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REVIEW

Probiotics in aquaculture: importance and future perspectives

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Abstract Aquaculture is one of the fastest developing growth sectors in the world and Asia presently contributes about 90% to the global production. However, disease outbreaks are constraint to aquaculture production thereby affects both economic development of the country and socio-economic status of the local people in many countries of Asia-Pacific region. Disease control in aquaculture industry has been achieved by following different methods using traditional ways, synthetic chemicals and antibiotics. However, the use of such expensive chemotherapeutants for controlling diseases has been widely criticized for their negative impacts like accumulation of residues, development of drug resistance, immunosuppressants and reduced consumer preference for aqua products treated with antibiotics and traditional methods are ineffective against controlling new diseases in large aquaculture systems. Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the aquaculture systems there by to maintain the health of the cultured organisms. Use of probiotics is one of such method that is gaining importance in controlling potential pathogens. This review provides a summary of the criteria for the selection of the potential probiotics, their importance and future perspectives in aquaculture industry.

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Introduction

Aquaculture has become an important economic activity in many countries. In large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines. However, the utility of antimicrobial agents as a preventive measure has been questioned, given the extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria [1].

Globally, tones of antibiotics have been distributed in the biosphere during an antibiotic era of only about 60 years duration. In the United States, out of the 18,000 t of antibiotics produced each year for medical and agricultural purposes, 12,600 t are used for the non therapeutic treatments of livestock in order to promote growth [2]. In the European Union and Switzerland, 1600 t of antibiotics, representing about 30% of the total use of antibiotics in farm animals, are similarly used for growth promotion purposes [2]. These amounts of antibiotics have exerted a very strong selection pressure towards resistance among bacteria, which have adapted to this situation, mainly by a horizontal and promiscuous flow of resistance genes [2]. Resistance mechanisms can arise one of two ways: chromosomal mutation or acquisition of plasmids. Chromosomal mutations cannot be transferred to other bacteria but plasmids can transfer resistance rapidly [3]. Several bacterial pathogens can develop plasmid-mediated resistance.

Plasmids carrying genes for resistance to antibiotics have been found in marine *Vibrio* species and they could be laterally exchanged. At the high population densities of bacteria found in aquaculture ponds, transfer via plasmids, transduction via viruses and even direct transformation from DNA absorbed to the particles in the water or on the sediment surfaces could all be likely mechanisms for genetic exchange [4]. For example, transference of multi-drug resistance occurred in Ecuador during the cholera epidemic (1991–1994) in Latin America and this began among persons who were working on shrimp farms. Although the original epidemic strain of *Vibrio cholerae* 01 was susceptible to the 12 antimicrobial agents tested, in coastal Ecuador, it became multi-drug resistant by the transference of resistance genes of non-cholera vibrios that are pathogenic to the shrimp [5]. In addition, other evidences of the transmission of resistance between aquaculture ecosystems and humans have been demonstrated, with a novel florofenicol resistance gene floR, in *Salmonella typhimurium* DT104, which confers resistance to chloramphenicol and it is almost identical by molecular sequence to the florofenicol resistance gene first described in *Photobacterium damsela*, a bacterium found in fish [6]. There is an increasing interest within the industry at present in the control or elimination of antimicrobial use. Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the aquaculture systems. One such method that is gaining importance within the industry is the use of probiotic bacteria to control potential pathogens.

What is probiotic?

Pro: favour, Bios: life. An antonym of antibiotic, probiotics involves in multiplying few good/useful microbes to compete with the harmful ones, thus suppressing their growth. These include certain bacteria and yeasts that are not harmful on continued use for a long time [7]. Administration of beneficial organisms to animals started in the 1920's and the name "probiotics" was introduced by Parker [7] when the production of bacterial feed supplements began on a commercial scale. A widely accepted definition is taken from Fuller [8], who considered that a probiotic is a cultured product or live microbial feed supplement, which beneficially affects the host by improving its intestinal (microbial) balance. The important components of this definition reflect the need for a living microorganism and application to the host as a feed supplement.

However, other workers have broadened the definition. For example, Gram *et al* [9]. proposed that a probiotic is any live microbial supplement, which beneficially affects the host animal by improving its microbial balance. In this example, there is no association with feed. Furthermore, Salminen *et al* [10]. considered a probiotic as any microbial (but not necessarily living) preparation or the components of microbial cells with a beneficial effect on the health of the host. Here, the need for live cells in association with feed has been ignored. In short, it is apparent that there are variations in the actual understanding of the term probiotic. Based on the observation that organisms are capable of modifying the bacterial composition of water and sediments, albeit temporarily, Moriarty [11] suggested that the definition of a probiotic in aquaculture should include the addition of live naturally occurring bacteria to tanks and ponds in which animals live, i.e. the concept of biological control as discussed by Maeda *et al* [12]. As a compromise, it would appear that a probiotic is an entire or component(s) of a micro-organism that is beneficial to the health of the host. This all-embracing concept could impinge on other areas of disease control, particularly vaccinology.

Of course, probiotics must not be harmful to the host [10] and they will need to be effective over a range of temperature extremes and variations in salinity [8]. Application could be via feed (as implied by the definition of Fuller [8]) or by immersion or injection (as could occur with the definition of Salminen *et al* [10]). This is where confusion could occur, i.e. what is the distinction between a probiotic applied by injection or immersion, and a vaccine? Any confusion could have legal implications for the registration of probiotics in some countries. Specifically, when licensing/registering probiotics for use in fish culture should the organisms be considered as feed additives (probiotic stricto sensu) or veterinary products (vaccines)? Notwithstanding, it is essential to determine whether the benefit of a probiotic is actual or perceived, i.e. could the probiotic really be only a placebo? It is worth emphasizing that, according to Fuller [8], a probiotic should provide actual benefit to the host, be able to survive in the digestive tract, be capable of commercialization, i.e. grown on an industrial scale, and should be stable and viable for prolonged storage conditions and in the field.

How do probiotics work?

Antibiotics often treat the disease, but not the underlying problem. In addition, antibiotic and chemical therapy, especially broad spectrum chemical use, kills most of the beneficial bacteria in the water column of the pond and not just the bacteria causing problems to the aquatic species. In contrast, there are many different mechanisms involved in the probiotics process in the pond. Aquaculture

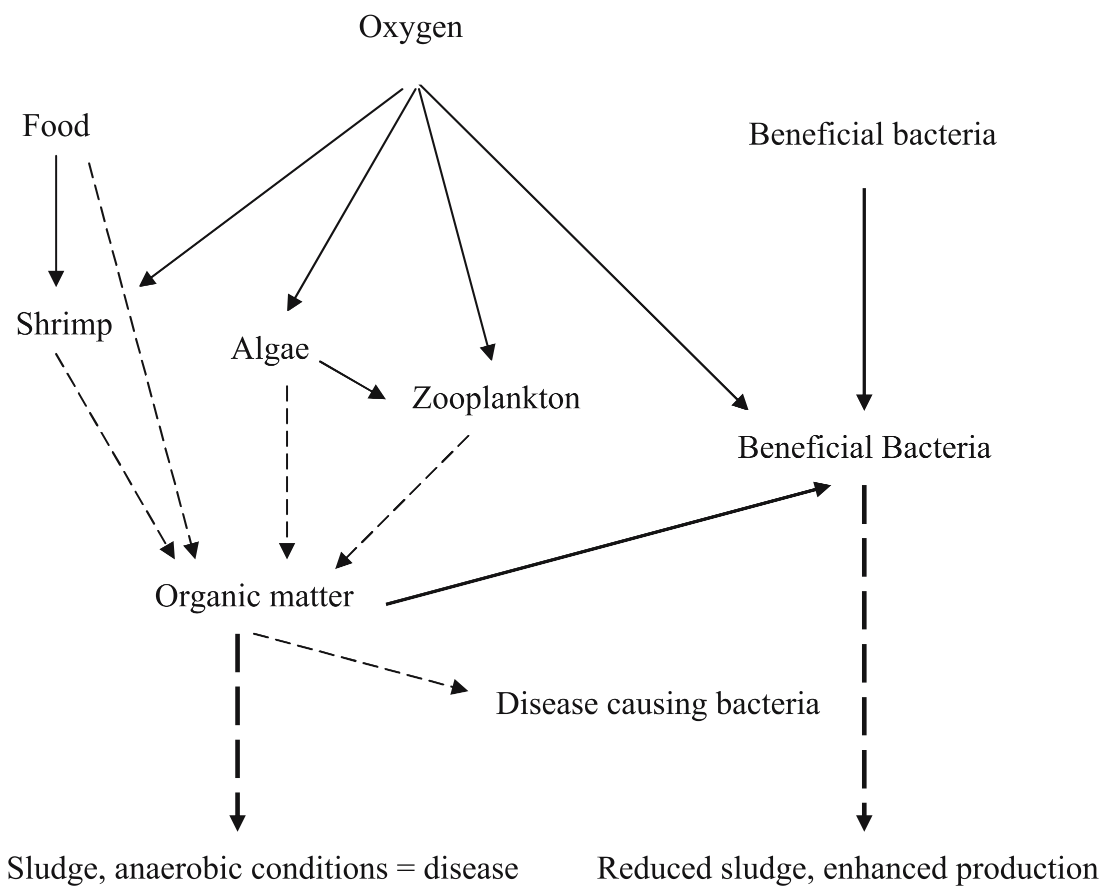

Fig. 1 Complex microbial interactions of aquaculture production (Adopted from Green and Green, 2003)

probiotics have a very important role to play in the degradation of organic matter thereby significantly reducing the sludge and slime formation. As a result, water quality would improve by reducing the disease (including *Vibrio* sp., *Aeromonas* sp. and viruses) incidences, enhancing zooplankton numbers, reducing odours and ultimately enhancing aquacultural production. By speeding up the rate of organic matter breakdown, free amino acids and glucose are also released providing food sources for the beneficial microorganisms. Inorganic forms of nitrogen, such as ammonia, nitrate and nitrite are also reduced. By improving total water quality and FCR, the overall health and immunity of the shrimp will be improved [13]. The complex microbial interactions of aquaculture production are highlighted in Fig. 1.

The assessment of the potential candidates for use as probiotics

Development of probiotics for commercial use in aquaculture is a multidisciplinary process requiring both empirical and fundamental research, full-scale trial and an economic assessment of its uses. Many of the failures in probiotic research can be attributed to the selection of inappropriate microorganisms. Selection steps have been defined, but they need to be adapted for different host species and environments. It is essential to understand the mechanisms of probiotic action and to define selection criteria for potential probiotics [14]. General selection criteria are mainly determined by biosafety (non-pathogenic) considerations, methods of production and processing, method of administration of the probiotic and the location in the body where the microorganisms are expected to be active [14]. Methods to select probiotic bacteria for use in the aquaculture might include the following steps.

1. Collection of background information: Before the start of research on development of probiotics, the activities about culture practices and economics of the development should be studied. A clear knowledge of the rearing practices used in the aquaculture farm is necessary to determine whether a probiotic application would be feasible or not.

2. Acquisition of putative probiotics: The acquisition of a good pool of candidate probiotics is of major importance in this process. It is vital in this phase that the choice of the strain is made as a function of the possible role of the probiotics to be developed. There is no unequivocal indication that putative probiotics isolated from the host or from their ambient environment perform better than isolates completely alien to the cultured species or those that originate from a very different habitat.

3. Screening of putative probiotics: A common way to select probiotics is to perform *in vitro* antagonism tests, in which pathogens are exposed to the candidate probiotics or their extracellular products in a liquid [15, 16] or solid [17, 18] medium. Candidate probiotics can be selected based on production of inhibitory compounds like bacteriocines, siderophores or when in competition for nutrients [17]. This has to be however done with extreme care.

4. Evaluation of pathogenicity and survival test: Probiotics should not be pathogenic to the hosts and this should be confirmed prior to acceptance. Therefore, the host must be challenged under stressed and non-stressed conditions. When probiotics are selected for larval rearing by green water technique, their possible interaction with algae should be considered. The probiotics should be evaluated for their survival to the transit through the gastrointestinal tract of the host (e.g. resistance to bile salts, low pH, and proteases) [19]. The probiotics strain should have efficient adherence to intestinal epithelial cells to reduce or prevent colonization of pathogens [20].

5. In vivo evaluation: Effect of candidate probiotics should be tested *in vivo* as well. It involves introducing candidate species to the host under culture and then monitoring the growth, colonization, survival and physico-chemical parameters [20]. However, when biological control of microbiota is desired, representative in vivo challenge tests seem to be the appropriate tool to evaluate the potential effect of the probiotics on the host. In addition, potential probiotics must exert its beneficial effects (e.g. enhanced nutrition and increased immune response) in the host. Finally, the probiotic must be viable under normal storage conditions and technologically suitable for industrial processes (e.g. lyophilized).

6. Effects in rearing conditions: The above test criteria are essential to select the candidate probiotics, but rearing experiments remain necessary to conclude that the strains are beneficial. The practical evaluation of the interest of probiotic treatments will require long-term surveys [21].

Types of probiotics

Probiotics are mainly of two types a) gut probiotics which can be blended with feed and administrated orally to enhance the useful microbial flora of the gut and, b) water probiotics which can proliferate in water medium and exclude the pathogenic bacteria by consuming all available nutrients. Thus, the pathogenic bacteria are eliminated through starvation [22].

Probiotics considered for use in aquaculture

The first probiotics discovered long time ago was *Lactobacillus* sp., the lactic acid producing bacteria. Thereafter, many probiotics such as *Aeromonas hydrophila* [23], *A. media* [24], *Altermonas* sp [25], *Bacillus subtilis* [26], *Carnobacterium inhibens* [27], *Debaryomyces hansenii* [28], *Enterococcus faecium* [29], *Lactobacillus helveticus* [30], *L. plantarum* [30], *L. rhamnosus* [31], *Micrococcus luteus* [23], *Pseudomonas fluorescens* [9], *Roseobacter* sp. [32], *Streptococcus thermopilus* [30], *Saccharomyces cerevisiae* [33], *S. exiguous* [33], *Vibrio alginolyticus* [34], *V. fluvialis* [23], *Tetraselmis suecica* [35] and *Weissella helenica* [36] were considered for use in aquaculture.

Methods of application of probiotics

Probiotics are marketed in two forms a) Dry forms: the dry probiotics that come in packets can be given with feed or applied to water and have to be brewed at farm site before application Each kit of dry probiotics contains a packet of dry powder and a packet of enzyme catalyst. Brewing has to be done in clean disinfected water after emptying the packets and blending thoroughly. Usually, it is brewed at 27–32°C for 16 to 18 hours with continuous aeration. The finished products must be used within 72 h. Maximum aeration is required in semi-intensive culture ponds. If aeration is less, the application of probiotics has to be spread for two consecutive days, applying 50% of the dose each time [37]. b) Liquid forms: The hatcheries generally use liquid forms which are live and ready to act. These liquid forms are directly added to hatchery tanks or blended with farm feed. The liquid forms can be applied any time of the day in indoor hatchery tanks, while it should be applied either in the morning or in the evening in outdoor tanks. Liquid forms give positive results in lesser time when compared to the dry and spore form bacteria, though they are lower in density [22]. There are no reports of any harmful effect for probiotics but it is found that the BOD level (biological oxygen demand) may temporarily be increased on its application; therefore it is advisable to provide subsurface aeration to expedite the establishment of probiotics organisms. A minimum dissolved oxygen level of 3% is recommended during probiotics treatment.

Benefits of probiotics in aquaculture

1. Production of inhibitory compounds: Probiotic bacteria release a variety of chemical compounds that are inhibitory to both gram-positive and gram-negative bacteria. These include bacteriocins, sideropheres, lysozymes, proteases, hydrogen peroxides etc. Lactic acid bacteria (LAB) are known to produce compounds such as bacteriocins that are inhibitory to other microbes [38].

2. Competition for adhesion sites: Probiotic organisms compete with the pathogens for the adhesion sites and food in the gut epithelial surface and finally prevent their colonization [39]. Adhesion capacity and growth on or in intestinal or external mucous has been demonstrated *in vitro* for fish pathogens like *Vibrio anguillarum* and *Aeromonas hydrophila* [40].

3. Competition for nutrients: Probiotics utilizes nutrients otherwise consumed by pathogenic microbes. Competition for nutrients can play an important role in the composition of the microbiota of the intestinal tract or ambient environment of the cultured aquatic organisms [41]. Hence, successful application of the principle of competition to natural situation is not easy and this remains as a major task for microbial ecologists.

4. Source of nutrients and enzymatic contribution to digestion: Some researches have suggested that probiotic microorganisms have a beneficial effect in the digestive processes of aquatic animals. In fish, it has been reported that Bacteroides and *Clostridium* sp. have contributed to the host's nutrition, especially by supplying fatty acids and vitamins [43]. Some microorganisms such as *Agrobacterium* sp., *Pseudomonas* sp., *Brevibacterium* sp., *Microbacterium* sp., and *Staphylococcus* sp. may contribute to nutritional processes in Arctic charr (*Salvelinus alpinus* L.) [44]. In addition, some bacteria may participate in the digestion processes of bivalves by producing extracellular enzymes, such as proteases, lipases, as well as providing necessary growth factors [45]. Similar observations have been reported for the microbial flora of adult penaeid shrimp (*Penaeus chinensis*), where a complement of enzymes exists for digestion and synthesis compounds that are assimilated by the animal [46]. Microbiota may serve as a supplementary source of food and microbial activity in the digestive tract may be a source of vitamins or essential amino acids [47].

5. Enhancement of immune response: The non-specific immune system can be stimulated by probiotics. It has been demonstrated that oral administration of *Clostridium butyricum* bacteria to rainbow trout enhanced the resistance of fish to vibriosis, by increasing the phagocytic activity of leucocytes [48]. Rengpipat *et al* [49] reported that the use of *Bacillus* sp. (strain S11) has provided disease protection by activating both cellular and humoral immune defenses in tiger shrimp (*Penaeus monodon*). Balcazar [1] demonstrated that the administration of a mixture of bacterial strains (*Bacillus* and *Vibrio* sp.) positively influenced the growth and survival of juveniles of white shrimp and presented a protective effect against the pathogens *Vibrio harveyi* and white spot syndrome virus. This protection was due to a stimulation of the immune system, by increasing phagocytosis and antibacterial activity. In addition, Nikoskelainen *et al* [50] showed that administration of a lactic acid bacterium *Lactobacillus rhamnosus* (strain ATCC 53103) at a level of 10^5 cfu g^{-1} feed, stimulated the respiratory burst in rainbow trout (*Oncorhynchus mykiss*).

6. Influence on water quality: Probiotics also help improve the water quality in aquaculture ponds [4]. This is due to the ability of the probiotic bacteria to participate in the turnover of organic nutrients in the ponds. However, there are few scientifically documented cases in which bacteria have assisted in bio-augmentation, with the notable exception of manipulating the $NH_3/NO_2/NO_3$ balance [51] in which nitrifying bacteria are used to remove toxic NH_3 (and NO_2). Fish expel nitrogen waste as NH_3 or NH_4+ resulting in rapid build up of ammonia compounds which are highly toxic to fish [52]. Nitrate, in contrast, is significantly less toxic being tolerated in concentrations of several thousand mg per litre. Several bacteria e.g. *Nitrosomonas,* convert ammonia to nitrite and other bacteria e.g. *Nitrobacter*, further mineralize nitrite to nitrate. Nitrifying bacteria excrete polymers [52], allowing them to associate with surfaces and form biofilms. Recirculating systems must employ biofilters to remove ammonia, and Skjolstrup *et al* [53] demonstrated a 50% reduction in both ammonia and nitrite in an experimental fluidised biofilter in a rainbow trout recirculating unit. Sulfur-reducing bacteria oxidize organic carbon using sulfur as a source of molecular oxygen. The hydrogen ion released when organic carbon fragments are oxidized is combined with sulfate to form sulfide which is less toxic to the aquatic animals. Methane-reducing bacteria use carbon dioxide as a source of molecular oxygen. Methane diffuses into the air and thereby improves the water quality.

7. Interaction with phytoplankton: Probiotic bacteria have a significant algicidal effect on many species of microalgae, particularly of red tide plankton [54]. Bacteria antagonistic towards algae would be undesirable in green water larval rearing technique in hatchery where unicellular algae are cultured and added, but would be advantageous when undesired algae species are developed in the culture pond.

8. Antiviral activity: Some bacteria used as candidate probiotics have antiviral activities. Though the exact mechanism by which these bacteria do this is not known, laboratory tests indicate that the inactivation of viruses can occur by chemical and biological substances, such as extracts from marine algae and extracellular agents of bacteria. It has been reported that strains of *Pseudomonas* sp., *Vibrios* sp., *Aeromonas* sp., and groups of coryneforms isolated from salmonid hatcheries, showed antiviral activity against infectious hematopoietic necrosis virus (IHNV) with more than 50% plaque reduction [55]. Girones *et al* [56]. reported that a marine bacterium, tentatively classified in the genus *Moraxella*, showed antiviral capacity, with high specificity for poliovirus.

Recent trends of probiotics research in aquaculture with special reference to shrimp culture

In aquaculture, probiotics have been tried in cultivation of shrimp larvae. Some of the good/beneficial microbes, e.g. non-pathogenic isolates of *Vibrio alginolyticus* [34], *B. subtilis* [26] etc. can be inoculated into shrimp culture with an aim to suppress the pathogenic vibrios, such as *Vibrio harveyi*, *V. parahaemolyticus* and *V. splendidus* thereby reducing the problem of opportunistic invasion by these bacteria.

In a study of tiger shrimp, the inoculation of *Bacillus* S11, a saprophytic strain, resulted in greater survival of the post-larval *P. monodon* that were challenged by pathogenic luminescent bacterial culture [57]. A mixture of *Lactobacillus* spp. isolated from chicken gastrointestinal tracts has improved the growth and survival rates of the juvenile *P. monodon* when fed with these strains for 100 days [58]. Recently, the growth of pathogenic *V. harveyi* was controlled by the probiotic effect of *Bacillus subtilis* BT23 under *in vitro* and *in vivo* conditions. Improved disease resistance was observed after exposing the juvenile *P. monodon* to *B. subtilis* BT23, isolated from shrimp culture ponds, at a density of 10^6–10^8 cells ml^{-1}, for 6 days before a challenge with *V. harveyi* at 10^3–10^4 cells ml^{-1} for 1 h infection with a 90% reduction in accumulated mortality [26]. The probiotic effect in *L. vannamei* has been reported using three strains isolated from the hepatopancreas of shrimp. These strains were identified as *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 and achieved inhibition percentages against *V. harveyi* S2 under *in vivo* conditions were 83, 60 and 58%, respectively. Histological analyses after the colonization and interaction experiment confirmed that the probiotic strains had no pathogenic effect on the host [59]. Also, *Pseudomonas* sp. PM 11 and *V. fluvialis* PM 17 have been selected as candidate probiotics isolated from the gut of farm reared tiger shrimp by the ability to secrete extra-cellular macromolecule digesting enzymes. However, when shrimps were treated with each of the candidate strains, the estimation of immunological indicators such as haemocyte counts, phenol oxidase and antibacterial activity showed declining trends [60]. Possibly, these bacteria did not colonize the gut, therefore, they did not help in improving the immune system of shrimp. It is known that colonization with specific microbiota in the gut may play a role in balancing the intestinal mucosal immune system, which may contribute to the induction and maintenance of immunological tolerance or to the inhibition of the deregulated responses induced by pathogens in host. Few multinational pharmceutical companies have introduced commercial preparations into the market as probiotics feed/food supplement in various commercial names as Aqualact, Spilac, Protexin etc.

Recommendations for the use of probiotics

SEAFDEC (South East Asia Fisheries Development Centre) combined with ASAN (Association of South East Asian Nations) have collaborated to research and publish guidelines for sustainable production of shrimp. Their publication, entitled "Environment-friendly schemes in intensive shrimp farming" [61], recommends the application of probiotics to both the grow out ponds and the reservoir for good water culture throughout the production cycle. In addition, both these organizations also recommend other pond management considerations including the stocking of fry that have been certified free from specific disease, such as white spot by PCR equipped diagnostic laboratories. Table 1 summaries the lower and upper pond parameters recommended, combined with the details on the efficacy range and benefits of commercially available probiotics.

Limitation of probiotics use

Probiotics can be used in advance as prevention tools. They can prevent the disease rather than treatment of the disease. They can be established well in static or low water exchange systems (re-circulatory system). They are effective if applied as soon as the water medium is sterilized before contamina-

Table 1 Lower and upper pond parameters recommended combined with details on efficiency range and benefits of a commercially available probiotic

Water	Protexin* probiotic range (Benefits)	Lower@ 100 cm depth	Upper @ 150 cm depth
Salinity	0–40 ppt	10 ppt	35 ppt
pH	6.5–9.0	7.5	8.5
Temperature	25°C–35°C	28°C	32°C
Alkalinity	>80 ppm	–	–
Transparency	(Balances)	30 cm	45 cm
Colour	(Balances)	Light green	Brownish green
DO	(Improves)	> 3.5 ppm	
Total ammonia	(Reduces)	< 1.0 ppm	
Nitrate	(Reduces)	< 0.2 ppm	
P as Orthophosphate	(Balances)	> 0.5 ppm	> 1.0 ppm
Total bacteria and *Vibrio* spp.	(Inhibits)	10^3–10^4 CFU/ml	
Total luminous bacteria (pathogenic *Vibrio*)	(Inhibits)	<10^2	
Beneficial algae	60%–90%	60%	90%

*Protexin is a probiotic produced by Probiotics International Ltd.

tion with other microbes [22]. In the process of application of probiotics, no other chemical or drug should be used for treating other diseases like fungal and protozoan diseases caused by those other than bacteria. These probiotics can easily be destroyed by any other chemical or drug which generally interferes with the establishment of useful microbes.

Future perspectives

Though several studies have shown that the probiotic concept has potential in the aquaculture sector, much work is still needed. Some of the most promising data stem from field trials where addition of probiotics to the water on a routine basis increased survival of fish or crustacean [57, 62–64]. Many questions remain unanswered regarding the use of probiotics in aquaculture. It is not yet clear if they are effective and if so, how they have an effect. Are they acting as a food or are they competing with potentially harmful bacteria? How will probiotics perform when a stressful situation arises and the larvae are weakened? Can they become pathogenic, since, for example, *V. alginolyticus* has been suggested as a probiont but other strains of this bacterium have been associated with vibriosis in shrimps? How can a probiotic strain be differentiated from a potentially pathogenic one? Many of these questions are still unanswered not only for probiotics but for bacteria associated with the aquatic organisms under culture conditions. It is crucial that the mechanisms involved in the in vivo probiotic effect be determined [65, 66].

Some go as far as stating that "without specific cause and effect relationships that can be substantiated scientifically, the use of probiotics remain controversial and should not be endorsed by the scientific community" [66]. Even with a slightly less rigorous attitude, understanding mechanisms is a requirement for any long-term commercial use as this is needed to determine any possible side effects on the environment, e.g. will the addition of probiotics alter the microbial community on a permanent scale and will this subsequently affect turnover of organic and inorganic compounds in the particular environment. Thus, the anti-microbial effect of some Bacillus and Pseudomonas species is caused by production of antibiotics [67–69] and this is obviously not a viable path in an attempt to find non-antibiotic substitutes for disease control. An understanding of the in vivo mechanism(s) would also allow for a much more efficient and intelligent selection of potential probionts.

As of today, not a single study has seriously compared in vitro and in vivo antagonism. Therefore, it is not known if the screening of thousands of isolates for antagonistic activity in in vitro assay has any importance for their in vivo effect. Determining mechanisms of activity is not an easy task, however, some options exists. Comparing phenotypic characteristics and disease suppressing abilities (against phytopathogenic fungi) of fluorescent pseudomonads has shown that for some strains, production of cyanide is important [70]. Mutant strains, e.g. constructed by random transposon mutagenesis, could allow for identification of clones with no disease preventive effect. Subsequent cloning and sequencing of the genes affected by the knock-out could help clarify mechanisms.

It has been hypothesised that iron chelation is important for the antagonism of pseudomonads in the rhizosphere and this hyposthesis has been tested by comparing the in vivo disease suppressing effects of a wild type strain and siderophore negative mutants [71]. A particular aspect concerns the testing of probiotic cultures. The use of field trials under real conditions is obviously the ultimate test. However, an intermediate step in terms of infection model systems using live hosts, is often needed. Due to the very high inherent (biological) variation in such systems, the model infection studies should be carried out with a sufficient number of replicates to allow for proper statistical treatment. Analyses normally used to describe and compare survival data must be used. Even with more appropriate statistical analysis, the development of the probiotic principle would benefit greatly from more stable infection models. It must also be recognised that a particular probiont which may work in one system [9, 72] may be completely ineffective in another host-pathogen system [9]. Therefore, more detailed knowledge of the pathogenic agents, their virulence factors and their interactions with the host would be of great importance.

Different approaches have been used for introducing the probiont to the system. The organism may be live or in a freeze dried state. It can be added directly to the water or incorporated in the feed; either pelleted or live feed. Nothing is known about how each of these treatments would affect the viability of the organisms or the probiotic effect. Knowledge of proliferation and invasion sites of the pathogen would assist in determining whether a water borne or food borne vehicle is the most appropriate. Such understanding is required for further technological developments.

Several studies have shown that a single treatment with probiotic culture is not enough and that the organism(s) must be added on a more continuous basis [9, 62, 63]; however, the robustness of the systems (e.g. required concentration of probiont, required frequency of addition, effects of changing temperature etc.) has not been documented.

Finally, legal matters must be resolved. Is probiotic treatment classified as a medical issue (treating animals) or an environmental issue (treating water) and in either case, who is responsible for control? Also, no cost-benefit analysis has yet been carried out. Whilst the application of probiotic technology is likely to increase costs per se, it must be emphasized that if used successfully, there may be tremendous benefits due to a more stable and therefore higher production. Also, as some uses of antibiotics may be prohibited, use of probiotics may gain wider interest.

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