Role of Gut Probionts in Enhancing Growth and Disease Resistance in Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) Fingerlings

MANPAL SRIDHAR[1*], N. SRIDHAR[1], P.A.W. ROBERTSON[2] and B. AUSTIN[2]

[1]*Physiology, Nutrition and Pathology Division*
Central Marine Fisheries Research Institute
Kochi – 682014, Kerala, India

[2]*Department of Biological Sciences*
Heriot – Watt University, E4 144AS
Edinburgh (U.K.)

Abstract

Seven out of 34 isolates were selected as putative probionts. These were mixed with an equal volume of a lipid oralizer and incorporated into compounded feed pellets to achieve a dose of 5 x 10^7 bacterial cells•g^{-1} of fish food and fed to Rainbow trout *Oncorhynchus mykiss* fingerlings (average weight 23.69 g) for a period of 25 days. Superior growth (SGR) ranging between 0.32 to 0.57 (P<0.05) as compared to 0.05 of the control group was obtained in fingerlings maintained on the probiotic incorporated diets. A comparatively lower growth of 0.12 was obtained in group F5 but this was still higher than that of the animals maintained on a feed devoid of probiotics. Feed efficiencies ranging between 0.36 for group F5 to 1.03 for group F7 (P< 0.05) as compared to 0.08 of the control group were obtained in animals fed the probiotic feeds.

A challenge of these probiotic fed rainbow trout with intra-peritoneal injections of an inoculum containing x 10^6 cells•ml^{-1} of *A. Salmonicida* (strain PR 107) recorded high survival rates ranging between 38% in group F5 to 78% in group F7 as compared to 20% survival in the animals fed the control diet devoid of any probiotic up to 14 days after challenge. The results of the present study clearly demonstrate the beneficial effects of orally administered probionts in enhancing growth of culture organisms as well as in increasing their resistance to diseases.

Introduction

In present day culture regimes, animals are constantly subjected to intense management practices which tell upon their ability to remain healthy as they are driven to achieve high production standards. The system of the body most likely to succumb to such stressful conditions is the intestinal tract which constitutes a doorway for pathogenic microorganisms. Control regimes often center on the use of vaccines and anti-microbial compounds, (Austin and Austin 1993), but pressure is mounting for greater control over the use of antibiotics and farmers are thus forced to look for alternative methods for healthier and quicker growth and disease control measures.

---

[*] Corresponding author.
  E-mail address**:** manpalsridhar@yahoo.co.uk

In agriculture, the value of probiotics, notably gram-positive bacteria such as *Lactobacillus,* has come to be appreciated as an alternative to antibiotics in disease control strategies (Fuller and Turvey 1971; Parker 1974; Fuller 1978; Roach and Tannock 1980; Smoragiewicz et al. 1993). However there is a dearth of published information about the potential of probiotics in aquaculture (Douillet and Langdon 1994), despite the unpublished observations of widespread use for primary disease control purposes in shrimp hatcheries within Ecuador.

By definition, *Aeromonas salmonicida* comprises fermentative, gram – negative, non-encapsulated coccobacilli, which produce catalase and oxidase, and grow optimally at 22 – 25°C. Typical isolates of *A. Salmonicida* have been inevitably associated with ulcerations in cyprinids (eg. goldfish ulcer disease and carp erythrodermatitis; Austin & Austin, 1993) and marine fish (Nakatsugawa 1994; Wiklund 1995; Wiklund and Dalsgaard 1995; Larsen and Pedersen 1996), while the typical *A. salmonicida* subsp. *Salmonicida* causes furnuculosis in salmonids (Austin and Austin 1993). These infections are of increasing economic importance to aquaculture worldwide.

The primary objective of the present study, therefore, was to study the effect of some putative probionts isolated from the gut and monitored orally *via* compounded feed on growth in Rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings. Moreover, the ability of these probionts to combat *A. salmonicida* infections was assessed in these animals by the help of *in vivo* challenge studies.

Materials and Methods

The role of putative probionts in enhancing growth along with their efficacy against bacterial infections was evaluated. Effective putative probionts isolated from guts were incorporated onto feed pellets as a suspension mixed with an equal volume of a lipid oraliser. Rainbow trout fingerlings maintained on these probiotic feeds were then challenged with *A. Salmonicida* as 0.1 ml intra-peritoneal injections of an inoculum containing x 10^6 cells•ml^{-1}.

Rainbow trout (*Oncorhynchus mykiss*, Walbaum) and Atlantic salmon (*Salmo salar*, Walbaum) were obtained from a commercial fish farm (Perth, Scotland). All trout were fingerlings and averaged 15 g in weight while the salmon averaged 18 g in weight. Both had been maintained in raceways with running river water on a commercial dry feed (Sweden). After transportation to the laboratory they were acclimatized for a week in circular 1000 liter capacity fiberglass tanks supplied with aeration at 15°C. The feed base used for the probiotic feeds was a commercial grower pelleted feed obtained from a commercial feed manufacturing plant at Edinburgh.

The strains of fish and human pathogens used in the antagonism tests (Table 1) as well as *A. Salmonicida* used in the challenge studies were all obtained from the stock cultures maintained at the microbiology unit of the Department of Biological Sciences, Heriot – Watt University, Edinburgh. Most of the isolates were maintained on slants of brain heart infusion agar (BHIA; Oxoid) at 4°C while a few were maintained as lyophilized ampoules.

Isolation of putative probionts from the gut

Five rainbow trout fingerlings (average weight 15g) and five Atlantic salmon fingerlings (average weight 18g) were dissected under aseptic conditions after exposure to an overdose of anaesthetic (MS 222; Sigma, Poole, England). The entire gut was removed aseptically

from each fish and aseptically macerated and vortexed with 0.85% saline in glass vials. Serially 10 fold diluted samples of 0.1 ml were spread onto Tryptone Soy Agar (TSA) plates. After incubation at 25°C for 48 h, colony forming units (CFU) were enumerated. Isolate were tested for morphological and biochemical characteristics and identification was based on Bergey's Manual of Systematic Bacteriology. Bacterial counts and flora in water and diets were also checked by the same method. All isolates were checked for catalase production (using 3% H_2O_2) and oxidase production (using Spot Test Oxidase reagent; Difco laboratories).

Table 1. The various fish and human pathogens used for the antagonism studies.

Sl. No.	Bacterial strain	Reference No
1.	*Aeromonas salmonicda*	Hooke
2.	*Vibrio harveyi*	V 645
3.	*Vibrio harveyi*	PR 188
4.	*Vibrio anguillarum*	PR 151=MSC 275
5.	*Yersinia ruckeri*	PR 128
6.	*Salmonella typhimurium*	S 206
7.	*Salmonella sp*	253
8.	*Salmonella enteritidus*	203
9.	*Enterococcus seriolicida*	PR 80
10.	*Photobacterium damsela Subsp. piscicida*	PR 84
11.	*Flavobacterium psychrophilum*	PR 33
12.	*Aeromonas salmonicida*	PR 107 (1 way resistant)
13.	*Staphylococcus aureus*	PR 117
14.	*Aeromonas hydrophila*	PR 81
15.	*Yersinia ruckeri*	PR 110 = EX5
16.	*Aeromonas salmonicida*	D 33
17.	*Vibrio alginolytics*	V 322 = PR 242

In vitro antagonism studies:

The isolated and selected bacterial strains were tested for antagonism against a range of fish and human pathogens *in vitro*. Pure cultures of the putative probionts and pathogens were streaked onto TSA media plates containing 1.0% NaCl. After 48 h incubation a loopful each of the fresh cultures of both pathogens and probionts were aseptically diluted and vortexed with 9 ml of 0.85% saline. Disruption of growth of the pathogens by the probionts was observed by cross streaking as by well as by drop plate techniques.

Toxicity studies of the putative probionts in vivo

The bacterial strains which showed maximum antagonism to the 17 pathogens were tested for toxicity studies *in vivo* before being finally selected for the feeding trials and challenge studies. Groups of 10 rainbow trout fingerlings averaging 18 g in weight were selected for testing the toxicity of each strain in this part of the study. They were maintained as separate groups in 50 liter capacity covered polypropylene tanks supplied with aerated, dechlorinated fresh water at an average temperature of 15°C. Fresh cultures of each of the selected strains of putative probiotic bacteria were diluted with PBS under aseptic conditions to yield final concentrations containing 10^7 cells•ml^{-1}. Five animals from each group were injected intraperitoneally while five animals were injected intramuscularly with 0.1 ml of each of the putative probiotic bacterial suspensions. They were fed a compounded feed every second day and water exchange was carried out daily. The fishes were observed for pathological symptoms and mortality over a period of 14 days. In case of mortalities animals were immediately dissected and after observation for any pathological symptoms, kidney and gut of these animals were dissected out aseptically and plated on TSA medium. Microbial flora was ascer-

tained after incubation at 15°C for up to 10 days. Surviving animals were killed by exposure to an overdose of anaesthetic (MS 222; Sigma, Poole, England) and examined for both external and internal pathologies.

Feed preparation and feeding trials with selected probionts

Fresh TSA plated culture of each of the selected probionts were inoculated into 100ml of Tryptone Soya broth containing 1.0% NaCl. After incubation at 15°C for 48h each culture was separately centrifuged under aseptic conditions at 10,000 rpm for 15 min at – 20°C. The supernatant was drained off and the cell biomass dispensed in 5millilitre of PBS. Five millilitre of fish oil was added to each and the whole was remixed thoroughly over a vortex mixer. One hundred grams of compounded extruded feed was added to each and mixed thoroughly in order to obtain evenly coated pellets of each feed containing 5 x 10^7 cells•g^{-1} of diet. The control feed contained only PBS and oil. The prepared feeds were stored in sterilized conical flasks and viability of the bacterial cells was determined at intervals and fresh coated feed was prepared accordingly.

For the feeding trials, groups of 20 rainbow trout fingerlings (average weight 23.69 g) were selected so that two replicated groups were maintained for each of the seven probionts plus the control group. The experimental set up was the same as that described under the toxicity studies. The animals were fed the probiotic coated feeds to satiation at 10.00 h and 16.00 hrs daily. Faecal material was siphoned off along with left over feed material and water exchange was done daily. After of 25 days, the animals of each group were weighed for estimation of the growth parameters.

Colonization of gut by probiotic bacteria

At the end of 25 days four fishes from each of the groups were sacrificed after anaesthetizing with MS – 222. The guts were dissected out aseptically and homogenized in 9 ml volumes of physiological saline (PS) in a tissue blender and then 10-fold dilutions were prepared to 10^{-5} in PS, and 0.1ml volumes were spread onto the surface of triplicate plates of tryptone soya agar (Difco, Detroit, Michigan, USA) supplemented with 1.0% (w•v^{-1}) sodium chloride and incubated for 24–72 h at 30°C. A representative of each colony type was purified by streaking and re-streaking on fresh media. Pure cultures were stored at 4°C in agar slants with sub culturing every 6-8 weeks. Identification was achieved after Baumann et al. (1984) and Austin and Lee (1993).

Fish challenge experiments

The remaining rainbow trout fingerlings from the feeding experiments with putative probionts were maintained in 50 liter capacity covered polypropylene tanks supplied with aerated, dechlorinated freshwater at an average temperature of 15°C. Each animal was given an intra peritoneal injection of PBS containing X10^6 cells of *A. Salmonicida* (strain PR 107) •ml^{-1}. Use of the probiont incorporated feeds to feed the fish was continued. Fish were observed for pathological symptoms and any dead moribund fish was examined promptly.

After 15 days of challenge five fish from each group were killed by exposure to an overdose of anaesthetic (MS 222: Sigma, Poole, England). The entire head kidney of each fish was removed aseptically, and each kidney was placed in a separate 5 ml volume of ice-cold holding medium (RPMI 1640; Gibco, Paisley, Scotland) supplemented with 0.1% fetal bovine serum (Gibco). Each tissue sample was drained of excess medium, weighed and divided into two approximately equal sections before being transferred to fresh 5ml volumes of ice-cold

holding medium. Half of each kidney was used to produce a tissue homogenate (10%, w•v^{-1}) in PBS. Homogenates were prepared immediately in micro centrifuge tubes with sterile (121°C for 15 min) micropestles (Eppendorf; Greliner, Stune house, England) and then returned to ice for 10 min to allow the settlement of any undisrupted tissue fragments. Then 200 µl of each homogenate was transferred to a fresh micro centrifuge tube. All manipulations were performed using filter pipette tips (Northumbria Biologicals Ltd., Cramlington, England) to reduce the risk of cross-contamination between samples. Duplicate volumes (20µl) of each homogenate were used to prepare 10 fold dilutions to 10^{7} in PBS, and two 20 µl volumes of each dilution served as inoculum for plates of TSA (Tryptone Soy Agar) following the drop plate method. Plates were sealed with laboratory film and incubated at 15°C for evidence of colony formation. Representatives of any colonies suspected of being *A. Salmonicida* were Gram stained and sub cultured onto TSA.

Statistical analysis

Tests for significant differences were analyzed using one-way analysis of variance (ANOVA) and multiple comparisons using a probability value of 0.05.

Results and Discussion

Isolation of putative probionts from the gut

Thirty-four isolates obtained from the guts of the five Atlantic salmon and five Rainbow trout samples were tested for their morphological and biochemical characteristics before being subjected to *in vitro* antagonism studies. *Vibrio anguillaram, Vibro vulnificus, Vibrio alginolyticus, Vibrio species; Pseudomonas; Alacaligenes; Enterobacteriaceae and Moraxella* comprised the major bacterial flora isolated from the guts of Rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) specimens (Table 2). While *Moraxella, Vibrio anguillaram* and *Pseudomonas* were recorded in higher percentages in *Oncorhynchus mykiss* specimens the *Salmo salar* guts reported higher percentages of *Vibrio* species in general followed by *Pseudomonas*. Presence of *Vibrio vulnificus* was identified in only one rainbow trout specimen gut. *Acinetobacter, Aeromonas* and *Cytophaga* bacterial general were however, not recovered in the guts of the five specimens each of Atlantic salmon and Rainbow trout used in the present study for isolation of putative gut probionts.

Table 2. Bacterial Flora (%) of fingerlings of Rainbow trout (R 1-5) and Atlantic salmon (A 1-5).

Sampling	Flora (%)											
	1	2	3	4	5	6	7	8	9	10	11	12
R1	60.7	-	-	-	-	14.8	-	-	1.1	-	-	23.4
R2	10.2	1.4	-	12.3	-	21.3	0.9	-	-	-	42.1	11.8
R3	28.2	-	-	-	-	6.3	14.0	-	2.8	-	34.1	14.6
R4	10.8	-	-	0.6	-	48.6	3.2	-	-	-	36.2	0.6
R5	4.9	-	0.9	10.2	-	71.6	-	-	0.8	-	-	11.6
A1	12.6	-	10.4	34.8	-	34.9	-	-	-	-	-	7.3
A2	21.8	-	0.8	32.6	-	42.2	-	-	-	-	-	2.6
A3	4.8	-	2.0	63.6	-	3.1	-	-	-	-	-	26.5
A4	7.6	-	-	65.8	-	22.8	-	-	0.8	-	-	2.0
A5	4.3	-	-	45.2	-	34.6	-	-	4.3	-	-	13.6

1. V. Anguillaram	4. Vibrio spp.	7. Alcaligenes	10. Cytophaga
2. V. Vulnificus	5. Acineto bacter	8. Aeromonas	11. Moraxella
3. V. Alginolyticus	6. Pseudomonas	9. Enterobacteriaceae	12. Others

Investigations on the aerobic bacterial flora in the intestine of larval and juvenile stages of red sea bream (*Pargrus major*), black sea bream (*Acanthopagrus schlegeli*), Japanese flounder (*Paralichthys olivaceus*), rock fish (*Sebastes schlegeli*), tiger puffer (*Takifugu rubripes*) and red grouper (*Epinephelus akaara*) recorded *Vibrio, Pseudomonas, Acinetobacter, Moraxella, Cytophaga* and *Alcaligenes*e genera of bacteria (Tanasomwang and Muroga 1990).

Fishes were continuously exposed to a wide range of microorganisms present in the environment, and the microbiota of fish have been the subject of several reviews. Moriarty (1998) evaluated lactic acid bacteria in fish, and demonstrated that *Streptococcus, Leuconostoc, Lactobacillus,* and *Carnobacterium* belonged to the normal microbiota of the gastrointestinal tract in healthy fish. However, it is well known that the population level of lactic acid bacteria associated with the digestive tract is affected by nutritional and environmental factors like dietary polyunsaturated fatty acids, chromic oxide, stress and salinity.

In vitro antagonism studies of the putative probionts

The 34 strains of putative probionts isolated from the guts of *Oncorhynchus mykiss and Salmo salar* specimens in the present study were subjected to *in vitro* antagonism against seventeen fish and human pathogens listed in Table 1. Disruption in growth of pathogens was observed by both cross streaking and drop plate techniques. Out of the 34 strains isolated as putative probionts only 11 strains recorded maximum antagonism in the form of a clear halo around the periphery and/ or excessive growth suppressing that of the pathogens. This observation was evidence that the strains could be used as probionts, and in keeping with the views of Olsson et al. (1992) who reported that native bacteria with inhibiting effect against *Vibrios* were especially promising, since they could colonize the gut of turbot.

Westerdahl et al. (1991) screened more than 400 isolates from the intestine and the external surface of farmed *Scophthalmus maximus* as well as from fish food and hatchery water for inhibitory effects against the fish pathogen *Vibrio anguillarum* and seven other fish pathogens. The bacteria with inhibitory effects were then characterized with based on their sites of colonization, especially the intestinal regions and sites within each region. Of the total number of bacterial isolates from the intestine, 28% were inhibitory against *Vibrio anguillarum.* A marine biochemical assay was used to order the inhibitory strains into different phena. Most inhibitory bacteria were found in the rinse and mucus fractions of the gastrointestinal tract. No correlations among the different phena, site of colonization and inhibitory effect could be found; however a biochemical diversity was noted in the strains with an inhibitory effect. Of the isolates with an inhibitory effect against *V.anguillarum,* 60% had an inhibitory effect on five other fish-pathogenic serotypes of *V.anguillarum*. Inhibitory effects of the isolates were also shown against *Aeromonas salmonicida* and *Aeromonas hydrophila*.

Toxicity studies of the putative probionts in vivo

The 11 strains recording antagonism to a maximum number of pathogens out of the 17 pathogens screened were evaluated for any possible sign of toxicity as evidenced from mortality of morbidity in Rainbow trout (*Oncorhynchus mykiss*) fingerlings. Intra peritoneal and intra muscular injections (0.1 ml) of inoculums containing 10^7 cells•ml^{-1} of these putative probionts in replicates recorded severe haemorrhages and necrosis at the sites of injections in two groups of the experimental animals while heavy mortalities were obtained upon injecting in another two groups of animals. Use of these four groups of probionts was therefore discontinued as they were found toxic. All the remaining seven groups of animals failed to elicit any mortality as well as external or internal pathologies upon injections with the putative probi-

onts. The characteristics of these seven probiotic bacteria isolated from the gut and selected for the feeding trials reported in Table 3 showed that two belonged to *Micrococcu*s genera, two belonged to Pseudomonas genera, two belonged to Bacillus while the last one belonged to *Moraxella* genera. Only one strain of *Micrococcus* was yellow pigmented while all others were colorless. The *Bacillus* recorded comparatively shorter generation times as compared to *Micrococcus* and *Moraxella* genera.

Table 3. The characteristics of the seven probiotic bacteria isolated from the gut and selected for the feeding trials.

Strain		Gram Strain	Mobility	Shape	Pigment	Doubling time (min)
Micrococcus	P1	+	-	Cocci	Yellow	70
Bacillus	P2	+	+	rods	-	49
Bacillus	P3	+	+	rods	-	32
Pseudomonas	P4	-	+	rods	-	43
Pseudomonas	P5	-	+	rods	-	60
Micrococcus	P6	+	-	Cocci	-	65
Moraxella	P7	-	-	Cocci	-	58

During growth of larval turbot in aquaculture the first food supplied is usually the rotifer, *Brachionus plicatilis* and algae and are commonly included in the system as food for the rotifers, thereby maintaining their nutrient quality. As bacteria are known to influence markedly the survival of larval turbot, the effect of bacteria, isolated from larval turbot, on growth of Pavlova lutheri was measured over a 3-d period. Of the 41 bacteria tested, 23 inhibited growth to various degrees, eight had no effect and 10 were weak growth stimulants. Four bacteria, identified as a *Flavobacterium, Vibrio fluvialis, Vibrio natrigens* and *Vibrio* sp., were strongly inhibitory and the *Flavobacterium* inhibited growth of Pavlova lutheri from an inoculum of 10 super (3) colony-forming units per ml. Inhibition was due to a heat-labile factor released by the *Flavobacterium* into the culture medium. The *Flavobacterium* also produced bacteriocin(s) which inhibited the growth of a range of *Vibrios.* Bacteria antagonistic towards algae would be undesirable in larval rearing and if bacteria are to be selected which are beneficial (probiotics) in larval rearing systems their possible interaction with algae must be considered (Munro et al. 1995).

Feeding trials with Rainbow Trout (Oncorhynchus mykiss) fingerlings

The seven putative probionts selected for the feeding trials were coated onto commercial pelleted fish feed as a suspension of bacterial cells mixed with an equal volume of a lipid oralizer to achieve a dose of 5×10^7 bacterial cells•g^{-1} of fish food. Analysis of the feed (Table 4) recorded 45% protein, 6% lipid, 10% moisture and 8% ash content. The feed had a crude fiber content of 3.00% and carbohydrate content of 28%. All these were in keeping with the nutritional requirements prescribed for fish.

Table 4. Proximate composition of the commercial pelleted feed used for feeding the rainbow trout fingerlings.

Nutrient	Percentage
Moisture	10.10
Protein	44.90
Lipid	5.50
Ash	8.20
NFE*	28.30
Fiber	3.00

*Nitrogen Free Extractives (NFE) calculated as 100 – (% moisture +%protein +% lipid +% ash +%fiber). All values are the mean of analysis carried out in triplicate..

After feeding the rainbow trout fingerlings for a period of 25 days the effect of the orally administered probionts on growth was evaluated (Table 5). A significant gain in weight was observed in all the rainbow trout fingerlings fed on/with the probiotic feeds as compared to the control group. High specific growth rates (SGR) ranging between 0.32 to 0.57 were observed on feeding the probiotic feeds while the rainbow trout fingerlings maintained on feed F5 gave a comparatively low SGR of 0.12.

Rainbow trout fingerlings maintained on the control feed showed a very poor SGR of only 0.05. Feed efficiencies (FE) were also greatly enhanced and ranged from 0.36 in rainbow trout fingerlings maintained on feed F5 to 1.03 in rainbow trout fingerlings maintained on feed F7. Rainbow trout fingerlings fed on diet devoid of any probiont gave a poor FE of 0.08. No mortalities were recorded in any of the experimental or control groups of animals during the feeding trial.

Table 5. Initial and final body weights, specific growth rates (SGR) and feed efficiencies (FE) of the groups in relation to diet. Treatment values are expressed as arithmetic means ± standard errors (S.E) (n 20).

Diets	Parameters			
	Initial Body Weight (g)	Final Body Weight (g)	SGR	FE
C	22.3 ± 4.10	23.3 ± 10[a]	0.05 ± 0.0[a]	0.08 ± 0.01[a]
F1	27.5 ± 2.36	29.7 ± 12[a]	0.32 ± 0.1[b]	0.56 ± 0.04[b]
F2	26.7 ± 3.21	40.3 ± 15[b]	0.56 ± 0.1[c]	1.01 ± 0.3[d]
F3	25.5 ± 2.82	38.8 ± 11[a]	0.56 ± 0.2[c]	1.02 ± 0.3[d]
F4	24.3 ± 6.00	36.1 ± 14[b]	0.52 ± 0.1[c]	0.95 ± 0.03[d]
F5	20.7 ± 3.80	33.9 ± 12[a]	0.42 ± 0.1[b]	0.36 ± 0.02[c]
F6	21.9 ± 4.63	33.6 ± 12[a]	0.41 ± 0.3[b]	0.71 ± 0.01[c]
F7	20.6 ± 2.98	41.6 ± 14[b]	0.57 ± 0.1[c]	1.03 ± 0.3[d]

Column means with the same superscript are not significantly different (P< 0.05).
± Standard error.

The most promising result of probiotic use was reported in the improvement of the survival rate of turbot due to rotifers fed with spores of *Bacillus* (Gatesoupe 1993).This improvement was observed in the event of an infection with *A. hydrophila*, whether the infection was casual or experimental. As the spores were fast ingested then digested by rotifers, the hypothesis was that a vibriostatic substance from *Bacillus* hindered the proliferation of *Vibrionaceae* in rotifers and thereby in turbot. The advantage of this treatment over antibiotics was that it worked at a very low concentration (about 20 spores per rotifer, daily), and the medium was not affected by any drug also.

Bacillus S11 bacterium isolated from black tiger shrimp habitats was added to shrimp feed as a probiotic in three forms: fresh cells, fresh cells in normal saline solution, and a lyophilized form (Rengpipat et.al. 1998). After a 100-day feeding trial with probiotic supplemented and non-supplemented (control) feeds, *Penaeus monodon* (from PL30) exhibited no significant difference (p>0.05) in growth, survival nor external appearance among all three probiotic treatments, but significant differences (p<0.05) occurred between probiotic and control groups. After challenging shrimps with a shrimp pathogen, *Vibrio harveyi*, by immersion for 10 days, all probiotic treatment groups had 100% survival; whereas the control group had only 26% survival. In addition, the control group had an unhealthy external appearance, and deformed texture of the hepatopancreas and intestine, while treatment group shrimp appeared healthy and normal.

Metaillier and Hollocou (1993) fed triplicate lots of young 30 g European sea bass (*Dicentrarchus labrax*) three different diets each including a different probiotic ad libitum. They were compared with lots fed on a standard diet. Food consumption was quite the same for all the lots. When compared with the standard, growth was slightly improved (5%) for two diets but not significantly. Nutritional parameters obtained with probiotics were not different from the standard. They concluded that only through a new study carried out on younger animals, during weaning period for example, would it be possible to point out a potential "probiotic effect" for this species

Adhesion to the digestive tract wall to prevent colonisation of pathogens, neutralisation of toxins, bactericidal activity and increased immune competence are some of the mecha-

nisms proposed for bacteria to act as probiotics. Experimental introduction of lactic acid bacteria in fish intestine by regular intake through feed is established (Ringo and Gatesoupe 1998). Adhesion is the first step in colonisation of a microorganism and the intestinal mucosa plays a vital role in this process. In endothermic animals there are reports that there are some degrees of host specificity in this adhesion process. Joborn et al. (1997) assayed *in vitro* adhesion to intestinal mucosa in fish. Intestinal bacteria of turbot adhere specifically to intestinal mucous than to any control surface. Specific adhesions have also been demonstrated in the adhesion of yeasts to the intestinal cell walls of rainbow trout (Vazquez- Juarez et al. 1997).

In our study colonization was obtained in all the groups (Table 6). Though counts as high as 7.2×10^5 were observed in group F_2 the other groups showed counts ranging between 1.1×10^2 for group F_7 to 6.8×10^5 in group F_1. Tanasomwang and Muroga (1990) observed an increase in the number of bacteria in direct proportion to the size of the fish up to 10^5 CFU •fish^{-1} in fish fed on live diets comprised of rotifer, brine shrimp and copepods. However, the number of bacteria decreased thereafter when the feeds were changed to minced fish and artificial feed.

Pathogenic lactic acid bacteria such as *Streptococcus, Enterococcus, Lactobacillus, Carnobacterium* and *Lactococcus* have been detected from ascites, kidney, liver, heart and spleen. Some antibiotic treatments and vaccinations have been proposed to cure or prevent these diseases that seem, however, to spread with the development of fish culture. It has also been reported that some lactic acid bacteria isolated from the gastrointestinal tract of fish can act as probiotics. These candidates are able to colonize the gut,

Table 6. Colonization of the putative probionts in the gut of rainbow trout (*Oncorhynchus mykiss*) fingerlings.

Group No.	Intestinal bacterial count (CFU*•fish^{-1}) [a] on TSA ** plates
F1	6.8×10^5
F2	7.2×10^5
F3	6.6×10^4
F4	3.4×10^4
F5	1.7×10^2
F6	2.6×10^4
F7	3.1×10^2

* Colony Forming Units.
** Tryptone Soya Agar.

and act antagonistic against Gram-negative fish pathogens. These harmless bacteriocin-producing strains may reduce the need to use antibiotics in future aquaculture (Moriarty, 1998).

Although the microbiology of the intestinal tracts of marine and freshwater fish have been investigated by many researchers few studies have addressed the production of inhibitory components by these bacteria. There is evidence that dense microbial populations occur within the intestinal contents, with the number of bacteria much higher than those in the surrounding water, indicating that the fish intestine provides a favorable ecological niche for these organisms. However, lactic acid bacteria are not dominant in the normal intestinal micro biota of fish, at variance with homeotherms, but some strains can colonize the gut. It has also been reported that the number of bacteria in freshwater salmonids increased between the stomach and the posterior portion of the intestine. It has been proposed that mucus may serve as a source of nutrients and that it may enhance colonization by serving as an initial attachment site for bacteria or as a matrix for permanent bacterial attachment. Conversely, the mucus layer may in some instances be an effective barrier, providing protection against penetration by invading microorganisms.

Challenge studies

Very few challenge studies have been carried out to date among aquatic animals. The Lactobacillus */ Carnobacterium strain* isolated from rotifers increased the resistance of turbot

larvae against a pathogenic *Vibrio spp.* At day 9 the inoculum concentration of lactic acid bacteria had a decisive effect on survival rate, and the optimum was between 10^7 and 2×10^7 CFU daily added per ml of the enrichment medium(53% survival rate after 72 h of challenge versus 8% for the infected control group without lactic acid bacteria) (Gatesoupe 1994). The addition of freeze- dried *C. divergens* to compounded feed did not improve the resistance of salmon fry challenged against pathogenic *A. hydrophila* (Gildberg et al. 1995). However, a similar dietary addition reduced the mortality rate of Atlantic cod fry when challenged against V. *anguillarum* (Gildberg et. al. 1997).

The effect of lactic acid bacteria in increasing disease resistance to *Vibrio* pathogens was tested experimentally in challenge studies by Griffith (1995) and Gomezgil (1995) who reported on the beneficial effects of nutritional probiotics in developing shrimp of high immunity. The studies of Garriques and Arevelo (1995) also agree with these observations and recommend the use of probiotics in increasing the disease resistance of animals. Probionts may therefore provide growth factors and inhibit the proliferation of pathogens by stimulating the non- specific immune response (Vanbelle et al 1989).

Rainbow trout fingerlings ranging between 23–41 g from the feeding experiments were injected intraperitoneally with 0.1 ml of an inoculum containing x 10^6 CFU•ml^{-1} of *A. salmonicida* (strain PR 107) and maintained for 15 days. Survival (%) of rainbow trout fingerlings challenged with *A. salmonicida* up to 15 days of challenge (Fig. 1) showed a beneficial effect of the orally administered probiotic bacteria. Only 20% survival was obtained in the control group of animals as compared to very high survival rates in all the treatment groups. Only group F$_5$ recorded 38% survival but this was partly attributed to the observation of low feed consumption in this group of rainbow trout as compared to other groups. Some off flavour of the bacterial biomass could be the only probable answer as all other conditions were favorable.
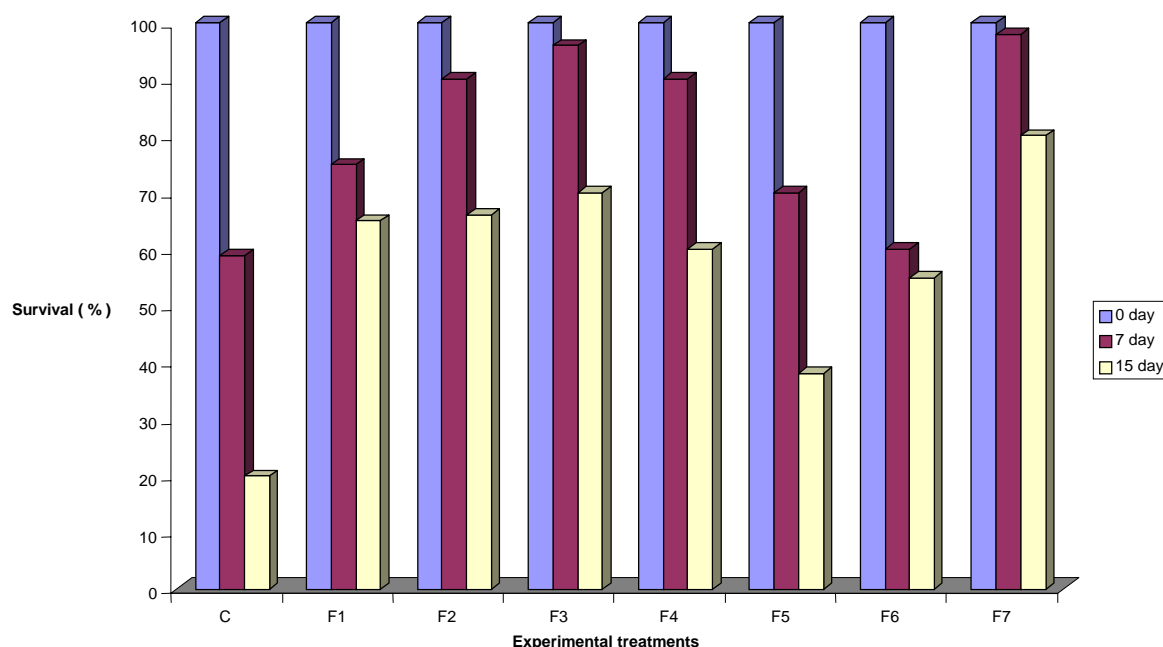

Fig. 1. Survival of rainbow trout (*Oncorhyncus mykiss*) fingerlings challenged with *A. salmonicida*.

Austin et al. (1995) demonstrated the overgrowth of fish pathogens by means of cross-streaking and there was no evidence for any zones of clearing. Use of freeze dried culture supernatant resulted in zones of clearing of 3mm in diameter in *Aeromonas salmonicida, Vibrio. Anguillarum* and *Vibrio ordalli* and to a much lesser extent ie. 1 mm in diameter in

Yersinia ruckeri. The probiont did not cause any harmful effect in salmonids. Indeed, the organism could not be found in the infected fish at the end of the experiment. It was established that the probiont entered salmonids, and was recovered from the intestine (but not from the stomach, kidney or spleen) within 1 hr of exposure by immersion. The evidence suggested that the probiont was capable of survival in the intestine, insofar as *V. alginolytics* was found 21 days after the initial application.

These experiments indicate that the application of probionts to Atlantic salmon led to a reduction in mortalities after challenge with *Aeromonas salmonicida,* and to a lesser extent *Vibrio. anguillarum* and *Vibrio ordalli.* Thus 18% of mortalities were recorded among fish which were immersed in *Aeromonas salmonicida* after exposure to the probiont, compared to 100% of the controls. With *Vibrio. anguillarum*, 90% of the Atlantic salmon controls died compared to 74% of the fish which also received the probiont. The data for *Vibrio ordalli* revealed that cumulative mortalities in the Atlantic salmon were 100% and 74% for the controls and probiont treated fish, respectively. The probiont was not beneficial in mediating infection by *Y. ruckeri,* as all the controls and probiont treated fish died. The observations with this probiont *vibrio* show that there is a potential role for the use of such probiotics in aquaculture as part of a disease control strategy. The possibility of freeze drying the probiotic is worthy of further consideration, in so far as this would allow a more efficient application to aquatic animals via bioencapsulation, such as with rotifers, or by inclusion in diets.

Conclusion

Probiotics have been successfully used in aquaculture to enhance both internal and external microbial environments. The role of beneficial bacteria to limit and to control environmental pathogens will become particularly important in the future of aquaculture, especially with regard to the increasing number of antibiotic resistant strains of bacteria, the tightening of governmental and environmental regulations of treatments, and the cost-effectiveness.

The use of probiotics in aquaculture is now an acceptable practice and is on the increase. The present investigation clearly demonstrates the role of putative probionts isolated from the gut and orally monitored via compounded feed on growth in rainbow trout *(Oncorhynchus mykiss)* fingerlings. It is thus possible to seed the gut with harmless bacteria which occupy the attachment sites and prevent infection by pathogenic bacteria by artificially maintaining these bacterial populations at high level by regular intake with feed. Such treatments would be beneficial to improve the health and quality of fish in culture.

Probiotics however, should not be considered as an alternative to antibiotics in disease therapy, but rather as a complementary therapy for restoring balance to the intestinal flora. Gatesoupe (1999) stated that the first question that remains unanswered in most cases is the fate of the probiotic organism in the rearing medium or in the gut. Investigations on the use of gut probionts against Gram-positive pathogens, and as sources of immunostimulants are also warranted in aquatic organisms. Further works on these lines using molecular and immunological approaches will provide conclusive evidence for these studies.

Acknowledgements

References

Austin, B. and D.A. Austin, 1993. Bacterial Fish Pathogens: Diseases of farmed and Wild Fish, 2nd ed Simon and Schuster, Chichester. 356 pp.

Austin, B. and J.V. Lee, 1993. *Aeromondaceae* and *Vibrionaceae*. Identification Methods in Applied and Environmental microbiology 29:163–182.

Austin, B., L.F. Stuckey, P.A.W. Robertson, I. Effendi and D.R.W. Griffitth. 1995. A probiotic strain of *Vibro alginolytics* effective in reducing disease caused by *Aeromonas salmonicida*, *Vibro anquillarum* and *Vibrio ordalii*. Journal of Fish Diseases 18:93–96.

Baumann, P., A.L. Furniss and J.V. Lee. 1984. Genus 1.Vibrio Pacini 1854, 411AL. In: Bergeys Manual of Systematic Bacteriology. (ed. N.R. Krieg and J.G. Holt), pp. 518–538. Williams and Wilkins, Baltimore, MD.

Douillet, P.A. and C.J. Langdon. 1994. Use of a probiotic for culture of larvae of the Pacific oyster (*Crossostrea gigas*). Aquaculture 119: 25–40.

Fuller, R. 1978. Epithelial attachement and other factors controlling the colonization of the intestine of the gnotobiotic chicken by lactobacilli. Journal of Applied Bacteriology 46:335–342.

Fuller, R. and A. Turvey. 1971. Bacteria associated with the intestinal wall of the fowl (*Gallus domesticus*). Journal Applied Bacteriology 34:617–622.

Garriques, D. and G. Arevalo. 1995. An evaluation of the production and use of five bacterial isolates to manipulate production of *P. vannamei* post larvae in Eucador. World Aquaculture meeting 1-14.

Gatesoupe, F.J. 1993. *Bacillus sp.* spores as food additive for the rotifer *B. plicatilius*: improvement of their bacterial environment and their dietary value for larval turbot, *S. maximus*. Fish Nutrition in Practice 61:561-568.

Gatesoupe, F.J. 1994. Lactic acid bacteria increase the resistance of Turbot larvae, *Scopthalmus maximus*, against pathogenic vibrio. Aquatic living resources 7:277-282.

Gatesoupe, F.J. 1999. The use of probiotics in Aquaculture. Aquaculture 180:147-165.

Gildberg, A., A. Johansen and J. Bøgwald. 1995.Growth and survival of Atlantic salmon (*Salmo salar*) fry given diets supplemented with fish protein hydrosolate and lactic acid bacteria during a challenge trial with *Aeromonas salmonicida*. Aquaculture 138:23-34.

Gildberg, A., H. Mikkelsen, E. Sandaker, E. Ringø. 1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*). Hydrobiologia 352:279-285.

Gomezgil, R. 1995. The use of bacteria as probiotics in shrimp larviculture. Larvi 95, Fish and shellfish larviculture symposium, Belgium 479-480.

Griffith, D.R.W. 1995.Microbiology and role of the probiotics in Eucadorian shrimp hatcheries. Larvi 95, Fish and shellfish larviculture symposium, Belgium. 478-481.

Joborn, A., C. Olsson, A. Westerdahl, P.L. Conway and S. Kjelberg. 1997. Colonisation in the fish intestinal tract and production of inhibitory substances in intestinal mucous and faecal extract by *Carnobacterium sp.* Strain K. Journal of Fish Diseases 20:383-392.

Larsen, J.L. and K. Pedersen. 1996. Atypical *A. salmonicida* isolated from diseased turbot (*Scopthalmus maximus*). Acta veterinaria Scandinavica 37:139–146.

Metaillier, R. and Y. Hollocou. 1993. Feeding of European sea bass (*Dicentrarchus labrax*) juveniles on diets containing probiotics. In: Fish nutrition in practice (ed. S.J. Kanshik and P. Luquet), pp. 429-432. France Institut National De La Recherche Agronomique, Paris.

13

Moriarty, D.J.W. 1998. Control of luminous Vibrio species in penaeid aquaculture ponds. Aquaculture 164:351-358.

Munro, P.D., H.A. McLean, A. Barbour and T.H. Birkbeck. 1995. Stimulation or inhibition of growth of the unicellular alga *Pavlova lutheri* by bacteria isolated from larval turbot culture systems. Journal of Applied Bacteriology 79:519-524.

Nakatsugawa, T. 1994. A typical *Aeromonas salmoncida* isolated from cultured spotted halibut. Fish pathology 29:193-198.

Olsson, J.C., A. Westerdahl, P.L. Conway and S. Kjelleberg. 1992. Intestinal colonization potensial of turbot (*Scopthalmus maximum*) – and dab (*Limanda limanda*) – associated bacteria with inhibitory effects against *Vibrio anquillarum*. Applied and Environmental Microbiology 5:551-556.

Parker, R.B. 1974. Probiotics the other half of the antibiotic story. Animal Nutrition and Health 29: 4–8.

Rengpipat, S., W. Phianphak, S. Piyatiratitivorakul and P. Menasveta. 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167:301-313.

Ringo, E. and F.J. Gatesoupe. 1998. Lactic acid bacteria in fish: a review. Aquaculture 160:177-203.

Roach, S. and G.W. Tannock. 1980. Indigenous bacteria that influence the number of salmonella *typhimurium* in the spleen of intravenously challenged mice. Canadian Journal of Microbiology 26:408-411.

Smoragiewicz, W., M. Bielecka, A. Babucgowski, D. Boutard and H. Dubeau. 1993. Les probiotiques. Canadian Journal of Microbiology 39:1089-1095.

Tanasomwang, S. and K. Muroga. 1990. Intestinal microflora of marine fishes at their larval and juvenile stages, In: The second Asian Fisheries Forum (ed. Hirano, R. and I. Hangu), pp. 991. Asian Fisheries Society, Manila, Philippines.

Vanbelle, M., E. Teller and M. Focant. 1989 Probiotics in animal nutrition: a review. Archiv für Tierernährung 40:543-567.

Vazquez-Juarez, R., T. Andlid and L. Gustaffsson. 1997. Adhesion of yeast isolated from fish gut to crude intestinal mucus of rainbow trout, *Salmo giardneri*. Molecular Marine Biology and Biotechnology 6:64-71.

Westerdahl, A., J.C. Olsson, S. Kjelleberg and P.L. Conway. 1991. Isolation and characterization of Turbot (*Scophtalmus maximus*) – Associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied and Environmental Microbiology 57:2223–2228.

Wilklund, T. 1995. Virulence of 'atypical' *Aeromonas salmoncida* isolated from ulcerated flounder *Platichthys flesus*. Disease of Aquatic Organism 21:145 -150.

Wilklund, T. and I. Dalsgaard. 1995. Atypical *Aeromonas salmonicida* associated with ulcerated flatfish species in the Baltic Sea and the North Sea. Journal of Aquatic Animal Health 7:218-224.