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Abstract

The aquaculture of bivalve molluscs has attained a considerable level of production but it is not enough to cover the demand of worldwide consumers. In the development of this sector, hatcheries play an important role, as suppliers of competent spat of different bivalves, including species with an aquaculture based on natural extraction present. Besides, these installations may help in the recovery of exhausted natural beds and in the obtaining of populations under genetic selection.

Unfortunately, the disease outbreaks caused by bacterial pathogens are frequent, with the loss of complete batches, compromising the regular production and the economic viability of the industry.

There are many descriptive studies about these outbreaks, but only a few focused on the control of microbiota. The particularities of bivalve aquaculture in hatchery must be taken into account to design methods of control. A common environment is shared by larvae and bacteria, including both beneficial and potentially pathogenic. The filter-feeding behaviour of larvae increases the strong influence of these populations.

The classical treatments are directed toward to the complete elimination of bacteria from culture seawater. That objective is unfeasible, because the cultures are not axenic, and undesirable, since some bacteria enhance larval development. Taking into account these considerations, the most promising alternative is the use of probiotic bacteria. In this review we summarize the scientific literature about this subject, considering the particularities of bivalve larval cultures and the need to adapt the concept of probiotic and the strategies to use in marine bivalve hatcheries.

Keywords: probiotic, aquaculture, bivalve, larvae, hatchery

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1. INTRODUCTION

Bivalves belong to the phylum Mollusca, a group that includes such diverse animals as chitons (chain shells), gastropods (abalones) and cephalopods (squid and octopus). The Class Bivalvia is formed by animals compressed laterally and with the soft body parts completely or partially enclosed by the shell, which is composed of two hinged valves. The gills are well developed organs, specialized not only for respiration but also for filter-feeding (Helm et al., 2004).

The main bivalve species used in marine aquaculture belong to families *Mytilidae* (genus *Mytilus* and *Perna*), *Ostreidae* (genus *Crassostrea*, *Ostrea* and *Saccostrea*), *Veneridae* (genus *Ruditapes* and *Mercenaria*) and *Pectinidae* (genus *Patinopecten*, *Argopecten* and *Pecten*), i.e., mussels, oysters, clams and scallops (<http://www.fao.org/docrep/w2333e/W2333E05.htm#51>).

In 2006, FAO estimated that the 65 percent of mollusc production was originated from aquaculture, being the second-largest share of global aquaculture production, with 14.1 million tones (www.fao.org/docrep/011/i0250e00.htm). Within the aquaculture of molluscs, bivalves are the most important group. Despite the advances in the production of certain bivalves species by marine aquaculture (4.2×10^6 Tons of *Crassostrea gigas*, Pacific oyster, and 3.0×10^6 Tons of *Ruditapes philippinarum*, Manila clam, according to FAO 2007), an increase is still needed to cover the demand of consumers. The worldwide decline of natural beds has appreciated the importance of the hatcheries to support, by supplying competent spat, the change of extractive activity to the aquaculture of complete cycle.

These installations vary greatly in their design, configuration and construction depending on species cultured, production levels and local conditions. However, the basic activities for any bivalve hatchery are conditioning and spawning adults, rearing and setting larvae, rearing juveniles to an acceptable size, together with the production of large quantities of food (microalgae) to feed all stages of the production cycle. Besides the physical plant, the water system is critical to guarantee the supply of high quality seawater (Helm et al., 2004).

Hatcheries have several advantages, as the possibility of cover seed requirements out of the natural growing season, the supply of genetic strains with improved biological characteristics or new bivalve (exotic) species. By the other side, the culture of bivalves in hatchery is frequently affected by serious disease outbreaks, mainly related to bacterial infections caused by members of genus *Vibrio*. The main consequence is the lost of complete batches of production, arising not only economic problems to the industry but also the lack of regularity in the supply of spat for the ongrowing in the open beds.

Despite of the numerous descriptions of outbreaks in hatcheries, the scarcity of systematic and deep studies, and therefore the lack of knowledge in the bacterial populations associated to these cultures have led to the search of partial solutions always focused on the elimination of the microbiota from the culture seawater. The different methods employed until present, from treatment of the rearing water to chemotherapy, have showed limited success in avoiding mortalities. This fact has forced to the consideration of alternatives to resolve the problem. Among them, the use of probiotic bacteria has appeared as the most promising one. The approach pursues the obtaining of an equilibrated bacterial population with ability of autoregulation.

The aim of the present review is to summarize the scientific literature about probiotics designed or applied in bivalve hatcheries, discussing the particular characteristics of these cultures, and their repercussion on the concept of 'probiotic' and on the strategy to use them.

2. THE CLASSICAL APPROACH FOR CONTROL OF PATHOGENS IN BIVALVE HATCHERIES

2. 1. Water treatment

The influence of the environment on hatchery cultures is paramount. The bivalve larvae live in seawater, sharing the environment with both beneficial bacteria and potential pathogens. Besides, they are released in early ontogenic stages, being hence especially sensitive to potential infections, favoured by the filter-feeding behaviour and the subsequent flow of seawater through their organisms.

Other factors increasing the risks are derived from the system design, a closed circuit with the same water along periods of 2-3 days or a semi-closed system with a low renovation rate. Besides, there is a regular supply of organic matter and bacteria with the food. Therefore, the microbiological quality of the water is the main point to control the appearance of bacterial diseases.

In hatcheries, the water is subjected to treatments with the aim to reduce the associated bacterial population. Generally, the first step is the decantation of the water pumped from the sea. Once most of the solid particles are eliminated, the water is filtered. Filtration is an expensive treatment, with incremental costs in function of the volume and the degree reached. In fact, only larval cultures and small-scale phytoplankton cultures receives maximum filtration.

The filtration reduces the number of viable bacteria, but its efficiency related with the total number of bacteria is irregular. The studies of Jeanthon et al. (1988) with hatchery cultured scallop, *Pecten maximus*, showed an increase in the counts of viable heterotrophic bacteria in the days following filtration, probably associated to the daily supply of phytoplankton. Anyway, it is an advisable practice to reduce contents of bacteria as well as organic matter, and its results are better than the obtained by other systems like pasteurization, as showed by Lewis et al. (1988) in a hatchery of the Pacific oyster, *Crassostrea gigas*. The main disadvantage of the pasteurization is the risk of contaminations due to the necessity of long pipes and the time of stay of the water there until it cools down.

The disinfection with chlorine has been employed too, but it shows many problems, like the interference in the larval mechanism of pumping, as described by Vasconcelos and Lee (1972) for cultures of *C. gigas*. Some studies suggested that the reactions among

chlorine and organic nitrogen in the water could produce toxic residues for marine organisms (Jorquera et al., 2002).

Other alternative is the ozonization, but its application to disinfect aquaculture systems can be complex and costly (Summerfelt, 2003), this last factor especially relevant for the case of bivalve hatcheries if is taken into account the cost of production stocks. The ozone forms oxidants in seawater which are relatively stable, resulting toxic to aquaculture species (Richardson et al., 1982; Summerfelt, 2003). These facts, together with the toxicity of ozone gas to humans, imply that care must be used in the utilization of this method. Moreover, the effective dose to achieve inactivation of different pathogens needs further studies. Among the scarce literature about the effects of ozone on bivalves, Richardson et al. (1982) investigated the ozone toxicity to *Crassostrea virginica*, the American oyster, and compared the results with previous studies on chlorine toxicity (Roosenburg et al., 1980). They established that the sensitivity of larval oysters to ozone- and chlorine-produced oxidants is greatest immediately following fertilisation and decreases with larval age. Besides, they suggested that variations in water quality can drastically modify the specification and reaction paths of residual oxidants and their by-products. Therefore, this method is not the most suitable for bivalve hatcheries at least until present.

One of the most usual treatments is the radiation of seawater with ultraviolet light, with unquestionable lethal power on bacteria. However there are disagreements about the true effects when the treatment is used on water culture in hatcheries. Vasconcelos and Lee (1972) found advantages in its use, like the decrease of different bacterial populations, (*Pseudomonas*, *Vibrio*, Coliforms and Gram-positive cocci), including bivalve pathogens. Among the microbiota studied by these authors, only the group *Acinetobacter-Moraxella* increased its level. Other authors, like Murchelano et al.

(1975) found important variations among samples of larval cultures of *C. virginica*, although with significant reduction in the average numbers of bacteria. There was a decrease of *Vibrio*, *Achromobacter* and *Flavobacterium*, but the *Pseudomonas* (dominant in the water with treatment too) rose. In general, the treated water presented less diversity and a reduction in pigmented bacteria. In larval cultures of *Ostrea edulis*, the population of vibrios decreased with UV treatment (Lodeiros et al., 1987). Nevertheless, Brown (1981) found that the same treatment with UV tested against two pathogenic *Vibrio* spp. was effective against one of them, while the other strain was able to grow after an initial inhibition.

Therefore, the effects of UV-radiation treatment are not homogenous against all the bacterial populations, and more variability is added with factors like the dose, the water flow and the individual efficiency of the radiation unit. Brown and Russo (1979) studied the effect of UV-treatment in seawater previously filtered and inoculated with bacterial pathogens (*Vibrio* and *Pseudomonas*) and using for hatch *C. virginica* larvae. Despite of the decrease of mortalities, they warned about the risk of the change from bactericide to only bacteriostatic effects if the dose is inappropriate.

This risk of temporary inhibition, with only delayed growth of some bacteria, was suggested in other studies (Brown, 1983). Once again, Liltved et al. (1995) observed the need to use different doses to inactivate completely distinct pathogens. Moreover, it is known that the efficiency of the treatment is affected by the organic content of water and the presence of small particles with attached bacteria (Liltved and Cripps, 1999). In summary, these big differences in the effectivity can led to the selection of undesirable populations, among those resistant to the treatment, which would find an ecological niche favourable to their growth. Other disadvantages are the high economic cost and the impossibility for the treatment of big volumes.

The use of a charcoal filter increased the effectiveness of UV radiation, removing organic material from the water. However, in experiments with the Eastern oyster, *Crassostrea virginica*, this treatment was occasionally detrimental after three weeks, when the culture water was not changed daily (Brown, 1983).

2. 2. Chemotherapy

The use of chemotherapeutant agents has been widespread in bivalve hatcheries, since Davis and Chanley (1956) showed their ability to reduce the larval mortalities in hard clam, *Mercenaria mercenaria*. However, in parallel with studies confirming the beneficial effects, other works described toxic effects, as well as a lack of effectiveness or consistency in the results obtained. From the 60's of the past century the most used antibiotics in bivalve hatcheries have been streptomycin and penicillin, alone or in combination, and chloramphenicol (see reviews D'Agostino, 1975; Le Pennec and Prieur, 1977; Prado, 2006).

Jeffries (1982) observed that the treatment with chloramphenicol achieved a slight recovery in a larval culture of *C. gigas*, but at the same time the larvae ceased swimming and therefore feeding. In experiments with the blue mussel, *Mytilus edulis*, the treatment with chloramphenicol resulted in good survival but slow development, showing an opposite effect to the combination of ampicillin-streptomycin, with excellent development but low survival (Hily, 1974). In other cases, the same antibiotic may have variable effect, toxic or beneficial, on the same species, like polymyxin B in *C. virginica* larvae (Brown, 1983). For this oyster species, the erythromycin was toxic at the effective dose (Brown and Tettelbach, 1988), while for *P. maximus* the results obtained with this antibiotic were inconsistent (Robert et al., 1996). In cultures of the

giant clam, *Tridacna derasa*, the rifampin provided a good initial survival but caused malformations (Fitt et al., 1992).

Especially interesting is the study of Hidu and Tubiash (1963). The use of Combistrep (dihydrostreptomycin-streptomycin sulphates) enhanced the growth rate of larval cultures of *M. mercenaria* and *C. virginica*. They observed a direct relation between the initial bacterial load in seawater and the effect obtained, including the lack of favourable effect on axenic cultures. The analysis of the changes in the bacterial populations showed a direct increase of the number of bacteria in relation to the dose of antibiotic. Therefore, it was suggested that the antibiotic selects a determinate bacterial population, which really has a positive effect on the larval growth, maybe by inhibition of toxin-producing bacteria.

Other objections derive from the characteristics of the substances, as the rapid degradation of some of them in solutions of high pH like seawater (tetracycline and penicillin), the appearance of resistances (streptomycin) or the toxicity to invertebrates (neomycin). Chloramphenicol, the most common antibiotic in bivalve hatcheries during years, has a long self-life but is extremely toxic, may cause larval malformations, imply known risks for human, and favour the development of resistances.

Other disadvantage is the economic cost of the antibiotics, which can be equal to the cost of the stock in aquaculture (Baticados et al., 1990). In this way, the simultaneous use of antibiotics, the more effective option, could not be applied in larval cultures of bivalves (Walne, 1958; Fitt et al., 1992).

The development of resistances deserves special mention, making possible that bacterial populations initially sensitive to an antibiotic may growth in its presence (Díaz-Granados et al., 2008; Prado et al. manuscript in preparation). Plasmids naturally present in different marine bacteria (including pathogenic vibrios) can confer resistance

to a wide spectrum of antibacterial agents, implying a risk of infection even for hatcheries where they are not used (Jeffries, 1982; Brown and Tettelbach 1988; Akinbowale et al., 2006). The appearance of resistances is favoured by the routine of use in hatcheries, as preventive treatment (Le Pennec and Prieur, 1977), due to the sudden mortalities and the impossibility of treatment once they are detected. The consequence is the rise and persistence of diseases that cannot be efficiently treated (McPhearson et al., 1991; Spanggaard et al., 1993; Karunasagar et al., 1994).

The possibility of transfer antibiotic-resistance factors to human pathogens also exists, with the subsequent risk for the hatchery workers (Jeffries, 1982; MCPhearson et al., 1991; Spanggaard et al., 1993). Moreover, the water exchange between the hatchery and the environment may spread resistant bacteria (McPhearson et al., 1991; Kemper, 2008).

Finally, the legislation is very restrictive with the use of antibiotics. Nowadays, the chloramphenicol is prohibited in any veterinary applications to avoid the health hazards to humans by food consumption at least within the EU (Annex IV of Directive 2377/90/EEC), due to its potential toxic effects in human as well as to the impossibility of determinate security levels of its residues.

All the treatments considered in this review (filtration, ultraviolet radiation, chemotherapy) are focused on the attempts to reach the complete elimination of the microbiota associated to the water. This aim is not reasonable, because the bacterial populations, or at least part of them, have a beneficial effect on the larval development. Hidu and Tubiash (1963) proposed the existence of a relation between larval growth and the species and number of bacteria present in the cultures, suggesting their role as nutrient source. The bacteria seem to satisfy metabolic requirements, providing vitamins

or other growth factors (Prieur et al., 1990). The production of extracellular enzymes may enhance the digestion of food or the elimination of toxic residues (Douillet and Langdon, 1993). In this sense, studies with *Argopecten purpuratus* (Chilean scallop) larvae cultured in seawater after different degree of filtration (5.0, 1.2 and 0.2 μm) showed that the best survival rates were reached in rearing water filtered through 5.0 μm . This water contained particles and bacterial aggregates which could be ingested by larvae and utilized as growth promoters (Riquelme et al., 1997).

The complete elimination of microbiota favours the colonization of the system by bacteria which may be not beneficial. By one hand, the natural competition among bacterial populations disappears and therefore a desirable ecological niche is empty. By the other, there is a regular supply of organic matter combined with the special culture conditions, together with the entry of bacteria in the system with the food and associated to larvae. In summary, the environment is appropriate for the proliferation of bacteria with fast growth rates, as the members of genus *Vibrio*, the opportunistic pathogens most frequently associated with bivalve larval cultures.

Therefore, the aim has to be to obtain an equilibrated bacterial population, with the ability to control of the proliferation of potential pathogens naturally present in the system.

The vaccination, an alternative with excellent results in fish cultures (Toranzo et al., 1996; Romalde et al., 2003), is not valid for molluscs, because their lack of an adaptive immune system.

All the facts above exposed make clear the need of searching for new solutions to control bacterial diseases in larval cultures of bivalves in hatcheries.

3. PROBIOTICS

3. 1. Definition of probiotic

The first reference to the term 'probiotic' dates back to 1965, when Lilly and Stillwell used it to define "substances produced by one protozoan that stimulated the growth of another". In 1974, Parker defined 'probiotic' as "organisms and substances which contribute to intestinal microbial balance". This definition may include antibiotics or short chain fatty acids, making necessary more precision, as proposed by Fuller (1989) "live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance". Tannock (1997) pointed out that the effect on intestinal balance is not demonstrated in most cases, suggesting "living microbial cells administered as dietary supplements with the aim of improving health".

All these definitions are designed for the use of probiotics in homeotermes, but their application in aquaculture is very difficult, taking into account the bacterial transit in aquatic environment. Therefore, the broadening of the concept proposed by Moriarty (1998) to include all the live microbial additives used to treat the cultures is justified, though made very imprecise the definition of Tannock.

In 1999, Gatesoupe gave an alternative for the use in aquaculture, "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". The treatment with probiotics would be a method of 'biological control' or 'biocontrol', term referred to the "limitation or the elimination of pests by the introduction of adverse organisms, like parasites or specific pathogens". In this sense, Maeda et al. (1997) had proposed to name 'biocontrol' those methods of treatment using "the antagonism among microbes (...) through which pathogens can be killed or reduced in number in the aquaculture environment".

Gram et al. (1999) widened the definition of probiotic to “a live microbial supplement which beneficially affects the host animal by improving its microbial balance”, eliminating the restriction to intestinal balance. Gómez-Gil et al. (2000) supported this consideration, distinguishing ‘probiotic’ from ‘biological control’, because a probiotic not necessarily makes a direct attack against a pathogen, may simply prevent the damages caused by it, impede its union to host, inhibit its growth... The authors separate the term ‘growth promoter’ too, since the probiotic action is associated to the general enhancement of target organism health, not only to the growth.

Finally, Verschuere et al. (2000) gave a definition that includes the narrow interaction in aquaculture between animal and environment, being the best adapted definition to application to larval cultures of bivalves. A ‘probiotic’ is a “live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment.”

Anyway, the controversy continued with the publication of new studies about the definition of ‘probiotic’ in relation to aquaculture. Irianto and Austin (2002) proposed the definition: “an entire or component(s) of a micro-organism that is beneficial to the health of the host”, taking up again the characteristics formerly suggested, like the effectiveness in extreme temperature and salinity ranges. Besides, they distinguish ‘probiotic’, additive supplied by food, from ‘vaccine’, referred to veterinary products supplied by injection or immersion. Once again there are difficulties to apply the term to bivalve aquaculture, because it is impossible to consider the establishment of a bacterial population in larvae different from those present in water culture (Jorquera et al., 2001) and to determine the suggested differences between vaccination and probiotic. Vine et

al. (2006) used in their review on probiotic in marine larviculture the definition of Gatesoupe (1999), but despite the title the work only tackle fish larviculture. Balcázar et al. (2006) considered a broad concept, “bacteria that promote the health of other organisms”, but again the approach is based on the characteristics of fish cultures. In a new review, Kesarcodi-Watson et al. (2008) supported the definition of Irianto and Austin (2002), indicating that the elimination of the requirement of being live cultures enable the inclusion of substances, bacterial derivatives, with immunostimulatory properties. By the other hand, they considered that the activity in the gastrointestinal tract is not necessary because the desired effects, like competition for nutrients or production of inhibitory substances, may occur in the water.

Taking into account all the definitions proposed for application in aquaculture, and specifically in bivalve larval cultures, the more suitable is the proposal of Verschuere et al. (2000). This definition includes the requirement of being a live organism, very important because makes possible the self-regulation of the microbial balance by the system, avoiding the troubles derived from the use of substances like antibiotics. It considers the special characteristics of larval cultures, because of their narrow interaction with the environment as well as the lack of a real immune system.

3. 2. Modes of action

There are many publications about probiotics in aquaculture, but the modes of action were rarely supported by strong experimental data (Verschuere et al., 2000). The beneficial effect may be reached by different mechanisms: nutritional complement, improvement of the use of feed, enhancement of the immune response, antibacterial activity, competence for substances, energy or adhesion sites, stimulation of biological processes and improvement of the quality of the water.

For the specific case of bivalve larval cultures, only some of these modes of action have been described (Table 1). The probiotics can work as nutritional complement (Douillet and Langdon, 1994) or play a stimulatory role in very important processes in bivalves like settlement or metamorphosis (Fitt et al., 1990; Szewzyk et al., 1991; Tritar et al., 1992; Leitz and Wagner 1993; Walch et al., 1999). Anyway, given the circumstance that the larvae are released to the environment in early ontogenic stages, and therefore they are exposed to microbiota-associated disorders before a complete development, the most promising mode of action seems the antibacterial activity, spread among marine bacteria (Dopazo et al., 1988; Barja et al., 1989; Lodeiros et al., 1989; Lemos et al., 1991; Riquelme et al., 1996; Riquelme et al., 1997; Gibson et al., 1998; Jorquera et al., 1999; Nakamura et al., 1999; Ruiz-Ponte et al., 1999; Riquelme et al., 2001; Prado, 2006). The inhibition enhances the water quality, with regard to the composition of bacterial population and the control of potential pathogens (Table 2), searching a balance dependent on the interactions among the microbiota and avoiding the risks related to the use of chemotherapeutants.

The desirable characteristics of a probiotic strain, apart from the condition of live microorganism, adapted to the application in bivalve aquaculture, are:

- a) Origin in the environment where it will be used, in other words, marine bacteria, better autochthonous from hatchery (Riquelme et al., 1996; Verschuere et al., 2000). This fact guarantees the ability of the strain to develop in the environment and minimize the risks due to the introduction of alochthonous organisms in the system.
- b) Beneficial effect on the target organism, by any of the modes of action detailed above.

- c) Lack of pathogenicity and toxicity, not only for the target organism, but for other live organisms also present in the environment, like phytoplankton. Besides, the strain must not proliferate until dangerous levels in the water culture.

3. 3. Application of probiotics in bivalve cultures

Most of the studies about probiotics in aquaculture are focused on fish and crustaceans cultures, with scarce literature considering their application in bivalve molluscs.

Lodeiros et al. (1989) published a first work on the antibiotic effect of marine bacteria on the larval survival of scallop *Pecten ziczac*. They compared the effects of three different strains, *Alteromonas* sp. x24, *Pseudomonas* sp. P12 and *Flavobacterium* sp. P14, assaying live cells as well as antibiotic extracts. The two first were toxic in all the experiments, but *Flavobacterium* sp. P14 was able to counteract the pathogenicity of *Vibrio anguillarum* EPp3. The best results were achieved by the use of live bacterial culture.

Douillet and Langdon (1993) carried-out a set of experiments with axenic oyster larvae (*C. gigas*), fed with a monoalgal axenic diet and inoculated with different marine bacteria. Most of the isolated assayed had negative, neutral or variable effects, and only the strain CA2 (*Alteromonas* sp.) showed a beneficial effect on the growth and survival of oyster larvae. The mode of action proposed was the nutritional supply to the insufficient monoalgal diet, enhancing the nitrogen fixation or the digestion of the ingested microalgae by the bacterial extracellular enzymes. In later experiments with this strain in non-axenic larval cultures, Douillet and Langdon (1994) found a variation of the effect depending on the bacterial concentration, being harmful with 10^7 cells per millilitre for larval growth and survival, whereas values around 10^4 - 10^6 cells/ml did not

affect survival and enhance larval growth. The optimal concentration, 10^5 cells/ml, was within the usual values for bacterial populations in bivalve cultures obtained by plate counting, between 10^4 and 10^6 cells/ml (Jeanthon et al., 1988). The authors suggested the same modes of action mentioned above. They pointed to the beneficial effect observed in the percentage of larvae metamorphosed to spat and their size after 30 days, a possible indirect effect of the benefits on growth and development.

The broadest works on the application of probiotic bacteria in bivalve cultures were developed by the group of Riquelme, with *Argopecten purpuratus* larvae. They assayed the effect of the strain INH, *Pseudoalteromonas haloplanktis* (formerly *Alteromonas haloplanktis*), isolated from gonad of broodstock (Riquelme et al., 1996). First, they determined the *in vitro* inhibition spectrum of the strain against different bacterial species, including members of genus *Vibrio*. The compound(s) with inhibitory activity, as observed in experiments in liquid medium with *Vibrio anguillarum*-related (VAR) and *V. alginolyticus*, was produced by live bacterial cells, being probably intracellular, a secondary metabolite(s) excreted in stationary phase of growth. The incubation of larvae in suspensions of strain INH conferred them protection against a further infection with VAR at moderate concentration (10^3 cells/ml). This fact suggests a possible use as a prophylactic method, against the usually low levels of opportunistic pathogens in aquaculture systems. In the following work, Riquelme et al. (1997) used for the first time the term 'probiotic'. The strain 11 (*Pseudomonas* sp.) was selected among 506 isolates from the larval cultures of *A. purpuratus*, on the basis of the antibacterial activity against pathogenic VAR-strain and the larval protection against the same pathogen by preincubation in suspensions of potential probiotic strain. Avendaño and Riquelme (1999) studied the use of axenic microalgal cultures (*Isochrysis galbana*) as administration route of bacteria with inhibitory activity. As result of the experiments,

the strain C33 (*Vibrio* sp.) was selected due to the ability to growth in presence of microalgal extracellular products, to inhibit the pathogenic VAR and to enhance the growth of phytoplankton, together with the efficient and significant ingestion by *A. purpuratus* larvae after inoculation with the microalga. In further experiments with pure bacterial cultures, they found that there is ingestion of the probiotic strain by adults (Avendaño-Herrera et al., 2001) but not by larvae (Riquelme et al., 2000). The spectrum of antibacterial activity of *Vibrio* sp. C33 and the production of bactericide substances were also studied (Jorquera et al., 1999). Finally, Riquelme et al. (2001) carried-out experiments adding the inhibitory-producer bacterial strains to massive larval cultures. The inoculation of mixtures of strains *Vibrio* sp. C33, *Pseudomonas* sp. 11 and *Bacillus* sp. B2, made possible the development of larval stages without antibiotics. They showed evidence that the antibiotics reduce the levels of bacteria in the water, but did not impede their proliferation into larvae, whereas the incorporation of selected bacteria allowed a modification of the microbiota associated to larvae.

The term 'probiotic' was also used by Gibson et al. (1998) in a study about the protection exerted by the strain A199 (*Aeromonas media*) on *C. gigas* larvae, against the infection by *Vibrio tubiashii*. The strain A199 showed a wide spectrum of *in vitro* inhibition, including members of genus *Vibrio* and *Aeromonas*. The conditioning of larvae with the probiotic strain (10^4 colony forming units (cfu)/ml) allowed the survival of larval cultures inoculated with the pathogen (10^2 , 10^3 y 10^5 cfu/ml). Nakamura et al. (1999) protected *C. gigas* larvae against *V. alginolyticus* inoculating the strain S21, isolated from seawater on the basis of the inhibition of pathogenic vibrios (*Vibrio* sp. ATCC 19107, *V. alginolyticus* ATCC 19108 and *V. tubiashii* ATCC 19109) in assays on solid medium. The inhibitory activity was confirmed by cocultures in seawater of strain S21 and *V. alginolyticus*. Finally, the authors demonstrated that the inoculation of

probiotic (10^4 and 10^5 cfu/ml) immediately after the pathogenic strain (10^5 cfu/ml) reduced the larval mortality from 91.6% to 53.1 and 78.0%, respectively.

The same mechanism of action was the basis of the study of Longeon et al. (2004) with *P. maximus* larvae. They isolated the marine bacteria *Pseudoalteromonas* sp. X153, with a wide spectrum of inhibitory activity *in vitro*. The antimicrobial compound (P-153) was purified, but the instability of this protein and the problems to obtain enough quantity prevent for doing direct assays with larval cultures. With the aim of the use of the live bacteria as probiotic strain, the toxicity was ruled out in a short term experiment with *Ruditapes philippinarum* larvae, at bacterial concentrations until 10^7 cells/ml. In a long-term experiment, they compared the evolution of larval cultures of *P. maximus* without any additive (control), inoculated with the strain X153 or with chloramphenicol. The results showed a higher survival in cultures with strain X153 than control, but slightly lower than with antibiotic. However, the growth rate of larvae with bacterial inoculum was lower than the other two cultures. Ruiz-Ponte et al. (1999) carried-out experiments with scallop larvae too, and the marine isolate BS107 *Phaeobacter gallaeciensis* (formerly *Roseobacter gallaeciensis*). The strain displayed *in vitro* activity against a number of bacteria, including aquaculture pathogens like *V. pectenicida* A496. The authors stated that the strain BS107 displays antibacterial activity only in the presence of other bacteria, which release a proteinaceous molecule that acts as effector inducing the inhibitory activity. However, it is necessary to take in account that the experiments were performed only with culture supernatants, growing alone or with pathogenic vibrios, considering that the inhibitory ability implies the massive release of a compound to the environment. This consideration leads to studies about the use of a substance, but not of application of a probiotic bacterium. The live cells of BS107 added to larval cultures were unable to reduce the mortality caused by *V. pectenicida*,

with worse results than the cultures with chloramphenicol or without any addition. The use of cell extracts was as effective as the treatment with chloramphenicol, in terms of larval survival, but did not confer protection against *V. pectenocida*.

More recently, and following the same steps described in previous works, the isolate PP-154 (*Phaeobacter* sp.) was selected by Prado et al. (2009) among a collection of isolates from bivalve hatcheries. This strain displayed the strongest ability of inhibition *in vitro*, as demonstrated by the spot method (Lemos et al., 1985). In the experiments, four different target strains were used, including the known aquaculture pathogen *Vibrio anguillarum* and the Gram positive *Staphylococcus aureus*. Besides, two bivalve larval pathogens isolated in parallel works (Prado et al., 2005), *Vibrio neptunius* PP-145.98 and *Vibrio ostreocida* PP-203, were selected. Assays with the double-layer method (Dopazo et al., 1988) confirmed the results obtained with cell spots.

A screening of the antibacterial activity was performed, showing the broad spectrum of inhibition of the strain PP-154. Among the susceptible bacterial species, there were all the species of genus *Vibrio* assayed, other aquaculture pathogens (*Tenacibaculum maritimum*, *Photobacterium damsela* subsp. *piscicida*, *Pseudomonas anguilliseptica*, *Edwardsiella tarda*, *Aeromonas hydrophila* and *Streptococcus parauberis*) and clinical strains (*Streptococcus faecalis*, *Proteus vulgaris*, *Citrobacter freundii* and *Escherichia coli*) (Prado et al., 2009).

Furthermore, the antibacterial activity was confirmed in seawater. The experiments demonstrated the inhibition of the growth of the two vibrios pathogenic for bivalve larvae (Prado et al., 2009). In both cases the pattern recorded was the same, the strain PP-154 needed to be incorporated to the system previous or simultaneously to the income of the pathogen and the increase of its population in the environment. In any case the potential probiotic maintained their numbers in the medium, avoiding the risk

of a dangerous proliferation. The assays extrapolated the ability described in solid medium to the seawater, the environment for probiotic application.

In the course of these studies, a new pathogen, *Vibrio* sp. PP-638, was obtained from a disease outbreak in flat oyster larvae. It was demonstrated *in vitro* its ability to cause severe mortalities, being dependent on the dose (concentrations higher than 10^3 cfu/ml, 100% mortality after 48 hours). Micro-scale probiotic experiments showed that the strain PP-154 added to the larval culture reduced the mortality rates caused by the vibrio, whenever the inoculated concentration of pathogen was lower than 10^4 cfu/ml (Prado, 2006; Prado et al., 2001).

Interestingly, the incorporation of strain PP-154 in seawater also enhanced the survival of naturally infected larvae compared to control without bacterial addition. In the same assays, marked differences in the vibrio population of seawater were observed, with a drastic reduction in seawater, according to the estimation by the growth in TCBS plates (Prado, 2006; Prado et al., 2001).

The design of a protocol for use in aquaculture systems was the next step. With this aim, the optimal growth conditions of temperature and salinity were investigated, together with the relation between growth and antibacterial activity. The results indicated that the use of PP-154 should be based on cultures at 25°C and salinities higher than 25‰. The strain showed inhibitory activity after culture in Marine Broth, even with agitation, but in other media too. The pellet recorded the strongest activity between days 4 and 8, while the supernatant was only weakly active at days 6-8, indicating that most of the substance(s) is not released and remains attached to the bacterial cells. The strain must be administered at final concentrations about 10^5 - 10^6 cfu/ml, allowing if possible their stabilization in the environment (Prado, 2006; Prado et al., 2009).

As complement, and in order to check the interactions of the strain PP-154 with other live organisms also present in the environment, assays with phytoplankton were carried-out. The supply of the potential probiotic strain to monospecific cultures (*Nanochloropsis*) or mixtures of microalgae used as food (*Tetraselmis*, *Isochrysis*, *Chaetoceros* and *Phaeodactylum*) prevented the proliferation of vibrios and therefore avoided this route as way of entry of these pathogens (Prado, 2006; Prado et al., 2002).

In summary, the results pointed to the potential use of the strain PP-154 as control measure in bivalve larval cultures, allowing its colonization before than the pathogens reach high concentrations.

3. 4. Larval settlement and metamorphosis

Settlement is an irreversible process of adherence to the substrate which ends larval stage. High mortalities are often recorded during settlement. It is followed by the metamorphosis, which implies the acquisition of adult structures and the beginning of the benthonic life.

The relation of bacteria with those processes in marine invertebrates is known. In most cases, there is an induction mediated by natural biofilms or even monospecific biofilms. In bivalves, the most complete studies began with the work of Weiner and Colwell (1982) and the demonstration of the stimulation of larval settlement of *C. virginica* and *C. gigas* by the biofilm of LST, a pigmented marine bacterium (Weiner et al., 1985). This strain produced a hydrosoluble exopolisaccharide (Abu et al., 1986) which promote the adhesion of microorganisms to surfaces. It was described as *Alteromonas colwelliana* (Weiner et al., 1989) and further reclassified as *Shewanella colwelliana* (Coyne et al., 1989). Fitt et al. (1989) demonstrated that settlement could be induced by the supernatant of *Sh. colwelliana*, or other bacteria as *V. cholerae*, suggesting the

presence of a soluble substance. Although the settlement behaviour appeared as a response to the supernatant of final-logarithmic cultures, later could be inhibited by the release of noxious compounds related to processes of lysis or pigment formation. In further studies, this group demonstrated that biofilms of *Sh. colwelliana* induced settlement in *O. edulis* larvae, but not in *P. maximus* (Tritar et al., 1992). Finally, Walch et al. (1999) demonstrated the effectiveness of *Sh. colwelliana* supernatant to induce larval settlement of *C. gigas* and *C. virginica* at large scale in hatchery.

Prado (2006) assayed the effect of strain *Phaeobacter* PP-154, diffusible-pigment producer, in the settlement of *O. edulis* larvae.

The conditioning of settlement surfaces, black PVC pieces, by incubation during 48-72 hours in a bacterial suspension did not enhance the settlement rate, at none of the temperatures assayed. The results obtained in different experiments were inconclusive, with worse percentages with treated surfaces than in controls without bacterial incubation.

However, the experiments with direct inoculation of strain PP-154 ($\geq 10^5$ cfu/ml) in the seawater enhanced the settlement percentages, at different temperatures. Moreover, in a set of assays the improvement of oyster survival was observed with all the concentrations of PP-154 tested, including those ones below 10^5 cfu/ml.

The results of settlement varies among different batches, in micro-scale experiments as well as in hatchery (Weiner and Colwell, 1982; Weiner et al., 1989), being affected by a high number of variables, from changes in physical condition of larvae to the bacterial physiology (Tritar et al., 1992). In fact, there were marked differences among the controls used in the experiments (Prado et al., 1999; Prado, 2006). Therefore, the induction of these processes and the enhancement of survival and settlement mediated

by bacteria may be considered as a mode of probiosis, with special relevance in bivalve larval cultures.

4. FINAL REMARKS

In bivalve hatcheries, there is a constant flow of bacteria through all the compartments (broodstock, seawater, phytoplankton, larvae and tanks), i.e., there are a lot of routes for the introduction of potential pathogens in the system.

The environment, i.e. seawater, determines to a great extent the microbiota of larval cultures. Bivalves are filter-feeders, with a constant flow of seawater through their organisms which difficult the establishment of a bacterial population in the digestive tract. Therefore, the colonization of this place, one of the requirements in most of the definitions of 'probiotic', is not important in bivalve larvae. The pathogens can proliferate in every part of the animal and spread easily. Hence, it is advisable a control of the microbiota present in the environment, maintaining a beneficial balance for the larval development and survival, or at least, preventing the proliferation of opportunistic pathogens.

At any time, potentially harmful bacteria can enter to the system, but usually they begin at low concentrations and proliferate only under favourable conditions. The presence of a strain, sharing the same ecological niche than pathogens and with the ability of inhibit them, allows a control of the microbiological quality of seawater without any alochthonous species. This mechanism avoids the use of chemotherapeutants and the subsequent risks of harmful effects to larvae, the development and transmission of resistances, and the indiscriminate elimination of the bacterial population associated to larval cultures. The course of disease in bivalves uses to be very quick, impeding the

identification of aetiological agents necessary to select the antibiotic appropriate to treat the outbreak. Moreover, bivalve larvae require in any case the presence of a bacterial population. The knowledge of different mechanisms to help the larval development constitutes the basis for probiotic use in these cultures.

More in depth works on probiosis in bivalve larval cultures are needed, studying in every case the interaction among bacteria and the other live organisms, establishing their ability to remain in the systems and the appropriate dosage to achieve the highest effectiveness, and finding a method to improve their conservation, storage and manipulation in hatcheries.

The goal of these studies should be to obtain a strain or a mixture of strains with probiotic activity that help to stabilize the microbiota of the larvae, together with protocols to be used in hatcheries in a simple way. Therefore, the achievement of these objectives should improve the larval production and hence increase the bivalve production from aquaculture.

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Table 1. Review of published studies about probiotics assayed in bivalve larval cultures.

authors	year	probiotic	effect	target species
Lodeiros et al.	1989	<i>Flavobacterium</i> sp. P14	antibacterial activity	<i>Pecten ziczac</i>
Douillet and Langdon	1993	<i>Alteromonas</i> sp. CA2	enhancement of growth rate enhancement of survival rate	<i>Crassostrea gigas</i>
Douillet and Langdon	1994	<i>Alteromonas</i> sp. CA2	enhancement of growth rate	<i>C. gigas</i>
Riquelme et al.	1996	<i>Alt. haloplanktis</i> ¹ INH	protection against infection antibacterial activity (<i>Vibrio anguillarum</i> -like)	<i>Argopecten purpuratus</i>
Riquelme et al.	1997	<i>Pseudomonas</i> sp. 11 <i>Vibrio</i> sp. C33	protection against infection antibacterial activity (<i>Vibrio anguillarum</i> -like) enhancement of survival rate antibacterial activity (<i>Vibrio anguillarum</i> -like)	<i>A. purpuratus</i> <i>A. purpuratus</i>
Gibson et al.	1998	<i>Aeromonas media</i> A199	enhancement of survival rate antibacterial activity (<i>Vibrio tubiashii</i>)	<i>C. gigas</i>
Avendaño and Riquelme	1999	<i>Vibrio</i> sp. C33	antibacterial activity (<i>Vibrio anguillarum</i> -like)	<i>A. purpuratus</i>
Nakamura et al.	1999	S21	enhancement of survival rate antibacterial activity (<i>Vibrio alginolyticus</i>)	<i>C. gigas</i>
Ruiz-Ponte et al.	1999	<i>Roseobacter gallaeciensis</i> ² BS107	enhancement of survival rate antibacterial activity (<i>Vibrio pectenecida</i>)	<i>Pecten maximus</i>
Riquelme et al.	2001	<i>Vibrio</i> sp. C33 <i>Pseudomonas</i> s. 11 <i>Bacillus</i> sp. B2	enhancement of survival rate	<i>A. purpuratus</i>
Longeon et al.	2004	<i>Pseudoalteromonas</i> sp. X153	enhancement of survival rate * decrease of growth rate	<i>P. maximus</i>
Prado	2006	<i>Phaeobacter gallaeciensis</i> 154	enhancement of survival rate antibacterial activity	<i>Ostrea edulis</i>

¹ Nowadays *Pseudoalteromonas haloplanktis*² Nowadays *Phaeobacter gallaeciensis*.

Table 2. Published studies about the spectrum of antibacterial activity of marine bacteria.

authors	strain and source	target strains ^{*,**}
Riquelme et al., 1996.	<i>Alteromonas haloplanktis</i> ¹ INH broodstock gonad <i>Argopecten purpuratus</i>	<i>V. alginolyticus</i> , <i>V. anguillarum</i> , <i>V. parahaemolyticus</i> , <i>V. damsela</i> ² , <i>V. ordalii</i> <i>Achromobacter</i> sp., <i>A. hydrophila</i> , <i>Escherichia coli</i> , <i>Morganella morgani</i> , <i>Proteus vulgaris</i> , <i>Ps. fluorescens</i> , <i>Salmonella typhimurium</i> , <i>Shewanella putrefaciens</i> , <i>S. aureus</i> <i>V. alginolyticus</i> , <i>V. anguillarum</i> , <i>V. cholerae</i> , <i>V. harveyi</i> , <i>V. parahaemolyticus</i> , <i>V. splendidus</i> , <i>V. tubiashii</i> , <i>V. vulnificus</i> <i>A. caviae</i> , <i>A. hydrophila</i> , <i>A. salmonicida</i> , <i>A. veronii var sobria</i> ³ , <i>Ph. damsela</i> subsp. <i>damsela</i> , <i>Yersinia ruckeri</i> <i>Enterococcus seriolicida</i> ⁴
Gibson et al., 1998.	<i>Aeromonas media</i> A199	<i>V. aestuarianus</i> , <i>V. alginolyticus</i> , <i>V. anguillarum</i> , <i>V. damsela</i> ² , <i>V. splendidus</i> , <i>V. ordalii</i> , <i>V. parahaemolyticus</i> , <i>A. hydrophila</i> , <i>Ph. damsela</i> subsp. <i>damsela</i>
Jorquera et al., 1999.	<i>Vibrio</i> sp. C33 seawater <i>A. purpuratus</i>	<i>V. aestuarianus</i> , <i>V. alginolyticus</i> , <i>V. anguillarum</i> , <i>V. damsela</i> ² , <i>V. splendidus</i> , <i>V. ordalii</i> , <i>V. parahaemolyticus</i> , <i>A. hydrophila</i> , <i>Ph. damsela</i> subsp. <i>damsela</i>
Ruiz-Ponte et al., 1999.	<i>Roseobacter gallaeciensis</i> ⁵ BS107 larval cultures and collectors <i>Pecten maximus</i>	<i>V. anguillarum</i> , <i>V. alginolyticus</i> , <i>V. damsela</i> ² , <i>V. pelagius</i> , <i>V. splendidus</i> , <i>V. tubiashii</i> , <i>V. vulnificus</i> <i>V. alginolyticus</i> CCM 2578, <i>V. vulnificus</i> ATCC 27562 <i>Acinetobacter</i> sp., <i>A. salmonicida</i> , <i>Pasteurella piscicida</i> ⁶ , <i>Ps. doudoroffii</i> ⁷ , <i>S. aureus</i> , <i>Xanthomonas</i> sp.
Robertson et al., 2000.	<i>Carnobacterium</i> sp. K1 digestive <i>Salmo salar</i>	<i>V. anguillarum</i> , <i>V. ordalii</i> , <i>V. alginolyticus</i> , <i>V. harveyi</i> <i>A. hydrophila</i> , <i>A. salmonicida</i> , <i>Flavobacterium psychrophilum</i> , <i>Ph. damsela</i> subsp. <i>piscicida</i> , “ <i>Streptococcus milleri</i> ” <i>Janthinobacterium lividum</i>, <i>Yersinia ruckeri</i>
Chythanya et al., 2002.	<i>Pseudomonas aeruginosa</i> I-2 seawater	<i>V. harveyi</i> , <i>V. vulnificus</i> , <i>V. damsela</i> ² , <i>V. fluvialis</i> , <i>V. parahaemolyticus</i>
Tirola et al., 2002.	<i>Pseudomonas</i> sp. MT5 <i>Salmo salar</i> <i>Oncorhynchus mykiss</i> with flavobacteriosis	<i>Cytophaga</i> sp., <i>E. coli</i> , <i>Flavobacterium</i> sp., <i>F. columnare</i> , <i>F. psychrophilum</i> , <i>Bacillus pabuli</i> ⁸ , <i>Rhodococcus erythropolis</i> , <i>Y. ruckeri</i> <i>Bacillus myeoides</i>
Longeon et al., 2004.	<i>Pseudoalteromonas</i> X153 marine pebbles	<i>V. alginolyticus</i> , <i>V. anguillarum</i> , <i>V. carchariae</i> ⁹ , <i>V. costicola</i> ¹⁰ , <i>V. damsela</i> ² , <i>V. haloplanktis</i> ¹ , <i>V. harveyi</i> , <i>V. mediterranei</i> , <i>V. natriegens</i> , <i>V. parahaemolyticus</i> , <i>V. pectenocida</i> , <i>V. pelagius</i> , <i>V. proteolyticus</i> , <i>V. splendidus</i> , <i>V. tapetis</i> , <i>V. vulnificus</i> <i>Cytophaga lytica</i> ¹¹ , <i>C. marinoflava</i> , <i>Deleya marina</i> ¹² , <i>E. coli</i> , <i>Halomonas elongata</i> , <i>Marinobacterium jannaschii</i> ¹³ , <i>Propionibacterium acnes</i> , <i>P. granulorum</i> , <i>Ps. doudoroffii</i> , <i>Ps.</i> <i>nautica</i> ¹⁴ , <i>S. aureus</i> , <i>S. epidermidis</i>
Prado et al., 2009	<i>Phaeobacter gallaeciensis</i> PP-154 bivalve hatchery	<i>V. aestuarianus</i> , <i>V. alginolyticus</i> , <i>V. anguillarum</i> , <i>V. fluvialis</i> , <i>V. harveyi</i> , <i>V. mimicus</i> , <i>V. natriegens</i> , <i>V. neptunius</i> , <i>V. parahaemolyticus</i> , <i>V. pelagius</i> , <i>V. proteolyticus</i> , <i>V. splendidus</i> , <i>V. tapetis</i> , <i>V. tubiashii</i> , <i>V. vulnificus</i> , <i>Vibrio</i> sp., <i>A. hydrophila</i> , <i>Aliivibrio logei</i> , <i>Al. fisheri</i> , <i>Edwardsiella tarda</i> , <i>Ph. damsela</i> subsp. <i>piscicida</i> , <i>Ps. anguilliseptica</i> , <i>Streptococcus</i> <i>parauberis</i> , <i>Tenacibaculum maritimum</i> , <i>Ps. fluorescens</i>, <i>Lactococcus garvieae</i>

* crossed out names correspond to resistant bacteria

** V=Vibrio, A=Aeromonas, Al=Aliivibrio, Ph=Photobacterium, Ps=Pseudomonas, S=Staphylococcus.

Nowadays ¹*Pseudoalteromonas haloplanktis* subsp. *haloplanktis*, ²*Photobacterium damsela* subsp. *damsela*, ³*A. sobria*, ⁴*Lactococcus garvieae*,⁵*Phaeobacter gallaeciensis*, ⁶*Ph. damsela* subsp. *piscicida*, ⁷*Oceanimonas doudoroffii*, ⁸*Paenibacillus pabuli*, ⁹*V. harveyi*, ¹⁰*Salinivibrio costicola*,¹¹*Cellulophaga lytica*, ¹²*Cobetia marina*, ¹³*Oceanospirillum jannaschii*, ¹⁴*Marinobacter hydrocarbonoclasticus*