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Review

The role of probiotics in aquaculture

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Abstract

The increase of productivity in aquaculture has been accompanied by ecological impacts including emergence of a large variety of pathogens and bacterial resistance. These impacts are in part due to the indiscriminate use of chemotherapeutic agents as a result of management practices in production cycles. This review provides a summary of the use of probiotics for prevention of bacterial diseases in aquaculture, with a critical evaluation of results obtained to date.
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1. 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 extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria. Globally, tonnes of antibiotics have been distributed in the biosphere during an antibiotic era of only about 60 years duration. In the United States, 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 (SCAN, 2003). In the European Union and Switzerland, 1600 t of antibiotics, representing about 30% of the total use of antibiotics in farm animals, were similarly used for growth promotion purposes in 1997 (SCAN, 2003). 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 (SCAN, 2003).

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 (Lewin, 1992). 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 (Moriarty, 1997). For example, transference of multidrug 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 multidrug resistant by the transference of resistance genes of non-cholera vibrios that are pathogenic to the shrimp (Weber et al., 1994). In addition, other evidence of the transmission of resistance between aquaculture ecosystems and human has been demonstrate, when a novel florofenicol resistance gene *floR*, in *Salmonella typhimurium* DT104, which also confers resistance to chloramphenicol, is almost identical by molecular sequence to the florofenicol resistance gene first described in *Photobacterium damsela*, bacterium found in fish (Angulo, 2000).

The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment. The use of probiotics in human and animal nutrition is well documented (see Fuller, 1992; Mulder et al., 1997 reviews; Rinkinen et al., 2003) and recently, they have begun to be applied in aquaculture (Gatesoupe, 1999; Gomez-Gil et al., 2000; Verschuere et al., 2000; Irianto and Austin, 2002; Bachère, 2003). The purposes of this review are to describe the principles, mechanisms of action and criteria for selection of probiotics, and to summarize their applications in aquaculture.

2. What is a probiotic?

The term probiotics is generally used to denote bacteria that promote the health of other organisms. Lilley and Stillwell (1965) described them as substances secreted by one microorganism, which stimulated the growth of another. An expert with the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), stated that probiotics are live microorganisms, which when consumed in adequate amounts, confer a health benefit for the host (FAO/WHO, 2001).

Generally, probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals. Gram-negative facultative anaerobic bacteria such as *Vibrio* and *Pseudomonas* constitute the predominant indigenous microbiota of a variety of species of marine fish (Onarheim et al., 1994). In contrast to saltwater fish, the indigenous microbiota of freshwater fish species tends to be dominated by members of the genera *Aeromonas*, *Plesiomonas*, representatives of the family *Enterobacteriaceae*, and obligate anaerobic bacteria of the genera *Bacteroides*, *Fusubacterium*, and *Eubacterium* (Sakata, 1990). Lactic acid producing bacteria, which are prevalent in the mammal or bird gut (*Bifidobacterium* in human, *Lactobacillus* in swine, rodent and bird, *Enterococcus* in carnivore), are generally sub-dominant in fishes and represented essentially by the genus *Carnobacterium* (Ringø and Vadstein, 1998).

It is important to note that the population of endogenous microbiota may depend on genetic, nutritional and environment factors. However, microorganisms present in the immediate environment of aquatic species have a much larger influence on the health status than with terrestrial animals or humans. The gut microbiota of aquatic animals is probably constituted by the indigenous microbiota jointly with artificially high levels of allochtonous microorganisms so maintained by their constant ingestion from the surrounding water (Hansen and Olafsen, 1989).

3. Mechanisms of action

Enhancement of colonization resistance and/or direct inhibitory effects against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Probiotic strains have been shown to inhibit pathogenic bacteria both in vitro and in vivo through several different mechanisms.

Several studies on probiotics have been published during the last decade. However, the methodological and ethical limitations of animal studies make it difficult to understand the mechanisms of action of probiotics, and only partial explanations are available. Nevertheless, some possible benefits linked to the administering of probiotics have already been suggested as: (i) competitive exclusion of pathogenic bacteria (Garriques and Arevalo, 1995; Moriarty, 1997; Gomez-Gil et al., 2000; Balcázar, 2003; Balcázar et al., 2004; Vine et al., 2004a); (ii) source of nutrients and enzymatic contribution to digestion (Sakata, 1990; Prieur et al., 1990; Garriques and Arevalo, 1995); (iii) direct uptake of dissolved organic material mediated by the bacteria (Garriques and Arevalo, 1995; Moriarty, 1997); and others are still being investigated as: (iv) enhancement of the immune response against pathogenic microorganisms (Andlid et al., 1995; Scholz et al., 1999; Rengpipat et al., 2000; Gullian and Rodríguez, 2002; Irianto and Austin, 2002; Balcázar, 2003; Balcázar et al., 2004); (v) antiviral effects (Kamei et al., 1988; Girones et al., 1989; Direkbusarakom et al., 1998).

3.1. Competitive exclusion

Bacterial antagonism is a common phenomenon in nature; therefore, microbial interactions play a major role in the equilibrium between competing beneficial and potentially pathogenic microorganisms. However, the composition of microbial communities can be altered by husbandry practices and environmental conditions that stimulate the proliferation of selected bacterial species. It is well known that the microbiota in the gastrointestinal tract of aquatic animals can be modified, for example by ingestion of other microorganisms; therefore, microbial manipulation constitutes a viable tool to reduce or eliminate the incidence of opportunist pathogens (Balcázar, 2002).

The first report of the existence in seawater of bacteria with an inhibitory effect against a *Vibrio* sp. has been attributed to Gaixa (1889). Subsequently, Rosenfeld and Zobell (1947) described a study of antibiotic-producing marine microorganisms, and

since then the research has started to develop biological control agents.

In aquaculture, *Thalassobacter utilis*, has shown inhibitory effects against *Vibrio anguillarum*. This strain increased the survival of larvae of the crab, *Portunus trituberculatus*, and also reduced the amount of *Vibrio* sp. in the water used to rear the larvae (Nogami and Maeda, 1992; Nogami et al., 1997). Subsequently, it has been reported that bacterial strains associated with intestinal and skin mucus of adult marine turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*), suppressed the growth of the fish pathogen *V. anguillarum* (Olsson et al., 1992). The use of *Vibrio alginolyticus* strains as a probiotics has been recommended to increase survival and growth of white shrimp (*Litopenaeus vannamei*) post-larvae in Ecuadorian hatcheries. Competitive exclusion of potential pathogenic bacteria effectively reduces or eliminates the need for antibiotic prophylaxis in intensive larviculture systems (Garriques and Arevalo, 1995). Recently a marine bacterial strain, *Pseudomonas* I2, was isolated from estuarine environmental samples that produced inhibitory compounds against shrimp pathogenic vibrios. This antibacterial compound was shown to be of low molecular weight, heat stable, soluble in chloroform, and resistant to proteolytic enzymes (Chythanya et al., 2002).

3.2. Source of nutrients and enzymatic contribution to digestion

Some researches have suggested that 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 (Sakata, 1990). 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.) (Ringø et al., 1995).

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 (Prieur et al., 1990). Similar observations have been reported for the microbial flora of adult penaeid shrimp (*Penaeus chinensis*), where a complement of enzymes for digestion and synthesize compounds that are assimilated by the animal (Wang et al., 2000). Microbiota may serve as a supplementary source of food and microbial activity in the tract digestive may be a source of vitamins or essential amino acids (Dall and Moriarty, 1983).

3.3. Influence on water quality

Improved water quality has especially been associated with *Bacillus* sp. The rationale is that gram-positive bacteria are better converters of organic matter back to CO_2 that gram-negative bacteria. During the production cycle, high levels of gram-positive bacteria can be minimize the buildup of dissolved and particulate organic carbon. It has been reported that use of *Bacillus* sp. improved water quality, survival and growth rates and increased the health status of juvenile *Penaeus monodon* and reduced the pathogenic vibrios (Dalmin et al., 2001).

3.4. Enhancement of the 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 (Sakai et al., 1995). Rengpipat et al. (2000) mentioned that the use of *Bacillus* sp. (strain S11) provided disease protection by activating both cellular and humoral immune defenses in tiger shrimp (*P. monodon*). Balcázar (2003) demonstrated that the administration of a mixture of bacterial strains (*Bacillus* and *Vibrios* 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. (2003) showed that administration of a lactic acid bacterium *Lactobacillus rhamnosus* (strain ATCC 53103) at a level of $\sim 10^5$ cfu g^{-1} feed, stimulated the respiratory burst in rainbow trout (*Oncorhynchus mykiss*).

3.5. Antiviral effects

Some bacteria used as candidate probiotics have antiviral effects. Although 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 (Kamei et al., 1988). Girones et al. (1989) reported that a marine bacterium, tentatively classified in the genus *Moraxella*, showed antiviral capacity, with high specificity for poliovirus. Direkbusarakom et al. (1998) isolated two strains of *Vibrio* spp. NICA 1030 and NICA 1031 from a black tiger shrimp hatchery. These isolates displayed antiviral activities against IHNV and *Oncorhynchus masou* virus (OMV), with percentages of plaque reduction between 62 and 99%, respectively.

4. Colonization

Colonization of the gastrointestinal tract of animals by probiotics is possible only after birth, and before the definitive installation of a very competitive indigenous microbiota. After this installation, only the addition of high doses of probiotic provokes its artificial and temporary dominance. In mature animals, the population of probiotic organisms in the gastrointestinal tract shows a sharp decrease within days after the intake had stopped (Fuller, 1992).

According to Conway (1996), a microorganism is able to colonize the gastrointestinal tract when it can persist there for a long time, by possessing a multiplication rate that is higher than its expulsion rate. For example, *Vibrio* sp. normally colonize the hepatopancreas of juvenile white shrimp; however, this normal microflora can artificially become dominated by *Bacillus* sp. (up to 50% of the total) if it is added to the water for 20 days (Gullian and Rodríguez, 2002).

The process of colonization is characterized by attraction of bacteria to the mucosal surface, followed by association within the mucous gel or attachment to epithelial cells. Adhesion and colonization of the mucosal surfaces are possible protective mechanisms against pathogens through competition for binding sites and nutrients (Westerdahl et al., 1991), or immune modulation (Salminen et al., 1998).

Factors known to influence the colonization of microorganisms can be grouped as follows: (i) host-related factors: body temperature, redox potential levels, enzymes, and genetic resistance. For example, bacteria may enter through the mouth, either with water or food particles, and pass down the alimentary tract, at which point some of them are retained as part of a resident microflora. Others are destroyed by the digestive process or pass through the gut, and are eliminated via the faeces. In addition, bacterial growth may be inhibited by any antimicrobial compound produced by the host. (ii) Microbe-related factors: effects of antagonistic microorganims, proteases, bacteriocins, lysozymes, hydrogen peroxide, formation of ammonia, diacetyl, and alteration of pH values by the production of organic acids (Dopazo et al., 1988; Gram et al., 1999; Chythanya et al., 2002; Sugita et al., 2002; Gullian et al., 2004). For example, lactic acid bacteria are known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms.

The same methods employed for bacterial detection are used to study colonization. Common techniques include immunoassays, (i) immunocolony blot ‘‘ICB’’ and (ii) enzyme-linked immunosorbent assay ‘‘ELISA’’; molecular techniques, (iii) random amplification of polymorphic DNA ‘‘RAPD’’, (iv) amplified fragment length polymorphism ‘‘AFLP’’, (v) terminal restriction fragment length polymorphism ‘‘T-RFLP’’, (vi) immunohistochemical methods, and (vii) denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA, which been recently introduced in microbial ecology (Spanggaard et al., 2000; Cunningham, 2002; Temmerman et al., 2003).

5. Selection of 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 (Sotomayor and Balcázar, 2003; Vine et al., 2004a) or solid (Dopazo et al., 1988; Chythanya et al., 2002) medium. However, Gram et al.

(1999) suggested that in vitro activity in well-diffusion assays and broth cultures cannot be used to predict a possible in vivo effect. For example, in vitro antagonism of *Pseudomonas fluorescens* (strain AH2) against *Aeromonas salmonicida* does not confer Atlantic salmon protection against furunculosis, but is an effective probiotic in rainbow trout, conferring protection against vibriosis (Gram et al., 2001). Therefore, it is essential to know the origin (it is preferable to use strains isolated from the host), safety (non-pathogenic) and ability of the strain to survive to the transit through the gastrointestinal tract of the host (e.g. resistance to bile salts, low pH, and proteases). The ability of microorganisms to colonize is often considered as one of the main selection criteria for potential probiotics, that is, the efficient adherence to intestinal epithelial cells to reduce or prevent colonization of pathogens (Vine et al., 2004b). 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).

In conclusion, the methods to select probiotic bacteria for use in aquaculture include: (i) collection of background information; (ii) acquisition of potential probiotics; (iii) evaluation of the ability of potential probiotics to out-compete pathogenic strains; (iv) assessment of the pathogenicity of the potential probiotics; (v) evaluation of the effect of the potential probiotics in the host; (vi) economic cost/benefit analysis (Gomez-Gil et al., 2000) (see Fig. 1).

Probiotics can be provided to the host or added to its aquatic environment in several ways: (i) addition via live food (Gomez-Gil et al., 1998); (ii) bathing (Austin et al., 1995; Gram et al., 1999); (iii) addition to culture water (Moriarty, 1998; Spanggaard et al., 2001); (iv) addition to artificial diet (Rengpipat et al., 2000). For example, it has been reported that daily inoculations of larval white shrimp (*L. vannamei*) tanks with probiotic bacteria at a density of 10^5 cfu ml^{-1} prevented colonization by pathogenic bacteria during larval culture (Peeters and Rodríguez, 1999).

5.1. Consideration of regulations on probiotics

In the last few years, the basis on which the utilization of feed additives was consolidated has been

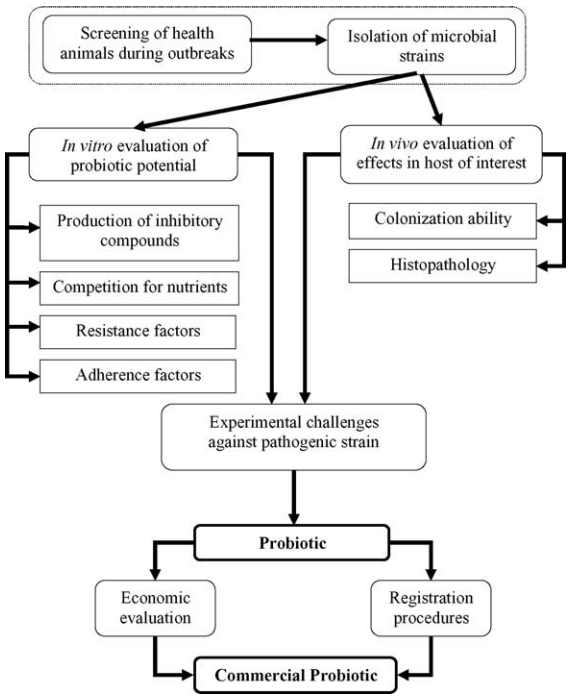

Fig. 1. Diagram for selection of probiotics as biocontrol agents in aquaculture.

modified in the European Union. In terms of the additives used in feed products, efforts have been directed towards providing assurance of a high level of protection of human health, animal health and welfare, and environmental user, and consumer interests. After a phase out of the use of antibiotics as growth promoters in animals, the White Paper on Food Safety and the Regulation (EC) No. 178/2002 of the European Parliament was published, in order to establish a policy of food safety for the European Union and the European Food Safety Authority (EFSA) was created (Regulation (EC) No. 178/2002). EFSA works on all stages of food production and supply, from primary production to the safety of animal feed, right through the supply of food to consumers. The authorization, marketing and use of feed additives are currently regulated under Council Directive 70/524/EEC. Before a feed additive may be marketed or used, it must be authorized in accordance with the provisions of the Directive. To obtain authorization, a manufacturer is required to submit a dossier containing data and studies demonstrating the efficacy and safety of the product for animals, consumers and the environment.

Table 1
List of microorganism authorized as probiotics in feedingstuffs under Council Directive 70/524/EEC

Probiotics
Bacillus cereus var. *toyoi*
Bacillus licheniformis
Bacillus subtilis
Enterococcus faecium
Lactobacillus casei
Lactobacillus farciminis
Lactobacillus plantarum
Lactobacillus rhamnosus
Pediococcus acidilactici
Saccharomyces cerevisiae
Streptococcus infantarius

Recently, some microorganisms have been authorized for use as probiotics in feedingstuffs in the European Union, and those appearing in the process of notification demanded by the regulation are detailed in Table 1. In addition, other probiotics are commercialized on the market that has been notified, but that do not appear in the last authorized list of feed additives published by the Commission (Council Directive 70/524/EEC, 2004).

In the US, the Food and Drug Administration (FDA) regulates the safety, labelling and health statements made on conventional foods, medicinal foods, food for special dietary use, and dietary supplements. The general requirements for a petition are detailed in the US Code of Federal Regulations (CFR, 2005) and proceed as follows. First, any interested person may petition FDA to issue a regulation regarding a health claim. For preliminary requirements, the petitioner must present a complete explanation of how the substance has been authorized for use in the food supply. For summary of scientific data, the summary must establish a significant scientific agreement among experts qualified on the health claim, and an optimum level of the particular substance to be consumed; potential adverse effects for any segments of the population, and other nutritional or health factors that could interact with substances or food components of interest. For analytical data, the amount of the substance that is present in foods must be obtained from representative samples using methods from the Association of Official Analytical Chemists (AOAC), if no AOAC method is available, the petitioner must submit the assay method used and data establishing the validity. For model health claims, one or more models that represent label statements must include a brief statement of the relevant conclusions of the summary, and how this substance helps the consumer.

In addition, the petition must include copies of computer literature searches done by the petitioner, copies of all articles cited, and all information on adverse consequences to any segment of the population.

Obviously, before a health claim petition can be prepared and submitted, substantial scientific evidence is needed of the health benefits of the product, safety, and other food science issues (Berner and O'Donnell, 1998).

Japan possesses a programme for the regulation of functional foods, named ''foods for specified health use'' (FOSHU). The approval process to obtaining a licence proceeds as follows. First, a good manufacturer compiles scientific data on the health effects, physicochemical properties, appropriated level of intake, safety, nutritional composition, and test methods for the food or compound of interest. Then, the application is submitted to the Ministry of Health and Welfare, with the above information plus descriptive information. The application is evaluated by the Japan Health Food and Nutrition Food Association, by academic experts, and finally by a committee appointed by the Ministry of Health and Welfare, which can approve the application (Arai, 1996; Shinohara, 1995; Berner and O'Donnell, 1998).

6. Probiotic strains studied in aquaculture

Most probiotics proposed as biological control agents in aquaculture belong to the lactic acid bacteria (*Lactobacillus* and *Carnobacterium*), to the genus *Vibrio* (*V. alginolyticus*), to the genus *Bacillus*, or to the genus *Pseudomonas*, although other genera or species have also been mentioned (*Aeromonas* and *Flavobacterium*) (Tables 2 and 3).

6.1. Fish eggs and larvae

The spawning and early life stages of fish larvae may have profound implications for the dynamics of microbial communities. These communities can also be influenced by inorganic and organic compounds.

Table 2
Probiotics considered as biological control agents in aquaculture of fishes

Probiotic strain	Source	Used on	Method of application	Reference
Streptococcus lactis and *Lactobacillus bulgaricus*	?	Turbot larvae (*Scophthalmus maximus*)	Enrichment of live food	García de la Banda et al. (1992)
Lactobacillus sp. and *Carnobacterium* sp.	Rotifers (*Brachionus plicatilis*)	Turbot larvae	Enrichment of rotifers	Gatesoupe (1994)
Vibrio alginolyticus	Commercial shrimp hatchery	Atlantic salmon (*Salmo salar* L.)	Bathing in bacterial suspension	Austin et al. (1995)
Carnobacterium divergens	Intestines of Atlantic salmon	Atlantic cod fry	Addition to diet	Gildberg and Mikkelsen (1998)
Bacillus megaterium, *B. subtilis, B. polymyxa*, *B. licheniformis*	Commercial product (Biostart)	Channel catfish	Addition to pond water	Queiroz and Boyd (1998)
Vibrio pelagius	Turbot larvae	Turbot	Addition to culture water	Ringø and Vadstein (1998)
G-probiotic	Commercial product	*Oreochromis niloticus*	Addition to diet	Naik et al. (1999)
Pseudomonas fluorescens	Iced freshwater fish (*Lates niloticus*)	Rainbow trout (*Oncorhynchus mykiss*)	Addition to culture water	Gram et al. (1999)
Carnobacterium sp.	Intestines of Atlantic salmon	Atlantic salmon	Addition to diet	Robertson et al. (2000)
Lactobacillus rhamnosus ATCC 53103	Culture collection	Rainbow trout	Addition to diet	Nikoskelainen et al. (2001)
Aeromonas hydrophila, *Vibrio fluvialis*, *Carnobacterium* sp., *Micrococcus luteus*	Digestive tract of rainbow trout	Rainbow trout	Addition to diet	Irianto and Austin (2002)
Enterococcus faecium SF68	Commercial product (Cernivet)	*Anguilla anguilla*	Addition to diet	Chang and Liu (2002)
L. rhamnosus JCM 1136	Culture collection	Rainbow trout	Addition to diet	Panigrahi et al. (2004)
Roseobacter sp. strain 27-4	Turbot larvae, *Tetraselmis* copepod-fed larvae	Turbot larvae	Addition to culture water	Hjelm et al. (2004)
Bacillus circulans	Intestines of *Labeo rohita*	*L. rohita*	Addition to diet	Ghosh et al. (2004)

Immediately after hatching process, fish larvae come into contact with their immediate environment that provides colonization by a wide variety of microorganism. It is therefore evident that health status will depend on management conditions to constitute the egg microbiota an important factor in the first establishment of an indigenous microbiota. Viable counts from water in the hatching units have been on the order of 10^3 ml^{-1} prior to hatching and 10^6 ml^{-1} 2 days after hatching during early life of fish larvae in intensive rearing systems (Hansen and Olafsen, 1989).

The composition of microbiota is influenced by many factors including the availability of nutrients, animal physiology and immunological factors. Under normal conditions, one of the basic physiological functions of the resident microbiota is that it functions as a microbial barrier against microbial pathogens and as a complement to the establishment of digestive enzymes.

During the initial feeding period, it is possible to manipulate the establishment of an artificial dominance of a determined group of bacteria in the fish-associated microbiota by adding a specific strain. A significant increase in the mean weight and survival rate of turbot larvae (*S. maximus*) fed rotifers enriched in lactic acid bacteria and these strains provided a significant protection against a pathogenic *Vibrio* compared to control larvae (Gatesoupe, 1994). Similarly, the addition of *Carnobacterium divergens*, showed a certain improvement in disease resistance in cod (*G. morhua*) fry after a challenge with *V. anguillarum* (Gildberg and Mikkelsen, 1998).

A study in the rearing of larvae turbot, 34 strains of 400 marine bacteria exhibited in vitro antibacterial activity against *V. anguillarum*, *Vibrio splendidus* and a

Table 3
Probiotics considered as biological control agents in aquaculture of crustaceans, molluscs, and live food

Probiotic strain	Source	Used on	Method of application	Reference
Crustaceans				
Bacillus sp. S11	*Penaeus monodon*	*P. monodon*	Addition to diet	Rengpipat et al. (1998)
Bacillus sp.	Commercial product (DMS)	*P. monodon*	Addition to culture water	Moriarty (1998)
Lactobacillus spp.	Digestive tract of chicken	*P. monodon*	Addition to diet	Phianphak et al. (1999)
Saccharomyces cerevisiae, *S. exiguus*, *Phaffia rhodozyma*	Commercial product	*Penaeus vannamei*	Addition to diet	Scholz et al. (1999)
Vibrio hepatarius, Vibrio sp., *Bacillus* sp.	*P. vannamei*	*P. vannamei*	Addition to diet	Balcázar (2003)
Vibrio P62, *Vibrio* P63, *Bacillus* P64	*P. vannamei*	*P. vannamei*	Addition to culture water	Gullian et al. (2004)
Pseudomonas sp., *Vibrio fluvialis*	*P. monodon*	*P. monodon*	Addition to culture water	Alavandi et al. (2004)
Molluscs				
Aeromonas media strain A199	?	*Crassostrea gigas*	Addition to culture water	Gibson et al. (1998)
Roseobacter sp. BS107	Scallop larval cultures	*Pecten maximus*	Addition to culture water	Ruiz-Ponte et al. (1999)
Alteromona haloplanktis	Microalgal cultures	*Argopecten purpuratus*	Addition to culture water	Riquelme et al. (2000)
Live food				
Flavobacterium sp.	*Chaetoceros gracilis* culture	*C. gracilis*, *I. galvana*, *P. lutheri*	Addition to culture water	Suminto and Hirayama (1997)
Lactococcus lactis AR21	Rotifer culture	Rotifers	Addition to culture water	Harzevili et al. (1998)
V. alginolyticus C7b	Seawater	*Chaetoceros muelleri*	Addition to culture water	Gomez-Gil et al. (2002)
Pediococcus acidilactici	Commercial product	*Artemia*	Addition to culture water	Gatesoupe (2002)
Lactobacillus casei, *L. brevis*, *L. helveticus, Lactococcus lactic* spp. *lactis*, *Leuconostoc, Mesenteroides* spp. *mesenteroides, Pediococcus acidilactici*	Culture collection	*Artemia* nauplii	Addition to culture water	Villamil et al. (2003)

Pseudoalteromonas. These strains were identified as *Roseobacter* spp., *Vibrio* spp., and *Pseudoalteromonas*. *Roseobacter* spp. were not lethal to egg yolk sac turbot larvae and in two of three trials, the mortality of larvae decreased in treatments where 10^7 cfu ml^{-1} *Roseobacter* sp. strain 27-4 was applied (Hjelm et al., 2004).

6.2. Fish juveniles and adults

In experiments performed by Queiroz and Boyd (1998), it was reported that a commercially prepared bacterial mixture of *Bacillus* spp. mixed into the rearing water increased survival and production of channel catfish (*Ictalurus punctatus*).

A strain of *Carnobacterium* sp. previously isolated from the intestine of Atlantic salmon, was effective at controlling infections caused by *A. salmonicida*, *Vibrio ordalii*, and *Yersinia ruckeri* in fry and fingerling salmonids, applied at 5×10^7 cells g^{-1} of feed (Robertson et al., 2000).

Cultures of *Aeromonas hydrophila*, *Vibrio fluvialis*, *Carnobacterium* sp. and an unidentified gram-positive coccus, have been beneficial for rainbow trout when these strains were administered as food additives,

since their application reduced significantly the impact of furunculosis by competitive exclusion and enhanced cellular immunity in the fish (Irianto and Austin, 2002). Similarly, a *V. alginolyticus* strain at 10^8 cells ml^{-1} was applied in a bath treatment to Atlantic salmon. Experiments revealed that application of the probiotic led to a reduction in mortality after exposures to *A. salmonicida*, and to a lesser extent after exposures to *V. anguillarum* and *V. ordalii* (Austin et al., 1995). Recently, studies have reported the presence of antifungal effects from various strains of probiotics. For example, a strain isolated from fresh water, *Aeromonas media* (strain A199) in culture of eels (*Anguilla australis* Richardson), presented antagonistic activity against *Saprolegnia* sp., supressing the growth of this opportunistic pathogen (Lategan and Gibson, 2003).

The antibacterial abilities of intestinal bacteria isolated from juveniles and larvae of Japanese flounder (*Paralichthys olivaceus*) have been studied, reporting that 53.3% of *Vibrio* spp. inhibited the growth of *Pasteurella piscicida* (Sugita et al., 2002). One hundred and six bacterial isolates from the stomach and intestine of common clownfish (*Amphiprion percula*) have been recovered. Of these, five isolates were inhibitory using its extracellular products to a wide variety of pathogen as *A. hydrophila*, *A. salmonicida*, *V. harveyi*, *V. anguillarum*, *V. damsela*, *V. alginolyticus* and *C. piscicola* (Vine et al., 2004a).

In fecal extract from turbot juveniles, the growth of *V. anguillarum* was inhibited by *Carnobacterium* cells. From the observations, it was concluded that the turbot intestinal tract and faeces can serve as an enrichment site for *V. anguillarum*, and the use of intestinal bacteria with antagonistic activity against vibrios may be used to reduce the load of fish pathogenic vibrios in turbot hatcheries (Olsson et al., 1998).

Strains that are generally used as human probiotics (e.g. lactobacilli and enterococci), have been considered for studies in fish as a novel and safe treatment in aquaculture. *L. rhamnosus*. Administration to rainbow trout for 51 days reduced the fish mortality caused by *A. salmonicida* from 52.6% in the control to 18.9 and 46.3% in the 10^9 cells g^{-1} feed and the 10^{12} cells g^{-1} feed groups, respectively (Nikoskelainen et al., 2001). Also, it has been demonstrated that survival rates of European eels (*Anguilla anguilla* L.) fed with *Enterococcus faecium* (strain SF68) were significantly higher than control groups after challenge with *Edwardsiella tarda* (Chang and Liu, 2002).

6.3. Crustaceans

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 (Rengpipat et al., 1998). A mixture of *Lactobacillus* spp. isolated from chicken gastrointestinal tracts has improved the growth and survival rates of juvenile *P. monodon* when fed these strains for 100 days (Phianphak et al., 1999). 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 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 (Vaseeharan and Ramasamy, 2003). 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 of 83, 60 and 58%, respectively. Histologic analyses after the colonization and interaction experiment confirmed that the probiotic strains had no pathogenic effect on the host (Gullian et al., 2004). 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 extracellular macromolecule digesting enzymes. However, when shrimp 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 (Alavandi et al., 2004). 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 disregulated responses induced by pathogens in host.

6.4. Mollusks

Studies in mollusks have determined that *Roseobacter* sp. (strain BS107) in co-culture with *V. anguillarum* (strain 408), displayed an inhibitory effect on *Vibrio*, enhancing the survival of scallop (*Pecten maximus*) larvae (Ruiz-Ponte et al., 1999). Similarly, cultures of *A. media* have controlled infections by *Vibrio tubiashii* in oyster (*Crassostrea gigas*) larvae (Gibson et al., 1998). Recently, the use of *Alteromona haloplanktis* (strain 77) and *Vibrio* sp. (strain 11) has been effective at controlling infections by *V. anguillarum* in scallop (*Argopecten purpuratus*) larvae (Riquelme et al., 2000).

7. Conclusions

It is essential to understand the mechanisms of action in order to define selection criteria for potential probiotics. Therefore, more information on the host/microbe interactions in vivo, and development of monitoring tools (e.g. molecular biology) are still needed for better understanding of the composition and functions of the indigenous microbiota, as well as of microbial cultures of "probiotics". The decision of using probiotics in aquaculture has been in large part a result of historical and empirical use and not based on scientific criteria. The use of probiotics is an important management tool, but its efficiency depends on understanding the nature of competition between species or strains.

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