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Probiotics: The New Ecofriendly Alternative Measures of Disease Control for Sustainable Aquaculture

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ABSTRACT

Diseases are considered to be the major constraint in aquaculture production. They cause mortality in shrimp larviculture and fish hatcheries. They are also a constraint on consistent production of fish and shell fish. Traditionally, the control of diseases in aquaculture has relied on the use of chemical compounds. More recently probiotic microorganisms and vaccination or other forms of immunostimulation have also been employed. The abuse of antimicrobials can result in the development of resistant strains of bacteria. Such resistance can be readily transferred to other strains, either following alterations to the existing genome or by transfer of genetic material between cells through plasmids or bacteriophages. The massive use of antibiotics for the control of diseases has been questioned by acquisition of antibiotic resistance in disease causing agents and the need of alternative measures to control these diseases is of prime importance. In recent years, probiotics have a center stage and are used as alternative measures to control the fish diseases. Probiotics have been used by man for millennia since the time humans first consumed fermented milk products. Probiotics can be essential for the normal digestive, endocrine and immunological functions of the bowel. They inhibit pathogenic microorganisms and have been used therapeutically to treat a variety of gastrointestinal and even systemic disorders. Probiotics transiently colonize the bowel and except when used to treat an acute disorder, must be regularly consumed to maintain benefit. Use of microbial probiotics to promote health maintenance and disease prevention and control is now widely accepted as the new ecofriendly alternative measures for sustainable aquaculture.

Key words: Aquaculture, fish, diseases, antibiotics, probiotics

INTRODUCTION

Aquaculture provides opportunities for the production of wide variety of aquatic foods including fish and shell fish. Fish is a vital source of food for man. It is very important protein source particularly in regions where livestock is relatively scarce. It is the most important single source of high-quality protein, providing approximately 16% of the animal protein consumed by the world's, population. The total animal protein intake to human diet, fish contribute 26.2% in Asia, 17.4% in Africa, 9.2% in Europe, 7.4% in North and Central America and 7.2% in South America (FAO, 2006). About one billion people world-wide depend upon fish as their primary source of animal protein. The value of fish traded internationally comes out to be US$ 51 billion per annum. Over 36 million people are employed directly through fishing and aquaculture (FAO, 2000) and as many as 200 million people derive direct and indirect income from fish (FAO, 2006).

Consumption of food fish is increasing, having risen from 40 million tones in 1970 to 86 million tones in 1998 and is expected to reach 110 million tones by 2010. Aquaculture has evolved as the fastest growing food-producing sector and has been developed as an important component in the global food security (FAO, 2000).

Aquatic diseases make the biggest constraint in aquaculture production. This is because; water makes a very suitable medium for the proliferation of several diseases in aquatic animals. Bacterial fish diseases like dropsy, hemorrhagic septicaemia, edwardsiellosis/edwardsiella septicemia, bacterial kidney disease, bacterial gill disease, pop eye, vibriosis, Epizootic Ulcerative Syndrome (EUS), ulcerative disease and fin and tail rot were reported by different workers from different parts of the globe in fresh water fishes (Gahlawat *et al*., 2006). Bacterial pathogens cause heavy mortality in both cultured and wild fish/shell fish species over the world (Bader *et al*., 2003; Ampofo and Clerk, 2002; Jakhar *et al*., 2010a).

In the past, several methods have been tried to control the diseases of aquatic animals. These include hygiene maintenance, use of chemicals and use of antibiotics. High stocking densities and over feeding become the cause of aquatic pollution. Entry of wild fish or restocking of ponds with unhealthy or sub quality eggs is another source of ponds becoming unhygienic. Hygien maintenance is, therefore, the most important part of prevention of aquacultural diseases (FAO, 2008). Several chemicals have been recommended for the prevention and control of aquacultural diseases. Simple disinfectants like permanganate, benzalkonium chloride solution or calcium cyanamide have been used as prophylactic measures. Besides these, there are other chemicals in use e.g., formalin (Klontz, 1979), organophosphates (Burridge and Haya, 1995), malachite green and copper sulphate (Gomez-Gil *et al*., 2000), quaternary ammonium compounds (Snieszkd, 1981), furazolidane (Samuelsen *et al*., 1998), sulphadiazine (Capone *et al*., 1996), epson salt and di-n-butyl tin oxide (Burridge and Haya, 1995). Some of these chemicals are highly disease specific whereas others are highly non-specific. Their over doses can pollute the water bodies too.

As a replacement to chemicals, later on, the control of aquacultural diseases was focused on the use of chemotherapeutants, such as erythromycin and deoxycycline (Munday, 1994). At present many antibiotics used in veterinary therapy are commonly used for the control of aquacultural diseases (Smith, 1996). The indiscriminate worldwide use of antibiotics in aquaculture has, however, led to the development of drug-resistant bacteria which are becoming increasingly difficult to control and eradicate (Aoki *et al*., 1980; Aoki and Watanabe, 1993; Hayashi *et al*., 1993; Aoki *et al*., 1995; De Paola, 1995; Sahul and Balasubramanian, 2000; Van der Waaij and Nord, 2000; Bruun *et al*., 2000; Miranda and Zemelman, 2002). Besides development of drug resistant bacteria and pathogens, the adverse effect of antibiotics is caused by their influence on the aquatic microflora and the retention of harmful residues in aquatic animals (Fuller, 1989; Esiobu *et al*., 2004; Sartar *et al*., 2007). Therefore, to keep a sustainable aquacultural growth, health management strategies must go beyond antibiotics and chemotherapeutics. Although, now focus is increasingly being shifted to vaccination (Eldar *et al*., 1997; Klesius *et al*., 2000; Evans *et al*., 2004), yet the dangers of ill effects of antibiotics cannot be ruled out. Of late, the method that is gaining recognition for controlling pathogens within the aquaculture industry is the use of beneficial or probiotic bacteria (Ringo and Gatesoupe, 1998; Gatesoupe, 1999; Ringo and Birkbeck, 1999; Verschuere *et al*., 2000; Irianto and Austin, 2002; Dahiya *et al*., 2010; Sihag, 2010). These probiotic bacteria prove harm less to the host as well as human being and result in improved resistance in the host against infectious diseases. Therefore, the objective of prevention and control of disease can be achieved by the use of probiotics. The use of probiotics will prove a new ecofriendly alternative measure for sustainable aquaculture.

DISEASES IN FISH

Several bacteria have been reported as pathogenic to fish and shell fish. These are either obligate or facultative pathogens. The facultative bacterial pathogens prove a potential threat when fish are under environmental and physiological stress (Wedemeyer, 1970). Six gram negative rods (*Aeromonas, Proteus, Citrobacter, Pseudomonas, Flavobacterium* and *Chromobacterium*) and three gram positive cocci (*Micrococcus, Streptococcus* and *Staphylococcus*) genera of bacteria have been characterized as potential pathogen *Aristichthys nobilis* and *I. idella* fingerlings (Shamsudin, 1986; Welker *et al*., 2005; Verma *et al*., 2006). Likewise, three bacteria (viz. *Vibrio anguillarum, V. alginolyticus* and *Aeromonas hydrophila)* have been reported to cause pathogenecity in Indian magur (*Clarias batrachus* L.) (Dahiya and Sihag, 2009; Dahiya *et al*., 2009). Fresh water prawns are also not the exception (Jakhar *et al*., 2010a). *Aeromonas hydrophila, Pseudomonas flourescens* and *Enterobacter aerogenes* were found to cause bacterial necrosis in *Macrobrachium rosenbergii* (Jakhar *et al*., 2010b) whereas *Micrococcus luteus, M. varians, Cellabiosococcus scuri, Streptococcus* grp Q1 and *Staphylococcus aureus* were found to cause tail rot disease in this animal (Jakhar *et al*., 2010c).

Over the past two decades, Epizootic Ulcerative Syndrome (EUS) has had serious impact on global fisheries resulting in heavy economic losses. It is one of the most destructive diseases amongst fresh and brackish water fish in the Asia-Pacific region. This disease has spread through rivers, reservoirs and paddy fields to neighboring states, causing considerable loss to fish farmers (Bondad-Reantaso *et al*., 1992). The first recognized account of a EUS-like condition occurred in Japan in 1971 (Egusa and Masuda, 1971). Later on, similar disease was reported in Australia (1972), Papua Guinea (1975), Indonesia (1980), Thailand (1980), Malaysia (1981), Burma (1984), Laos (1984), Kampuchea (1984) and Srilanka (1988) (Cerro *et al*., 2002). In natural outbreaks, several fish species, especially air breathing fishes, had been affected by EUS (Roberts *et al*., 1994). A diverse group of biotic agents such as viruses, bacteria and cutaneous ectoparasites may initiate skin lesions which are subsequently colonized by a fungus (*Aphanomyces invadans)* and ultimately lead to EUS (Lilley, 1992; Cerro *et al*., 2002). Different pathogenic organisms, including bacteria (Karunasagar *et al*., 1995; Pal, 1996), fungus (Roberts *et al*., 1993) and viruses (Frerichs *et al*., 1985; Davies and Frerichs, 1989) have been isolated from naturally infected fish. Different bacterial species isolated from the diseased fish include *A. punctatus, Flavobacterium* sp., *Pseudomonas* sp., *E. tarda, V. parahaemolyticus* and *Streptococcus* sp. (Kumar *et al*., 1986).

DISEASES AS CONSTRAINT IN AQUACULTURE PRODUCTION

Disease outbreak is being increasingly recognized as one of the most important constraints in aquaculture production in many countries (Nicolas *et al*., 1989; Grisez *et al*., 1997; Riquelme *et al*., 1997). Commercial fish farming creates favorable conditions for the development of infectious diseases in fish. With the increasing fish culture activities, several bacterial diseases, causing morbidity and mortality in fish have been reported in fresh water aquaculture. Pathogenic microorganisms (bacteria, fungi, viruses and protozoan) generally enter the fish through the gills, the skin or gastrointestinal tract (Birkbeck and Ringo, 2005). These three routes of entry represent physical and immunological barriers against pathogens. Thus, their integrity, both at the cell and tissue levels, is vital for the later outcome of the host-pathogen interaction.

Bacterial diseases in fishes were broadly classified as surface ulcerative, acute systemic and chronic granulomatous types. Surface ulcerative types of diseases were characterized by haemorrhagic surface ulcers and were caused by species of *Aeromonas*, *Pseudomonas*, *Vibrios*, *Flexibacter* and *Myxobacter*. These infections lead to acute systemic diseases with passage of time.

Systemic disease is characterized by the presence and proliferation of bacteria in internal organs like kidneys, heart, spleen, blood and other visceral organs. These diseases produce necrotic changes like bacterial haemorrhagic septicaemia in all affected organs and cause mass mortality. Chronic granulomatous type of disease conditions are characterized by formation of granulomas with the initiating bacteria in the centre of glands (Kumar *et al*., 1986; Dey, 1989).

Histopathology of EUS affected fishes were studied by various workers in different species of fishes in India and abroad (Kumar *et al*., 1986; Dey, 1989; Mukherjee *et al*., 1991; Nayak, 1993; Roberts *et al*., 1994; Cerro *et al*., 2002). The changes associated with the disease include cellular infiltration in the hypodermis and epidermis, oedema, degeneration of kidney tubules, depletion of lymphoid cells of spleen and congestion of cerebral tissue. In the initial stages, the disease is marked by little red spots on the skin surface which progresses in size until eventually, a circular to oval deep haemorrhagic ulcer exposing the skeletal musculature is visible. Studies reveals that there is complete loss of epidermis in the ulcerative area of the skin where hypodermis shows characteristic granulomatous changes (Dey, 1989). Most of the hepatocytes showed cloudy swellings. In case of Chinese carp (grass carp and common carp), the affected fish showed ulcer and deep red hemorrhagic lesions in caudal peduncles. Ecological studies carried on *Aeromonas* sp. and *Pseudomonas* sp. present in mrigal (*Cirrihinus mrigala*) in cultured ponds revealed occurrence of high percentage of these bacterial species in EUS affected fishes (Iqbal *et al*., 1999). The microbial flora of the liver, gills, intestine and muscle of murrel (*Channa striatus*), infected with Epizotic Uncreative Sgndrome (EUS), was estimated quantitatively and qualitatively. The most predominant bacterial isolates in all the samples were *Aeromonas hydrophila, Enterobacter* sp., *Vibrio* sp., *Pseudomonas* sp., *Escherichia coli,* along with *Aphanomyces invadans* and *Aspergillus* sp. Total viable microbial count was highest in gills ($5.9\pm0.5\times10^{7}$ cfu (colony forming units) g^{-1}) and lowest in intestine ($8.7\pm1.8\times10^{4}$ cfu g^{-1}) which could reveal the presence of bacterial and mycotic species. This observation confirms the association of some bacteria with fungus in EUS disease (Dhanaraj *et al*., 2008). This disease was reported to occur due to a mixed infection of *A. hydrophila* and *P. fluorescens* which had been identified as the most commonly occurring bacterial agents of fish disease (CIFA, 2003). Besides bacteria and fungi, other microorganism like viruses and parasites were also found to be associated with epizootic ulcerative syndrome (Dey, 1989; Kumar *et al*., 1991; Chowdhury *et al*., 2003).

The extensive observational studies made during EUS outbreaks in Karnataka and Tamil Nadu (South India) revealed that Indian Major Carp (IMC) present in many water bodies were not affected by this disease (Vishwanath *et al*., 1997, 1998). IMC appeared resistant in South India but not in North India. There was an anomaly in susceptibility of IMC with regard to geography (Chinabut and Roberts, 1999). This perhaps was due to the fact that the temperature in South India was high enough for the IMC to resist infection (Roberts *et al*., 1994). Surprisingly, during severe EUS outbreaks in several North and Northeastern states, IMC were found mildly affected whereas other species like *Puntius*, *Channa*, *Mastacembalus* etc. were found severely affected in southern states (Kumar *et al*., 1991; Das, 1997). EUS outbreaks in freshwater fishes generally occur during winter months and after periods of heavy rainfall coinciding with a fall in water temperature (Lilley *et al*., 1998). These conditions favoured sporulation of *Aphanomyces invadans* (Lumanlan-Mayo *et al*., 1997) and low temperature had been shown to delay the inflammatory response of fish to fungal infection (Chinabut *et al*., 1995; Catap and Barry, 1998). In all the North and Northeastern states of India, during winter months the temperature normally goes below 20°C which is ideal for EUS outbreaks. The fingerlings and advanced fingerlings of IMC as compared to adult suffer heavy mortality during disease outbreak (Mohan, 2003).

The higher concentration of certain chemicals in water was the predisposing factor for bacterial fin and tail rot disease in fishes (Chakrabarti, 1994). Bacterial gill disease or gill rottening was caused by *A. hydrophila*, *Flavobacterium columnaris* and *Myxobaterial* organisms in common carp (*Cyprinus carpio*) which showed gill hyperplasia (CIFA, 2003). Fin and tail rot disease was reported in large number of fresh water fishes in India (Kumar *et al*., 1986; Karunasagar *et al*., 1989; Jhingran, 1991). During this infection, a white line was observed on the margin of fin, spreading as an imparting frayed appearance on the appendages which later on eventually putrefies and disintegrates. In gill rot disease, gills showed hyperplasia of the secondary lamellar epithelium, fusion of lamella, clubbing of gill tips, necrosis of primary and secondary lamellae of the gill tubular epithelial cells and in some cases oedema and hypotrophy changes in the affected tissues (Dey, 1989).

TRADITIONAL METHODS OF DISEASE CONTROL IN AQUACULTURE

Hygiene maintenance: Hygien maintenance is the most important part of prevention of fish dieases. In traditional methods, pond hygiene used to be the first priority of pond management. Pond must be periodically dried and then refilled with abundant water of good quality. Pond should be well maintained to avoid silting and weeds are controlled. High stocking densities, over feeding and pollution are avoided. Entry of wild fish or restocking of ponds with unhealthy or sub quality eggs and young fish are prevented. Too frequent handling, transfer or transport of pond fish is avoided. The condition of gills must be periodically checked in random samples of the selected fishes. As soon as the onset of a disease is noticed, the seriously affected, moribund or the dead fish must be removed from ponds and buried with quicklime (FAO, 2008).

Use of chemicals: Prophylactic measures are taken to disinfect the pond and the gears. The nets and other tools are routinely disinfected with benzalkonium chloride solution. Ponds having fish and threatened with disease outbreak are disinfected with either potassium permanganate (0.5 g/100 L) or benzalkonium chloride solution (600 ppm) or calcium cyanamide.

Formalin (40% formaldehyde) is a traditionally tried chemical. It is very effective at concentrations of 167 to 250 mg L^{-1} (Klontz, 1979) against parasitic infections of protozoan ectoparasites (*Costia*) and trematodes like *Discocotyle*. Organophosphates are useful for such ectoparasites where formalin fails. These are effective against crustacean parasites like *Lernaea* and *Argulus* and such trematodes as *Gyrodactylus* (Burridge and Haya, 1995). Malachite green is the next most tried chemical, especially effective against fungal infections including *Saprolegnia*. It is used at a concentration of 1-2 mg L^{-1}. Copper sulphate is effective against bacterial infections but it is not favoured for reasons of its toxic effects (Gomez-Gil *et al*., 2000). Quaternary ammonium compounds are very effective against bacterial infections. These are generally used at concentrations of 1-4 mg L^{-1} (Snieszkd, 1981). Furazolidane, a nitrofuran, popular in veterinary therapy, is used in USA for incorporation in fish food to control/prevent bacterial infection; the recommended dose is 11 g/100 kg fish/day for about a week (Samuelsen *et al*., 1998). Sulphadiazine, a sulphonamide, mixed with trimethoprim, is also found to be equally effective against bacterial diseases when administered through diet; the recommended dose is $5^{1}/_{2}$ g/100 kg fish/day for a week (Capone *et al*., 1996). These chemicals are, however, non specific with low efficacy, that is why, these are less reliable. Epson salt (magnesium sulphate) mixed with diet when given is found very useful in treatment of protozoan parasites of the gut. Di-n-butyl tin oxide also

administered via diet with a dose of 25 g/100 kg fish/day for three days is effective against most parasites of the gut other than protozoans (Burridge and Haya, 1995).

Use of antibiotics: Earlier, the control of fish diseases was focused on the use of chemotherapeutants, such as erythromycin and deoxycycline (Munday, 1994). Oxytetracycline an antibiotic used in veterinary therapy is commonly used for bacterial therapy of vibriosis and ulcer disease in fishes; the recommended dose is $7^1/_2$ g/100 kg fish/day for one to two weeks (Smith, 1996). But now focus is increasingly being shifted to vaccination (Eldar *et al*., 1997; Klesius *et al*., 2000; Evans *et al*., 2004). Combined with the problem of antibiotic contamination of aquaculture facilities and livestock, the indiscriminate worldwide use of antibiotics in aquaculture has led to the development of drug-resistant bacteria which are becoming increasingly difficult to control and eradicate (Aoki *et al*., 1980; Aoki and Watanabe, 1993; Hayashi *et al*., 1993; Aoki *et al*., 1995; De Paola, 1995; Sahul and Balasubramanian, 2000; Van der Waaij and Nord, 2000; Bruun *et al*., 2000; Miranda and Zemelman, 2002). To keep a sustainable growth pattern, health management strategies must go beyond antibiotics and chemotherapeutics which create resistance in the bacteria and immunosuppression in the host. Besides development of drug resistant bacteria and pathogens, the adverse effect of antibiotics is caused by their influence on the aquatic microflora and the retention of harmful residues in aquatic animals (Fuller, 1989; Esiobu *et al*., 2004; Sartar *et al*., 2007). Subsequently, certain antibiotics such as chloramphenicol have been banned in many countries (Roberts, 1997; FAO, 2000).

Use of probiotics in aquaculture: A growing concern for the high consumption of antibiotics in aquaculture has initiated a search for alternative methods of disease control. One of the methods gaining recognition for controlling pathogens within the aquaculture industry is the use of beneficial or probiotic bacteria (Ringo and Gatesoupe, 1998; Gatesoupe, 1999; Ringo and Birkbeck, 1999; Verschuere *et al*., 2000; Irianto and Austin, 2002). Use of probiotics has been highlighted by many workers (Yazid *et al*., 1999; Chang and Chen, 2003; El-Naggar, 2004; Ghafoor *et al*., 2005; Igbasan *et al*., 2005; Kabir *et al*., 2005; Trachoo and Boudreaux, 2006; Anukam, 2007; Deeseenthum *et al*., 2007; Anukam and Koyama, 2007; Raj *et al*., 2008; Hung *et al*., 2008; Radfar and Farhoomand, 2008; Capcarova *et al*., 2008; Fazeli *et al*., 2008; Patel *et al*., 2008; Soundarapandian and Sankar, 2008; Vamanu *et al*., 2008; Vijayabaskar and Somasundaram, 2008; Abdelhamid *et al*., 2009; Al-Otaibi, 2009; Raja *et al*., 2009; Nasrollah, 2009; Dahiya *et al*., 2010; Nikfar *et al*., 2010; Sihag, 2010; Vamanu and Vamanu, 2010; Agouz and Anwer, 2011; Bansal *et al*., 2011; Gharaei-Fathabad and Eslamifar, 2011; Yesillik *et al*., 2011). Improved resistance against infectious diseases can be achieved by the use of probiotics.

Probiotics are live microorganisms administerted in adequate amounts as feed or food supplements which have beneficial effects on the intestinal microbial balance of the host. These are emerging as significant microbial food supplements in the field of prophylaxis (Geovanny *et al*., 2007). In aquaculture, the term Aprobiotics@ is often loosely used to describe a microbial formulation responsible for biocontrol or bioremediation of pathogens. The term probiotics comes from the Greek word "pro bios" meaning "for life". The original definition of probiotics given by Lilly and Stillwell (1965) "substances produced by one protozoan that stimulated the growth of another" was expanded from an agricultural perspective and redefined as "a live microbial feed supplement which beneficially affects the host animal its intestinal microbial balance" (Fuller, 1989). Probiotics are commonly defined as mono- or mixed cultures of live microbes that,

when applied to animal or human, generate a beneficial effect on health of the host. These beneficial effects include disease treatment and prevention as well as improvement of digestion and absorption in the host (Havenaar and Huis, 1992).

However, according to Salminen *et al.* (1999), probiotics include microbial cell preparations or microbial cell components. Gatesoupe (1999) redefined probiotics for aquaculture as microbial cells that are administered in such a way so that these could enter the gastrointestinal tract of the aquatic animal to be kept there alive, with the aim of improving its health. According to Verschuere *et al.* (2000), "the ability of a probiotic is to modify the host-associated or ambient microbial community and to improve the quality of its surroundings".

Various bacteria commercially used as probiotics are: *Lactobacillus* species viz. *L. acidophilus, L. casei, L. fermentum, L. gasseri, L. johnsonii, L. lactis, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus, L. salivarius*; Bifidobacterium species viz. *B. bifidum, B. breve, B. lactis, B. longum* and *Streptococcus* species viz. *S. thermophilus*. Likewise, non-bacterial sources of probiotics are yeast. The yeast, *Saccharomyces cerevisiae*, has also been commonly identified as probiotic. It stimulates immune system of fish for the production of inhibitory substances against foreign antigen (Dahan *et al.*, 2003). Yeasts are not affected by antibiotics. This is advantageous in probiotic preparations used for preventing disturbances in the normal microflora in the presence of antibacterial metabolites. Strains of *Saccharomyces cerevisiae* and *Dermocystidium hansenii* have been shown to attach and grow in the intestinal mucus of fish.

Several reports suggest that dietary yeast or nucleotides act as probiotics in fishes enhancing their immune function and disease resistance (Ramadan *et al.*, 1994; Wang and Zhang, 2000; Burrells *et al.*, 2001a, b), besides improving their growth performance (Adamek *et al.*, 1996; Kubitza *et al.*, 1997; Burrells *et al.*, 2001b). It has been proposed that *de novo* synthesis and salvage pathway of nucleotides is a costly metabolic process; a dietary supply of nucleotides or precursors may have a protein sparing effect (Sanderson and He, 1994).

Probiotics protect their host against neighboring or invading pathogens by interfering with their cellular functions. Probiotics may protect their host from pathogens by producing metabolites which inhibit the colonization/growth of other microorganisms or by competing with them for resources such as nutrients or space (Ouwehand *et al.*, 1999; Ouwehand *et al.*, 2001; Forestier *et al.*, 2001; Pinchuk *et al.*, 2001; Mukai *et al.*, 2002; Fiorillo *et al.*, 2002; Servin and Coconnier, 2003; Vine *et al.*, 2004a, b).

The addition of potentially probiotic microorganisms to culture water in larval fish systems is a means of biocontrol. It is possible that some of these may be ingested and have a probiotic effect on the host animal (Nogami and Maeda, 1992; Jory, 1998; Moriarty, 1998; Verschuere *et al.*, 1999; Ruiz-Ponte *et al.*, 1999; Douillet, 2000; Chythanya *et al.*, 2002).

PROBIOTIC SELECTION CRITERIA

***In vitro* antagonism tests:** A common way to select a probiotic is to perform *in vitro* antagonism tests, in which pathogens are exposed to the probiotics or their extracellular products in liquid (Gram *et al.*, 1999) or solid (Austin *et al.*, 1992) medium. Depending on the exact arrangement of the tests, candidate probiotics can be selected based on the production of inhibitory compounds (Gram *et al.*, 1999) or siderophores, or on the basis of competition for nutrients (Dopazo *et al.*, 1988). The preselection of candidate probionts based on these *in vitro* antagonism tests has often led to the finding of effective probiotics.

Colonization and adhesion: A probiotic should either be supplied on a regular basis or be able to colonize and persist in the host or in its ambient environment. The ability of a strain to colonize in the gut or an external surface of the host and adhere to the mucus layer may be a good criterion for preselection among the putative probiotics (Shi and Walter, 2004). This involves the viability of the potential probiotic within the host and/or within its culture environment, adherence to host surfaces and the ability to prevent the establishment of potentially pathogenic bacteria (Panigrahi and Azad, 2007).

***In vivo* evaluation of effects of potential probiotics on the host:** The effect of candidate probiotics should be tested *in vivo* as well. When the probiotic effect is supposed to be nutritional, the candidate probiotics could be added to the culture of the aquatic species and their effect on growth and survival parameters could be assessed. However, when biological control of the microbiota is desired, representative *in vivo* challenge tests seem to be the appropriate tool to evaluate the potential effect of the candidate probiotic on the host (Gildberg *et al.*, 1995). The approach outlined above should result in formulating a set of strains with a well-established probiotic effect on the target organism without affecting other possibly involved trophic levels. Comparative pilot experiments under hatchery or grow out conditions in the farms should be performed to estimate the economic consequences of the probiotic application. An important factor in the economic evaluation is the mass production of the probiont. Also, effective legislation, if any, should be taken into account before commercial application is begun. Finally, a cost-benefit analysis will determine whether the probiotics could be applied in practice or not (Verschuere *et al.*, 1999; Ruiz-Ponte *et al.*, 1999; Douillet, 2000). In general, the selection criteria for a bacterium to be used as a probiotic should be followed. It should be non-pathogenic; withstand incorporation into a delivery vehicle at high cell counts and remain viable throughout the shelf-life of the product; withstand transit through the gastrointestinal tract, i.e., show acid and bile tolerance; be able to adhere to cells of the intestinal epithelium and/or colonize the lumen of the tract; show antagonistic activity towards enteric pathogens and/or provide demonstrated health benefits (Chythanya *et al.*, 2002).

A probiotic must possess certain properties in order to aid in correct establishment of new, effective and safe products (Verschuere *et al.*, 2000). It should not be harmful and be acceptable to the host through ingestion, potential colonization and replication within the host. It should reach the location where the effect is required to take place. It should actually work *in vivo* as opposed to *in vitro* findings. It should preferably not contain virulence resistance or antibiotic resistance genes.

POSSIBLE MODES OF ACTION

Production of antagonistic compounds: Antagonistic compounds are described as chemical substances produced by bacteria which are toxic or inhibitory towards other micro organisms (Ramirez and Dixon, 2003). These substances may be produced as either primary or secondary metabolites and, therefore, have different modes of inhibitory action. Microbial populations may release chemical substances that have a bactericidal or bacteriostatic effect on other microbial populations which can alter interpopulation relationships by influencing the outcome of competition for chemicals or available energy (Fredrickson and Stephanopoulos, 1981). The presence of bacteria producing inhibitory substances in the intestine of the host, on its surface, or in its culture medium is thought to constitute a barrier against the proliferation of opportunistic pathogens.

In general, inhibitory effect of bacteria is due to many factors, either singly or in combination with others. These include production of antibiotics (Williams and Vickers, 1986), bacteriocins (Bruno and Montville, 1993), siderophores, lysozymes, proteases, and/or hydrogen peroxide and also the alteration of pH values by the production of organic acids (Sugita *et al.*, 1997). Lactic acid bacteria are known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms (Vandenbergh, 1993). There are many reports of inhibitory activity of lactic acid bacteria mediated by bacteriocins. Many bacteria have been identified to produce bacteriolytic enzymes against *Vibrio parahaemolyticus* (Nair *et al.*, 1985). Bacteriocins are proteinacious agents produced by bacteria to inhibit or kill other bacteria. Other inhibitory compounds produced by bacteria include organic acids, hydrogen peroxide, carbon dioxide and siderophores. Siderophores are iron-complexing chemicals secreted by bacteria and fungi. Siderophore-producing bacteria can survive in nutrient-poor environments (Vine *et al.*, 2004a, b).

Competition for chemicals or available energy: Competition for chemicals or available energy may determine how different microbial populations co-exist in the same ecosystem (Fredrickson and Stephanopoulos, 1981). The microbial ecosystem in aquaculture environments is generally dominated by heterotrophs competing for organic substrates as both carbon and energy sources. Specific knowledge of factors governing the composition of microbiota in aquaculture systems is required to manipulate the latter. Verschuere *et al.* (1999) selected several strains of bacteria with a positive effect on the survival and growth of *Artemia juveniles*. It was suggested that the selected bacteria exerted their protective action by competing with the pathogen for chemicals and available energy.

Competition for iron: Virtually all microorganisms require iron for growth (Reid *et al.*, 1993). Siderophores are low-molecular-weight (<1,500), ferric ion-specific chelating agents which can dissolve precipitated iron and make it available for microbial growth. The ecological significance of siderophores resides in their capacity to scavenge an essential nutrient from the environment and deprive competitors of it. Successful bacterial pathogens are able to compete successfully for iron in the highly iron-stressed environment of the tissues and body fluids of the host. The ecological significance of siderophores in soils as important tools for iron acquisition by microorganisms and plants and their involvement in suppression of plant root pathogens have been established (Wang and Zhang, 2000). Harmless bacteria which can produce siderophores could be used as probiotics to compete with pathogens whose pathogenicity is known to be due to siderophore production and competition for iron or to out compete all kind of organisms requiring ferric iron from solution (Gullian *et al.*, 2004). The possible effectiveness of siderophore-producing probiotics can be illustrated by the fact that the addition of bacterial siderophore to live food (rotifers) increased the resistance of turbot (*Scophthalmus maximus*) larvae challenged with the pathogenic *Vibrio* strain. The addition of a siderophore-producing *Vibrio* strain protected the turbot larvae slightly more than without such addition (Gatesoupe, 1997).

Competition for adhesion sites: One possible mechanism for preventing colonization by pathogens is competition for adhesion sites on gut or other tissue surfaces. The ability to adhere to enteric mucus and wall surfaces is necessary for bacteria to become established in fish intestines (Westerdahl *et al.*, 1991). Adhesion can be non specific, based on physicochemical factors, or specific, involving adhesin molecules on the surface of adherent bacteria and receptor molecules on

epithelial cells. Adhesion capacity and growth on or in intestinal or external mucus has been demonstrated *in vitro* for fish pathogens like *Vibrio anguillarum* and *Aeromonas hydrophila* (Krovacek *et al*., 1987) and for candidate probiotics such as *Carnobacterium* strain K1 and also for several isolates inhibitory to *V. anguillarum*. In one of these studies, the aim was to measure the *in vitro* capacity of the strains to adhere to and grow in turbot intestinal mucus in order to investigate their potential to colonize the intestine of farmed turbot as a means of protecting the host from infection by *V. anguillarum* (Chabrillon *et al*., 2006; Watson *et al*., 2008). The intestinal isolates generally adhered much better to a film of turbot intestinal mucus, skin mucus and bovine serum albumin than did *V. anguillarum*, indicating that they could compete effectively with the pathogen for adhesion sites on the mucosal intestinal surface (Olsson *et al*., 1992; Isolauri *et al*., 2001).

Enhancement of the immune response: Immunostimulants are chemical compounds that activate the immune systems of animals and provide them more resistance to infections by viruses, bacteria, fungi and parasites (Raa, 1996). Fish larvae, shrimps and other invertebrates have immune systems that are less well developed than adult fish and are dependent primarily on nonspecific immune responses for their resistance to infection (Sakai *et al*., 1999; Balcazar, 2003). Observations obtained in various experiments with warm-blooded animals indicate that probiotic (lactic acid) bacteria administered orally may induce increased resistance to enteric infections (Nikoskelainen *et al*., 2003). The ingestion of bacteria and subsequent endocytosis in cod and herring larvae have been suggested to be involved in the stimulation of their developing immune system (Brunt and Austin, 2005; Selvaraj *et al*., 2005).

Improvement of water quality: In several studies, water quality has been recorded to be improved during the addition of the probiotics, especially *Bacillus* spp. The rationale is that gram-positive *Bacillus* spp. are generally more efficient in converting organic matter back to CO_2 than are gram-negative bacteria which would convert a greater percentage of organic carbon to bacterial biomass or slime (Stanier *et al*., 1993). It is reasoned that by maintaining higher levels of these gram-positive bacteria in the production pond, farmers can minimize the buildup of dissolved and particulate organic carbon during the culture cycle while promoting more stable phytoplankton blooms through the increased production of CO_2 (Nogami and Maeda, 1992; Jory, 1998; Moriarty, 1998; Verschuere *et al*., 1999; Ruiz-Ponte *et al*., 1999; Douillet, 2000; Chythanya *et al*., 2002).

Interaction with phytoplankton: When probiotic bacteria are selected to be used in a culture environment comprising algae, their possible interaction with these unicellular algae must be taken into consideration when the mode of action is being investigated (Ramirez and Dixon, 2003). Recent reports demonstrate that some bacterial strains have a significant algicidal effect on many species of microalgae, particularly on red tide plankton (Fukami *et al*., 1997). Some bacterial strains inhibited the growth of the unicellular alga (*Pavlova lutheri)* to various degrees (Munro *et al*., 1995). However, bacterial antagonism toward algae would be undesirable in the situations of larval rearing where unicellular algae are added (e.g., the green-water technique). It would be advantageous when undesired algal species develop in the culture pond. Conversely, positive effects of bacteria on cultured microalgae have also been observed (Fukami *et al*., 1997). Therefore, it is conceivable that bacteria can indirectly influence the health or the zootechnical performance of the cultured aquatic animals through their effect on the microalgae used as food.

Vitamin production: Lactic acid bacteria produce small amounts of certain B vitamins, including foliates and vitamin B (Kumari and Sahoo, 2005; McFarland, 2006). Microbial synthesis of vitamin K and vitamin C in the intestine appears to have nutritional significance in most animal species (Conway, 2001; Nayak *et al*., 2007). Bifidobacteria, streptococci and enterococci have been shown to produce vitamin K (Bentley and Meganathan, 2007).

Reduction of cholesterol: Some probiotics can lower total serum cholesterol and low density lipoprotein cholesterol. *In vitro* studies have shown that *Lactobacillus casei* and *L. acidophilus* effectively remove cholesterol from culture media. It is postulated that lactic acid bacteria assimilate cholesterol in the gut or deconjugate bile acids, thus disrupting the intestines-to-liver circulation of cholesterol (Anderson and Gilliland, 1999; Dambekodi and Gilliland, 1998; Sanders, 2000).

Short-chain fatty acid (SCFA) production: Probiotics, especially the bifidobacteria (*Bifidibacterium bifidum, B. breve, B. lactis, B. longum*) are able to break down and metabolize non-digestible carbohydrates such as fiber. The major by-products of this process are Short-chain Fatty Acids (SCFA) such as lactate, acetate, propionate and butyrate. SCFA lower down intestinal pH and create an environment inhospitable to pathogenic bacteria such as *E. coli* and *Salmonella* species. SCFA nourish mucosal cells of colon supplying 60-70% of colonocyte energy needs. Butyrate is the preferred energy source for colonocytes. Studies in animals and humans have found that SCFA directly stimulated colonic calcium, magnesium and potassium absorption, increased colonic blood flow, enhanced tissue oxygenation and transport of nutrients and might be of therapeutic value for various intestinal disorders (Scharrer and Lutz, 1990; Topping and Clifton, 2001; Zhenming *et al*., 2006; Jha *et al*., 2007).

Mode of application of the probiotics: The probiotics can be added to the host or its ambient environment in different ways e.g., (1) addition to the artificial diet (2) addition to the culture water and (3) bathing and addition through live food (Verschuere *et al*., 2000; Gomez-Gil *et al*., 2000).

The concept of inoculating the culture system and adding to live food is advisable when the small volumes are used. The inclusion in the artificial diet is more adapted when the greater volumes are used; however, this requires sufficient number of probiotics to last when they reach the gastrointestinal tract and exhibit capacity to adhere to the epithelial mucosa.

ROLE OF PROBIOTICS IN AQUACULTURE

Although plenty of work has been done on the role of probiotics in aquaculture during the past decade, yet the research has generally been of an applied nature with few discussions on the bacterial mode of action.

The use of lactic acid bacteria as probiotic (Perdigon *et al*., 1995; Salminen *et al*., 1998; Kontula, 1999; Tannock, 1999) and knowledge regarding aspects of this organism's biology, culture and suitability has been of benefit to aquaculture (Gildberg and Mikkelsen, 1998; Ringo and Gatesoupe, 1998; Nikoskelainen *et al*., 2003). A number of general review articles on the use of probiotics in aquaculture are also available (Ringo and Gatesoupe, 1998; Gatesoupe, 1999; Skjermo and Vadstein, 1999; Ringo and Birkbeck, 1999; Verschuere *et al*., 2000; Irianto and Austin, 2002). In aquaculture, the exposure to a diverse bacterial microflora is limited to the available resources. Therefore, the gastrointestinal flora usually resembles the microflora initially present in the rearing water, microalgae and livefood (Tanasomwang and Muroga, 1989; Munro *et al*., 1994; Ringo *et al*., 1996; Gatesoupe, 1999; Riquelme *et al*., 2001). The development of the microbial community in

Artemia populations is influenced by both deterministic and stochastic factors (Verschuere *et al.*, 1997). Deterministic factors include salinity, temperature, feed quality etc., while organisms in the right place at the right time to enter the habitat and proliferate make up the stochastic factors. The combination of controllable and chance factors determines the composition of the resulting microflora, making research on probiotics for larviculture both interesting and challenging (Gildberg and Mikkelsen, 1998; Calo-Mata *et al.*, 2008).

Manipulation of beneficial bacterial population present in the bivalve larval cultures is a potentially useful strategy for the enhancement of oyster production. Douillet and Langdon (1994) have reported the use of probiotics for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunbeerg). They added the probiotic bacteria as a food supplement to the larval cultures of this species which consistently enhanced the growth of larvae during different seasons of the year. They suggested that the action of probiotic bacteria were to provide essential nutrients that were not present in the algal diets or to improve the oyster's digestion by supplying digestive enzymes to the larvae or to remove the metabolic substances released by the bivalves or the algae. Likewise, based on the photosynthesis of micro algae mainly, it was clarified that the bacteria, protozoa and other microorganisms from microbial food assemblages play a significant role in the aquatic food chain and help in increasing the crustacean production (Maeda and Liao, 1994).

Cell free extracts of three strains of Lactic Acid Bacteria (LAB) i.e., *Lactobacillus acidophilus, Lactobacillus bulgaricus*-56 and *Lactobacillus bulgaricus*-57 inhibited growth of *Vibrio alginolyticus* in nutrient broth (Ajitha *et al.*, 2004). The antagonism of lactic acid bacteria to *Vibrio alginolyticus* was further confirmed by streak plating wherein suppression of growth of *Vibrio* was reported. Animals such as shrimp maintained on a diet devoid of bacterial biomass exhibited 80% mortality while no external or internal pathological changes were observed in shrimp fed with the lactic acid bacteria incorporated diets. These results showed an inhibition of *V. alginolyticus* by the lactic acid bacteria and the stimulation of the non-specific immune response resulting in the resistance to the disease in the shrimp fed on the lactic acid bacteria incorporated diets. *Bacillus* spp. has been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation (Duc *et al.*, 2004; Barbosa *et al.*, 2005). The strains appear to be the effective probiotics and the commercial products containing such strains have been demonstrated to improve the shrimp production (Hong *et al.*, 2005; Ghosh *et al.*, 2008).

The marine *Bacillus subtilis* AB65, *Bacillus pumilus* AB58 and *Bacillus licheniformis* AB69 bacteria, isolated from the local marine environment could be used for bioremediation in the shrimp hatcheries and were observed to reduce total nitrogen in Artemia cysts as an alternative to antibiotics. The action of the marine bacteria appeared to be significant in protecting the host shrimp against pathogenic bacteria (Misra *et al.*, 2006). Adenosine 5-diphosphoribose (ADP-ribose); a hydrolysis product of Beta-nicotine Adenine Dinucleotide (NAD) acted as a significant contributor to the anti-cytotoxic activity of probiotic *Lactobacillus bulgaricus* (John *et al.*, 2007).

The probiotic bacterium acted as an effective treatment against vibriosis and white spot diseases in the giant tiger shrimp (*Penaeus monodon* Fabricius). Inhibitory activity of two *Lactobacillus* sp. against *Vibrio* sp., *E. coli*, *Staphylococcus* sp. and *Bacillus subtilis* was established (Jiravanichpaisal *et al.*, 1997).

Likewise, *Vibrio* spp. were focused to act as probiotics against some shrimp pathogens (Direkbusarakom *et al.*, 1998). Two isolates of *Vibrio* spp. which were the dominant composition

of the flora in shrimp hatchery showed the antiviral activities against Infectious Haematopoietic Necrosis Virus (IHNV) and Oncorhynchus Masou Virus (OMV) by reducing the number of plaques. The result demonstrates the possibility of using the *Vibrio* flora as probiotic against the pathogenic viruses in shrimp culture. The use of *V. alginolyticus* as a probiotic agent may increase survival and growth in *Penaeus vannamei* postlarvae by competitively excluding potential pathogenic bacteria and can effectively reduce or eliminate the need for antibiotic prophylaxis in intensive larvae culture system (Austin *et al*., 1995; Garriques and Arevalo, 1995).

Higher survival and molt rates of larvae of prawn (*P. monodon*) were observed in the experiment treated with soil extract (Maeda and Liao, 1992). It was assumed that if a specific bacterium was cultured and added to the prawn ecosystem to the level of 10 million cells mL^{-1}, other bacteria might hardly inhabit the same biotype because of protozoan activity which would be one of the ways to biologically control the aquacultural pathogenic biotypes.

Microbial food assemblages have been reported to have great utility in the culture of a crab, *Portunus trituberculatus* (Maeda *et al*., 1997). Assemblages of microorganisms were produced by adding several nutrients, urea, glucose and potassium phosphate to the natural seawater with gentle aeration in which bacteria and yeast were prevailing. The crab larvae were fed on these microorganisms successively. It was found that some strains of bacteria promoted larval growth. By adopting these assemblages of microorganisms, a high yield was obtained in the larvae of prawn *P. japonicus*.

Both marine and freshwater fishes have been shown to have a specific indigenous microflora in gastrointestinal tract (Sakata, 1990; Ringo and Strom, 1994; Ringo *et al*., 1997; Ringo and Gatesoupe, 1998; Kennedy *et al*., 1998a,b). The composition of microflora may change with environmental stresses, diet (Munro *et al*., 1993; Ringo and Strom, 1994; Douillet and Langdon, 1994; Gildberg *et al*., 1995; Ringo *et al*., 1997) and fish age (Bergh *et al*., 1994; Prayitno and Latchford, 1995; Olafsen, 2001). The most common members of the microflora of healthy fish are *Vibrio* spp., *Pseudomonas* spp., *Acinetobacter* spp. (Muroga *et al*., 1987; Tanasomwang and Muroga, 1989; Nicolas *et al*., 1989; Cormack and Fraile, 1990; Bergh *et al*., 1994; Munro *et al*., 1994; Gatesoupe, 1997), *Aeromonas* spp., *Plesiomonas* spp., *Pseudomonas* spp. and members of the family Enterobacteriaceae (Ugajin, 1979; Sugita *et al*., 1988; Sakata, 1990; Sugita *et al*., 1991; Ringo *et al*., 1995).

It has also been reported that the bacteria isolated from intestine of seven kinds of freshwater cultured fish possess the antibacterial abilities and the presence of the intestinal bacteria can protect the fish against the infection by pathogenic bacteria (Sugita *et al* 1996).

Lactic acid bacteria were found to produce many kinds of metabolites which affected the other microbes in the mid gut of fish and reduced pH in their luminal contents in the stomach. These bacteria showed higher antagonistic activity by releasing extra cellular proteins or bacteriocins against *A. hydrophila* and enhanced the production of rotifers which act as biocarriers of probiotic (Vijayabaskar and Somasundaram, 2008).

Bandyopadhyay and Mohapatra (2009) analyzed the effect of a probiotic bacterium *Bacillus circulans* PB7 in the formulated diets on growth, nutritional quality and immunity of *Catla catla* (Ham.) The effect of supplement on the growth performance, feed utilization efficiency and immune response was also evaluated.

Likewise, a strain of *Carnobacterium* sp., isolated from the intestine of Atlantic salmon, was evaluated for potential use as a probiotic for salmonids. *In vitro* studies demonstrated antagonism of this bacterium against *Aeromonas hydrophila, A. salmonicida, Flavobacterium psychrophilum, Photobacterium damselae* subsp. piscicida, *Streptococcus milleri, Vibrio anguillarum* and

V. ordalii. Feeding salmonids with diets containing the probiotic having a strain of *Carnobacterium* sp., revealed that the isolate remained viable in the gastrointestinal tract (Robertson *et al*., 2000).

Some fluorescent strains of pseudomonad bacteria can also competitively inhibit the growth of fish pathogen, *A. salmonicida*. The fluorescent pseudomonad is capable of inhibiting the growth of *A. salmonicida* in culture media and that this inhibition is probably due to competition for free iron (Smith and Davey, 1993).

The micro algae (*Tetraselmis suecica*) too inhibited *Aeromonos hydrophila, A. salmonicida, Serrstia liquefaciens, Vibrio anguillarum, V. salmonicida* and *Yersnia ruckeri* bacteria. When used as a food supplement, the algal cells inhibited laboratory-induced infection in Atlantic salmon. When used therapeutically, the algal cells and their extracts reduced mortalities caused by *A. salmonicida, Ser. liquefaciens, V. anguillarum, V. salmonicida* and *Yersnia ruckeri*. It was suggested that there might be some bioactive compounds in the algal cells and there appeared to be a significant role for *Tetraselmis* in the control of fish diseases (Austin *et al*., 1992).

Micrococcus luteus and *Pseudomonas* species from the gonads and intestine of Nile tilapia, *Oreochromis niloticus* were found to antagonize *Aeromonas hydrophila* with inhibition zone of 4 and 9 cm diameter, respectively; *M. luteus* acted as a probiotic *in vivo*, while *Pseudomonas* species showed probiotics effects *in vitro* only (Azza *et al*., 2009).

The intestinal microflora of turbot (*Scophthalmus maximus*) larvae was strongly affected by the addition of *Vibrio pelagius* to the rearing water (Ringo *et al*., 1996). Similarly, 70% of the intestinal microflora of cod (*Gadus morhua*) larvae was comprised of *Lactobacillus plantarum* when exposed to the bacteria in the larval rearing water compared to 1% in unexposed tanks (Strom and Ringo, 1993).

Studies were made to selection, identification and characterization of bacteria. To control a disease in the rearing turbot (*Scophthalmus maximus*) larvae, thirty-four out of 400 bacterial strains that mainly identified as *Roseobacter* spp. based on phenotypic criteria exhibited *in vitro* anti-bacterial activity against three fish larval pathogens (*V. anguillarum, V. splendidus* and *Pseudoalteromonas*). The mortality of larvae decreased significantly in treatments where *Roseobacter* sp. strains at 10^7 cfu mL^{-1} was added, indicating a probiotic potential of this bacterium (Hjelm *et al*., 2004).

Two probiotics viz. lactic acid bacterium (*Pediococcus acidilactici*) and yeast (*Saccharomyces cerevisiae* var. boulardii) were tested as alternative treatment to limit the prevalence of the Vertebral Column Compression Syndrome (VCCS) in rainbow trout, compared with a preventive treatment with florfenicol. Either the antibiotic or a lactic acid bacterium was introduced into the experimental diets which were compared with the control diet without supplementation. *Pediococcus acidilactici* provided the same level of protection as did the antibiotic, without any adverse effect on survival of rainbow trout. The long-term dietary supplementation with *P. acidilactici* seemed promising as a preventive treatment against this syndrome (Aubin *et al*., 2005).

To determine the influence of probiotics on the growth and gut microbial load of juveniles of goldfish, three probiotics (sporolac, lactobacil and yeast) were added to the basal diets at 0.5% level and the juvenile goldfish were fed for a period of 60 days. Sporolac incorporated feed recorded a maximum mean weight gain of 0.673 g and a six-fold increase in growth compared to control feed. The percentage composition of *Lactobacillus* in the *Carassius auratus* fed with control feed was comparatively low (2.7%) than those supplemented with probiotics (Ahilan *et al*., 2004).

A variety of microorganisms have been used as probiotics to improve the growth or survival of larvae of aquatic species (Table 1).

Table 1: Intestinal probiotics used in fish/shellfish and their effects on the host

Microbe	Host species	Effect on host	References
Molluscs			
Roseobacter sp. (strain BS107)	*Pecten maximus*	Short-term improvement in survival was observed	Ruiz-Ponte *et al*. (1999)
Arthrobacter sp. (Strain 77)	*Argopecten purpuratus*	Produced inhibitory compounds and replaced resident microflora within 24 h	Riquelme *et al*. (2000)
Strains 11 and C33	*A. purpuratus*	Added with microalgae and colonized the digestive tract	Avendano and Riquelme (1999)
Vibrio sp. 33, *Pseudomonas* sp. 11 and *Bacillus* sp. (strain B2)	*A. purpuratus*	Effect was compared with antibiotic treatment; the addition of probiotics increased number of eyed larvae	Riquelme *et al*. (2001)
Strain CA2	*Crassostrea gigas*	Enhanced the growth	Douillet and Langdon (1994)
Aeromonas media (strain A199)	*C. gigas*	Reduced host mortality when challenged with *Vibrio* tubiashii	Gibson *et al*. (1998)
Crustaceans			
Vibrio alginolyticus	*Litopenaeus vannamei*	Host survival improved after challenge with *Vibrio* parahaemolyticus	Garriques and Arevalo (1995)
Lactobacillus sporogenes	*Macrobrachium rosenbergii*	Fed as bio-encapsulated probiotic via Artemia, improved growth rate and feed efficiency ratio of post-larvae	Venkat *et al*. (2004)
Bacillus S11	*Penaeus monodon* (PL-10)	Post-larvae survival was higher when challenged with *V. harveyi*. Probiont provided cellular and humoral immune defence responses	Rengpipat *et al*. (2008)
VKM-124 *Pseudoalteromonas undina*	*Penaeus* sp.	Reduced the host mortality by controlling pathogenic viruses	Maeda *et al*. (1997)
Bacterial strains F3 and PM-4	*Portunus trituberculatus*	Improved survival of crab larval PM-4 repressed growth of *Vibrio* spp. in culture water	Nogami and Maeda (1992)
Fish			
Bacillus toyoi	*Scophthalmus maximus*	Improved turbot larval growth when added to disinfected rotifers	Gatesoupe (1989)
Bacillus strain IP5832 spores	*S. maximus*	Improved weight gain of larvae and reduced mortality was observed after challenging with *Vibrio*	Gatesoupe (1991a,b)
S. thermophilus, L. helveticus and *L. plantarum*	*S. maximus*	Added to rotifer cultures which were fed to turbot larvae. Larval survival increased	Gatesoupe (1989b)
Vibrio Pelagius	*S. maximus*	Higher larval survival than controls was observed when added alone or in combination with *Aeromonas caviae*	Ringo and Vadstein (1998)
Vibrio mediterranei Q40 strain	*S. maximus*	Reduced the colonization of gut by opportunistic microflora	Huys *et al*. (2001)
V. alginolyticus	*S. maximus*	Presence of probiotic correlated with better survival	Gatesoupe (1999)
L. plantarum and *Carnobacterium* sp.	*S. maximus*	Improved survival was observed after challenge with *Vibrio* sp.	Gatesoupe (1994)
Vibrio (strain E)	*S. maximus*	Improved survival and growth was observed after challenge with *Vibrio splendidus*	Gatesoupe (1997)
Carnobacterium divergens and *Vibrio Pelagius*	*S. maximus*	Beneficial effect of *C. divergens* on survival was inconclusive from *in vivo* study	Ringo (1999)
Streptococcus lactis and *Lactobacillus bulgaricus*	*S. maximus*	Six times increase in survival of larvae host observed	De la Bande *et al*. (1992)

Table 1: Continued

Microbe	Host species	Effect on host	Reference
Roseobacter strain 27-4	*S. maximus*	Improved larval survival was observed over first five days.	Hjelm *et al.* (2004)
L. plantarum	*Gadus morhua*	Opportunistic colonization reduced by 70%	Strom and Ringo (1993)
Carnobacterium divergens	*G. morhua*	Improved survival was observed after challenge with *Vibrio* anguillarum	Gildberg *et al.* (1997)
V. salmonicida strain	*Hippoglossus hippoglossus*	Larval survival increased (to 72.8 per cent) compared to control (58.2 per cent) after 32 days post-hatching	Ottesen and Olafsen (2000)
Vibrio strains PB 1-11 and PB 6-1	*H. hippoglossus*	No difference in gut bacterial CFUs or growth between controls and bacteria bioencapsulated in *Artemia franciscana* was observed	Makridis *et al.* (2001)
Mixture of *Pseudomonas* and *Cytophaga/Flavobacterium*	*H. hippoglossus*	Improved larval survival and reproducibility of treatment was observed	Skjermo and Vadstein (1999)
Microbiologically matured water	*H. hippoglossus*	Improved survival of yolk-sac larvae was observed	Vadstein *et al.* (1993)
Bacillus no. 48	*Centropomus undecimalis*	Reduced *Vibrio* spp. in the microflora	Kennedy *et al.* (1998a,b)
Pediococcus acidilactici and *Saccharomyces cerevisiae*	*Pollachius pollachius*	Addition of *P. acidilactici* with *Artemia* improved growth of larvae	Gatesoupe (2002)
VKM-124 *Pseudoalteromonas undina*	*Sparus auratu*	Reduced infection with various pathogenic viruses	Maeda *et al.* (1997)
Arnobacterium sp. (strain K1)	*Oncorhynchus mykiss* fry	Detected in intestine ten days after being administered	Robertson *et al.* (2000)
Carnobacterium sp. Strain 1	*Oncorhynchus mykiss* fry	Fed with pellet diet; no adverse affect on survival was observed	Joborn *et al.* (1997)
Streptococcus faecium M74	*Cyprinus carpio*	Improved growth and food conversion ratio in fry over six weeks was observed	Bogut *et al.* (1998)
Kocuria AP4 *Pseudoalteromonas* AP5	*Amphiprion percula*	Improved clownfish larval survival and reduced *Vibrio* spp. in the culture water microflora were observed	Vine *et al.* (2004a, b)

PROBIOTICS AND HAEMATOLOGICAL PARAMETERS

Hematological parameters of fish are used as indicators of their physiological state and their study has become widespread in the control of pathogens and manipulation of stress in fish (Bansal *et al.*, 1979). *Bacillus subtilis*, a Gram-positive, aerobic, endospore forming bacterium, evaluated for its probiotic potential in Indian major carp, *Labeo rohita* (Rajesh *et al.*, 2006, 2008). The fish (15±2 g) were given a feed containing *B. subtilis* in three concentrations for 2 weeks, e.g., 0.5×10^7, 1.0×10^7 and 1.5×10^7 cfu g^{-1} feed. The control group was given feed without *B. subtilis* for the same period. Haematological and serum parameters were monitored at weekly intervals. The response variables were total erythrocyte count, Total Leucocyte Count (TLC), haemoglobin, total protein, albumin, globulin, albumin-globulin ratio, alkaline phosphatase activity, alanine aminotransferase activity and aspartate aminotransferase activity. Fish were challenged intraperitoneally with a virulent strain of *Aeromonas hydrophila* after 2 weeks of feeding to the treatment groups and positive control group, while the negative control group was challenged with phosphate-buffered saline only. Clinical signs and symptoms and mortality/survival percentage were noted in each group. The *B. subtilis*-treated fish at 1.5×10^7 cfu g^{-1} feed showed maximum percent survival (87.50%), weight gain (35.5%), TLCs (3.23×10^4 cells mm^3), haemoglobin content

(7.4 g/100 mL), total protein (2.37 g dL^{-1}) and globulin content (1.28 g dL^{-1}) during the pre-challenge. Enzymes showed higher activities during post challenge. The result suggests that *B. subtilis* can be used effectively as a commercial product for use in aquaculture.

However, nonsignificant changes in the hematological and the biochemical parameters were revealed after 1 day of infection with the fungus (*Saprolegnia parasitica*) on the hematological, serum biochemical and pathological in *Tilapia nilotica* but after 7 days of post-infection and 10 days of post-treatment, a significant decrease in red blood cell, haemoglobin, packed cell volume and significant increase in aspartate aminotransferase, urea, creatinine, sodium, cortisol, insulin and glucose were seen (Mona-Mori and Matsumoto, 2008). Iron showed a significant decrease during the same period of sampling. The pathological examination revealed a massive fungal growth resembling a tuft of cotton wool threads in eyes, gills, fins and in localized areas of the skin. Microscopically, the fungal hyphae and spores appeared on eyes, gills, skin and underlying muscles with marked degenerative, necrotic and inflammatory reactions. These reactions were evident after 7 days of post-infection but the severity of the lesions were markedly decreased after 10 days of post-treatment. It could be concluded that fungus infection induced marked tissue alterations as well as some hematological and serum biochemical changes.

Likewise, decrease in the level of blood in the common carp after exposure to *Cyanobacteria* extract was observed (Palikova *et al*., 2004). When the spleen, liver and kidney of unhealthy Nile tilapia (*Oreochromis niloticus*) held fairly severe infection, the haematopoiesis was also severely affected. The latter, in turn, affected the peripheral blood by decreasing erythrocyte volume (Ishikawa, 1998). The erythrocytes count and haematocrit of Nile tilapia decreased when inoculated with *Mycobacterium marinum*. Perhapes this decrease led to a tendency to develop hypochromic and microcytic anaemia in this fish (Ranzani-Paiva *et al*., 2004).

But there was an increase in the erythrocyte count in some other fish fed on probiotic bacteria (Irianto and Austin, 2002). Rajesh *et al*. (2006) also reported that the probiotics used in carps increased the blood parameter values as a result of hemopiotic stimulation. Similarly, an increase in total leukocyte counts and proportion of neutrophils and monocytes was observed when the carp (*Cyprinus carpio*) were fed with *Saccharomyces cerviase* (Selvaraj *et al*., 2005). Irianto and Austin (2002) used dead probiotic cells to control disease and observed higher number of leucocytes, erythrocytes, macrophages in rainbow trout (*Oncorhynchus mykiss*). Rengipipat *et al*. (2000) in black tiger shrimp (*Penaeus monodon*) and Siwicki *et al*. (1994) in rainbow trout (*Oncorhynchus mykiss*) found disease resistance or protection in use of probiotics *Bacillus* sp. and also reported increase in the level of selected hematological parameters in the blood (Red Blood Cell count (RBC), Hematocrit (Ht), Hemoglobin (Hb), various leukocyte counts, the total leukocyte level and MCV, MHC and MCHC counts.

CONCLUSION

Diseases take a heavy toll of the aquaculture production. Traditionally, these diseases are controlled by using chemical compounds. The use of antimicrobials is a common practice in practically all types of hatcheries. The latter can result in the development of resistant strains of bacteria. Such resistance can be readily transferred to other strains. Therefore, the need of alternative measures to control these diseases is of prime importance. In recent years probiotics have a center stage and are used as alternative measures to control the aquatic diseases. Probiotics inhibit pathogenic microorganisms and have been used therapeutically to treat a variety of gastrointestinal and even systemic disorders. The use of probiotics will prove a new ecofriendly alternative measure for sustainable aquaculture.

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