

The selection and potential use of aquatic bacteria as fish probiotics

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Fish probiotics can be defined as live microbial cultures which upon addition to the rearing systems through feed or water improves the health of the host. Typically, the efficiency of probiotics is measured as increased survival rates. Both terrestrial and aquatic microorganisms have been suggested and/or tested as fish probiotics, however, the findings are not conclusive. This paper discusses strategies for selection of probiotics from the natural fish/fish larvae environment including evaluation of the dominant microflora and occurrence of pathogen-antagonising strains. Further steps in the selection procedure have often focused on the ability of the potential probiotic to attach and colonize, however, the present paper discusses possible shortcomings of such focus. Instead, model and full scale *in vivo* trials must be carried out and examples are provided where this approach has been followed. Determining the mechanisms of action of a probiotic culture is essential for evaluation of stability and safety. The need for more detailed, molecular studies in fish probiotics is emphasised. Finally, suggestions are made for areas in aquaculture where the probiotic principle may be applicable.

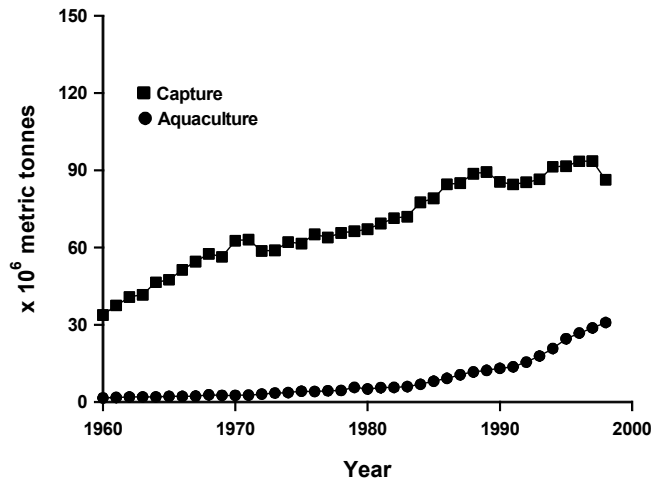
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Introduction

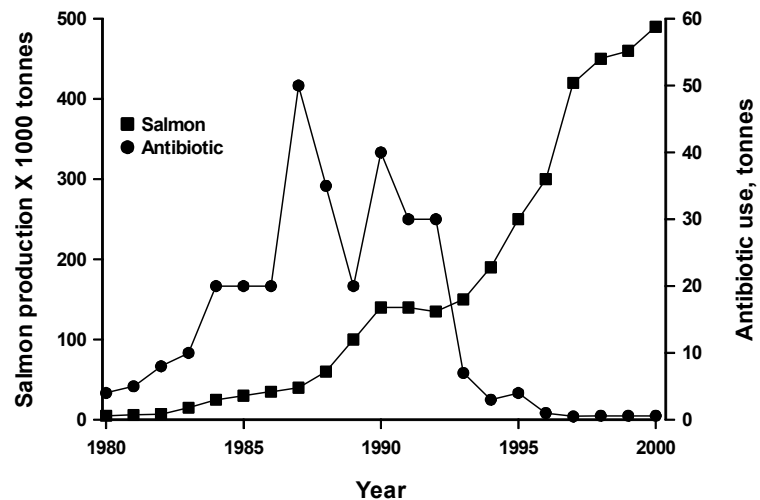
Farmed fish, crustaceans and molluscs constitute a major part of the world seafood resources. Thus, approx. 1/3 of the fish used for human consumption are produced in aquaculture. Catches from wild stocks have stagnated at approx. 90 mill tonnes / year (FAO 2000) and although an increased catch is possible for some species, fish from aquaculture remains the only area of long-term potential increase in fish resources (Fig 1)

Figure 1
Total world fish catches and aquaculture production from 1960 to 1997 (FAO 2000)



Intensive rearing of any animal or plant organism may result in stressful conditions with rapid spread of disease in the stock. Fish suffer from several bacterial, viral and parasitic diseases. Prevention of diseases is an important prerequisite for further increase in the aquaculture sector, and environmentally friendly techniques are needed to ensure the sustainability of aquaculture. Several fish diseases have successfully been controlled by vaccination and, for instance, in Norway, the use of antibiotics has dropped from 50 tonnes to less than 1 tonne parallel to an increase in salmon production from 60,000 tonnes to over 400,000 tonnes (Fig 2).

Figure 2
Increase in production of farmed salmon and decrease in use of antibiotics in Norway from 1984 to 2000 (modified from Buchman and Larsen (2001))



However, not all (bacterial) diseases can be controlled by vaccination. The immune system is only partially developed in the young fish (eggs, fry, larvae), and molluscs and crustaceans are difficult to vaccinate. The use of antibiotics may lead to resistance in both fish pathogens and in bacteria that potentially can transfer the resistance to human pathogens in the environment or in the seafood chain.

Some disease causing microorganisms may be controlled by the addition of non-pathogenic, pathogen-antagonising microorganisms to the host or host environment. This concept has been widely studied in the horticulture sector where microorganisms are surveyed for their bio-control potential. For instance, fluorescent pseudomonads may control several plant pathogenic fungi (Walsh et al. 2001, Shoda 2000). Intensive research is also ongoing in humans and other mammals, where probiotic organisms are investigated (Holzapfel and Schillinger 2002, Saavedra and Tschernia 2002). Microorganisms that are ingested, and possibly able to control intestinal pathogens, are of particular importance as mammalian probiotics, although the use of lactic acid bacteria for control of certain vaginal diseases is also investigated (Ocana et al. 1999, Reid and Burton 2002).

The use of beneficial microorganisms has also emerged as an area of intensive research in the field of aquaculture (Gram and Ringø 2002, Verschuere et al. 2000, Gatesoupe 1999). The current paper discusses how organisms have been – and may be selected – for this purpose and suggests future directions for the research.

Definitions

The term probiotics originate from the potential use of beneficial microorganisms in human nutrition and Fuller (1989) defined a probiotic as: “a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance”. This definition does not completely cover the use in aquaculture, where infectious agents also invade through skin and gills. Therefore addition of the probiont to the host environment, i.e. the water may also control disease. Further, the notion “microbial balance” is imprecise. We have defined (fish) probiotics as: “a live microbial preparation that when added to the fish, crustaceans or molluscs (larvae, fry, young or adult animals) has a beneficial effect on the health of the host” (Gram and Ringø 2002). Whilst one could argue that also the term “health of the host” is imprecise, this is typically measured as increased survival during periods of disease.

Live microorganisms are also used in aquaculture for improvement of water quality such as the conversion of ammonia to nitrite and nitrate (Hagopian and Riley 1998) or as feed for fish (McCausland et al. 1999). Such treatments may also cause improvement of disease survival, although the organisms are not added specifically to control pathogens. Therefore, they are not defined as probiotics *sensu stricto*.

Strategies for selecting fish probiotic bacteria

Fish probiotic bacteria (microorganisms) have originated either from culture collections or have been sampled from the fish/water environment. In the first case, it has typically been organisms such as lactic acid bacteria, which have a history of success as antagonists and/or as mammal probionts (Nikoskelainen et al. 2001a). Selection of potential fish probiotic

bacteria from fish or water has resulted in a broad collection of bacterial species, both Gram-negative bacteria (*Pseudomonas*, *Aeromonas*, *Roseobacter* a.o.) and Gram-positive bacteria (*Bacillus*, *Carnobacterium*).

Almost all studies have (pre-)selected the potential probionts based on their ability to inhibit fish pathogenic bacteria, typically in agar-based assays (Gram 1993, Bly et al. 1997, Lemos et al. 1985, Dopazo et al. 1988, Westerdahl et al. 1991, Bergh 1995, Smith and Davey 1993). A word of caution should be included since no studies have documented i) that inhibitory substances produced *in vitro* are actually effective *in vivo* (Atlas 1999) or ii) that *in vitro* antagonism is a predictor of *in vivo* effect.

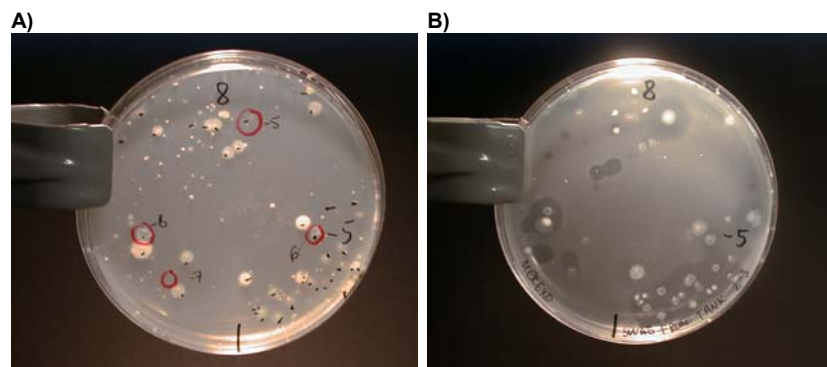
Some studies have further characterised the strains for the ability to survive in the fish gastro-intestinal tract or their adhesion to fish mucus (Olsson et al. 1992; Jöborn et al. 1997). Whilst these parameters are clearly likely to be important for probiotics acting in the gastro-intestinal tract, they are less likely to determine the success of a probiont supplied to the water. Here, characteristics such as the ability to survive in tank water a.o. may be more important. The final “evaluation” of a potential probiont, namely its ability to suppress disease in fish has, not been included in all studies.

The “normal” rate of antagonism as measured by *in vitro* assays is 1-4% of a mixed microbial (culturable) population from fish (Spanggaard et al. 2001) but some studies have found that almost 1/3 of the culturable microflora inhibited selected fish pathogens (Westerdahl et al. 1991). A high proportion (20%) of bacteria from intertidal seaweeds was also inhibitory to other bacteria (Lemos et al. 1985).

With a rate of antagonism of approx. 1-4%, it follows that many hundreds (or thousands) of pure cultures of bacteria need to be isolated and tested to provide a selection of potential probiotic bacteria. We have (Hjelm et al. 2002) demonstrated that a pre-selection step significantly accelerate the process of isolating potential probiotic bacteria. In brief, samples from the environment in question (fish, water, feed,...) are surface plated onto an appropriate agar and the colonies replica-plated onto an agar into which the pathogen is incorporated. Colonies causing clearing zones in the turbid agar – which is turbid due to growth of the pathogen – are isolated (Fig 3). Since antagonism is not a stable trait in all organisms, further testing of the isolates are required in agar-well-assays. Using this procedure, we have tested approx. 8,500 colonies from turbot larvae farms. Approx. 200 colonies were isolated and pure cultured resulting in 32 highly antagonistic strains (Hjelm et al. 2002).

Figure 3

Replica plating (B) from a colony count plate (A) from a fish larvae rearing unit. (B) contains *Vibrio anguillarum*. Potential probiotic bacteria cause clearing zones as a result of growth inhibition of *V. anguillarum* (Hjelm et al. 2002).



Following the *in vitro* selection of organisms, they must be tested for potential pathogenicity. Some species of bacteria such as *Vibrio* spp. can be highly antagonistic but may also be pathogenic to the host.

In vivo testing of the potential probiotics in model infection studies is an important step before field trials are commenced. Infection models should be based in bath infection or, preferably, on co-habitant infection. Infection by injection does not reflect the natural infection and will not allow the probiotic to interact with the pathogen. A sufficient number of replicates must be included and appropriate statistics applied (Spanggaard et al. 2001). Depending on the set-up, e.g. the variation between tanks, duplicate or triplicate measurements may be sufficient (Fig 4). However, when large variations are experienced (Fig 5) as much as 8 replicates may be required to allow for statistical analysis (Gram and Ringø).

Figure 4

Accumulated mortality of Atlantic salmon following co-habitant infection with *Aeromonas salmonicida*. In six tanks, fish were infected with furunculosis and three tanks were treated with *Pseudomonas fluorescens* AH2 three times per day (Gram et al. 2001, Gram and Ringø 2002).

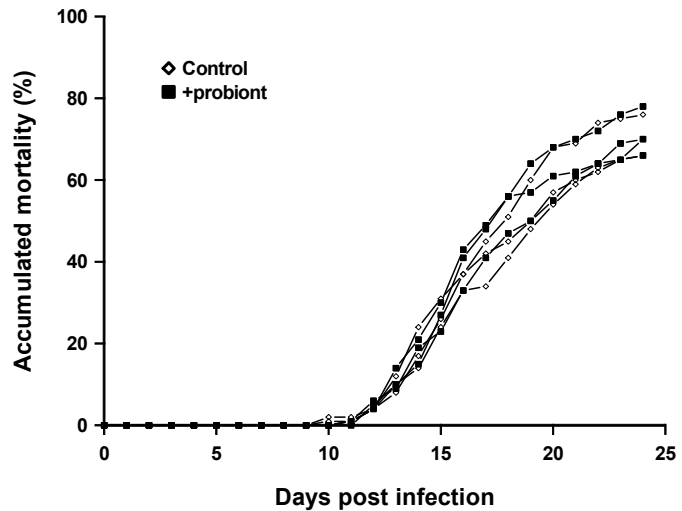
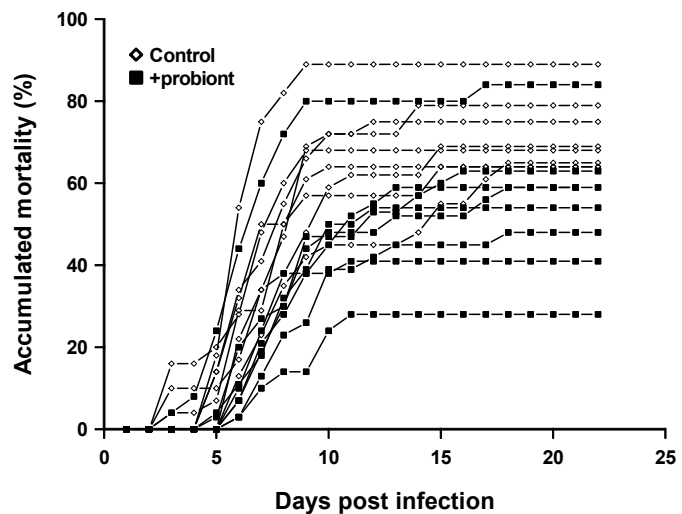


Figure 5

Accumulated mortality of rainbow trout following immersion infection *Vibrio anguillarum*. Approx. 480 fish were infected; half of which were also submerged in *Pseudomonas fluorescens* strain AH2 and subsequently treated with AH2 on a daily basis. Fish were divided into 16 tanks – eight served as controls and eight were treated with AH2 (Slierendrecht and Gram, 2001 (unpublished data), Gram and Ringø 2002).



As mentioned the main criteria for selecting potential probionts so far have been the detection of *in vitro* antagonism. However, even if such antagonism is important little is known about the stability of the antagonistic traits in the environment and it is not known if a potential effect can be enhanced. Both these aspects would be facilitated by a better understanding of the mechanisms underlying both the *in vitro* antagonism and the *in vivo* probiotic effect. Studies of rhizosphere biocontrol organisms have demonstrated how, for instance, the construction of mutants deficient in one or more specific traits (siderophore synthesis or antibiotic production) can help reveal the importance of each trait (Buysens et al. 1996). Also, the identification, at the molecular level may allow the mechanism important for inhibition to be boosted. For instance, Schnider et al. (1995) amplified the expression of a σ -factor that regulates 2,4 diacetylphloroglucinol (Phl) in *Pseudomonas fluorescens*. Phl is important for disease suppression and the over-producing strain showed increased protection of cucumber against the fungal disease caused by *Pythium ultimum* (Schnider et al. 1995). Also, promoter fusions can be used to determine if the gene (trait) in question is expressed *in vivo*.

The selection of (fish) probionts would benefit from systematic studies where a particular trait, such as *in vitro* antagonism, was evaluated with respect to its importance for disease suppression. This could be done using the molecular approach described above where mutants deficient in *in vitro* antagonism are compared to the antagonistic wild type strain with respect to disease suppression. If molecular techniques for some reason are not available, comparisons could be made between the probiotic effect of antagonistic strains and “similar” strains (same species, same isolation, same habitat, same sample) having no *in vitro* antagonism

Examples of use of fish probiotics

As mentioned, many studies have documented the *in vitro* antagonism of aquatic (and other) bacteria against fish (larval) pathogens. Some studies include *in vivo* assessment of a potential probiotic effect.

Several studies have demonstrated that the addition of live bacteria to tank or pond water can reduce mortalities during bacterial infections in grown fish. Smith and Davey (1993) demonstrated that pseudomonads could reduce stress-induced furunculosis in Atlantic pre-smolts and *Pseudomonas* spp. reduced mortality of rainbow trout bath infected with *Vibrio anguillarum* (Gram et al. 1999, Spanggaard et al. 2001). Also, application of a strain of *Vibrio alginolyticus* (Austin et al. 1995) and *Bacillus* species (Queiroz and Boyd 1998) have improved fish survival. Several studies have assessed the probiotic potential of lactic acid bacteria which are typically incorporated into the fish feed. Some studies have imported improved survival (Robertson et al. 2000) whilst other trials have been inconclusive (Nikoskelainen et al. 2001b).

The trials mentioned above with grown fish probably primarily serve to demonstrate the viability of the probiotic principle. Evaluation of the probiotic principle under standardised conditions has been possible due to the existence of several bacteria infection models in grown fish. However, the development and use of vaccines are probably a much more efficient disease control strategy in grown fish than bacterial probiotics.

In contrast, probiotics may serve a purpose in larval rearing units and in rearing of crustaceans or bivalve molluscs. Moriarty (1998) found that addition of *Bacillus* species reduced levels of potential shrimp pathogens (luminescent vibrios) in ponds, and *Bacillus* also improves shrimp survival (Table 1, Rengpipat et al. 1998, 2000). Both scallop, oyster and finfish larvae may have some level of disease protection when probiotics are applied in model infection trials (Table 2). However, the ultimate test of a probiotic strain is the application of the principle in field trials. Nogami et al. (1997) clearly demonstrated the disease suppressing effect of adding probiotics to larval rearing units (Table 3).

Table 1 Survival and production of prawns when comparing un-treated systems to systems treated with the *Bacillus*. The bacteria were applied to a final concentration of 10^4 cfu per ml once every 1 to 3 days (modified from Moriarty 1998).

Year	Addition of probiont	No. of experiments	Larval no. at start	Avg. survival rate (%) up to 143-200 days	Final production (kg/ha)
1995	-	ND ¹	ND	poor production or complete collapse on day 60-80	< 5.000 (poor production)
	+	7	ND	38 - 63	5.300 – 11.500

¹ND: not determined

Table 2 Small scale *in vivo* trials

Host organism	Potential probiont	Pathogen	No. of exp.	% mortality		Days	Reference
				probiонт + pathogen	pathogen		
Oyster larvae, 2 – 6 days old	<i>A. media</i>	<i>V. tubiashii</i> , 10 ² -10 ⁵ cfu/ml	5	0-2	96	6	Gibson <i>et al.</i> 1998
Rainbow trout fry, 1.5-2.0 g	<i>Carnobacterium</i>	<i>A. salmonicida</i> dip in 10 ⁷ cfu/ml	3	4-18	76 –94	14	Irianto and Austin 2002
	<i>V. fluvialis</i>	do	3	0-6	76 -94	14	
	<i>A. hydrophila</i>	do	3	12-16	76-94	14	
	<i>Micrococcus</i> ¹	do	3	0-16	76-94	14	
Scallop larvae	<i>Vibrio</i> spp.(C9) <i>Pseudomonas</i> <i>Vibrio</i> (C33)	not challenged	1 ²	probiонт 60	untreated 97	14	Riquelme <i>et al.</i> 1997
		do	1 ²	80	97	14	
		do	1 ²	50	97	14	
Turbot larvae 0-5 day post hatch	<i>V. mediterranei</i> <i>Pseudomonas</i>	not challenged	5	18-45	39-86	7	Huys <i>et al.</i> 2001
		do	2	43-56	39-50	7	

¹ ID based on API-reactions

² One trial in triplicate

Table 3. Survival and production of a swimming crab (*Protunus trituberculatus*) in five consecutive years when comparing un-treated systems to systems treated with the bacterium *Thalassobacter utilis*. The bacteria were added at a density of 10^5 - 10^6 per ml once every 6 to 8 days (modified from Nogami et al. 1997, Gram and Ringø 2002).

Year	Addition of probiont	No. of experiments	Larval no. at start	Avg. survival rate (%) to 1 st crab stage	Final production (ind/m ³)	No. of production failures
1989	-	10	46,960,000	22.0	5,158	0
	+	4	20,300,000	30.4	7,703	0
1990	-	9	42,930,000	6.8	1,617	4
	+	7	30,570,000	26.7	5,838	0
1991	-	7	34,150,000	10.4	2,543	4
	+	7	34,790,000	27.9	6,938	0
1992	-	8	35,710,000	17.8	3,963	3
	+	9	37,410,000	28.8	5,994	1
1993	-	8	33,200,000	21.6	4,474	1
	+	6	26,610,000	27.7	6,150	0
Total	-	42	192,950,000	15.7	3,605	12
	+	33	149,680,000	28.2	6,397	1

Concluding remarks

Several studies have demonstrated that non-pathogenic microorganisms when added to a fish or the fish environment may serve a disease suppressing effect. This is clearly an area where research should be pursued, in particular, use of the probiotic principle in larval rearing. However, the field of fish probiotics would benefit from inclusion of more bacterial physiology and molecular techniques. Many studies are empirical in nature and just describe the observations without addressing the question that brings forward science: Why? does the principle work (or not work). These questions from which knowledge of the mechanisms of action will arise will allow evaluation of the robustness of the technique as well as determining any possible environmental side effects. Both are required to evaluate the benefits, the risks and the costs of a probiotic treatment

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