Vol. 56: 105–113, 2003 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published September 24

Bacterial influences on Atlantic halibut *Hippoglossus hippoglossus* yolk-sac larval survival and start-feed response

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ABSTRACT: A bacteria-free halibut larval rearing system was used to test 20 bacterial isolates, from British halibut hatcheries, for their toxicity towards halibut yolk-sac larvae under microbially controlled conditions. The isolates tested spanned a range of genera and species (*Pseudoalteromonas*, *Halomonas marina*, *Vibrio salmonicida*-like, *Photobacterium phosphoreum* and *V. splendidus* species). A pathogen of turbot, *Scophthalmus maximus*, *V. anguillarum* 91079, and 2 isolates from adult halibut were also included. Isolates were inoculated, at a concentration of 5×10^2 cfu ml^{-1}, into flasks containing 25 recently hatched axenic halibut larvae, using a minimum of 3 flasks for each treatment. Control survivals to 38 d post-hatch for the 3 experiments averaged 84, 51.5 and 49%, respectively. With the exception of *V. anguillarum* 91079, which was highly pathogenic towards halibut yolk-sac larvae, there was no statistically significant difference in survival between the controls and the different treatments. This suggests that most of the bacteria routinely isolated from halibut hatcheries are not harmful to yolk-sac larvae, even though most flasks contained in excess of 5×10^6 cfu ml^{-1} of the inoculated organism when the experiments were terminated. Three organisms previously shown to inhibit growth of bacteria *in vitro* were tested for their ability to protect halibut yolk-sac larvae against invasion by *V. anguillarum*. In 4 separate challenge experiments none of the test isolates, a *Pseudoalteromonas* strain and 2 *Carnobacterium*-like organisms, showed any protective effect. To investigate how particular bacteria influence their start-feed response, larvae were fed axenic and gnotobiotic *Artemia* colonized with a range of different *Vibrio* spp., and examined after 8 d. There were no statistically significant between-treatment differences in the proportion of *Artemia*-containing larvae, indicating that bacterial contamination of the live food does not appear to influence initiation of the feeding response.

KEY WORDS: Halibut larvae · Bacteria · Probiotic · *Vibrio anguillarum*

INTRODUCTION

Atlantic halibut are unique among commercially reared marine fish in having a long larval yolk-sac stage following hatching (>35 d), when they are wholly reliant on endogenous reserves (Haug 1990). Under current UK hatchery protocols, halibut eggs are hatched *in situ* in large (>500 l) upwelling silos and the hatched yolk-sac larvae are reared in the dark at a low temperature (<6°C), for at least $^2/_3$ of the yolk-sac absorption phase. They are then transferred to illuminated first-feeding tanks where the temperature is raised to 10–12°C, and are presented with live prey (*Artemia* or marine copepods) (Shields et al. 1999).

In the UK, mortalities through these initial phases are often high; on average less than 50% of hatched larvae survive the yolk-sac absorption period. Of the survivors, frequently less than 20% initiate a feeding response and the remainder consequently starves to death (Shields et al. 1999).

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The specific causes of the high mortalities are unclear, as both the variation in survival and first-feed response from tank to tank can be high. It has been postulated that this variable survival is due to opportunistic pathogens able to take advantage of high densities of hosts of reduced immunocompetence (Hansen & Olafsen 1999, Ringo & Birkbeck 1999, Skjermo & Vadstein 1999). There is indirect evidence that bacteria have a role in larval mortalities, in that addition of antibiotics such as oxytetracycline enhances survival of yolk-sac larvae reared in small-scale static systems (Lein et al. 1997, Ottesen & Bolla 1998). Also, addition of antibiotics has been shown to improve survival in commercial-scale incubators and to reduce tank-to-tank variation in their survival (Verner-Jeffreys 2000). Although pathogens such as *Vibrio anguillarum*, *Flexibacter ovolyticus* and *Aeromonas salmonicida* ssp. *salmonicida* can cause mortalities in experimentally reared larvae (Bergh et al. 1992, 1997), it is unclear which of the bacteria, other than *F. ovolyticus*, occurring in commercial rearing systems for Atlantic halibut larvae are harmful, as *V. anguillarum* and *A. salmonicida* are rarely isolated from such tanks.

To test the hypothesis that bacteria affect halibut yolk-sac larval performance, the effect of individual bacterial isolates, originally isolated from commercial rearing systems, on larval survival was tested under bacterially defined conditions.

Adverse bacterial floras in rearing systems may be controlled by addition of probiotic organisms which colonize the gut of developing larvae and prevent colonization by pathogenic micro-organisms (Gatesoupe 1999, Hansen & Olafsen 1999, Ringo & Birkbeck 1999, Skjermo & Vadstein 1999). Therefore, in conjunction with the above experiments with bacterially defined small-scale rearing systems, the potential of 3 organisms, previously shown to inhibit growth of bacteria in *vitro*, were tested for their ability to protect halibut yolk-sac larvae against invasion by the pathogen *Vibrio anguillarum.* Also, to test whether high levels of bacteria commonly associated with the live prey inhibit the start-feed response in halibut larvae we offered such larvae *Artemia* colonized with defined bacteria to investigate how specific bacteria influence the start-feed response.

MATERIALS AND METHODS

Bacteria. The bacterial isolates used in this study are shown in Table 1. The 26 isolates shown include 22 tested for their effects on larvae (see Table 2) and 4 which were only tested in first-feeding experiments. Bacteria were routinely grown in shake cultures of marine broth (MB, Difco) or on marine agar (MA, marine broth plus 1.5% technical agar) at 15°C. For long-term storage cultures were stored using the Protect system (Technical Service Consultants) at –70°C, according to the manufacturer's instructions except that 100 µl 20% NaCl was added to the culture medium to raise the salinity.

Supply and preparation of halibut eggs for infection experiments. Eggs were collected from a commercial hatchery after incubation at 5°C for 13 d (65 degree-days post-fertilization). All the eggs (approx. 1 l) from a conical incubator were removed and treated with a solution of 8 ml of 25% glutaraldehyde (Sigma) in 5 l of l00 µm filtered, UV-sterilized hatchery water for 10 min (glutaraldehyde working concentration 0.125% v/v). Eggs were then rinsed thoroughly in seawater (SW) from the same source. Some eggs were transferred to 2 l stoppered conical flasks containing 1.5 l of seawater and antibiotics (oxolinic acid, kanamycin and erythromycin (Sigma), all at 0.0l g l^{-1}; penicillin G, 0.15 g l^{-1}; streptomycin, 0.075 g l^{-1}) added. The eggs were transported on ice in antibiotic-containing flasks, normally within 2 h of collection, to a controlled environment room in our laboratory. After overnight incubation, eggs were rinsed in sterile seawater (SSW), treated for 20 s in 0.02% peracetic acid-based solution (Kick Start; RS Hygiene) and rinsed again in SSW; 25 eggs were then distributed into each 2 l conical flask containing 1.5 l 100 µm filtered 32‰ hatchery SW. The SW was allowed to mature for at least 2 wk prior to distribution into flasks stoppered with compressed paper plugs (Fisher Scientific), sterilization by autoclaving and equilibration to 6°C.

Single strain addition infection Expts 1 to 3. All bacterial isolates were incubated overnight in MB shake cultures at 15°C. Bacteria were collected by centrifugation, washed 3 times in 25‰ artificial seawater (ASW) before estimation of bacterial concentrations by spectrophotometry, based on an optical density $(OD)_{650mn}$ reading of 1.0 being equivalent to 10^9 bacteria ml^{-1}. For Expts 1 to 3, washed cultures were added to the experimental flasks 3 to 5 d after the eggs hatched, to give final bacterial cell densities of 5×10^2 cells ml^{-1}.

Experiments with probiotic bacteria: infection Expts 4 to 6. Three potential probiotic candidates were tested in 3 further rearing experiments for their ability to protect halibut yolk-sac larvae against infection by *Vibrio anguillarum*: these were a *Pseudoalteromas* species, TG15-07, and 2 Gram-positive *Carnobacterium* isolates, AN1 and AN2. These isolates had previously been shown to inhibit the growth of many other bacteria including the pathogen V. *anguillarum* 91079, *in vitro* following standard meth-

Table 1. Bacterial isolates used in this study. The rearing trial investigated the effects of halibut yolk-sac rearing conditions on larval survival and gut flora development (Treatment F: flow-to-waste with disinfected eggs; Treatment RD: recirculated water with disinfected eggs; Treatment RN: recirculated water with non-disinfected eggs [Verner-Jeffleys 2000]). The survey investigated the types of bacteria associated with Atlantic halibut in UK hatcheries (Verner-Jeffreys et al. 2003). All isolates were Gram-negative rods, except for AN1 and AN2. Characterization followed the scheme of Verner-Jeffreys et al. (2003), except for Phenons 4 to 7 and where otherwise described. Isolates assigned to Phenons 4 to 7 were genetically and biochemically distinct groups of Gram-negative organisms not reported in the survey (Verner-Jeffreys 2000)

Isolate	Source	Description (and EMBL accession number for partial 16 S rDNA sequence, where available)
SYS6-10	Survey; healthy yolk-sac larvae	*Vibrio* sp.
TG2-11	Rearing trial, Treatment F; poorly performing yolk-sac larva	Fermentative, catalase-positive, oxidase-negative (Phenon 5)
TG2-12	Rearing trial, Treatment F; poorly performing yolk-sac larvae	Weakly fermentative, oxidase-negative (Phenon 4)
TG2-14	Rearing trial, Treatment F; poorly performing yolk-sac larvae	Fermentative, oxidase-negative (Phenon 4)
TG2-18	Rearing trial, Treatment F; poorly performing larvae	Oxidase-negative (Phenon 7)
TG4-02	Rearing trial, Treatment RN; healthy yolk-sac larvae	*Vibrio splendidus* gp. 3
TG4-07	Rearing trial, Treatment RN; healthy yolk-sac larvae	*Vibrio splendidus* gp. 3 (AJ414125)
TG4-11	Rearing trial, Treatment RN; healthy yolk-sac larvae	*Pseudoalteromonas* sp. (Phenon 3)
TG7-01	Rearing trial, Treatment F; poorly performing larvae	*Oceanospirillum*-like; weakly fermentative (Phenon 6) (AJ414130)
TG8-01	Rearing trial, Treatment RD; healthy yolk-sac larvae	*Pseudoalteromonas* gp. 3 (AJ414131)
TG10-01	Rearing trial, Treatment F; healthy yolk-sac larvae	*Pseudoalteromonas* gp. 1
TG11-16	Rearing trial, Treatment RD; healthy yolk-sac larvae	*Pseudoalteromonas* gp. 3
TG15-07	Rearing trial, Treatment RN; healthy yolk-sac larvae	*Pseudoalteromonas* gp. 4 (AJ318941). Strongly inhibits growth of other bacteria *in vitro*
TG15-08	Rearing trial, Treatment RD; healthy yolk-sac larvae	*Pseudoalteromonas* gp. 2
TG15-19	Rearing trial, Treatment RD; healthy yolk-sac larvae	*Pseudoalteromonas* gp. 3
TG16-05	Rearing trial, Treatment RD; healthy yolk-sac larvae	*Oceanospirillum*-like, weakly fermentative, oxidase-negative (Phenon 6) (AJ41426)
Vibrio anguillarum 91079	Moribund juvenile turbot	Horne et al. (1977)
AE1-26	Survey; enriched *Artemia* (A318954)	Hemolytic *Vibrio splendidus* gp. 1
OFE1-19	Survey; halibut eggs	RFLP group VII
E1-03	Survey; freshly fertilized halibut eggs	*Photobacterium phosphoreum*
AN1	Adult turbot gut from survey	*Carnobacterium* sp., inhibiting growth of bacteria *in vitro*
AN2	Adult turbot gut from survey	*Carnobacterium* sp., inhibiting growth of bacteria *in vitro*
OF2-05	Survey; healthy first-feeding halibut maintained on calanoid copepod diet	*Vibrio salmonicida*-like (AJ318950)
AF6-01	Survey; moribund first-feeding halibut larvae maintained on *Artemia*	*Photobacterium phosphoreum*
AF2-14	Survey; first-feeding halibut larvae maintained on *Artemia*	Hemolytic *Vibrio splendidus* gp. 1 (AJ414123)
DMC-1	Pathogen isolated from moribund larval turbot in commercial Spanish turbot	Hemolytic *Vibrio splendidus*
OF1-02	Survey; first-feeding halibut larvae maintained on mixed *Artemia* and copepod diet	*V. alginolyticus*

ods (Smith & Davey 1993, Gram et al. 1999). Isolates were incubated and harvested as described above, with the exception of AN1 and AN2, which were incubated for 2 to 3 d in static Tryptose Soya Broth (Oxoid) + 2% NaCl + 1% glucose cultures at 20°C.

For infection Expt 4, strains AN1, AN2 and TG15-07 were tested. *Pseudoalteromonas* TG15-07 was added to larvae once to a final concentration of 5×10^5 cfu ml^{-1}. For AN1 and AN2 treatments, both isolates were added 3 times, at Days 1, 4 and 10 post hatch, to a final

concentration of 1×10^6 cfu ml^{-1}. The larvae were then challenged with *Vibrio anguillarum* at 1×10^3 cfu ml^{-1} 3 d later. Appropriate controls were also included (TG15-07, AN1 and AN2 added alone, *V. anguillarum* alone, and no bacteria added). Four replicate flasks were used for each treatment.

For Expt 5, TG15-07 was added to larvae at 2 concentrations 1 d post hatch, 1×10^2 and 5×10^5 cfu ml^{-1}. The larvae were challenged with *Vibrio anguillarum* at 1 cfu ml^{-1} 3 d later. Appropriate controls were also included (TG15-07 added alone at the 2 concentrations, *V. anguillarum* alone and no bacteria added). Four replicate flasks were used for each treatment.

In Expt 6, isolates AN1 and AN2 were tested with a lower *Vibrio anguillarum* inoculum than in Expt 4. Each probiotic candidate was added as described for Expt 4, and *V. anguillarum* was added to the test flasks 4 d post hatch to a concentration of 10 cfu ml^{-1}. Appropriate controls (AN1, AN2 and *V. anguillarum* alone, or no bacteria added) were again included. Four replicate flasks were again used for each treatment.

Maintenance of yolk-sac larvae. For all experiments, flasks were maintained in the dark at 5.5 to 6.0°C for 30 to 42 d. At the end of the incubation period surviving larvae in all flasks were counted. The control flasks, where no bacteria were added, were assessed for bacteriological sterility by direct plating of 100 µl water samples onto MA and addition of 5 ml water to 5 ml MB. Selected control flasks were also analyzed for the presence of bacteria not culturable on MA by microscopic examination of glutaraldehyde-fixed 4′, 6-diamidino-2-phenylindole (DAPI)-stained water samples as described elsewhere (Enger et al. 1989). For flasks inoculated with bacteria, serial 10-fold dilutions of water samples were made in 25‰ ASW and 100 µl plated onto MA in duplicate. Viable counts were made after 4 wk incubation at 6°C to calculate the average final flask density of bacteria for each treatment.

Effect of bacterial isolates on the start feed response: infection Expts 7 to 8. The effect of bacterial isolates on the start feed response was investigated using the flask-rearing model in 2 experiments, infection Expts 7 and 8. The system used was not bacteria-free, although live food and eggs were treated to remove bacteria. Twenty-five disinfected eggs were placed into 2 l flasks and hatched larvae reared as previously described until Day 35, except for an 80% water exchange at Day 28. At this point the flasks were transferred to a separate controlled environment room where the temperature was gradually raised from 6 to 9°C over 4 d. At this point (approximately 40 d post hatch) light from a fluorescent source was provided in the controlled environment room, and *Pavlova lutheri* was added to each flask to a final density of 3.5×10^3 cells ml^{-1}. *P. lutheri* were cultured as described previously (Munro et al. 1995), except that the cultures used were not free of bacteria, containing some which were culturable on MA but not on Thiosulphate Citrate Bile Salt Sucrose agar (TCBS, Difco). Visual assessment of the number of living halibut larvae in each flask was made prior to addition of 750 monoxenic or axenic freshly hatched *Artemia*, to each flask.

Preparation of monoxenic *Artemia*. Decapsulation to remove the external chorions from *Artemia* cysts under axenic conditions was done as follows. Initially, cysts (EG grade cysts, INVE Aquaculture) were rehydrated in aerated distilled water for 1 h, before collection on a 6 sterile 100 µm sieve followed immediately by immersion in alkaline 10% sodium hypochlorite solution in 0.25 M NaOH over ice. The solution was kept on ice and stirred with a sterile glass rod until the outer chorion had dissolved, as indicated by the colour of the cysts turning from brown through white to orange. The reaction was then quenched by addition of 0.1 M HCl. The cysts were rinsed thoroughly in sterile seawater and approximately 0.3 g amounts of the decapsulated cysts were distributed into sterile containers for later use. Decapsulated cysts were used within 1 wk of preparation. When required, decapsulated cysts were hatched overnight in stoppered 2 l flasks containing sterile SW aerated with 0.22 µm filtered air supplied to each flask under conditions of high light intensity. After 24 h the *Artemia* nauplii were collected on a sterile 100 µm filter, the concentration adjusted to 10 nauplii ml^{-1} and the nauplii dispensed into separate sterile, stoppered, 100 ml Erlenmeyer flasks. Individual bacterial strains to be tested were grown overnight and harvested as previously described, before addition to the separate flasks to a final concentration of 5×10^5 cfu m^{-1} in each case. *Artemia* and bacteria were incubated together for a minimum of 2 h prior to adding 750 *Artemia* nauplii to each of the specified treatment flasks (0.5 *Artemia* ml^{-1}). Larvae were fed on 4 further occasions with axenic *Artemia* before termination of the experiments after 8 d. No water exchange was attempted during this period. At this point, all larvae were anaesthetised by immersion in 0.1% (w/v) benzocaine in SW for 30 s and assessed for the 24 presence of *Artemia* in their guts, by observation under a binocular dissecting microscope. All 25 larvae were then fixed in buffered formol saline. Water samples from each flask were plated onto MA and TCBS. Water quality parameters (ammonia, nitrate and nitrate concentrations) were assessed using aquarium test kits (Tetra Werke).

Data analysis. Survivals in the different treatments were analyzed by comparing numbers of larvae present in the control, uninfected flasks, and the infected flasks using Mann-Whitney tests. Analysis was done on a PC using the program Minitab v13 (Minitab Ltd.).

Table 2. *Hippoglossus hippoglossus.* Effect of specific bacteria on the survival of Atlantic halibut yolk-sac larvae. In Expt 1, all control flasks were apparently sterile (as assessed by both DAPI staining of water samples and inoculation into marine broth, MB). In Expt 2, 7 out of 8 control flasks were free of marine agar (MA)-culturable organisms. DAPI staining of these water samples showed $^{3}/_{7}$ contained $>10^5$ cfu ml^{-1} rod-shaped bacteria. In Expt 3 all control flasks were contaminated and by inference most other flasks were probably also contaminated. It should not be concluded that the final bacterial density in those flasks was dominated by the bacterial inoculates. ND: no data

Expt	Length of experiment (d)	Treatment	No. of flasks per treatment	Mean % survival (±SEM)	Bacteria density at end of experiment Mean cfu ml^{-1}± SEM
1	34	None	3	84 (±9.8)	<1
		SYS6-10	4	41 (±10.7)	$6.1 \times 10^6 \pm 9.8 \times 10^5$
		TG11-16	3	52 (±12.2)	$>1 \times 10^6$
		TG15-07	3	41.3 (±14.5)	$5.7 \times 10^6 \pm 1.9 \times 10^5$
		TG4-07	5	57.6 (±11.6)	$1.6 \times 10^6 \pm 4.0 \times 10^5$
		TG7-01	4	64 (±19.1)	$2.9 \times 10^6 \pm 1.5 \times 10^6$
2	38	Control	8	49 (±14)	<1
		AE1-26	4	45 (±5.4)	$4.8 \times 10^6 \pm 3.2 \times 10^6$[a]
		TG10-01	4	60 (±10.2)	$9.1 \times 10^5 \pm 1.1 \times 10^6$
		TG15-19	4	20 (±9.5)	$2.5 \times 10^7 \pm 7.9 \times 10^6$
		TG16-05	4	49 (±2.8)	$2.0 \times 10^6 \pm 1.26 \times 10^6$
		TG2-14	4	55 (±8.2)	$9.1 \times 10^5 \pm 1.1 \times 10^6$
		TG2-18	4	45 (±10.6)	$1.2 \times 10^7 \pm 3.6 \times 10^6$
		TG4-02	4	83 (±15.7)	$1.4 \times 10^7 \pm 6.2 \times 10^6$
		TG8-01	4	47 (±15.6)	$1.7 \times 10^7 \pm 1.0 \times 10^7$
3	38	None	7	51.5 (±7.8)	$>10^6$ in all flasks
		Vibrio anguillarum 91079	4	0[b]	$>10^6$
		El-03	4	43 (±11.8)	$>10^6$
		OFE1-19	4	47 (±5.7)	$>10^6$
		OFE1-05	4	47 (±13.3)	$>10^6$
		TG15-08	4	52 (±1.6)	$>10^6$
		TG2-11	4	50 (±12.1)	$>10^6$
		TG2-12	4	34 (±18)	$>10^6$
		TG4-11	4	27 (±9.3)	$>10^6$
4	34	AN1	5	31.2 (±9.8)	ND
		AN1 + *Vibrio anguillarum*	5	0	ND
		AN2	5	32.0 (±9.7)	ND
		AN2 + *Vibrio anguillarum*	5	0	ND
		TG15-07	5	22.4 (±8.1)	ND
		TG15-07 + *Vibrio anguillarum*	5	0	ND
		Vibrio anguillarum	5	8 (±8.9)	ND

[a]Two of the 4 flasks to which AE1-26 was added were apparently free of TCBS- and MA-culturable bacteria; in the other two, presumptive AE1-26 was present at high density ($>10^7$ cfu ml^{-1})
[b]Survival of larvae in flasks inoculated with *Vibrio anguillarum* 91079 was significantly lower than in the control (Mann-Whitney; $p < 0.05$)

RESULTS

Single strain addition infection experiments (Expts 1 to 3)

Table 2 summarizes the results of testing *Vibrio anguillarum* and 20 bacterial strains isolated from halibut of varying developmental stages from UK hatcheries (Verner-Jeffreys 2000, Verner-Jeffreys et al. 2003) for their toxicity towards halibut yolk-sac larvae in 3 separate rearing experiments in the gnotobiotic 2 l flask-rearing system. To illustrate the flask-to-flask variability in survival, Fig. 1 shows the results for individual flasks for the third experiment.

Unlike *Vibrio anguillarum* 91079, none of the 20 isolates from halibut rearing were significantly pathogenic, including organisms recovered from poorly performing larvae in rearing trials, a luminescent *Photobacterium phosphoreum* isolate E1-03, originally isolated from halibut eggs, or a hemolytic *V. splendidus* strain, AE1-26, originally isolated from *Artemia* cultures in a UK halibut hatchery. The experimental system generally produced a high proportion of halibut yolk-sac larvae free of culturable

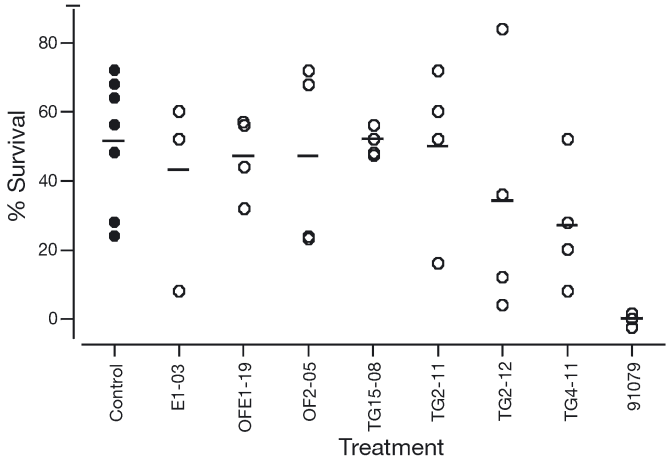

Fig. 1. *Hippoglossus hippoglossus*. Effect of addition of specific bacteria to yolk-sac larvae, illustrating intra-treatment variation. All flasks were stocked with 25 axenic halibut eggs; 5 d later they were inoculated with 5×10^2 cfu ml^{-1} of a range of bacteria isolated from halibut-rearing systems. Individual survivals to 38 d post hatch for all the treatment flasks are shown; horizontal lines indicate the treatment averages. For this experiment, control flasks had no bacteria added; however, they were all contaminated by the end of the experiment with a range of Gram-negative organisms

bacteria. Control flasks in Expts 1 and 2 were almost all free of culturable bacteria immediately before addition of test bacteria, and similar results have been achieved in other rearing experiments not reported here. In Expt 3, high numbers of bacteria were found in all control flasks (Table 2). However, these results are included as the contaminating bacteria appeared to have no deleterious effect with the percentage survival in control flasks being similar to those in Expts 1 and 2.

It is clear that *Vibrio anguillarum* 91079 is highly pathogenic to halibut yolk-sac larvae at the low infection level used in Expt 2 (5×10^2 ml^{-1}). No larvae survived in any of the flasks infected with *V. anguillarum*, whether contaminating bacteria were present or not; the difference in survival in *V. anguillarum*-infected flasks was significant, compared to the non-infected controls (Mann-Whitney; $p < 0.05$). Levels of *V. anguillarum* free-living in the flask water were lower than the levels of contaminating organisms in 3 of the flasks. In one flask, *V. anguillarum* concentration was below the detection limit. Living yolk-sac larvae were present in all *V. anguillarum*-infected flasks 29 d after hatching (approximately 150 degree-days post infection), indicating a slow pathogenic process at this low incubation temperature (6°C).

Probiotic addition infection experiments (Expts 4 to 6)

In a further series of trials, 3 bacterial strains (AN1, AN2, and TG15-07) which inhibited growth of *Vibrio anguillarum in vitro* were tested for their ability to protect against *V. anguillarum in vivo.* Table 2 shows that in all treatments where the test probiotic bacteria were added in conjunction with 10^3 cfu ml^{-1} *V. anguillarum* 91079, no larvae were alive by Day 34 (Expt 4). By contrast, surviving larvae were present in all the control flasks, where only the test probiotic bacteria or no other bacteria were added. There were significant differences in survival between the *V. anguillarum*-infected and other treatments, even between the controls and the flasks that were only infected with *V. anguillarum*, which had larvae surviving in 1 flask at the end of the experiment (Mann-Whitney; $p < 0.05$). Fig. 2 shows the cumulative mortality for the TG15-07 plus *V. anguillarum*, *V. anguillarum* alone and control treatments in Expt 4. A similar pattern was also seen for the AN1 and AN2 treatments (data not shown). Although larvae exposed to TG15-07 + *V. anguillarum* appeared to die faster than those that were only exposed to *V. anguillarum* alone, the difference was not significant. In this experiment larvae in 3 of 5 flasks were all dead by Day 20, but in the other 2 flasks larvae survived for longer.

In 2 further trials the concentration of *Vibrio anguillarum* was reduced to 1–10 cfu ml^{-1} to determine whether the putative probiotics could protect larvae from very low levels of challenge bacteria. However, larval survivals in the control flasks to which only *V. anguillarum* had been added were similar to those of

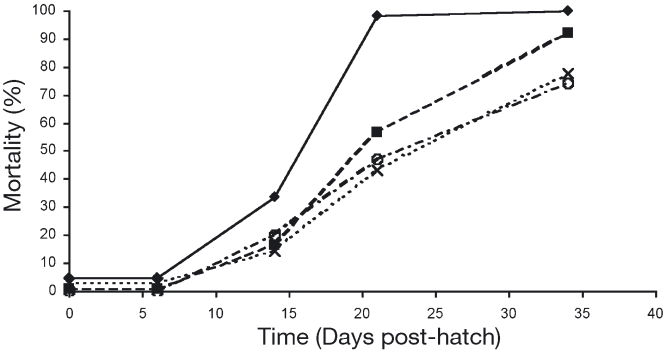

Fig. 2. *Hippoglossus hippoglossus*. Cumulative mortality of halibut yolk-sac larvae following challenge with 1×10^3 cfu ml^{-1}. *Vibrio anguillarum* 91079 Day 5 post hatch, in the presence or absence of TG15-07, a bacterial strain that inhibits 91079 *in vitro*. Mean cumulative mortality for each group is shown until 35 d post hatch (±SEM). Each treatment consisted of 5 replicate flasks, individually stocked with 25 axenic halibut eggs. TG15-07 + *V. anguillarum* (◆), *V. anguillarum* (■), TG15-07 only (×), Control (○)

the other treatments. This indicated that the lethal dose $(LD)_{50}$ for *V. anguillarum* 91079 in the model system was greater than 10 cfu ml^{-1} (data not shown). When *Pseudoalteromonas* TG15-07 was used at 2 concentrations (infection experimentExpt 5), bacterial concentrations were monitored until termination of the experiment. Even when added to only 5×10^2 cfu ml^{-1}, levels rose to a peak of in excess of 1×10^6 cfu ml^{-1} within 3 d of addition and remained at this level until the end of the experiment. In all cases where presumptive TG15-07 reisolates were tested for their continued ability to inhibit *V. anguillarum* 91079 *in vitro*, they retained their inhibitory properties.

First-feeding infection experiments (Expts 7 to 8)

Fig. 3, which combines data from Expts 7 and 8, shows that *Artemia* loaded with a range of bacterial isolates, including a turbot larval pathogen *Vibrio splendidus* DMC-1, did not inhibit the start-feed response in Atlantic halibut. There were no significant between-experiment or between-treatment differences in the average start-feed response or in the average number of surviving larvae, whether the data from each experiment was treated separately or combined. The generally observed response was of a good uptake onto feed by Day 4 of the experiment, independent of treatment. However, the water quality and general condition of the larvae later deteriorated. By the end of the experiment (Day 8), the few flasks containing larvae with guts packed full of *Artemia* were generally confined to the control or OF2-05 treatments (data not shown). All the controls were contaminated with a range of non-TCBS-culturable organisms by the end of the 2 experiments.

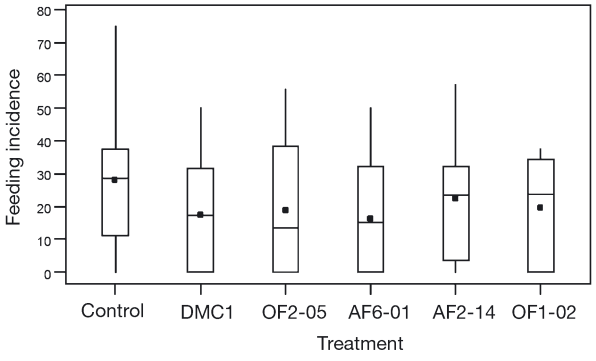

Fig. 3. *Hippoglossus hippoglossus*. Box plots of average% first-feed incidence of larvae fed gnotobiotic *Artemia* colonized with a range of different *Vibrio* isolates. Results are pooled from the infection experiments, Expts 7 and 8. Central horizontal line in each box is the median; squares are mean values. Horizontal boundaries above and below the box are the 1st and 3rd quartiles; whiskers show the within-treatment range in feeding incidence. Number of samples analyzed for each treatment group was 12, except for OF2-2 (n = 60) and the controls (n = 19)

Water quality

Total ammonia, nitrite and nitrate water measurements were taken from selected flasks at the end of all the experiments. Levels of ammonia were below 0.25 mg l^{-1}. Nitrite and nitrate levels were less than 0.1 mg l^{-1}.

DISCUSSION

Atlantic halibut are a good model for analyzing the effects of bacteria on larval development and survival under microbially controlled conditions. Their eggs are resistant to an aggressive disinfection protocol and high apparent rates of sterility could be obtained at the end of the yolk-sac absorption phase, as assessed by inoculating water samples into MB. For the control treatments, survival through yolk-sac absorption (>50%) and average start-feed response (21.1% for the 2 experiments) were within the range encountered in commercial hatcheries. This indicates that the axenic flask-rearing model may also be useful for investigating parameters such as water quality, temperature and salinity, particularly as the variable factor of the microbial population is removed.

A high rate of deformities in the larvae was observed in all the treatments, whether bacteria were present or not (data not shown). It has been speculated that the lockjaw deformity, commonly observed in halibut hatcheries, may result from bacterial infection (Morrison & Macdonald 1995), but since we found this condition in larvae in flasks which appeared bacteria-free, as assessed by both DAPI-stained direct counts and inoculating water samples into MB, it does not appear to be due to bacteria. A further condition often observed in the hatcheries, in which larvae acquire opaque patches in the intestinal cavity ('white gut') at the end of their development, was also observed in some of these apparently bacteriologically sterile control flasks (results not shown). This indicates that it too does not have a bacterial cause and demonstrates the value of model systems in helping to isolate the cause of specific conditions. Although it is possible that the aggressive disinfection regime may have contributed to the high deformity rate, it was within the range experienced by hatcheries. To test this further, experiments could be done to test the effect of different handling and disinfection regimes on the deformity rate.

Munro et al. (1995) challenged turbot larvae with a range of bacterial isolates under monoxenic conditions, and none of the bacteria tested appeared to be very pathogenic, with the exception of *Vibrio anguillarum* 91079. Similar results were found here (Table 2), and most of the bacteria introduced into flasks at a low initial inoculum were present at levels in excess of 10^5 cfu ml^{-1} by the end of the experiments. Thus, yolk-sac larvae appeared to be generally unaffected by high densities of culturable bacteria isolated from different halibut rearing systems. Bergh et al. (1992) found that *Vibrio* species other than *V. anguillarum* can be pathogenic towards Atlantic halibut yolk-sac larvae. Also, in commercial-scale rearing trials it was noted that there were correlations between the survival of yolk-sac larvae and the presence, or absence, of particular bacterial isolates (Verner-Jeffreys 2000). However, none of these isolates appeared to be very pathogenic when tested individually against larvae in the model rearing system and when compared to *V. anguillarum.* The results on *V. anguillarum* pathogenicity are in agreement with previous infection studies (Bergh et al. 1992, Bricknell et al. 2000). Bergh et al. (1992) used a different challenge strain (NCIMB 6, which was originally isolated from cod) and a higher inoculum (2 to 3×10^6 organisms ml^{-1} 2 d before the eggs hatched, as opposed to 5×10^2 to 1×10^3 organisms ml^{-1} 4 d after hatch). It was interesting that in trials where the larvae had presumably been invaded and killed by *V. anguillarum* it was not readily isolated from the flask water, even at the termination of the experiments. Munro et al. (1995) showed that *V. anguillarum* was present at very low levels in heavily inoculated flasks containing turbot larvae until periods of high mortality, which presumably coincide with infected larvae shedding the pathogen into the water. These results together reinforce speculation (Enger et al. 1990) that certain *V. anguillarum* serotypes are specialized invasive pathogens that are not well suited to a free-living aquatic existence.

Larval mortalities were not noticeable until late in development (Fig. 2). This could have been a temperature-related effect, with the pathogen multiplying slowly inside the larvae at the low rearing temperature (6°C) used in this study. Alternatively, the larvae may be resistant early in their development, but when their maternally derived host defences are exhausted they may then become more susceptible to invasion.

There was a wide range in the percentage survival of larvae within treatments (Fig. 1), often irrespective of treatment. This was particularly noticeable in Expt 2, where survivals in the control flasks varied between 0 and 88%. Culturable bacteria were only isolated from one of these flasks, so a bacterial etiology is unlikely, particularly as water samples from 1 flask which had no surviving larvae appeared bacteriologically sterile by DAPI staining.

The high intra-treatment variation in survival observed could have masked possible treatment effects. However, observed mortalities in the hatcheries are often catastrophic, suggesting that, if the mortality events are caused by bacteria, similarly high mortality rates would be reproduced by the responsible organisms in the flask-rearing system. It is possible that mortalities in the flask-rearing system were caused by a viral agent present in some of the eggs, despite the aggressive surface-disinfection procedure. However, in one experiment, moribund larvae from one 'crashing' flask were transferred to flasks containing sterile freshly hatched larvae, but no mortalities ensued in larvae in these flasks (data not shown). Although this does not rule out the possibility that a transmissible agent was responsible for the mortalities, it lessens the likelihood that this was the cause.

The results of the probiotic addition experiments show the importance of testing organisms showing *in vitro* promise in small-scale model experimental systems first, before committing substantial resources to field experiments. However, *Vibrio anguillarum* is a particularly virulent fish pathogen and may be an inappropriate test pathogen for such experiments. Strains TG15-07, AN1, AN2 and other probiotic candidates may confer protection against other pathogens such as *Flexibacter ovolyticus* (Bergh et al. 1992) or less specialized, opportunistic pathogens that, it has been speculated, may be a greater problem in marine hatcheries (Vadstein et al. 1993, Skjermo et al. 1997, Hansen & Olafsen 1999, Ringo & Birkbeck 1999). However, as indicated here, the inability to isolate readily obvious bacterial pathogens indicates that even 'opportunistic' pathogens may be rare components of the hatchery microbiota.

Poor, or variable, start-feeding rates have been commonly reported by commercial Atlantic halibut hatcheries (Shields et al. 1999) and the data presented here suggest that bacterial contamination of the live feed is unlikely to be the major cause of this problem. Accordingly, research efforts may be better focused on other potential biological and environmental causes, such as the tank environment, as a way of resolving this key bottleneck. However, live-food-associated pathogenic micro-organisms do seem to cause serious mortalities in turbot after the larvae have initiated feeding (Gatesoupe et al. l999). Axenic larvae flask rearing models might be usefully deployed in determining the cause of such losses in Atlantic halibut and other cultured marine fish.

Acknowledgements. This study was funded by the British Marine Finfish Association (formerly known as the British Halibut Association) and MAFF/LINK FIN22 project 'Rearing protocols for Atlantic halibut larvae during transition from endogenous to exogenous feeding'. We thank Berit Adam for technical assistance. Eggs for the infection experiments were kindly supplied by Otter Ferry Seafarms, Argyll, Scotland.

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Editorial responsibility: David Bruno, Aberdeen, UK

Submitted: November 5, 2002; Accepted: May 16, 2003
Proofs received from author(s): August 25, 2003