enzyme potential capacity. Most species of Bacillus can grow anaerobically at the expense of sugars. They carry out a distinctive fermentation, in which 2,3-butanedioil, glycerol and CO₂ are the major end products accompanied by small amounts of lactate and ethanol. The fermentation can be represented as:

\[3 \text{ glucose} \rightarrow 2,2,3\text{-butanediol} + 2 \text{ glycerol} + 4 \text{ CO}_2.\]

Stainer (1976) reported glucose is initially dissimilated through Embden and Meyerhof pathway to the level of triose phosphate at which point a metabolic divergent occurs. Pyruvate is formed from part of the triose phosphate from which butanediol and CO₂ are produced. *Bacillus subtilis* cannot grow anaerobically at the expense of glucose as it cannot reduce triose phosphate to glycerol where as *B. licheniformis* can grow anaerobically at the expense of organic substrate when furnished with
nitrate it is the only bacillus with a vigorous denitrifying capacity under anaerobic condition.

In the present study all the strains of *B. subtilis* from Valappu and Cherai fermented glucose while only 85.5% of strain from Valappu and 86.05% of strains from Cherai exhibited a high glucose fermentation capacity. Chandrika (1999) stated that all strains isolated from Cochin backwaters exhibited fermentation of glucose while *Bacillus polymyxa* and *B. macerans* form spores with distinct star shaped profile to the spore. Both are fermentative organisms dissimilating starch and pectin as well as monosaccharides and good growth will occur only in the presence of utilisable carbohydrate like glucose. Another distinctive property of *B. polymyxa and B. macerans* is the ability to fix nitrogen when grown under anaerobic conditions. They are the only Bacillus species known to possess this property.

Arabinose fermentation was exhibited high, by *B. subtilis* (77.7%), *B. circulans* (71.4%), *B. polymyxa* (83.3%), *B. pumilus* (54.5%). While only few strains showed arabinose fermentation for other species in Cherai. While xylose and mannitol fermentation was exhibited by 77.7% and 61.11% of *B. subtilis*, 81.81% and 36.3% of *B. pumilus*, 71.42% of *B. circulans*, 100% and 33.3% of *B. polymyxa*, 100 and 33.3% of *B. licheniformis*, from Cherai station while in Valappu station 100% *B. macerans*, 75% *B. polymyxa*, 40% *B. licheniformis*, 62.5% of *B. pumilus*, 136
50% *B. subtilis* fermented arabinose, while xylose and mannitol fermentation was low in most of them except 75% of *B. circulans* which exhibited a high xylose fermentation.

According to Chandrika (1999) 95% of bacillus exhibited Gelatin liquefaction and 100% hydrolysis of casein was exhibited by the isolated strains. 83.3% of isolates hydrolysed starch in 24 hours incubation time. While in the present study 89.2% of strains from Cherai and 72.14% of strains from Valappu exhibited a starch hydrolysis capacity showing their amylolytic potential. The starch hydrolysing enzymes are usually inducible but the ability of microorganism to form amylolytic enzymes depends on the type of starch.

In the present study gelatin liquefaction was exhibited by all strains of *B. subtilis* from both Cherai and Valappu and more than 80% of *B. megaterum*, *B. steareothermophilus*, *B. licheniformis* from Valappu station exhibited gelatin hydrolysis while more than 80% of strain of *B. pumilus*, *B. megaterum*, *B. circulans*, *B. polymyxa*, *B. brevis*, *B. licheniformis* and *B. sphaericus* from Cherai exhibited gelatin hydrolysis showing their versatility for proteolytic activity.

In relation to the present study, Hitchfeld and Wood (1996) found that by proteolytic activity of bacillus higher enzyme yield was obtained.
50% *B. subtilis* fermented arabinose, while xylose and mannitol fermentation was low in most of them except 75% of *B. circulans* which exhibited a high xylose fermentation.

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In relation to the present study, Hitchfeld and Wood (1996) found that by proteolytic activity of bacillus higher enzyme yield was obtained.
When protein was suspended in medium than when dissolved in the medium. Chandrika (1983) studied that 36 bacillus from sediments of Cochin backwaters showed high proteolytic, ureolytic and caseinolytic activity. Wood (1959) isolated proteolytic bacillus from surface water and bottom sedimentary 1 metre depth, the genus Bacillus dominated predominated and formed 22% of surface water 39.5% from 1m and 45% from bottom sediments. New species produce specific enzymes are being studied with reclassification of certain species and variants for use as probiotics and DMS-range of bacteria to enhance intensive shrimp fish culture. In the present study casenolytic activity was exhibited by 80.4% of the strain from Cherai while only 78.1% of strains from Valappu exhibited caseinolytic activity. Murchelano and Brown (1970) reported that in all the seasons proteolytic, amylolytic activity of marine bacteria isolated from long island Sound was highly stable even though there was variation in Genera obtained.

In the present study ureolytic activity was exhibited by only 51.04% of isolates from Valappu, while only 43.9% of the isolates from Cherai exhibited ureolytic activity parallel to the present study. Chandrika (1999) recorded 44.4% of the total flora isolates from Cochin backwaters.

\[ \text{CO}(\text{NH}_2)_2 + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3 \]
The ecological advantage of spore formers is their strong ureolytic activity. In an enrichment medium wide variety or chemo heterotrophlis can grow in pure culture on this medium. While incorporating these flora in the preparation of probiotic the pH has to be taken care of as high concentration of free NH$_3$ can make the medium alkaline.

Many microorganisms possess the enzyme urease, the catalyse responsible for hydrolysing urea. Urease is a constitutive enzyme in other it is an inducible enzyme. Ammonium carbonate is an intermediate compound in the urea hydrolysis. *Bacillus, micrococcus, pseudomonas, klebsiella, pseudococcus, clostridium* are the flora actively hydrolising urease. 32.69% of the flora are capable of hydrolysing urea from sediment. (Table 25 and 26)

In the studies of Austin, (1988) *B.maximus* isolate did not degrade urea at all. Voges Proskauer reaction was also negative. While in the present study, 70.07% of *B.subtilis*, 43.75% of *B.pumilus*, 50% of *B.alvei* and *B.polymyxax* and 40% of *B.licheniformis* and all strains of *B.cereus* degraded urea at Valappu while in Cherai only lesser no: of strains degraded urea besides *B.subtilis* 61.1%, 54.54% of *B.pumilus*, 66.6% of *B.licheniformis* and 50% of *B.polymyxax*. Studies of Chandrika (1999) reported that 95% of the isolate showed positive Voges Proskanes reaction showing the ability to produce acetoin. Tyrosine decomposition
was reported only in 33.15% of isolates from Cherai while 50.27% isolates from Valappu station decomposed tyrosine.

The procedure for identification of lactobacillus was exemplified by Bergy's manual of determinative bacteriology (1976). (Table 30 and 31). All the strains of lactobacillus from Cherai and Valappu shows gram-positive reaction with long slender rods which were non motile and without the enzyme catalase. Temperature tolerance at 15°C was exhibited by 91.7% of lactobacillus strain from Valappu and 80.6% of strains from Cherai while 45°C was tolerated by 95% of L.acidophilus and 86.15% of L.fermentum only from both the stations.

Nitrate was reduced to nitrite by 25% of L.buchnerii from Cherai station while 36.67% of lactobacillus strains from Valappu station reduced nitrated. Glucose fermentation was found in almost all strains of lactobacillus isolated from both Cherai and Valappu. As many complex compounds must be transformed to simpler forms prior to use by the organisms.

\[
C_6H_{12}O_6 \rightarrow 2C_3H_4O_3 + 4H
\]

Glucose  Pyruvic acid

B.amylase are not common in micro-organisms. The maltose and low molecular weight oligo saccharides that are converted to glucose by
mediation of the enzyme $\alpha$-glucosidase so that starch is transformed ultimately to glucose. The simple sugars are water soluble and penetrate the cells to be used as energy sources for growth and protoplasmic synthesis.

Mannose is essentially the only sugar which are present in some marine plants apart from galactose. The Mannose : Glucose ratio in the environment will be always 2:1. In the present study 75.9% of the strains from Cherai and 70% of strains from Valappu fermented mannose. 66.5% of strains from Valappu and 72.3% of strains from Cherai showed cellobiose fermenting capacity. Fructose and galactose, lactose and maltose was fermented by almost all the strains of lactobacillus from both Cherai and Valappu. While rhamnose and xylase fermentation was weaker with most of the strains.

Mannitol was fermented by 81.25% of L.plantarum and 92.8% of L.casei from Cherai and Valappu. Ribose was fermented by 61.8% of strains from Valappu and 79.2% of strains from Cherai while xylose and rehalose fermentation was weaker in all the strains. In 1947, Rosenfeld and Zobell carried out a detailed study of antibiotic producing marine microorganisms. Although they did not attempt an isolation of specific antibiotic produced by marine bacteria. It was evident from their work that various aspect of microorganisms, indigenous to the sea, released anti-
microbial substance. Since then, there have been several reports of bacteria with inhibitory effects isolated from seawater; the main purpose of these mostly have been to characterise the specific antagonistic compounds or bacteriocins produced. Only recently have bacteria been isolated from other marine habitats.

Although the microbiology of the intestinal tracts of marine and fresh water fish has been investigated by many researchers, few studies have addressed the production of inhibitory components by these bacteria based on the proteolytic activity, enzyme potential, alkali tolerance, temperature tolerance and halotolerance. The highly potential species of \textit{Bacillus subtilis} and \textit{Lactobacillus acidophilus} was selected for the study. (Table 37 and 38)

In the present study, growth and survival of \textit{Penaeus monodon} juveniles were greater treated with probiotic bacteria (\textit{Bacillus subtilis} and \textit{lactobacillus acidophilus}) were greater when compared to the control. Significant difference were found between the treatment control tanks. The bacillus subtilis shrimps showed higher growth and survival rate than lactobacillus treated shrimps.

Parallel to the present study (Rengipipat et. al., 1998, a,b) reported that growth and survival difference between bacillus – treated and non
treated *P. monodon* were more pronounced when Rengpipat et. al., 2000 reported that no significant difference was obtained with all treatments.

In the present study the better growth rate may be due to the effect of probionts on nutritional profile and also on their ability to colonise the intestine and improve their feeding efficiency by producing some microbial enzymes or artemia were reared exclusively on a diet of bacteria while *B. plicatilis* has also grown with bacteria (Yasuda and Tata, 1980; Gatesoupe et. al., 1989). (Fig 18 and 19)

Intriago and Jones (1993) reported best growth of artemia to pre-adult stage on a mixture of flexibactis and algae and concluded that bacteria acted not only as food, but also aided digestion of algae through the presence of exo enzyme. Recently their apparatus have been used to improve shrimp health and yields. First, use of specific disease-resistant shrimp (SPF) second, vaccination or immuno-stimulation of shrimp to promote immune response. Lastly probiotic used to stimulate immunity and to exclude pathogen. In addition, stress reduction is used to improve disease susceptible to less virulent pathogen. In these cases, the pathogens are often considered secondary infections (Song et. al., 1993) since shrimps possess a non-specific immune response (Anderson, 1992), vaccination or immuno stimulation may provide only short term protection against specific pathogen (Sing and Song, 1996, Sing et. al., 1996).
Effective probiotic treatment on the other hand may provide broader-spectrum and greater non-specific disease protection as a result of both serological immunity enhancement and competitive exclusion in shrimp gut. At variance is the study of Gildberg et. al., (1998) who observed highest mortality in fish given the diet containing lactic acid bacteria, where as no significant difference was observed between fish given feed supplemented with cod muscle protein and hydrolysed cod muscle protein. Besides the production of anti-microbial substance, a great variety of mechanism have been proposed for the action of probiotics (Monter and Pugh, 1993). Eg. Competition for adhesion receptor in the intestine, competition for nutrients and immuno-stimulation. Further investigation on these lines throw more insight into the actual mechanism of probiont action. (Fig 20 and 21)

The present study showed the presence of viable *Bacillus subtilis* and *Lactobacillus acidophilus* in intestine and faeces of shrimp fed with these probionts Competitive exclusion most likely occurred in this case. Water quality is not affected by probiotic feed additive. The water quality is maintained throughout the experiment (Rengpipat et. al., 1998 a) but shrimp health. Prophylactic functions can be enhanced (Austin et. al., 1992, 1995; Rengpipat et. al., 1998 a, b; Phianphak et. al., 1999). At the same time, probiont will proliferate in rearing water thus providing a better
environment for shrimp by reducing the level of certain pathogens in the culture water (Moriarty, 1998). (Fig 22 and 23) *Bacillus subtilis*, a saprophytic strain appears harmless to shrimp survival during normal culture (Rengipipat et. al., 1998 a). This suggests that probiotic treatment is an effective alternative for enhancing shrimp health.

The research on 'probiotics' has formed the focal point of the present study as well as representing the development of knowledge required by serious and responsible fish farmers. The global fish farming industry is characterised by dynamism and change. There is a constant stream of new knowledge and the result of research are published all the time. New species are being used in production and a combination of indigenous new Bacillus species *B. subtilis* - enzyme potential, *B. polymyxa* – pH tolerance, alkali tolerant *B. licheniformis* - thermo tolerant and halotolerant showed all the 4 Bacillus isolated in the present study can be used in probiotic production as unique production methods are seeing the light of the day.