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Review

The use of probiotics in aquaculture

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Abstract

The research of probiotics for aquatic animals is increasing with the demand for environment-friendly aquaculture. The probiotics were defined as live microbial feed supplements that improve health of man and terrestrial livestock. The gastrointestinal microbiota of fish and shellfish are peculiarly dependent on the external environment, due to the water flow passing through the digestive tract. Most bacterial cells are transient in the gut, with continuous intrusion of microbes coming from water and food. Some commercial products are referred to as probiotics, though they were designed to treat the rearing medium, not to supplement the diet. This extension of the probiotic concept is pertinent when the administered microbes survive in the gastrointestinal tract. Otherwise, more general terms are suggested, like biocontrol when the treatment is antagonistic to pathogens, or bioremediation when water quality is improved. However, the first probiotics tested in fish were commercial preparations devised for land animals. Though some effects were observed with such preparations, the survival of these bacteria was uncertain in aquatic environment. Most attempts to propose probiotics have been undertaken by isolating and selecting strains from aquatic environment. These microbes were Vibrionaceae, pseudomonads, lactic acid bacteria, *Bacillus* spp. and yeasts. Three main characteristics have been searched in microbes as candidates to improve the health of their host. (1) The antagonism to pathogens was shown in vitro in most cases. (2) The colonization potential of some candidate probionts was also studied. (3) Challenge tests confirmed that some strains could increase the resistance to disease of their host. Many other beneficial effects may be expected from probiotics, e.g., competition with pathogens for nutrients or for adhesion sites, and stimulation of the immune system. The most promising prospects are sketched out, but considerable efforts of research will be necessary to develop the applications to aquaculture.

Keywords: Microbial ecology; Digestive tract; Feed additives; Disease control; Antibacterial activity; Water quality

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1. Introduction

Long before their discovery, microbes have been unawarely used to preserve food, and these empirical methods contributed to improve human health (Bengmark, 1998). Early in the century, Metchnikoff (1907, 1908) (cited by Tannock, 1997) proposed to implant lactic acid bacteria into the human intestine, with a view to suppressing the detrimental activity of other microbes. The modern concept of probiotics was formulated only 25 years ago (Parker, 1974), then its pertinence was challenged for many years among the scientific community. Several definitions of probiotics were successively proposed. Parker (1974) originally referred to ''organisms and substances which contribute to intestinal microbial balance'' as probiotics. The definition was then restricted to ''a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'' (Fuller, 1989). Tannock (1997) noted that the effect on the ''intestinal microbial balance'' has not been demonstrated in most cases, and he proposed to speak of ''living microbial cells administered as dietary supplements with the aim of improving health''.

To my knowledge, the first application of probiotics in aquaculture seems relatively recent (Kozasa, 1986), but the interest in such environment-friendly treatments is increasing rapidly. Scientific evaluation corroborated seldom the first empirical trials, and the information was mainly spread by ''grey literature''. However, a growing number of scientific papers have dealt explicitly with probiotics, and it is now possible to survey the state of the art, from the empirical use to the scientific approach. A previous review was devoted to lactic acid bacteria in finfish (Ringø and Gatesoupe, 1998), but many other microbes have been tested as probiotics for various aquatic animals, appealing for a general overview.

It is essential to remind some definitions of ecological concepts to clear away the treatments termed improperly ''probiotic''. The goals of this paper are (1) to examine the pertinence of such terminology applied to the aquatic environment, (2) to draw the different trends of applications, and (3) to point out needs for further research.

2. Is the intestinal environment of aquatic animals favourable to probiotics?

Aquatic animals are quite different from the land animals for which the probiotic concept was developed, and a preliminary question is the pertinence of probiotic applications to aquaculture.

Man and terrestrial livestock undergo embryonic development within an amnion, whereas the larval forms of most fish and shellfish are released in the external environment at an early ontogenetic stage. These larvae are highly exposed to gastrointestinal microbiota-associated disorders, because they start feeding even though the digestive tract is not yet fully developed (Timmermans, 1987), and though the immune system is still incomplete (Vadstein, 1997). Thus, probiotic treatments are particularly desirable during the larval stages.

Gram-positive obligate or facultative anaerobes are dominant in the gastrointestinal microbiota of man and terrestrial farm animals (Gournier-Chateau et al., 1994). In human feces, the major bacterial groups are *Bacteroides*, Gram-positive anaerobic cocci, *Eubacterium*, and *Bifidobacterium* (Hume, 1997), whereas the predominant groups in pig feces are ''streptococci'' and ''lactobacilli'' (Stewart, 1997). Most probionts belong to dominant or sub-dominant genera among these microbiota, e.g., *Bifidobacterium*, *Lactobacillus*, *Streptococcus* (Gournier-Chateau et al., 1994). Gram-negative facultative anaerobes prevail in the digestive tract of fish and shellfish, though symbiotic anaerobes may be dominant in the posterior intestine of some herbivorous tropical fish (Clements, 1997). *Vibrio* and *Pseudomonas* are the most common genera in crustaceans (Moriarty, 1990), marine fish (Sakata, 1990) and bivalves (Prieur et al., 1990). *Aeromonas*, *Plesiomonas* and Enterobacteriaceae are dominant in freshwater fish (Sakata, 1990). A consequence of the specificity of aquatic microbiota is that the most efficient probiotics for aquaculture may be different from those of terrestrial species.

The resident microbes benefit from a fairly constant habitat in the gastrointestinal tract of man and terrestrial livestock, whereas most microbes are transient in aquatic animals (Moriarty, 1990). These animals are poikilothermic, and their associated microbiota may vary with temperature changes (Lésel, 1990). Salinity changes may also influence microbiota (Hamid et al., 1978; Sakata et al., 1980; Ringø and Strøm, 1994), and marine finfish are obliged to drink constantly to prevent water loss from the body. This continuous water flow increases the influence of the surrounding medium, in the same way as the water flow observed in filter-feeders, like bivalves, shrimp larvae and live food organisms. This influence is particularly important in larvae, when the gastric barrier is absent. Therefore, the intestinal microbiota of aquatic animals may change rapidly with the intrusion of microbes coming from water and food. In bivalves, the associated microbiota is very similar to those found in seawater and sediment (Sugita et al., 1981; Prieur et al., 1990). The same kinds of bacteria were found in the gut of *Penaeus japonicus* and in seawater, but normal members of microbiota may be introduced via the diet (Moriarty, 1990). In larval and juvenile fish, the influence of food has been clearly demonstrated (Tanasomwang and Muroga, 1989; Ringø et al., 1995). The influence of bacteria brought by live food organisms is particularly dramatic during first feeding (Munro et al., 1993; Bergh et al., 1994).

3. Applications of the ''probiotic'' concept in a broad sense

The transience of aquatic microbes may legitimate the extension of the probiotic concept to living microbial preparations used to treat aquaculture ponds. Moriarty (1998) proposed to extend the definition of probiotics to microbial ''water additives''. However, this extension would make too vague definition of Tannock (1997). I suggest an alternative definition of probiotics as: microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health. This latter definition will be used in the following to sort the microbial preparations which can be designated as probiotics.

In 1991, Porubcan reported on two attempts at bacterial treatments to improve water quality and production yield of *Penaeus monodon*. (1) Floating biofilters pre-inoculated with nitrifying bacteria decreased the amounts of ammonia and nitrite in the rearing water. This treatment increased shrimp survival (Porubcan, 1991a). (2) The introduction of *Bacillus* spp. in proximity to pond aerators reduced chemical oxygen demand, and increased shrimp harvest (Porubcan, 1991b). Several commercial products have sought to exploit the same idea that bacteria which improve water quality may be beneficial to animal health (e.g., ''bacteria'', Aquatic Warehouse, San Diego, CA; ''Biostart'', Advanced Microbial Systems, Shakopee, MN; ''BRF-1A, BRF-13A, PB-32, PBL-44'', Enviro-Reps International, Camarillo, CA; ''Liqualife'', Cargill, Animal Nutrition Division; ''microbial and enzymic products'', Alliance Bioremediation and Composting, Encinitas, CA; ''PondPro-VC'', Biomanagement Systems, Wellington Point, Australia; ''probiotics'', Contessa, ZB Industries, San Pedro, CA). These products are referred to as ''probiotics'' and most of them contain nitrifying bacteria and/or *Bacillus* spp. These two kinds of bacteria are quite different. The nitrifying bacteria have strict ecological niches, and they have not been detected in the gastrointestinal tract of animals. The strains of *Bacillus* spp. used as probiotics for terrestrial livestock have telluric origins, and they are not autochthonous in the gastrointestinal tract, but they may be active during intestinal transit (Gournier-Chateau et al., 1994). Moreover, there are many reports of isolation of *Bacillus* strains from fish (Hamid et al., 1978; Strøm and Olafsen, 1990; Nedoluha and Westhoff, 1995; Sadhukhan et al., 1997; Kennedy et al., 1998; Sugita et al., 1998), crustaceans (Austin and Allen, 1982; Sharmila et al., 1996; Sugita et al., 1996a), and bivalves (Sugita et al., 1981). Queiroz and Boyd (1998) confirmed that a commercial inoculum of *Bacillus* spp. increased survival and production of channel catfish, but these authors focused their attention on water quality criteria, which were poorly affected by the treatment. Kennedy et al. (1998) isolated a strain of *Bacillus subtilis* from the common snook, *Centropomus undecimalis*. The inoculation of this strain into the rearing water resulted in the apparent elimination of *Vibrio* sp. from whole larvae of snook, after decreasing salinity from ca. 30 to ca. 3 practical salinity units. Moriarty (1998) noted an increase of prawn survival in ponds where some strains of *Bacillus* spp. were introduced. This treatment decreased the proportion of pathogenic luminous *Vibrio* spp. in the sediments, and to a lesser extent, in the water. The effect on prawn intestinal microbiota was not studied. The strains of *Bacillus* were selected because of their antibiotic activity against luminescent *Vibrio* sp., but the author emphasized the multiplicity of the possible probiotic effects, e.g., enzymatic excretions, competition for nutrients and for space. These various mechanisms of action might prevent the emergence of resistant strains, a well-known risk of antibiotic treatments. However, these speculations need to be confirmed by experimental evidence, in particular, the reference to the principle of competitive exclusion, frequently put forward in advertisements for probiotics. This principle is based on the observation that experiments in chemostats ''usually result in the replacement of all members of an initial community by one organism best adapted to those highly selective substrate-limited growth conditions'' (Dolfing and Gottschal, 1997). The antibiotic activity of the strains of *Bacillus* would interfere, making uneasy to demonstrate competitive exclusion in this case. The actual data of Moriarty (1998) showed the inhibitory activity of *Bacillus* spp.

against luminous *Vibrio* sp. in pond sediment, but the effect on prawn survival might be due either to a probiotic effect, or to an indirect effect on animal health. For instance, the degradation of organic matter by *Bacillus* spp. might improve water quality. Therefore, the use of *Bacillus* spp. as pond supplement needs further investigation to be considered as a probiotic treatment, with particular attention to a possible intestinal transience.

The probiotic treatments may be considered as methods of biological control, the so-called ''biocontrol'' that termed the limitation or the elimination of pests by the introduction of adverse organisms, like parasites or specific pathogens. Maeda et al. (1997) proposed to designate as biocontrol the methods of treatment using ''the antagonism among microbes (...) through which pathogens can be killed or reduced in number in the aquaculture environment''. In this acceptation, the pond treatment proposed by Moriarty (1998) is indisputably relevant to biocontrol, as well as many other microbial treatments, including those whose target organisms are not animals, but microalgae (Rico-Mora et al., 1998). Another terminology should designate the applications of nitrifying bacteria that are related to the bioremediation concept. This concept refers to the treatment of pollutants or waste by the use of microorganisms that break down the undesirable substances (Fig. 1). The same concept is sometimes named bioaugmentation (Moriarty, 1997, 1998).

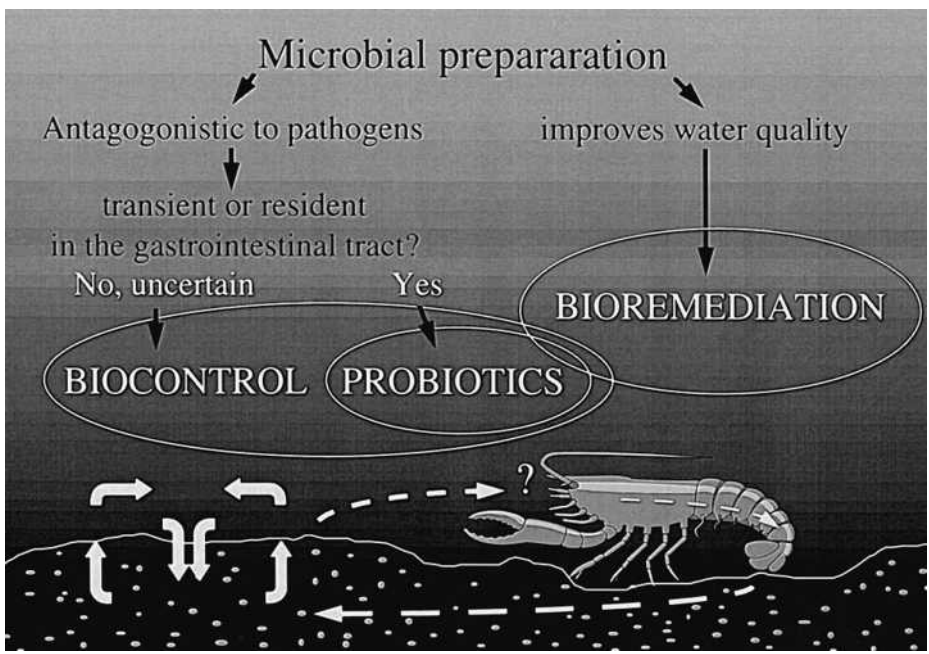

Fig. 1. Tentative classification of microbial treatments used in aquaculture, according to current terminology. The term ''probiotics'' is reserved to strains transient or resident in the gastrointestinal tract (Section 3), ''biocontrol'' implies only that the strains are antagonistic to pathogens (Maeda et al., 1997), and ''bioremediation'' refers to breakdown of pollutants or waste by the microbes (Moriarty, 1997, 1998).

4. Application of commercial products for terrestrial livestock

The first trials of incorporation of probiotics into aquaculture feeds used commercial preparations designed for land animals. Spores of *Bacillus toyoi* isolated from soil reduced the mortality of Japanese eel which were infected by *Edwardsiella* sp., (Kozasa, 1986). The same feed additive increased the growth rate of yellowtail (Kozasa, 1986). Spores may be easily incorporated into compound food, but their fate in the gastrointestinal tract of fish was not followed in these experiments. It would be particularly interesting to know whether the spores may germinate in the gut, depending on transit time and rearing temperature. The same strain of *B. toyoi* used by Kozasa (1986) was later tested on rotifers, *Brachionus plicatilis*, which were left to filter the spores for 2 h (Gatesoupe, 1989). This treatment increased the growth rate of larval turbot, but the microbiota were studied neither in the larvae nor in the rotifers. Later, a study was performed with the food additive ''Paciflor 9'' containing spores of *Bacillus* IP5832 by counting and characterising bacteria associated with spore-fed rotifers and turbot larvae (Gatesoupe, 1993). Most spores of *Bacillus* sp. were filtered by the rotifers within less than half an hour, but the number of cultivable cells of the strain decreased sharply in the rotifers 1 h after introduction of the spores into the water. These cells were thus recovered alive in the rotifers, but for a period probably too short to allow an actual probiotic effect, according to the definition suggested above (Section 3). Many bacilli produce antibiotics, especially in relation to the sporulation process (Brock, 1974), and some antibiotics may be produced by proteolysis of the vegetative cells (Vitkovic and Sadoff, 1977). When rotifers were fed with spores, the decrease of the Vibrionaceae normally dominant in the rotifers might be due to such a release of antibiotic from the cells of *Bacillus* sp. (Gatesoupe, 1993). Few cultivable cells of the strain of *Bacillus* sp. were recovered from turbot fed for five days with the spore-treated rotifers. A direct probiotic effect was therefore unlikely, though this treatment increased the resistance of turbot larvae exposed to pathogenic *Vibrio* sp.

Commercial preparations with live lactic acid bacteria have also been introduced into the medium of live food organisms for larval flatfish. Some of these treatments increased the production of rotifers and the growth of turbot and Japanese flounder (Gatesoupe, 1989, 1991; Gatesoupe et al., 1989). Some preparations with lactic acid bacteria limited also the proliferation of bacteria in rotifers, but the fate of the lactic acid bacteria was not studied in these experiments (Gatesoupe et al., 1989; Gatesoupe, 1991). Other commercial preparations of *Streptococcus faecium* improved the growth and feed efficiency of Israeli carp (Noh et al., 1994; Bogut et al., 1998). *Escherichia coli* disappeared from the intestinal microbiota of carp after 14 days of feeding with the probiotic preparation (Bogut et al., 1998). These authors stated that *S. faecium* ''has high adhesive ability in the epithelium of carp digestive tract'', but without any experimental evidence.

These trials with commercial probiotics for land animals were important to show the interest of bacterial additives in aquaculture feeds, but the survival of these microbes was uncertain in the gastrointestinal tract of aquatic animals. Most attempts have been afterwards aimed at seeking autochthonous strains with probiotic properties.

5. The search for autochthonous aquatic probiotics

5.1. Isolation and characterization of autochthonous microbes

In juvenile fish and shellfish, the autochthonous microbes may be isolated from the digestive tract after dissection, while distinguishing stomach and intestine regions. The microbes adherent to epithelial cells can be separated from those adherent to mucus, and from those transient in the lumen (Westerdahl et al., 1991). These methods are not applicable to larvae and live food organisms, but the external surface of larval fish may be washed with a 0.1% benzalkonium chloride saline solution to differentiate the microbes adherent to the external surface from those present in the gut (Blanch et al., 1997). Many microbes may be isolated on selective media (Pratt and Reynolds, 1973; Flint, 1985; Jeppesen, 1995; Donovan and van Netten, 1995). Then the isolates are characterized by proper methods (Roth et al., 1962; Hansen and Sorheim, 1991; Holt et al., 1994; Bertone et al., 1996; Austin et al., 1997; Tannock, 1999).

5.2. Pioneering studies

The first successful report seems to be attributed to Maeda and Liao (1992), who isolated a strain ''PM-4'' from the rearing water of larval *Pen. monodon*, with good survival and molting rate. The bacterium, identified as *Thalassobacter utilis* (Nogami et al., 1997), was used for the biocontrol of the larval rearing of *Pen. monodon* (Maeda and Liao, 1992; Maeda et al., 1997) and the swimming crab, *Portunus trituberculatus* (Nogami and Maeda, 1992; Nogami et al., 1997). This biocontrol treatment increased the survival of the larvae, and repressed the growth of *Vibrio anguillarum* (Nogami and Maeda, 1992) and *Haliphthoros* sp. (fungus, Lagenidales; Nogami et al., 1997). It would be worth studying whether *T. utilis* can survive in the gut of the larval crab, since *V. anguillarum* infection can start via the intestinal route (Colorni, 1985; Grisez et al., 1996; Olsson et al., 1996; Garcia et al., 1997).

Griffith (1995) reported that shrimp larvae reared in Ecuadorian hatcheries were affected by a disease characterized by a change in the bacterial population. The proportion of *Vibrio alginolyticus* decreased, whereas *Vibrio parahaemolyticus* increased. The strain of *V. alginolyticus* was isolated and used as probiotic in many hatcheries, where shrimp survival was restored to the level obtained before disease outbreak. Austin et al. (1995) investigated the probiotic effect of this strain, and these authors reported that cells of *Vibrio ordalii* lost their viability within 3 h after the introduction of freeze-dried supernatant of probiotic culture into the suspension medium. *V. anguillarum* and *Aeromonas salmonicida* were also inhibited, but to a lesser extent. The probiont survived in the intestine of Atlantic salmon for at least 3 weeks, and a preliminary bath with this probiont improved the survival of salmon challenged with pathogens. This provides an example of what might be expected from probiotics: (1) antagonism to pathogens, (2) gut colonization, with possible adhesion to intestinal mucus, and (3) increased resistance of the host to pathogens.

5.3. Antagonism to pathogens

Antagonism seems to be common among marine bacteria (Table 1). For example, over 60% of isolates from zooplankton were bacteriolytic (Nair et al., 1985), and up to 75% of the isolates from sponges produced antibacterial compounds (Marty and Martin, 1992). A pathogenic *Vibrio* sp. was inhibited by a variable proportion (0–100%) of the bacteria isolated from first-feeding halibut larvae (Bergh, 1995). Most marine ant-

Table 1
Antagonism of aquatic microbes to fish and shellfish pathogens. *Ae.*: *Aeromonas*; *Ae.h.*: *Ae. hydrophila*; *Ae.s.*: *Ae. salmonicida*; *Ed.t.*: *Edwardsiella tarda*; *En.s.*: *Enterococcus seriolicida*; IHNV: infectious hematopoietic necrosis virus; OMV: *Onchorhynchus masou* virus; *Ps.d.*: *Ps. doudorofii*; *Pa.p.*: *Pasteurella piscicida*; *V.*: *Vibrio*; *V.al.*: *V. alginolyticus*; *V.an.*: *V. anguillarum*; *V.o.*: *V. ordalii*; *Y.r.*: *Yersinia ruckeri*

Antagonist	Source	Pathogens tested	Reference
Freshwater bacteria	fish intestine	*Aeromonas* spp.	(Sugita et al., 1996b)
Freshwater bacteria	salmonid hatchery	IHNV	(Kamei et al., 1988)
Marine bacteria	invertebrates	*Vibrio* spp.	(Marty and Martin, 1992)
Marine bacteria	seaweeds	*Ed.t.*, *Pa.p.*, *Ae.* spp., *V.* spp., *Y.r.*	(Dopazo et al., 1988)
Marine bacteria	*Scophthalmus maximus*	*Ae.h.*, *Ae. salmonicida*, *V.an.*	(Westerdahl et al., 1991)
Marine bacteria	various	*Ae.h.*, *V.an.*	(Ivanova et al., 1998)
Marine bacteria	various	*En.s.*; *Pa.p.*; *V.an.*; *Vibrio vulnificus*	(Sugita et al., 1996a)
Marine bacteria	various	IHNV	(Kamei et al., 1987)
Marine bacteria	various	''*V. anguillarum*-related''	(Riquelme et al., 1997)
Aeromonas media	seawater	*Ae.* spp., *V.* spp., *Y.r.*	(Gibson et al., 1998)
Alteromonas haloplanktis	*Argopecten purpuratus*	*Ae.h.*, *V. al.*, *V.an.*, *V.o.*	(Riquelme et al., 1996)
Alteromonas sp.	*Palaemon macrodactylus*	*Lagenidium callinectes*	(Gil-Turnes et al., 1989)
Alteromonas sp.	*Pecten maximus*	*Ps.d.*, *Pa.p.*, *Vibrio* spp.	(Ruiz et al., 1996)
Alteromonas-like	*Pen. monodon* hatchery	*Vibrio* spp.	(Tanasomwang et al., 1998)
Bacillus sp.	*Callionymus* sp.	*V. vulnificus*	(Sugita et al., 1998)
Carnobacterium divergens	*Salmo salar*	*V.an.*, *V. salmonicida*	(Strøm, 1988)
Carnobacterium sp.	*Sa. salar*	*Ae.s.*, *V.an.*	(Jöborn et al., 1997)
Lactococcus lactis	*Br. plicatilis*	*V. anguillarum*	(Shiri Harzevili et al., 1998)
Lactobacillus sp.	*Paralichthys olivaceus*	*Ae.h.*, *Ed.t.*, *Pa.p.*, *V.an.*	(Byun et al., 1997)
Pseudoalteromonas undina	marine environment	IHNV, *V.an.*	(Maeda et al., 1997)
Ps. fluorescens	*Lates niloticus*	*V. anguillarum*	(Gram et al., 1999)
Ps. fluorescens	*Salmo trutta*	*Ae. salmonicida*	(Smith and Davey, 1993)
Roseobacter sp.	*Pec. maximus*	*Ae.* sp., *Ps.d.*, *Vibrio* spp.	(Ruiz-Ponte et al., 1998)
T. utilis	*Pen. monodon*	*Haliphthoros* sp.	(Nogami et al., 1997)
T. utilis	*Pen. monodon*	*V. anguillarum*	(Nogami and Maeda, 1992)
V. alginolyticus	*Pen. monodon*	*Vibrio harveyi*	(Ruangpan et al., 1998)
V. alginolyticus	shrimp hatchery	*Ae.s.*, *V.an.*, *V.o.*, *Y.r.*	(Austin et al., 1995)
Vibrio spp.	marine environment	*V. parahaemolyticus*	(Nair et al., 1985)
Vibrio spp.	shrimp hatchery	IHNV, OMV	(Direkbusarakom et al., 1998)
Vibrionaceae	*Hippoglossus hippoglossus*	*Vibrio* sp.	(Bergh, 1995)

agonistic strains are members of the *Pseudomonas–Alteromonas* and/or *Vibrio* groups (Lemos et al., 1985; Nair et al., 1985). Antibacterial activity is also common in freshwater microbiota (Sugita et al., 1996b). Some lactic acid bacteria, such as *Ca. divergens* and *Lactobacillus* sp. are antagonistic to fish pathogens (Strøm, 1988; Byun et al., 1997; Jöborn et al., 1997). Sugita et al. (1998) isolated a strain of *Bacillus* sp. that was antagonistic to 63% of the isolates from fish intestine. Pathogenic strains of *Vibrio* or *Aeromonas* have been targeted in most in vitro tests, but some other fish pathogens were also tested, e.g., *Ed. tarda*, *En. seriolicida*, *Pa. piscicida*, *Y. ruckeri* (Dopazo et al., 1988; Austin et al., 1995; Ruiz et al., 1996; Sugita et al., 1996a; Byun et al., 1997; Gibson et al., 1998). Some bacteria are antagonistic to viruses (Kamei et al., 1987, 1988; Direkbusarakom et al., 1998), and they may be efficient for the biocontrol of viral diseases (Maeda et al., 1997).

It is important to remind that antagonism may be mediated not only by antibiotics, but also by many other inhibitory substances, for example: organic acids, hydrogen peroxide (reviewed by Ringø and Gatesoupe, 1998), siderophores (Gram and Melchiorsen, 1996). The inhibition due to such compounds is highly dependent on the experimental conditions, which are different in vitro and in vivo. Therefore, the expression of antagonism in vitro is not a sufficient criterion to select candidate probiotics (Riquelme et al., 1997), nor is sufficient the absence of antagonism to rule the strains out (Rico-Mora et al., 1998).

5.4. Intestinal colonization and transience

The colonization potential is another important criterion to characterize probiotics, but transient bacteria may be also efficient if the cells are introduced at high dose, continuously or semi-continuously (Gournier-Chateau et al., 1994). In practice, it is therefore essential to evaluate the persistence of the probiotic in the gut (Table 2). The experimental introduction of lactic acid bacteria has been already reviewed (Ringø and Gatesoupe, 1998). The concentration of *Ca. divergens* was higher in the pyloric caeca than in the intestine of Atlantic cod juveniles (Gildberg and Mikkelsen, 1998). Isolates of lactic acid bacteria seemed able to survive for several days in the intestine of larval and juvenile fish (Strøm and Ringø, 1993; Jöborn et al., 1997). Vibrionaceae may also persist for days or weeks in fish (Austin et al., 1995; Munro et al., 1995; Ringø and Vadstein, 1998) and in Pacific oyster larvae, *Cr. gigas* (Gibson et al., 1998). Yeasts seemed particularly persistent in rainbow trout (Andlid et al., 1995).

Adhesion to intestinal mucus was also assayed in vitro. Such tests indicated that *Carnobacterium* sp. adhered indifferently to the intestinal mucus of rainbow trout or to control surface treated with bovine serum albumin (Jöborn et al., 1997). Autochthonous intestinal bacteria of turbot seemed to adhere specifically to intestinal mucus, since their adhesion potential was stronger to mucus than to control surface, whereas bacteria isolated from skin mucus were poorly adhesive (Olsson et al., 1992). Yeasts also adhere to the intestinal mucus of rainbow trout (Vazquez-Juarez et al., 1997), and the involvement of specific adhesins has been demonstrated (Vazquez-Juarez, 1996). Yeasts have therefore a great potential to adhere and to colonize the intestine of fish, and their application as probionts in aquaculture deserves more attention.

Table 2
Transience and artificial colonization of candidate probiotics in the gut of fish and shellfish; j: juveniles; l: larvae; n.d.:. minimum persistence time not determined (probiotic introduced daily or continuously)

Microbe	Host	Minimum persistence	Reference
Intestinal bacterium	*Sc. maximus* (j)	7 days (15°C)	(Olsson, 1995)
Ae. media	*Crassostrea gigas* (l)	2 days (20°C)	(Gibson et al., 1998)
Aeromonas sp.	*Sc. maximus* (l)	14 days (15–20°C; gnotobiotic)	(Munro et al., 1995)
Bacillus sp.	*Pen. monodon* (j)	n.d.	(Rengpipat et al., 1998)
Ca. divergens	*Gadus morhua* (j)	n.d.	(Gildberg and Mikkelsen, 1998)
Ca. divergens	*G. morhua* (l)	9 days (8°C)	(Strøm and Ringø, 1993)
Ca. divergens	*Sa. salar* (j)	n.d.	(Gildberg et al., 1995)
Carnobacterium sp.	*Onchorhyncus mykiss* (j)	4 days (11°C)	(Jöborn et al., 1997)
Carnobacterium sp.	*Sc. maximus* (l)	n.d.	(Gatesoupe, 1994)
Lactobacillus sp.	*Par. olivaceus* (j)	n.d.	(Byun et al., 1997)
V. alginolyticus	*Sa. salar* (j)	21 days (15°C)	(Austin et al., 1995)
Vibrio pelagius	*Sc. maximus* (l)	14 days (17–20°C)	(Ringø et al., 1996)
Vibrio sp.	*Sc. maximus* (l)	n.d.	(Gatesoupe, 1997)
Debaryomyces hansenii	*O. mykiss* (j)	30 days (15°C)	(Andlid et al., 1995)
Rhodotorula glutinis	*O. mykiss* (j)	65 days (8°C)	(Andlid et al., 1995)

5.5. Pathogens challenged in vivo

The improvement of the response of aquatic animals to pathogens was shown in a few challenges that followed probiotic treatments (Table 3). The animals tested were rotifers, larvae of turbot, scallop (*Ar. purpuratus*) and oyster, and juveniles of cod, Atlantic salmon, rainbow trout and *Pen. monodon*. All the pathogens were Vibrionaceae, but the probiotics were various, including Vibrionaceae, pseudomonads and Gram-positive bacteria. The probiotic-pathogen confrontation in the host is particularly important to characterize the probiotic effect, but the experimental conditions are difficult to fix. There are many factors which may influence the sensitivity of the animal to pathogens, and the efficiency of probiotic protection. These effects were sometimes difficult to observe repeatedly (Gildberg and Mikkelsen, 1998; Shiri Harzevili et al., 1998). In some studies, the mortality was only delayed, in comparison with the control without probiotic treatment (Gatesoupe, 1994, 1997; Gildberg and Mikkelsen, 1998).

Such tests were particularly useful to point out the peculiarity of the application of probiotics to bivalve larvae. Significant improvement of the survival of scallop larvae was observed after preliminary treatment with *Al. haloplanktis* for 1 h, followed by challenge with *V. anguillarum* (Riquelme et al., 1996). However, the growth of *Al. haloplanktis* in scallop larval cultures increased mortality, unlike a probiotic strain of *Vibrio* sp. (Riquelme et al., 1997). These authors assumed that the probiotic strain produced inhibitory substances that blocked bacterial growth in the larval rearing medium, including the growth of the probiotic itself (''autoinhibition''). Gibson et al. (1998) observed that the decrease of the probiotic, *Ae. media*, was even faster than that of the pathogen, *Vibrio tubiashii*, in Pacific oyster larvae, though the larvae were efficiently protected. Bivalve larvae are particularly sensitive to bacterial growth, and it seems essential that the probiotic was eliminated after a short transit time. However, some bacterial strains may increase the survival of bivalve larvae when they are

Table 3
Challenge tests of fish and shellfish treated with probiotics that improved the response to pathogens; j: juveniles; l: larvae. *Ae.s.*: *Ae. salmonicida*; *V.*: *Vibrio*; *V.an.*: *V. anguillarum*; *V.o.*: *V. ordalii*

Probiotic	Host	Pathogen	Reference
Ae. media	*Cr. gigas* (l)	*Vibrio tubiashii*	(Gibson et al., 1998)
Al. haloplanktis	*Ar. purpuratus* (l)	''*V. anguillarum*-related''	(Riquelme et al., 1996)
Bacillus sp.	*Pen. monodon* (j)	*V. harveyi*	(Rengpipat et al., 1998)
Ca. divergens	*G. morhua* (j)	*V. anguillarum*	(Gildberg et al., 1997)
Carnobacterium sp.	*Sc. maximus* (l)	*Vibrio splendidus*	(Gatesoupe, 1994)
L. lactis	*Br. plicatilis*	*V. anguillarum*	(Shiri Harzevili et al., 1998)
Ps. fluorescens	*O. mykiss* (j)	*V. anguillarum*	(Gram et al., 1999)
Ps. fluorescens	*Sa. salar* (j)	*Ae. salmonicida*	(Smith and Davey, 1993)
V. alginolyticus	*Sa. salar* (j)	*Ae.s.*, *V.an.*, *V.o.*	(Austin et al., 1995)
V. pelagius	*Sc. maximus* (l)	*Aeromonas caviae*	(Ringø and Vadstein, 1998)
Vibrio sp.	*Ar. purpuratus* (l)	''*V. anguillarum*-related''	(Riquelme et al., 1997)
Vibrio sp.	*Sc. maximus* (l)	*Vibrio splendidus*	(Gatesoupe, 1997)

introduced into the rearing medium (Riquelme et al., 1997), probably as food supplement (Douillet, 1994).

5.6. Effects in rearing conditions

Tests of antagonism, adhesion or challenge are essential to select the candidate probiotics, but rearing experiments remain necessary to conclude that the strains are harmless. Among these rearing trials, there are few reports of improvement of either survival (Ringø and Vadstein, 1998) or growth (Byun et al., 1997; Gatesoupe, 1997). This paucity of information is not surprising, since the protective effect of probiotics may be observed only when the conditions for disease outbreak are gathering. The practical evaluation of the interest of probiotic treatments will require long-term surveys.

6. Perspectives of development

The advantage of probiotics over antibiotics was discussed by Moriarty (1998), but most attention has been hitherto directed towards the production of inhibitory substances by the probiotics. The risk to select probiotic-resistant pathogens must not be underestimated, and it is particularly important to search for diversified antagonistic properties, which may lower the risk of multi-resistance. For example, the ability of some probiotics to adhere to intestinal mucus may block the intestinal infection route common to many pathogens (Evelyn, 1996).

Antagonism may be also due to competition for nutrients that favour the growth of probiotics, or the expression of their inhibitory effects. Competitive exclusion has been mentioned as a possible mechanism for probiotic effects, in reference to ''highly selective substrate-limited growth conditions'' (Dolfing and Gottschal, 1997). Iron is required by most organisms, and its availability in animal tissues may be a virulence factor for pathogens. Smith and Davey (1993) suggested that the growth inhibition of *Ae. salmonicida* by *Ps. fluorescens* was due to competition for free iron. The inhibitory activity of many *Pseudomonas* strains seemed indeed mediated by siderophores (Gram, 1993; Gram et al., 1999). Iron uptake seemed to be involved in the competition among *Vibrio* spp. (Pybus et al., 1994), and a purified bacterial siderophore could partly mimic the probiotic effect of *Vibrio* sp. in turbot larvae (Gatesoupe, 1997). The antibacterial activity of *Bacillus* sp. isolated by Sugita et al. (1998) was also attributed at least partly to a siderophore. It may be therefore important to favour the expression of such siderophore-mediated probiotic effects by adjusting the dietary supply to meet but not exceed the requirement of the host. Iron is often supplemented in excess in fish diets, and for example, iron limitation changed the microbiota without detrimental effect on seabass larvae (Gatesoupe et al., 1997).

Other nutrients may affect the intestinal microbiota, though they are essential to aquatic animals but not to microbes. For example, the dietary polyunsaturated fatty acids seemed to influence the proportion of lactic acid bacteria in the gastrointestinal tract of Arctic charr (Ringø, 1993; Ringø et al., 1998).

Gibson and Roberfroid (1995) defined ''prebiotic'' as ''a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health''. Fructooligosaccharides are thus used as food additives to stimulate bifidobacteria and/or lactobacilli in human and terrestrial animal microbiota (Bailey et al., 1991; Howard et al., 1995; Oli et al., 1998; Crittenden, 1999). Lactosucrose was found to be fermented by the hindgut microbiota of red seabream, and the thickness of tunica muscularis was increased in the anterior and posterior intestine of fish fed with the oligosaccharide (Kihara et al., 1995). This preliminary experiment suggested that some fermentation products may reinforce the intestinal barrier in fish, likewise in man (Vanderhoof, 1998). It may be interesting to seek substances specifically digestible by candidate probiotics, which might not only antagonize pathogens but also stimulate the tissular defence of the host.

The hypothesis of stimulation of the immune system of aquatic organisms may be also considered. Many immunostimulants have been tested on fish and shellfish, and some of them originated from microbial cell walls, e.g., muramyl dipeptide, glucans, lipopolysaccharides (Anderson, 1992). It is possible that autochthonous microbiota may stimulate the immune response of aquatic animals to enteric pathogens, as reported in land animals (Gaskins, 1997). However, the stimulation of the immune system by probiotic lactic acid bacteria is still in dispute, despite many reports (Tannock, 1997; Gill, 1998; Pouwels et al., 1998; McCracken, 1999).

7. Conclusion

The application of probiotics in aquaculture shows promise, but needs considerable efforts of research. The first question, unanswered in many cases, is the fate of the probiotic in rearing medium and in gastrointestinal tract. Immunological and molecular probes will be useful tools to trace the probiotic cells (Ringø et al., 1996; Austin, 1998; O'Sullivan, 1999). It is essential to investigate the best way of introduction and the optimal dose, and technical solutions are required, especially to keep the probiotic alive in dry pellets.

The spores of *Bacillus* spp. are especially easy to introduce in dry food, and this is an additional advantage of these promising candidate probiotics (Moriarty, 1998; Queiroz and Boyd, 1998; Kennedy et al., 1998; Rengpipat et al., 1998; Sugita et al., 1998). Lactic acid bacteria are also good candidates, and further studies are necessary to evaluate the interest of yeasts as probiotics. The bacteria normally dominant in healthy animals may be sources of probiotics, but there are many potential pathogens among Vibrionaceae and pseudomonads. It may be wise to carry out long-term surveys to make sure that the bacteria keep innocuous, without risk of apparition of potentially detrimental mutants.

The influence of probiotics on gastrointestinal microbiota remains poorly described, but further investigation may be expected with the propagation of molecular approaches to analyse bacterial communities (Raskin et al., 1997; Wallner et al., 1997; Hugenholtz et al., 1998).

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