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The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms

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Abstract

Research in probiotics for aquaculture is at an early stage of development and much work is still needed. The principal bacterial groups tested as probionts in the culture of shrimp, crab, oyster and fish have been *Vibrio*, *Pseudomonas*, *Bacillus*, and several lactobacilli. The available information is inconclusive, since few experiments with sufficiently robust design have been conducted to permit critical evaluation. Experiments have mainly been conducted with fish larvae, where significant reductions in mortality have been obtained. Most of the work reviewed in this article describes commercial hatchery experiments rather than rigorous laboratory investigations and the focus is principally shrimp larviculture.

Keywords: Probiotics; Larviculture; Bacteria

1. Introduction

Bacterial diseases are considered to be a major cause of mortality in shrimp larviculture (Wyban and Sweeney, 1991; Wilkenfeld, 1992) and fish hatcheries (Grisez and Ollevier, 1995). They are also a constraint on consistent larval production (Daniels, 1993; Nicolas et al., 1996). It is common for hatchery managers to try to control bacterial infections or even the presence of potentially pathogenic bacteria in the system.

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Traditionally, the control of bacterial problems in penaeid hatcheries has relied on the use of chemical compounds; more recently probiotic microorganisms and "vaccination" or other forms of immunostimulation have also been employed. The use of antimicrobials is a common practice in practically all shrimp hatcheries in Latin America and Southeast Asia, where there are few restrictions on their use.

The abuse of antimicrobials can result in the development of resistant strains of bacteria (Weston, 1996). Such resistance can be readily transferred to other strains, either following alterations to the existing genome or by transfer of genetic material between cells (Towner, 1995) through plasmids or bacteriophages. This is even more likely if chemotherapeutants have been used prophylactically in the culture of penaeid shrimps (Brown, 1989).

There is an increasing interest within the industry in the control or elimination of antimicrobial use. Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the larval rearing tanks. One such method that is gaining acceptance within the industry is the use of probiotic bacteria to control potential pathogens.

2. The definition of probionts

Elie Metchnikoff's work at the beginning of this century is regarded as the first research conducted on probiotics (Fuller, 1992). He described them as "microbes ingested with the aim of promoting good health". This same definition was modified to "organisms and substances which contribute to intestinal microbial balance" (Parker, 1974), and later by Fuller (1989) to "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". These definitions originally applied to farm animals (ruminants, poultry and pigs) or humans, since the first studies were carried out in these species.

Probiotics are now also being used in aquaculture and therefore, the definition may have to be modified. In aquatic animals, not only the digestive tract is important but also the surrounding water. Gatesoupe (1999) defines 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". Gram et al. (1999) broadened the definition by removing the restriction to the improvement to the intestine: "a live microbial supplement which beneficially affects the host animal by improving its microbial balance".

Biological control has been described as the utilisation of natural enemies to reduce the damage caused by noxious organisms to tolerable levels (Debach and Rosen, 1991) or more precisely, the control or regulation of pest populations by natural enemies (Smith, 1919). Strictly speaking, a probiotic ought not to be classified as a biological control agent, since a probiotic microorganism does not necessarily attack the noxious agent (pathogen). It need not necessarily be a natural enemy of the pathogen, but it merely prevents damage to the host caused by the pathogen, usually through competition but at most, it may produce substances that inhibit the growth or attachment of the harmful microorganism. Neither should probiotics be classified as growth promoters,

since their action is not confined to improved growth but is associated with a general improvement in health.

The microbial community inside the gut of some animals confers some degree of resistance to or protection against disease (Fox, 1988). In natural populations of aquatic animals, the microflora of the gut might reflect that of the aquatic environment. However, in massive artificial larval cultures, the balance can be altered by the use of disinfected water, microalgae, *Artemia* nauplii, rotifers, and antibacterials. As a result, a protective microbial community may not develop either in the environment or the digestive system of the larvae. The postlarvae reared in a relatively sterile environment of a hatchery do not grow well and show poor survival when they are exposed to the complex microbial populations of the nursery or grow-out ponds. They are particularly susceptible to diseases when exposed to environmental stress and potentially pathogenic bacteria.

3. Probiotic studies in the larval culture of shrimps

Several bacteria have been used in the larval culture of aquatic organisms (Table 1), either delivered directly into the water freezed-dried, or via live carriers such as *Artemia* nauplii or rotifers. There is almost no scientific information on the use of bacteria as probiotics in shrimp larviculture systems, although some work has been done in South America. Early experiments started when hatchery production managers wanted to improve the levels of "good vibrios", namely the sucrose fermentors (Garriques and Wyban, 1993), in the systems. To accomplish this, commercial table sugar was added to the larval rearing system in some hatcheries to stimulate the growth of the sucrose-fermenting *Vibrio* spp. Later, these bacteria were cultured in a similar manner to microalgae and then added to the larval rearing tanks (Garriques and Wyban, 1993; Daniels, 1993). Griffith (1995) reported that following the introduction of probiotics in Ecuador in 1992, hatchery down-time between batches was reduced from 7 days per month to 21 days annually, production volumes increased by 35%, and overall antimicrobial use decreased by 94%.

In Asia, there is one report of several species of bacteria being used in the larviculture of *P. monodon* and *P. penicillatus* with promising results (Anonymous, 1991), however, the report is very brief with almost no data. Maeda (1988, 1992b) tested the addition of sterile "soil extract" plus diatoms to larval rearing tanks of *P. monodon*. Apparently, high survival was achieved with this treatment over the first 4 days of the experiment when compared to the addition of diatoms alone. By the end of the experiment, however, both treatments produced similar results. Presumably, the purpose of adding the soil extract was to permit the growth of other microorganisms that might have had a beneficial effect on larval performance. However, interpretation is difficult since only very limited data were presented.

Garriques and Arevalo (1995) tested a strain of *V. alginolyticus* isolated from the seawater next to their hatchery on *Litopenaeus vannamei* larvae. No mortalities were observed in a bath challenge pathogenicity test, whereas 100% mortality was obtained

Table 1
Bacterial probionts employed in the larval culture of aquatic organisms

Species of bacteria	Target organism	Reference
Vibrio alginolyticus	Shrimp (*Penaeus vannamei*)	(Garriques and Arevalo, 1995; Zherdmant et al., 1997)
T. utilis (PM-4)	Shrimp (*Penaeus monodon*)	(Maeda and Liao, 1991)
V. harveyi, *Pseudomonas* sp., *Nitrobacter* sp., *Nitrosomonas* sp. and *Bacillus* sp.	Shrimp (*P. monodon* and *P. penicillatus*)	(Anonymous, 1991)
T. utilis (PM-4)	Crab (*Portunus trituberculatus*)	(Nogami and Maeda, 1992; Maeda, 1994; Nogami et al., 1997)
V. pelagius	Turbot (*Scophthalmus maximus*)	(Ringø and Vadstein, 1998)
Bacillus toyoi and *Bacillus* sp. spores	Turbot via rotifers (*Brachionus plicatilis*)	(Gatesoupe, 1989, 1991b)
Lactic bacteria	Turbot via rotifers	(Gatesoupe, 1990)
Lactobacillus plantarum and *Lactobacillus helveticus*	Turbot via rotifers	(Gatesoupe, 1991a)
Lactobacillus bulgaricus and *Streptococcus lactis*	Turbot via *Artemia*	(Garcia-de-la-Banda et al., 1992)
Alteromonas sp.	Oyster (*Crassostrea gigas*)	(Douillet and Langdon, 1993, 1994)
Aeromonas media	Oyster	(Gibson et al., 1998)
Roseobacter sp. (BS107)	Scallop (*Pecten maximus*)	(Ruiz-Ponte et al., 1999)
Vibrio sp.	Chilean scallop (*Argopecten purpuratus*)	(Riquelme et al., 1997)

with a *V. parahaemolyticus* strain after 96 h. The concentration of bacteria added to challenge the larvae was 2×10^3 cells ml^{-1}. In a trial, repeated three times on different dates, the probiotic strain was introduced into the larval rearing tanks and resulted in an average survival of 90.1% and a wet weight of 7.8 mg. Tanks that were treated with antimicrobials had 83.8% survival and a weight of 6.0 mg, and the controls averaged 74.5% and 7.1 mg. *Vibrio* counts on TCBS agar showed non-fermenting colonies (green colonies) in the probiotic tanks, with some appearing in the control and antimicrobial treatments. No statistical analysis was carried out by the authors, but statistical analysis of the data presented in the paper, taking into account pseudoreplication in time, no significant difference was observed in the survival and wet weight (one-way ANOVA, $p = 0.204$ and $p = 0.101$, respectively). Garriques and Wyban (1993) obtained similar results, but they observed that *L. vannamei* larvae grown with probiotics were larger and more active, and no luminous bacteria were observed.

Zherdmant et al. (1997) suggested that the guts of protozoea larvae were already colonised by bacteria from the surrounding environment, which might have interfered with probiotic experiments. The inoculation of a probiotic bacterial strain in a tank with nauplii stage V of *L. vannamei* at a density of 10^3 cells ml^{-1} prevented colonisation by a pathogenic strain, even when challenged at a density of 10^7 cells ml^{-1}. Again, the lack of data in the publication prevents any critical analysis of the results.

Yeast and fungi have also been used to improve the growth rate and performance of *L. vannamei* larvae (Intriago et al., 1998). A red pigmented yeast and a chitin-degrading fungus isolated from the marine environment were used, but very little additional information was supplied.

From the literature on probiotics in shrimp hatcheries, *V. alginolyticus* is a frequently tested bacterium with promising results. The work by Austin et al. (1995) and by Garriques and Arevalo (1995) suggest that *V. alginolyticus* may have characteristics capable of conferring some degree of protection against disease. Gatesoupe (1990) also detected *V. alginolyticus* in healthy rotifers and he established a positive correlation between the survival rate of turbot larvae and the proportion of *V. alginolyticus* in the rearing environment. These data support the hypothesis that *V. alginolyticus* could be a probiotic candidate for shrimp larviculture, although use may require some caution since some strains could be pathogenic (Lightner, 1993).

4. Probiotic use in crabs

Nogami and Maeda (1992) and Maeda (1992a) isolated a bacterial strain, coded PM-4 and identified as *Thalassobacter utilis* (Nogami et al., 1997), from seawater and inoculated it into blue crab (*P. trituberculatus*) larval rearing tanks at concentrations of 10^6 cells ml^{-1}. They obtained a survival of 27.2% in the test tanks compared with 6.8% in the control tank, with no bacteria inoculated. This was followed by 33 trials in which strain PM-4 was inoculated, resulting in an average survival rate of 28.3%, compared with 15.6% in control tanks over 42 trials (Nogami et al., 1997). All these trials were conducted over a 4-year period. They also noted that PM-4 could inhibit the growth of a

"supposedly pathogenic" strain of *Vibrio anguillarum* in in vitro experiments; it suppressed the presence of other *Vibrio* spp. and pigmented bacteria in the larval tanks, and inhibited the growth of the fungus *Haliphthoros* sp.

Despite repeated inoculation of strain PM-4 into the tanks, the bacterial level did not exceed 10^6 cells ml^{-1} (Nogami and Maeda, 1992). The authors attributed this effect to the grazing behaviour of protozoa which grow quickly and eat the bacteria, keeping the bacterial concentration at around 10^6 cells ml^{-1} (Maeda et al., 1997). Another possibility, not mentioned in the paper, is that there may have been a lack of necessary nutrients in the system. In 1994, Maeda in a related paper included some additional information. He stated that an aquacultural ecosystem cannot support a population of bacteria greater than 10^6 cells ml^{-1}. He suggested that if this is true, then maintaining the population at that level might diminish or disadvantage other bacteria. He considered that controlling the bacterial population would not be difficult, although the effect of the added bacteria may not last long. Gomez-Gil (1998) determined that inoculation of sterile shrimp hatchery seawater with high concentrations of bacteria (10^7 cells ml^{-1}), rarely resulted in bacterial levels greater than those at the time of inoculation and that bacterial counts decreased after 72 h, although the rate of decline was dependent on the strain.

Other experiments have been carried adding microbial nutrients (urea, glucose and potassium phosphate) to produce bacterial mixtures (Maeda et al., 1992) that might improve the survival of blue crab (*P. trituberculatus*) larvae. Results are not clear and the reported survival, when compared with the control, was variable depending on the composition of the microbial flora. Sometimes it was better than the control, e.g. 75% when a bacterial strain (F-3) was inoculated, compared with 45% for the control. In other tests the survival was lower than controls, for example, when yeasts were used (11%). No variation was reported for any treatment, so little can be concluded.

The work reported relating to *P. trituberculatus* larvae generally does not have a clear description of experimental design, and the data is presented without statistical analysis and the two replicates reported are too low to draw any inference in an experiment dealing with crustacean larvae.

5. Bivalves

Bacteria, especially *Vibrio* spp., are important pathogens in the culture of the scallops *P. maximus* (Nicolas et al., 1996), *A. purpuratus* (Riquelme et al., 1996) and oyster larvae (Elston et al., 1981), and probionts have been used in an attempt to control them. A bacterium (CA2), probably an *Alteromonas* spp. (Douillet and Langdon, 1993, 1994), was used in Pacific oyster (*C. gigas*) larviculture. Oyster larvae fed with algae and this bacterium showed enhanced survival (21–22%) and growth (16–21%) compared with those fed on algae alone. They found that 10^5 cells ml^{-1} was optimal for the enhancement of larval culture. The authors' experimental evidence suggests that the bacteria may have provided essential nutrients not present in the algae, or improved larval digestion by contributing enzymes (Douillet and Langdon, 1994). A clear distinction has to be made between the probiotic function of a bacterium and that of a feed

additive. A microorganism cannot be considered to be a probiotic if its role is confined to supplying essential nutrients.

Gibson et al. (1998) isolated a strain of *A. media* (A199) capable of producing bacteriocin-like inhibitory substances. This strain displayed antagonistic activity against several potentially pathogenic bacteria and was used as a probiont to control infections of *C. gigas* larvae with a *Vibrio tubiashii* strain; significant differences were observed between treatments, demonstrating the probiotic characteristics of this strain.

For the larval culture of the Chilean scallop (*A. purpuratus*), pre-treatment with a *Vibrio* sp. protected the larvae against subsequent challenges with a *V. anguillarum*-related strain (Riquelme et al., 1997). The authors isolated a large number of strains from laboratory and hatchery sources and screened them for production of inhibitory substances against the *V. anguillarum*-related strain. The ingestion of potential probionts (PP) by the scallop was shown to depend on the type of strain employed, as some strains were ingested significantly better than others were (Riquelme et al., 2000).

For the scallop *P. maximus*, a *Reseobacter* sp. was found to confer protection only when bacterial cell extracts were inoculated into larval cultures and not when the cells where added (Ruiz-Ponte et al., 1999). This species (BS107) had antibacterial activity only in the presence of another bacterium that produced a proteinaceous molecule that acts as an effector.

Since Douillet and Langdon (1994) found adverse, neutral, and beneficial effects depending on the strain of bacteria employed, they concluded that "no generalisation about the beneficial effects of specific bacterial strains can be made, i.e. each strain must be tested again with each new target".

6. Finfish

The digestive tract of fish contains a much higher number of microorganisms than the surrounding water, as many as 10^8 cells g^{-1} (Ringø et al., 1995). After hatching, the gastrointestinal tract of Atlantic cod (*Gadus morhua*) larvae is colonised by almost the same bacterial genera as found in the eggs (Hansen and Olafsen, 1989), most important being *Pseudomonas*, *Cytophaga* and *Flexibacter*. At the yolk sac stage of cold-water fish, ingestion of bacteria results in the establishment of a primary intestinal microflora which persists beyond first feeding. This is followed by a bacterial succession until the adult microflora is established (Hansen and Olafsen, 1999). It is therefore important to add the PP after hatching as soon as possible, in order to effectively colonise the larval gut before the introduction of live food (Ringø and Vadstein, 1998).

It has been demonstrated that adult marine flatfish, turbot (*S. maximus*) and dab (*Limanda limanda*), harbour bacteria capable of suppressing the growth of *V. anguillarum* (Olsson et al., 1992), that might therefore act as probionts against such pathogens. The gut flora also plays an important role in determining the survival of larval turbot, although no correlation between the number of bacteria in the gut and larval survival rates was observed (Munro et al., 1994). Recently, Austin et al. (1995) reported that a strain of *V. alginolyticus* was effective in reducing disease caused by *Aeromonas*

salmonicida and two pathogenic *Vibrio* species in Atlantic salmon. Better survival rates were achieved with the use of this probiont, sometimes as much as an 82% improvement. It is important to note that the strain was isolated from a shrimp hatchery in Ecuador and tested against strains from temperate waters (Scotland, England and Norway) and one from Tasmania. Kennedy et al. (1998) showed that the addition of a gram-positive probiotic bacterium increased survival, size uniformity, and growth rate of marine fish larvae (snook, red drum, spotted seatrout and stripped mullet). They also noted that the external and internal bacterial environments of the fish moved from the predominance of vibrios to greater numbers of other gram-negative and gram-positive bacteria. Gram et al. (1999) reported that a *Pseudomonas fluorescens* strain (AH2) reduced the mortality of 40 g rainbow trout (*Oncorhynchus mykiss*) infected with a pathogenic *V. anguillarum* (90-11-287, serotype 01) strain. Controls inoculated with the pathogenic bacterium had a cumulative mortality of 47% after 7 days, whereas in the treated fish the mortality was 32%.

Freeze-dried diets containing lactic acid bacteria (*Carnobacterium divergens*) isolated from *G. morhua* intestines were given to cod fry and confirmed a certain degree of resistance when subsequently challenged with *V. anguillarum* (Gildberg et al., 1997). On the other hand, lactic acid bacteria isolated from salmon intestines did not improve the resistance of Atlantic salmon (*Salmo salar*) fry challenged with a pathogenic strain of *A. salmonicida* (Gildberg et al., 1995).

Rotifers are a live food employed mainly in the rearing of fish larvae; thus some research has been directed towards improving their microbial qualities. Gatesoupe (1989) demonstrated that bacteria associated with rotifers could be detrimental to turbot larvae, but inoculation of a probiotic bacterium (*B. toyoi*) to disinfected rotifers, enhanced the growth rate of turbot. *V. alginolyticus* was detected wherever healthy rotifers and turbot were raised and *Aeromonas* spp. were dominant in tanks where high mortalities occurred (Gatesoupe, 1990). *L. plantarum* and *L. helveticus* were also used as feed additives for rotifers. The addition of *L. plantarum* increased the population density of rotifers, reduced aerobic bacterial loads, and increased the dietary value of the rotifers, with concurrent inhibition of *A. salmonicida* (Gatesoupe, 1991a). Another lactic acid bacterium, *Lactococcus lactis*, enhanced the growth of *B. plicatilis* and had an inhibitory effect against *V. anguillarum* (Harzevili et al., 1998). *Bacillus* spp. spores inhibited the growth of *Vibrio* spp. in the culture of rotifers, with an improvement in the mean weight of turbot (Gatesoupe, 1991b).

Bogaert et al. (1993) applied *Enterococcus faecium* to the culture of rotifers, referring to them as a probiotic. In this case, only their role as a nutrient source was examined, but it was not possible to determine whether this bacterium acted as a probiont.

Inoculating the guts of target animals with probiotic bacteria through bioencapsulation in *Artemia* is an interesting approach under some circumstances. However, literature dealing with the subject is scarce. Bioencapsulated lactic acid bacteria have been introduced into turbot larvae, apparently with improvements in survival. Garcia-de-la-Banda et al. (1992) added *S. lactis* and *L. bulgaricus* to rotifers and *Artemia* nauplii. They recorded larval survival up to six times higher than the control group at the end of the weaning period, although there was no conclusive difference in larval growth if

inactivated or if live bacteria were inoculated. The lack of treatment replicates also makes it difficult to draw any conclusions.

7. Selection of probiotic bacteria

Selection of probiotic bacteria has usually been an empirical process based on limited scientific evidence. Many of the failures in probiotic research can be attributed to the selection of inappropriate microorganisms. Selection steps have been defined, but they need to be adapted for different host species and environments.

It is essential to understand the mechanisms of probiotic action and to define selection criteria for potential probiotics (Huis in'T Veld et al., 1994). General selection criteria are mainly determined by biosafety considerations, methods of production and processing, the method of administration of the probiotic, and the location in the body where the microorganisms are expected to be active (Huis in't Veld et al., 1994).

Methods to select probiotic bacteria for use in the larviculture of aquatic animals might include the following steps: (1) collection of background information, (2) acquisition of PP, (3) evaluation of the ability of PP out-compete pathogenic strains, (4) assessment of the pathogenicity of the PP, (5) evaluation of the effect of the PP in larvae, and (6) an economic cost benefit analysis.

8. Further work

It is important to provide larvae with a healthy environment that includes a beneficial microbial community. Eliminating bacteria from rearing tanks or employing antimicrobials to control bacterial populations can lead to serious problems. Therefore probionts have a great deal of potential. However, many questions remain unanswered regarding the use of probiotics in aquaculture. It is not yet clear if they are effective and if so, how they have an effect. Are they acting as a food or are they competing with potentially harmful bacteria? How will probiotics perform when a stressful situation arises and the larvae are weakened? Can they become pathogenic, since, for example, *V. alginolyticus* has been suggested as a probiont but other strains of this bacterium have been associated with vibriosis in shrimps? How can a probiotic strain be differentiated from a potentially pathogenic one? Many of these questions are still unanswered not only for probiotics but for bacteria associated to aquatic organisms under culture conditions.

Many species of bacteria have been employed as probiotics for the larval culture of aquatic organisms, but their selection has been based on empirical observations rather than on scientific data. The results obtained so far, in the majority of cases, are dubious and no definitive conclusions can be drawn from the information. It would be useful if some bacterial characteristics were identified to allow easy identification of probiotic strains. Unfortunately, almost no work has been conducted in this area and therefore it is still largely based on trial and error.

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