Probiotic effect *in vivo* of *Roseobacter* strain 27-4 against *Vibrio (Listonella) anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae

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Abstract

The purpose of this study was to evaluate the probiotic effect of the marine bacterium *Roseobacter* strain 27-4 in turbot larvae infected with the pathogen *Vibrio (Listonella) anguillarum*. Initial trials demonstrated that cells of *Roseobacter* were not harmful to larvae whereas, large amounts of bacterial culture supernatant caused rapid mortality (70% at day 10 compared to 20% in the control). A similar high mortality was, however, also seen, when sterile marine broth was added to the larvae. Presumably both types of medium enhanced growth of opportunistic pathogens. In subsequent trials, both a pathogen, *Vibrio anguillarum*, and the probiont, *Roseobacter* strain 27-4, were delivered to the larvae bioencapsulated in rotifers. Accumulated mortality of *Vibrio* infected larvae increased to 80-90% over 10 days, whereas, mortality in non-infected controls was significantly lower (60-70%). Feeding larvae with rotifers enriched with *Roseobacter* 27-4 parallel to *V. anguillarum* infection, brought the accumulated mortality to the level of control indicating a clear *in vivo* effect. *Roseobacter* 27-4 could be detected in larvae both by agar plating and by immunohistochemistry, being located in the gastrointestinal lumen, and apparently did not colonise the larval gut and intestinal epithelium. Plate counts decreased when enriched feed was no longer added, suggesting that the probiont, *Roseobacter* 27-4, should be supplied repeatedly to exert its positive effect.

Introduction

Probiotics have been defined by WHO/FAO (2001) as "live microorganisms which when administered in adequate amounts, confer a health benefit on the host". The use of probiotics has emerged as a potential tool in the reduction of mortalities in the rearing of aquatic organisms (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Gómez-Gil *et al.* 2000; Verschuere *et al.* 2000; Gram and Ringø, 2005). In fish, probiotics have been studied in the prevention or reduction of disease outbreaks in larvae, fry or adults (Kozasa, 1986; Gatesoupe, 1999; Austin *et al.* 1995; Gildberg *et al.* 1997; Gram *et al.* 1999).

The development of the intestinal microbiota in marine fish larvae depends basically on the bacteria colonising in the live prey (in larviculture, mainly rotifers and *Artemia*) and, to a lesser extend, the rearing water (Nicolas *et al.* 1989; Munro *et al.* 1994; Bergh, 1995; Blanch *et al.* 1997; Grisez *et al.* 1997; Reitan *et al.* 1998). Consequently, attention has been focused on the delivery of bacterial additives or bacteria cells to live food as a vehicle for introducing beneficial bacteria to the fish larvae. Several studies have been conducted on turbot (*Scophthalmus maximus*) larvae due to the economic importance of this fish. The effects of commercially available lactic acid bacteria, including extracts of terrestrial lactic acid bacteria or live bacteria additives, were tested with varying results (Gatesoupe 1991, 1999; García de la Banda *et al.* 1992). Also, probiotic candidates have been selected among isolate strains from commercial hatcheries (Gatesoupe, 1997; Huys *et al.* 2001; Hjelm et al, 2004a,b).

We recently isolated bacteria antagonising fish larval pathogens from a turbot hatchery in Spain and the most prominent among the antagonists strains were identified as *Roseobacter* (Hjelm *et al.* 2004a,b). *Roseobacter* species belong to the so-called *Roseobacter* clade that are very important members of the procaryotic communities of marine environments (Selje *et al.* 2004) where they are believed play a major role in sulphur cycling (Moran *et al.* 2003). *Roseobacter* is typical of the marine environment (Shiba, 1991) and have been isolated from green seaweed (Shiba, 1992), marine aggregates (marine snow particles) (Bano and Hollibaug. 2002) and dinoflagellates (Töbe *et al.* 2001). Ruiz-Ponte *et al.* (1998) described *R. gallaeciensis* and later demonstrated that addition to tank water of cell extracts from cultures at particular cell densities enhanced survival of scallop larvae (Ruiz-Ponte *et al.* 1999). A member of the *Roseobacter* group was at one point associated with disease in juvenile oysters

(Boettcher *et al.* 2000). However, this strain was later grouped as a new genus and species *Roseimarina crassostreae* (Boettcher 2002).

From the screening performed by Hjelm *et al.* (2004a) on different groups of bacteria for inhibitory activity *in vitro*, *Roseobacter* 27-4 was selected as the most promising candidate probiotic. This strain showed 99.1% alignment with *R. gallaeciensis* (Hjelm *et al.* 2004a). Strain 27-4 did not oxidise glucose and it differed from the type description of *R. gallaeciensis* (Ruiz- Ponte *et al.* 1998). In our study, the *in vivo* ability of *Roseobacter* 27-4 to protect turbot larvae by the pathogenic strain *Vibrio anguillarum* 90-11-287 serotype O1 was evaluated. The strain was found to be promising as fish larvae probiotic.

It is known that *Roseobacter* strain 27-4 enhances survival of egg yolk sac larvae and is highly inhibitory to *Vibrio* species (Hjelm *et al.* 2004a). However, its effect has not been studied in model challenge trials. The aim of our work was to study the probiotic effect of the bacteria *Roseobacter* strain 27-4 in turbot larvae infected with the pathogen *Vibrio anguillarum* (Skov *et al.* 1995). Both bacteria were delivered to the larvae bioencapsulated in rotifers. Potential side effects of *Roseobacter* 27-4 (both bacteria cells and supernatant of bacteria cultures) to turbot larvae was investigated prior to the determination of the probiotic effect.

Materials and methods

Bacterial strains

Roseobacter 27-4 strain was isolated from the tank walls in healthy rearings from a turbot hatchery (Stolt Sea Farm) in Galicia (Nothwest Spain) and identified by Hjelm *et al.* (2004a). The strains were kept at – 80°C in TSB (Oxoid CM129) (30 g l^{-1}) with glucose (5 g l^{-1}), skimmed milk (20 g l^{-1}) and glycerol (40 g l^{-1}). The strain *Vibrio (Listonella) anguillarum* 90-11-287 serotype O1 was used as the target organism. The strain was isolated from rainbow trout (Skov *et al*. 1995) and obtained from K. Pedersen (Royal Veterinary and Agricultural University, Copenhagen, Denmark).

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117 **Bacterial culture and preparation of the inocula**

118

119 *Vibrio anguillarum* was grown for 24 hours in 10 ml of Marine Broth (MB, Difco, 2216) on a
120 rotary shaker at 200 rpm and 22°C. Culture (1 ml) was added to a flask with 100 ml of MB,
121 grown for 24 hours, and subcultured twice under the same conditions. Growth was monitored
122 by optical density (700 nm) and by plate counting (reference!!!!!!).

123

124 *Roseobacter* 27-4 was cultured according to Hjelm *et al.* (2004a). Bacteria were pre-cultured
125 in 3-4 ml of MB and incubated at 20°C for three days in the dark and stagnant aerobic
126 conditions. Culture (1 ml) was used to inoculate a 1 l flask with 100 ml of MB. After two
127 days, bacteria were harvested by centrifugation at 2,500 x g for 15 min and resuspended in
128 100 ml sterile seawater. The concentration was verified by serial dilutions in sea water and
129 plating on Marine Agar (Difco). These conditions ensured a bacterial concentration of 5 x 10^8
130 to 1 x 10^9 cfu ml^{-1}. When *Roseobacter* 27-4 was added to the water of the larval tanks, the
131 bacteria were centrifuged and washed as described. However, when *Roseobacter* 27-4 added
132 to the water of the rotifer enrichment, the bacteria were added with the culture supernatant.

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134 **Rotifer culture and bioencapsulation of bacteria**

135

136 Rotifers (*Brachionus plicatilis*) were cultured on baker's yeast and subsequently enriched
137 (200 rotifers ml^{-1}) on *Isochrysis galbana* (2 x 10^6 cells ml^{-1}) for 24 h. Two types of
138 bioencapsulation were carried out. For bioencapsulation of *V. anguillarum* (Rotifer-V): The
139 rotifers (200 rotifers mL^{-1}) were enriched on *Isochrysis galbana* (2 x 10^6 cells ml^{-1}) for 24 h in
140 10-20 l tanks at 23°C. Rotifers were then filtered (30 µm Nylon mesh), washed and
141 transferred (200 rotifers ml^{-1}) into 5 L buckets containing seawater and *V. anguillarum* (1x10^8
142 cfu ml^{-1}). The rotifers were maintained in this bacterial suspension for 3 hours and filtered,
143 washed and delivered to turbot larvae.

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145 For bioencapsulation of *Roseobacter* 27-4 (Rotifer-R), rotifers (200 rotifers ml^{-1}) were
146 enriched on *Isochrysis* (4 x 10^6 cells ml^{-1}) and *Roseobacter* (10^7 cfu ml^{-1}) for 24 h in 10 L
147 tanks at 23°C. Rotifers were then filtered, washed with seawater and delivered to the larvae.

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149 **Turbot larval rearing**

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151 Newly hatched larvae (day 0) of turbot were obtained from Stolt Sea Farm (Merexo, Galicia,
152 Spain). Larvae were transferred at day 2 (30-35 larvae l^{-1}) to 60-L tanks previously disinfected
153 with Dismozon Pur (Bode) (1 %, 4 h). The temperature was progressively raised from 15 to
154 18 °C during the following 3 days, the water of the tanks was moderately aerated (>90%
155 oxygen saturation) and light (day light provided by fluorescent lamps) intensity at the surface
156 of the larval tanks was adjusted to 3.5 µE . sec^{-1} . m^{-2}. The larvae were fed on enriched rotifers
157 from day 3 until day 10. For the different experimental trials, the larvae were fed on alternate
158 days with enriched rotifers with *Roseobacter* 27-4 or *V. anguillarum*. The density of rotifers
159 was adjusted daily (3-5 rotifers ml^{-1}) and the water of the rearing tanks was partially (30-40
160 %) changed every 2 days from first feeding with a subsequent addition of 2.5 L of *Isochrysis*
161 *galbana* culture (2 x 10^5 cells ml^{-1}). The bottom of the tanks was siphoned daily to remove
162 and count dead larvae. All the trials were conducted in duplicate. Samples of larvae and/or
163 water were taken for microbiological analyses. Dry weights of larvae were obtained at the end
164 of the experiments after collecting 100 larvae from each tank on 150 µm mesh, washing with
165 tap water and drying at 60°C for 48 h. A total of three trials were carried out with turbot
166 larvae.

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168 **Challenge A: Innocuous effect of *Roseobacter* 27-4 for turbot larvae**

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170 Hjelm *et al.* (2004a) demonstrated that *Roseobacter* strain 27-4 was not harmful to egg yolk
171 sac larvae. However, a preliminary trial was carried out to determine whether *Roseobacter*
172 was harmful to the turbot larvae at the feeding stage. The trial was carried out in duplicate in
173 eight 60-L tanks with four treatments. In treatment C (control), larvae were reared as
174 described above. In treatment SR (single addition of *Roseobacter*), the larvae were reared as
175 controls and 100 ml of bacterial cells re-suspended in sterile seawater were delivered (10^6 cfu
176 ml^{-1}) to the water of the larval rearing tanks at mouth opening (day 3). A continuous addition
177 of *Roseobacter* 27-4 (CR) was similar to the SR treatment, except that bacterial cell
178 suspension (10^6 cfu ml^{-1}) was added to the water of the larval tanks at days 3, 5 and 7. In the
179 last treatment (CS_{100}), a continuous addition of 100 ml *Roseobacter* free culture supernatant
180 was added to the water of the larval tanks at days 3, 5 and 7.

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182 **Challenge B: Effect of *Roseobacter* 27-4 supernatant or Marine Broth**

Challenge A demonstrated that the culture supernatant of *Roseobacter* strain 27-4 was toxic to turbot larvae, and the following treatments were applied to asses the effects of marine broth and the supernatant of *Roseobacter* 27-4 cultures on larvae. The control (C) larvae were reared as described above. In treatment MB, larvae were reared as controls with the addition of 100 ml of Marine Broth to the water of the larval rearing tanks at days 3, 5 and 7. The treatment described above CS_{100} was repeated and paralleled by a similar treatment CS_5 in which larvae were reared as controls with the addition of 5 ml of bacteria-free supernatant of *Roseobacter* culture to the water of the larval rearing tanks at days 3, 5 and 7.

Challenge C: Probiotic effect of *Roseobacter* 27-4 against V. *anguillarum*

Three trials were performed to determine the probiotic effect of *Roseobacter* 27-4 in turbot larvae challenged with the pathogen *V. anguillarum*. Turbot larvae were reared by duplicate for 10 days as reported above under three different conditions. Control (C) larvae were fed from day 3 to day 10 with normally enriched rotifers. During challenge with *V. anguillarum*, the larvae (V) were fed on days 4, 6 and 8 with rotifers enriched with *V. anguillarum*. In the probiotic test (VR), the larvae were fed with rotifers enriched with *Roseobacter* 27-4 (days 3, 5 and 7) and with rotifers enriched with *Vibrio* (days 4, 6 and 8).

Further details on the infection of turbot larvae by *V. anguillarum* have been published previously (Planas *et al.* in press, 2005, Aquaculture).

Microbiological methods

Samples from larvae, rotifer and water were taken under aseptic conditions during the trials. Ten larvae or 400 rotifers were separated using a 250 µm or 30 µm Nylon mesh, respectively. Larvae were anaesthetised with 3-Aminobenzoic acid ethyl ester (concentration!MS22, Sigma). Larvae and rotifer were washed with sterile seawater and homogenised. Processed samples were serially diluted in seawater, plated on Marine Agar (MA, Difco 2216) and incubated for 3 days at 20ºC in the dark. Plates with 30 to 300 colonies were counted. *Roseobacter* 27-4 colonies were identified by their dark brown pigmentation and confirmed by absence of growth on TSA plates (Oxoid CM131) (Hjelm *et al.* 2004a). For Vibrionaceae

counting, appropriate dilutions were replica-plated from MA onto TCBS (Cultimed 413817), incubated one day at 20ºC and colonies were counted. *Vibrio anguillarum* colonies were recognized and verified using the agglutination test MONO-VA (Bionor, Norway).

Immunohistochemistry

The primary antiserum was polyclonal rabbit antiserum against *Roseobacter* 27-4. Vaccines were produced by cultivation of *Roseobacter* 27-4 in filtered, autoclaved MB for 1-3 days. The culture was treated with formalin at 0.5% for minimum 3 hours and the cells harvested by centrifugation at 5000 g for 10 min. The cells were washed twice with phosphate buffered saline (PBS, Oxoid) and re-suspended to a density of 1 – 4 x 10^9 cells ml^{-1}. The vaccine was stored at -20°C until used. A rabbit was vaccinated repeatedly by 3 intravenous injections per week of bacterial cells. The doses were from 0.1 ml at the start, increasing gradually up to 1.0 ml after 3 weeks. In the 4^{th} week a booster of 1.0 ml was given, and in week 5 blood were collected and serum separated. The antiserum was tested for cross-reaction against related species by immuno colony blotting, and adsorbed with cross-reacting species. The serum was stored at -20°C. An antiserum against *Vibrio anguillarum*, kindly provided by Dr. Jens Laurits Larsen was also used as primary antibody.

The immunohistochemical protocol was modified from Evensen & Rimstad (1997) and Bergh *et al.* (1997). Turbot larvae were fixed in neutral phosphate-buffered 3.7% formaldehyde, and kept until processing. The larvae were dehydrated through a graded ethanol series and embedded in paraffin. Sections, approximately 3 µm thick were cut on a Reichert-Jung Biocut, incubated for 30 min at 56ºC, dewaxed in xylene, rehydrated through a graded ethanol series (100%, **Øivind** check this 96%, 70%, 50%), and brought to distilled water. Nonspecific antibody binding sites were blocked by covering the sections with a solution of 5% bovine serum albumin (BSA, Sigma Co., London, UK) in Tris-buffered saline (TBS, ph 7.4) for 20 min. The solution was blotted off the slides and the primary rabbit antiserum was incubated at a dilution of 1:900 in 2.5% BSA in TBS for 30 minutes. After washing for 5 min. in TBS, the secondary antibody, biotinylated goat anti-rabbit immunoglobulin, diluted 1:300 in 2.5% BSA in TBS (Dakopatts,Glostrup, Denmark) was added and incubated at room temperature for 30 min. After washing in TBS, streptavidin alkaline phosphate complex was added, and incubated for 30 min. After washing, New Fuchsin Chromogen (K698, Dako, CA, US) with 1 mM levamisole (Sigma) as inhibitor in TBS was added and allowed to develop for 5 min.

After washing in tap water, sections were counterstained with Mayer's haematoxylin and mounted in an aqueous mounting medium (Aquamount, BHD Laboratory Supplies, UK). All incubations were performed at room temperature (approximately 20ºC) in a humidity chamber. Tissue sections from larvae not exposed to *Roseobacter* 27-4, and exposed larvae were incubated with immune and nonimmune (normal rabbit serum) as controls.

Statistical analyses

Differences in final survivals and weights of larval challenges were analysed using one-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple range test at 5% level of significance. Survival data were previously transformed to arc sin (square root).

Results

Challenge A: Innocuous effect of *Roseobacter* 27-4 for turbot larvae

Single (SR) or repeated (CR) delivery of *Roseobacter* to the water of the rearing tanks was not detrimental to turbot larvae and the patterns of accumulated mortality were identical to that of controls (Figure 1). However, a significantly higher mortality occurred from day 2 when the larvae were exposed to *Roseobacter* culture supernatant (ANOVA: $p=0.035$; SNK test: $p>0.05$).

The level of culturable bacteria in the water was constant, at approx. 10^6-10^7 cfu ml^{-1} (Table 1). In the larvae, the number of culturable bacteria increased progressively from 10^3 cfu ml^{-1} at day 3 (first feeding day) up to 10^5-10^6 cfu ml^{-1} at day 8. A single addition of *Roseobacter* 27-4 kept concentration constant in values around 10^6 cfu ml^{-1} from day 3 to day 5, being the predominant bacteria in water. After day 5, *Roseobacter* 27-4 concentration diminished constantly, reaching 10^4 cfu ml^{-1} at day 9. Repeated addition of *Roseobacter* 27-4 resulted in maintained levels between 10^6 and 10^7 cfu ml^{-1}. After day 8, the concentration diminished sharply to 10^4 cfu ml^{-1}, which was similar to the level reached with a single addition. *Roseobacter* 27-4 was detected in larvae at day 6 in similar concentration (10^2 cfu $larvae^{-1}$) in

both challenges, decreasing slightly at day 8 with single addition and increasing significantly up to 10^4 cfu larvae^{-1} with repeated addition.

Challenge B: Effect for larvae of *Roseobacter* 27-4 supernatant in water

To elucidate the cause of the mortalities observed when culture supernatant was added to the rearing tank (challenge A), a challenge was performed by testing the addition of the supernatant and the bacteria culture medium (Marine Broth). The addition of 100 ml marine broth (MB) or 100 ml of *Roseobacter* supernatant (CS_{100}) reduced the survival and the growth of turbot larvae drastically. In contrast, growth and survival in larvae submitted to the low concentration of *Roseobacter* supernatant (CS_5) was high, similar to those in control tanks. The pattern of accumulated mortalities show that the highest mortalities in treatments MB and CS_{100} occurred between days 5 and 6 post hatching, just after the second delivery at day 5 (Figure 2).

At day 4, the total bacterial numbers in the rearing water in controls and CS_5 samples were about one log unit lower than in MB and CS_{100} treated samples. In addition, an ominous turbidity appeared in the tanks submitted to these treatments. Total concentration of Vibrionaceae was higher (10^5-10^6 cfu ml^{-1}) in tanks that showed high mortality (MB and CS_{100}) than in tanks with low mortality (control and CS_5) (10^3-10^4 cfu ml^{-1}).

Challenge C: Probiotic effect of *Roseobacter* 27-4 against *V. anguillarum*

In rotifers enriched with algae (*Isochrysis galbana*) and *Roseobacter* 27-4, the levels of *V. anguillarum* were about 3 x 10^2 cfu ml^{-1}, whereas in rotifers supplemented with *V. anguillarum*, the mean level was 2.5 x 10^3 cfu ml^{-1}.

The accumulated survivals were lower in larvae fed rotifers enriched with *V. anguillarum* than in larvae fed non-enriched rotifers in all trials (Table 2). In larvae that received *Roseobacter* and *Vibrio*, survivals were intermediate or similar to those of controls. These relative differences also apply to growth of the larvae. The addition of *Roseobacter* significantly reduced the mortalities caused by *V. anguillarum* (Table 3). With respect to controls, survival in larvae challenged with both *Roseobacter* and *V. anguillarum* was 68%,

double than that of larvae challenged only with *V. anguillarum*. Accumulated mortality patterns were different among trials (Figure 3). However, the main differences in survivals between larvae infected, and those infected but treated with *Roseobacter* seem to occur preferentially after day 8 post hatching.

The bacterial counts were followed in the first challenge trial (Table 4). The level of culturable bacteria remained at 10^6-10^7 cfu ml^{-1} water during the three trials. The level of Vibrionaceae in water and larvae during the experimental period were similar in all treatments, reaching a final level of about 10^5 cfu ml^{-1} both in water and larvae. *Roseobacter* was identified in the water of the larval rearing tanks when rotifers with *V. anguillarum* and *Roseobacter* were added, at levels of about 10^3-10^4 cfu ml^{-1}, but not inside the larvae (Figure 4). The pathogen was isolated from water (10^3-10^4 cfu ml^{-1}) and larvae (higher than 10^3 cfu $larvae^{-1}$).

Immunohistochemistry

Øivind arrange this and modify. Make reference to ALL figures (in Fig. 4)!! Larvae to which cultures of *Roseobacter* 27-4 were added generally showed positive immunohistochemical staining of bacterial cells in the gut and intestinal lumen (Figure 4). The bacteria appeared to aggregate in the lumen, often forming relatively large particles composed of positively stained cells (Figure 4 e,f). Few bacteria were present on the gut and intestinal surfaces, and with single exceptions (see arrow in Figure 4 f) they did not display positive immunostaining. No bacterial cells could be visualised on gills and skin, and no positive immunohistochemistry was detected on these surfaces. As visualised in Figure 4 d, small numbers of anti-*Roseobacter* 27-4 positive bacteria were also found in the gut and intestinal lumen following the addition of culture supernatant without bacterial addition. No indications of damages to larval gut or intestine, or other indications of harmful effects of the bacterial addition were detected in the larvae. Application of anti-*V. anguillarum* antibody caused positive (red) staining (Figure 4 c), indicating the presence of either this bacterium or serologically similar strains in the cultures.

Discussion

The *in vitro* inhibitory activity of *Roseobacter* 27-4 was previously analysed by Hjelm *et al.* (2004a) in co-culture assays with the pathogens *V. anguillarum* and *V. splendidus*. It was demonstrated that both pathogens were inhibited when *Roseobacter* 27-4 reached high densities and that *Roseobacter* produced a soluble sulphur-containing anti-bacterial factor produced under stagnant conditions when the organism was also producing a brown pigment (Bruhn *et al.* 2005a).

The probiotic concept obviously requires that the bacterial strains are not pathogenic. In the present study, it was found that *Roseobacter* 27-4 did not cause any detrimental effects in turbot larvae when added supernatant-free to the water of the larval rearing tanks. However, a harmful effect was noticed when bacterial culture supernatant added at a high dose. The same dose of Marine Broth had similar effect so probably the nutrients in Marine Broth remaining in the supernatant promoted growth of opportunistic pathogenic bacteria, as Vibrionaceae, in the water of the rearing tanks and, consequently the high mortalities recorded

The usual way of entry for pathogens is orally, via prey (Muroga *et al.* 1987; Nicolás *et al.* 1989; Cahill, 1990; Bergh *et al.* 1994; Blanch et al. 1997; Ringø and Birkbeck, 1999), and therefore, we have studied in this work the delivery of *Roseobacter* 27-4 via rotifers. It was noticeable that rotifers were not affected by high doses of bacterial supernatant, which makes the incubation of rotifers with *Roseobacter* 27-4 during long time enrichments possible. We also found less variability in the positive effect (survival) on larvae when *Roseobacter* 27-4 was delivered orally via rotifers rather by bath. Taking into account these facts, we consider bioencapsulation as a preferable way of delivery of *Roseobacter* 27-4 to larvae.

One of the reviewers said that this text (in red) is very speculative. Suggestions??? I think that we only give some ideas and explanations (to investigate in the future) but important. *Vibrio anguillarum* was better than *Roseobacter* strain 27-4 at colonizing rotifers and larvae. The presence of *Roseobacter* 27-4 in the intestinal lumen of larvae, but not in the gut or intestinal epithelium, indicates that the mode of action of this bacterium as a probiotic probably does not involve adhesion and colonisation of turbot larvae. Furthermore, it seems that the main protective function of the *Roseobacter* 27-4 could be more related to disallowing the proliferation or adhesion of pathogens, rather than adhering to distinct larval

surfaces and colonising them. As seen in Challenge C (Table 3; Figure 3), the presence of *Roseobacter* reduced mortality but not *V. anguillarum* counts. *Roseobacter* might act by reducing the pathogenicity of *V. anguillarum* rather than diminishing the numbers of *Vibrio*. However, this hypothesis is contradictory with the findings of Hjelm *et al.* (2004a) in co-cultures. These authors showed that presence of *Roseobacter* 27-4 (initial level of 10^6 – 10^7 cfu ml^{-1}) inhibited growth of *V. anguillarum* and *V. splendidus* during the first 5 days. The reduction of *V. anguillarum* concentration was seen when *Roseobacter* reached a concentration of 10^9 cfu ml^{-1}. *Roseobacter* 27-4 was present in the rotifers and appeared in the water, gut and intestinal lumen forming aggregates. *V. anguillarum*, when administrated to the larvae via infected rotifer, appeared in the epidermis of the larvae, which was severely affected, and in the gut of the larvae, associated to rotifers, but not on the intestinal epithelium (Ø. Bergh *et al.* unpublished results.). *V. anguillarum* has also been demonstrated to be taken up via the brush border of turbot larvae (Grisez *et al.* 1996). Therefore, *Roseobacter* 27-4, even not reducing the total counts of *V. anguillarum* in larvae, could perform the antagonistic effect at specific sites, and therefore improve survival of larvae. Further work should be done to elucidate this point.

In non-infected larvae, the presence of a low number of cells showing positive immunostaining following application of the anti-*V. anguillarum* antiserum could imply the natural presence of such bacteria. However, the absence of adhesion of immunolabelled bacteria to larval surfaces, and the generally normal appearance of the larvae indicate that this could be due to a cross-reaction with serologically similar bacteria. *V. anguillarum* is a well known pathogen to many species of fish, including turbot (Egidius, 1987; Myhr *et al.* 1991; Larsen *et al.* 1994; Toranzo et al. 1994) and it seems unlikely that the presence of such bacteria in significant amounts would not lead to pathological effects that would have been visible on the immunohistochemistry slides (Figure 4).

For turbot larvae challenged with *V. anguillarum*, the addition of *Roseobacter* 27-4 caused a reduction in mortalities. However, the mortality patterns during growth seemed to be different among trials as larval grow (Figure 3), but the causes are unknown at the present. On the other hand, microbiological analysis on the challenge systems showed little evidence of *Roseobacter* in the larval gut but high concentrations in the water (Table 4; Figure 4). This suggests that this probiotic does not colonise the turbot larval digestive tract but may act in the water or in surface biofilms from which it was isolated. Continuous additions (each 48-72

h) are probably necessary to maintain a minimum level of *Roseobacter* 27-4 in the culture water and rotifers. Therefore, another practical approach to investigate in the future would be the artificial production of a bio-film of such bacteria in the rearing system throughout the year (Bruhn *et al.* 2005b).

The use of *Roseobacter* 27-4 has been shown to be safe in the hatchery live food environment and it fulfils the requirements of a probiotic, although, clearly, much remains to be done to optimise the quantity and frequency of addition of *Roseobacter* 27-4, in which case greater benefits should be expected.

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551 **Table 1:**

552 Challenge A - Changes with time in total bacteria and *Roseobacter* 27-4 in water (log cfu ml^{-1})
553 and turbot larvae (log cfu larvae^{-1}). Mean (2 parallel tanks) ± SD. SR: Single addition of
554 *Roseobacter* 27-4 (day 3); CR: Continuous addition of *Roseobacter* 27-4 (days 3, 5 and 7);
555 CS_{100}: Continuous addition of 100 ml *Roseobacter* 27-4 free culture supernatant. ND: Not
556 detectable.

557

		Day	C	SR	CR	CS_{100}
WATER	Total Bacteria	3	6.50 ± 0.08	6.73 ± 0.05	6.77 ± 0.02	6.62 ± 0.11
		5	6.32 ± 0.35	6.25 ± 0.10	6.67 ± 0.06	6.13 ± 0.76
		7	6.32 ± 0.47	6.57 ± 0.06	6.58 ± 0.14	6.67 ± 0.06
		9	5.97 ± 0.25	5.93 ± 0.11	5.67 ± 0.00	6.83 ± 0.00
	Roseobacter 27-4	3	ND	6.06 ± 0.08	6.19 ± 0.16	ND
		5	ND	5.98 ± 0.85	6.28 ± 0.03	ND
		7	ND	4.89 ± 0.16	6.00 ± 0.00	ND
		9	ND	3.72 ± 0.17	4.08 ± 0.18	ND
LARVAE	Total Bacteria	3	3.14 ± 0.00	2.67 ± 0.00	2.83 ± 0.00	2.71 ± 0.00
		6	5.22 ± 0.38	4.89 ± 0.15	5.21 ± 0.54	6.69 ± 0.26
		8	5.78 ± 0.01	5.13 ± 0.04	5.53 ± 0.53	4.85 ± 0.23
	Roseobacter 27-4	3	ND	0.00 ± 0.00	0.00 ± 0.00	ND
		6	ND	2.69 ± 0.26	1.53 ± 0.92	ND
		8	ND	2.18 ± 0.14	3.97 ± 0.61	ND

558 **Table 2:**

559 Challenge C - Survivals and final dry weights (µg larva^{-1}) in the challenges performed to
560 assess the probiotic effect of *Roseobacter* 27-4 against *V. anguillarum*. First feeding: day 3.
561 Mean (2 parallel tanks) ± SD. Different letters superscript mean significant differences (SNK
562 test: $p<0.05$) between treatments (ANOVA: p=0.470, 0.001 and 0.001 in challenges C1, C2,
563 and C3, respectively).

564

Trial	Day	Treatment	% survival		Dry weight, µg larva^{-1}
			absolute	relative to control	
C1	14	Control	34±13^{a}	100	337±13
	14	Vibrio + Roseobacter	35±4^{a}	103	505±66
	14	Vibrio	15±8^{a}	44	388±110
C2	8	Control	29±1^{a}	100	40±3
	8	Vibrio + Roseobacter	17±0^{b}	52	41±3
	8	Vibrio	8±0^{c}	28	37±2
C3	10	Control	32±1^{a}	100	121±0
	10	Vibrio + Roseobacter	17±1^{b}	53	122±10
	10	Vibrio	10±0^{c}	31	101±9

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570 **Table 3:**

571 Challenge C - Effect of the delivery of *Roseobacter* 27-4 on the final survivals in turbot larvae
572 infected with *Vibrio anguillarum* (Pooled data from trials C1 – C3). Mean ± SD. Different
573 letters superscript mean significant differences (SNK test: $p<0.05$) between treatments. n:
574 number of trials.

575

		% survival	
Treatment	n	absolute	relative to control
Control	3	32±3[a]	100±0 [a]
Vibrio + Roseobacter	3	23±10[a]	68±27 [a]
Vibrio	3	11±4[b]	34±9 [b]
ANOVA-p		0.018	0.008

576

Table 4:

Challenge C – Changes on the microflora in water (Log cfu.ml^{-1}) and larvae (Log cfu.larva^{-1}) in Trial C1. Mean (2 parallel tanks) ± SD. VR: larvae were fed with rotifers enriched with *Roseobacter* 27-4(days 3, 5 and 7), with rotifers enriched with *V. anguillarum* (days 4, 6 and 8); V: larvae fed on rotifers enriched with *V. anguillarum* (days 4, 6 and 8). ND: Not detectable.

		Day	Control	VR	V
WATER	Total Bacteria	3	5.95 ± 0.16	6.01 ± 0.09	6.29 ± 0.05
		5	6.45 ± 0.07	6.30 ± 0.08	6.37 ± 0.07
		7	6.45 ± 0.04	6.34 ± 0.06	6.39 ± 0.00
		9	6.78 ± 0.03	6.78 ± 0.11	6.67 ± 0.06
	Roseobacter 27-4	3	ND	3.15 ± 0.21	ND
		5	ND	4.15 ± 0.21	ND
		7	ND	ND	ND
		9	ND	2.74 ± 0.37	ND
	V. anguillarum	3	ND	ND	ND
		5	ND	3.94 ± 0.14	4.00 ± 0.00
		7	ND	3.00 ± 0.06	2.42 ± 0.60
		9	ND	3.94 ± 0.14	3.66 ± 0.26
LARVAE	Total Bacteria	3	2.62 ± 0.02	3.83 ± 0.01	2.72 ± 0.07
		5	4.07 ± 0.82	4.07 ± 0.03	4.27 ± 0.45
		7	5.23 ± 0.04	4.85 ± 0.04	5.01 ± 0.54
		9	5.58 ± 0.21	5.40 ± 0.13	5.52 ± 0.39
	Roseobacter 27-4	3	ND	ND	ND
		5	ND	ND	ND
		7	ND	ND	ND
		9	ND	ND	ND
	V. anguillarum	3	ND	ND	ND
		5	ND	2.92 ± 1.05	1.61 ± 2.28
		7	ND	1.57 ± 2.21	2.68 ± 0.71
		9	ND	4.19 ± 1.01	1.59 ± 2.25

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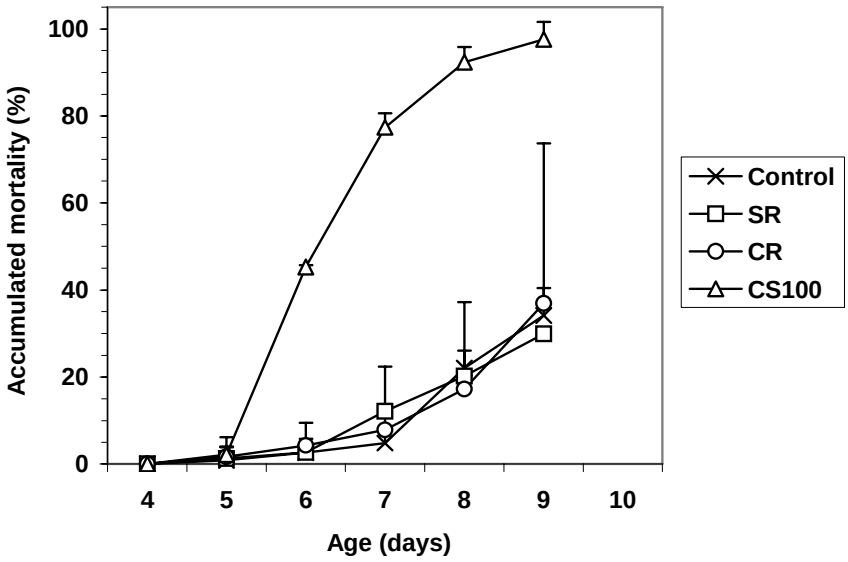

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589 **Figure 1:**

590 Challenge A - Accumulated mortality in turbot larvae from challenge A. Mean (2 parallel
591 tanks) ± SD. SR: Single addition of *Roseobacter* 27-4 (day 3); CR: Continuous addition of
592 *Roseobacter* 27-4 (days 3, 5 and 7); CS_{100}: Continuous addition of 100 ml *Roseobacter* 27-4
593 free culture supernatant.

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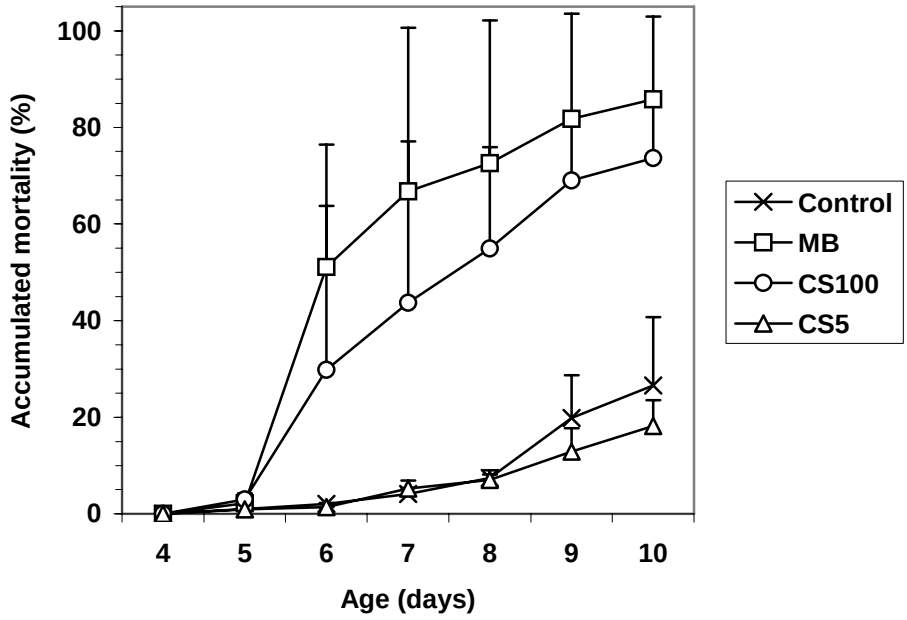

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600 **Figure 2:**

601 Challenge B - Accumulated mortalities in turbot larvae in the presence of marine broth and
602 supernatant of *Roseobacter* cultures. Mean (2 parallel tanks) ± SD. MB: Addition of 100 ml
603 of Marine Broth to the water; CS_{100} and CS_5: Continuous addition of 100 and 5 ml
604 *Roseobacter* 27-4 free culture supernatant, respectively.

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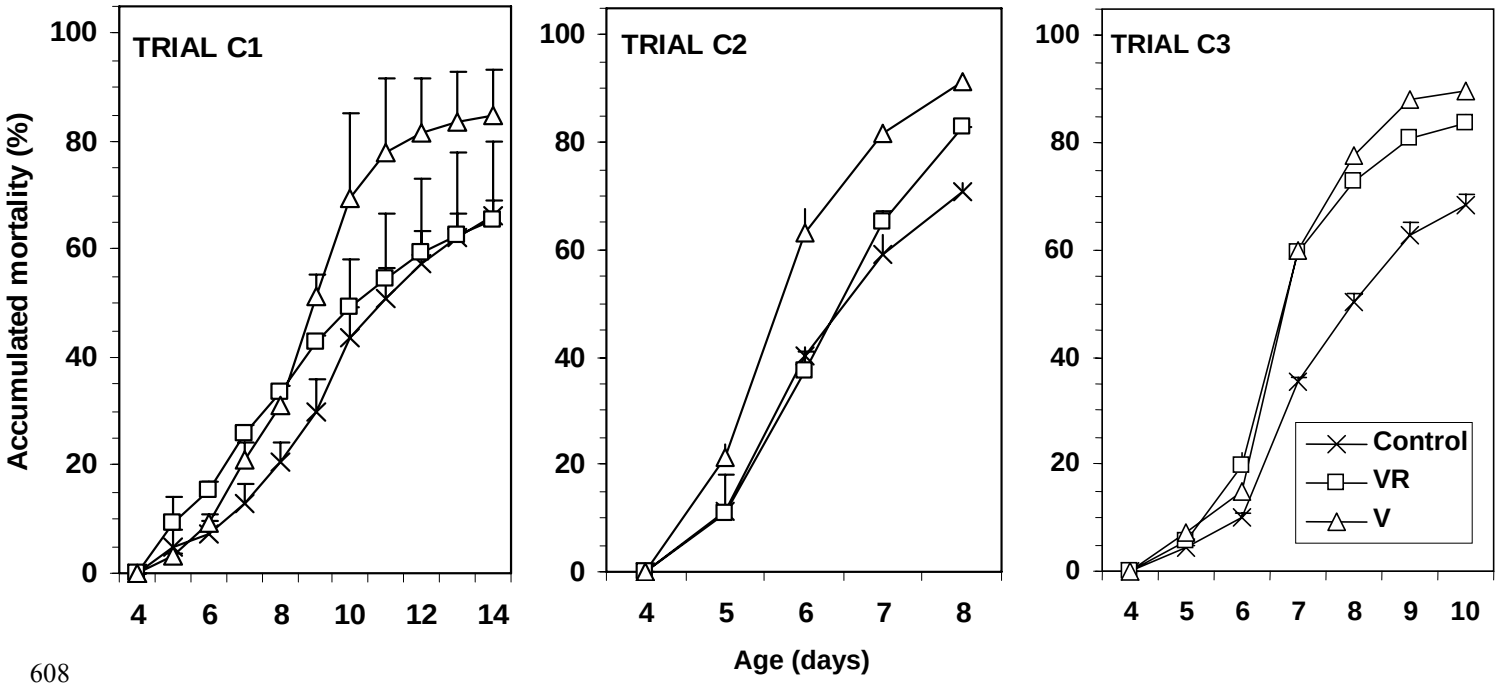

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612 **Figure 3:**

613 Challenge C - Accumulated mortalities in turbot larvae from Trials C1, C2 and C3. Mean (2
614 parallel tanks) ± SD. VR: larvae were fed with rotifers enriched with *Roseobacter* 27-4(days
615 3, 5 and 7), with rotifers enriched with *V. anguillarum* (days 4, 6 and 8) and with non-
616 enriched rotifers (days 9 and 10); V: larvae fed on rotifers enriched with *V. anguillarum* (days
617 4, 6 and 8).

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Figure 4:

Oivind: can you rearrange the text (from a to f)!!! Immunohistochemistry of turbot larvae. Primary antibodies against *Roseobacter* 27-4 (a,b,d,e,f) and *V. anguillarum* (c). Larva from control group to which no bacterial strain was added is shown in (a). Note the presence of particles (arrow) in the lumen of the gut not stained by the immunohistochemical protocol. Larvae from groups added continuously *Roseobacter* 27-4 are shown in b, c, e and f. s b, e and f all displayed positively stained (red) bacterial cells (arrow) in the lumen of the larval gut following application of the anti-*Roseobacter* 27-4 primary antibody. **Fig. d nor defined!!!!**