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Selection of bacteria and the effects of bacterial treatment of Atlantic halibut (*Hippoglossus hippoglossus* L.) eggs and larvae

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ABSTRACT

Bacteria dominating the cultivable gut community of overall successful first feeding halibut (*Hippoglossus hippoglossus* L.) larvae were tested for their *in vitro* growth inhibition activity against selected fish pathogenic bacteria and isolates dominating the cultivable gut community of larvae with an overall poor success. A mixture containing equal numbers of three isolates was selected for the treatment of halibut eggs through repeated bathing, and larvae through grazing of live prey in a mixture of the selected isolates prior to offering to larvae. The isolates were found as a part of the dominating bacterial community of treated eggs and treatment was not found to affect egg survival. Improved larval survival was observed as a result of offering bacteria-treated live prey to larvae, and improved larval growth was observed in one of the two experiments that were carried out in commercial size production units. The bacterial community structure of the live prey, analysed using PCR and denaturing gradient gel electrophoresis, was only partly reflected in larvae after one week in feeding. A successful colonization of fertilized eggs by the isolates used for treatment entails the possibility to establish a favourable bacterial environment already prior to hatching.

1. Introduction

Atlantic halibut (*Hippoglossus hippoglossus* L.) is considered a valuable candidate for commercial marine cold water farming. High mortality rates are observed during intensive larval production, with mean survival rates of 10–20% experienced at a commercial production site, when calculated from hatching of yolk sac larvae (Fiskey Ltd., Hjalteyri, IS-601 Akureyri, Iceland). Late maturation of the gastrointestinal tract and the specific immune system, in addition to the intimate relationship that exists between fish larvae and their external environment, represent significant problems caused by opportunistic and pathogenic bacteria (Gatesoupe, 1999; Magnadottir et al., 2005; Olafsen, 2001). A more extensive knowledge of the bacterial community and the potential role of defined bacterial groups is therefore needed for developing measures to manipulate the bacterial community of larvae and their environment.

The use of probiotics for improved nutrition and disease prevention in aquaculture has gained progressive interest, not least due to spread of antibiotic resistance genes and the increasing emphasis on fish welfare and environment-friendly aquaculture (Balcazar et al., 2006; Gatesoupe, 1999; Hong et al., 2005; Wang et al., 2008). Reduced mortality and improved growth and quality of fish larvae are among the beneficial effects that have been obtained by the use of probiotics, through enhanced immunological response and reduced adherence of pathogenic strains or other modulation of the gut microbiota at specific locations, as has been reviewed (Wang et al., 2008). The use of beneficial bacteria based on the principle of competitive exclusion is therefore viewed as a promising preventive method developed in the fight against diseases (Verschuere et al., 2000). Evidence furthermore implies that bacterial groups that are established as a part of a healthy gut community have a better chance of survival and involving in enhanced colonization in their native environment (Morelli, 2007). Repeated treatments may, however, be needed whereas beneficial effects have been observed without successful colonization of the probiont in the gastrointestinal lumen (Planas et al., 2006).

In a previous study, we isolated bacteria dominating the cultivable gut community of overall successful first feeding halibut larvae from a large number of commercial production units (Bjornsdottir et al., 2009).

The aim of the present study was to select isolates for treatment during early production stages of halibut. The selection was based on growth inhibition activity towards selected test strains and we investigated the effects of treatment on the bacterial community structure and overall success of halibut larvae and eggs.

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2. Materials and methods

The experiments were carried out in collaboration with a commercial producer of halibut larvae (Fiskey Ltd.). A considerable variation in the overall success of individual spawning groups is commonly observed and the experiments were therefore carried out in the normal spawning group (2007) and repeated in the advanced spawning group (2008). The study was approved and performed according to the Icelandic Animal Research Authority (approval no. YDL03080041/023BE).

2.1. Production methods and evaluation of larval success

Fertilized eggs were kept in 0.25 m^3 incubators at 5.0–5.3 °C and gentle aeration for 14 days prior to surface disinfection using 400 ppm glutaraldehyde for 7 min at 5.0–5.3 °C prior to transferring to 10 m^3 silos where the eggs hatched. The yolk sac larvae were held at 5.0–5.3 °C for ~50 days prior to transferring to first feeding incubators (3.5 or 7.0 m^3) with enriched 24 h *Artemia franciscana* nauplii offered in two daily feedings for ~60 days at 11 °C when weaning onto formulated feed was started. The *Artemia* nauplii (Intstar III stage) were obtained from decapsulated and hatched *Artemia* cysts (Great Salt Lake, Utah, USA) according to standard procedures. The cyst chorion was removed through hydration for 1 h at 22 °C followed by decapsulation in 2.45% NaOCl for 5–8 min followed by deactivation with sodium thiosulphate (5 $g L^{-1}$), thorough washing using fresh water and finally dehydration for 18 h in saturated brine. Hatching was then carried out through incubation in seawater (2–5 $g L^{-1}$) at 27–30 °C for 24 h under constant light (2000 lx) and intensive aeration for maintaining the oxygen levels above 2 mg L^{-1}. The pH was maintained at ~8.0 with the addition of sodium bicarbonate (0.5–1.0 $g L^{-1}$). After careful rinsing in 4 °C freshwater, the newly hatched *Artemia* were transferred to new containers (~300 animals mL^{-1}) followed by enrichment for 24 h using a fish oil emulsion containing vitamins, antioxidants and emulsifiers (Fiskey Ltd.), with 0.3 $g L^{-1}$ of the emulsion added in two rations, at 0 h and 12 h. For water conditioning, 0.4 $g L^{-1}$ of ACE (Inve, Belgium) was added to each container at 0 h. Enriched *Artemia* naupli were offered to larvae in the first of two daily feedings at 300 nauplii mL^{-1} (24 h *Artemia*) and the remaining 2/3 of the cultures stored at ~15 °C for 8 h prior to offering to larvae in the second of two daily feedings (32 h *Artemia*).

Survival and quality of unfed yolk sac larvae were estimated at transfer to first feeding incubators at 50 days post hatch (dhp). Survival through first feeding and success of metamorphosis was calculated at transfer of juveniles to weaning at ~60 days post onset of first feeding (dpff) as previously described (Bjornsdottir et al., 2009). Growth of larvae was monitored by measuring the dry weight of ~150 (0 dpff) to ~15 (50–60 dpff) larvae from individual incubators at approximately weekly intervals throughout the first feeding period.

2.2. Selection and preparation of bacteria for treatment

In our previous work, we studied the dominating bacterial community of surface sterilized halibut larvae by collecting samples at weekly intervals from a large number of production units at a commercial production site (Bjornsdottir et al., 2009). The larvae were killed by an overdose of Hypnodil (51 µg mL^{-1}) prior to surface sterilization through immersion in 0.1% benzalkonium chloride solution for 30 s, rinsing and subsequent homogenization in a ten-fold dilution of peptone-seawater (0.1% w/v Bacto peptone (Difco) dissolved in 70% v/v aged seawater stored in the dark at room temperature for a minimum of three weeks prior to filtration through 0.22 µm and subsequent dilution using distilled H_2O and the pH then adjusted to 8.6). Serial dilutions were then made in peptone-seawater and 100 µL aliquots plated in duplicates onto MA plates (Marine Agar 2216, Difco) and Thiosulphate Citrate Bile Salt Sucrose Agar plates (TCBS, Difco). The agar plates were incubated at 15 °C for 5–7 days prior to a random selection of twelve colonies from each sample, picked from MA plates containing 25–250 colony forming units (CFU) $plate^{-1}$. A total of 540 isolates were collected from the dominating gut community of larvae, immediately prior to the onset of feeding from unfed yolk sac larvae in 9 incubators (24 isolates) and from feeding larvae at various days post onset of first feeding (dpff) in a total of 13 incubators, with 5 of the incubators resulting in an overall poor (132 isolates) and 8 incubators in an overall high larval success (384 isolates) (Bjornsdottir et al., 2009). The isolates were sub-cultured on MA to ensure purity and single colonies were inoculated into 5 mL of TSB-sw (Tryptic Soy Broth, Difco, dissolved in 70% v/v of filtered aged seawater). The cultures were grown at 15 °C for 24–48 h prior to screening for *in vitro* inhibitory properties against test isolates, using a modified version of the previously described disc-diffusion method (Chythanya et al., 2002). Briefly, lawn cultures were prepared of the test cultures by spreading 200 µL over the surface of TSA-sw (Tryptic Soy Agar, Difco, dissolved in 70% v/v filtered aged seawater) plates. Sterile 6 mm paper discs (BBL Becton, Dickinson & Company, USA) were then placed on the lawn of the test cultures (4 discs $plate^{-1}$) and 20 µL aliquots of the cultures transferred to each disc. The test strains consisted of two fish pathogens, *Vibrio anguillarum* (F-139-03) isolated from diseased cod and identified phenotypically and serologically, according to standard protocols, at the Fish Disease laboratory, Institute for Experimental Pathology, University of Iceland, Reykjavik and *Aeromonas salmonicida* subsp. *salmonicida* (NCIMB 1102) type strain (National Collections of Industrial and Marine Bacteria, Aberdeen, UK) in addition to 132 bacterial isolates that were randomly selected from the dominating gut community of larvae originating from four incubators resulting in an overall poor larval success.

Based on the criteria of >2 mm zone of clearing against the test isolates, thirteen isolates were selected and identified by partial 16S rDNA sequencing. The 16S rRNA gene was amplified by PCR using primers F9 (5′-GAGTTTGATCCTGGCTCAG-3′) and R1544 (5′-AGAAAGGAGGTGATCCA-3′) (Skirnisdottir et al., 2000). The PCR products were sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems), R805 (5′-GACTACCCGGGTATCTAATCC-3′) sequencing primer internal of the 16S rRNA gene (Skirnisdottir et al., 2000) and the 3730 DNA analyzer (Applied Biosystems) (work carried out by Matis-Prokaria Ltd.). The sequences were processed, edited and classified using the sequencher 4.0.5 software. A BLAST search in GenBank was used to identify the species of the isolate or its closest relative (Altschul et al., 1990).

The thirteen isolates belong to 6 groups with three of the groups observed only in larvae from incubators resulting in overall high larval success. One isolate was then selected from each of the three groups for bacterial treatment.

Freeze-dried preparations of the selected isolates were obtained by culturing the isolates in TSB-sw, with gentle shaking for 48 h at room temperature, followed by centrifugation at 4700 rpm for 10 min, washing the pellet in a 4 °C solution of 1% NaCl and suspending it in 5–6 mL of peptone-seawater. Aliquots of 50–60 mL were collected into sterile jars with the addition of 0.1% glucose and frozen at −70 °C over night prior to freeze drying at −59 °C under 0.08 mbar pressure for 24–30 h (Alpha 1–4, Christ, Germany). Numbers of CFU in the freeze-dried preparations were determined using the standard plate count method on TSA-sw with incubation for 3–5 days at 15 °C. Based on numbers of CFU g^{-1}, a mixture containing equal CFU numbers of the three isolates was prepared for treatment of halibut eggs and larvae.

2.3. Bacterial treatment of halibut eggs and larvae

For treatment of eggs, freeze-dried preparations containing 10^7 CFU were suspended in 1 L of seawater from the respective

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incubators and then added to the incubators immediately post fertilization (0 dpf) and repeated on 6 dpf and 13 dpf. Treatment immediately post fertilization included turning off the water for 24 h to ensure sufficient contact time. Eggs from individual incubators were surface disinfected prior to hatching on 14 dpf and then collected to 1 L of seawater containing 10^9 CFU of the bacterial mixture and incubated for 5 min prior to transfer to yolk sac incubators (~200,000 eggs incubator^{-1}). Similar numbers of untreated eggs were collected to separate yolk sac incubators (controls).

Larvae of a common silo origin were equally divided into two tanks at ~50 dph, with treatments carried out in one tank and the sibling tank units used as controls, as detailed in Table 1. The live prey was incubated in seawater containing the bacterial mixture (10^9 CFU L^{-1}) for 30 min at 30 °C prior to offering 1/3 of the cultures to larvae in the first of two daily feedings on 0, 1, 17 and 18 days post onset of first feeding (dpff). The remaining 2/3 of the cultures were grown for additional 8 h and 10^7 CFU then added to the cultures and incubated for 30 min at 30 °C prior to offering to larvae in the second daily feeding. Untreated live prey was offered to larvae in the sibling tank units that were used as controls.

2.4. Sampling and analysis of the bacterial community

Samples of eggs were collected prior to treatment immediately post fertilization and then repeated on 7 dpf and prior as well as post surface disinfection of eggs at transfer to yolk sac incubators on 14 dpf. Larval samples were collected prior to offering bacteria-treated live prey to larvae on 0 and 1 dpff, and then repeatedly at weekly intervals throughout the first feeding period. Samples of untreated eggs and larvae were collected on the same dpf and dpff. Untreated and treated *Artemia* were collected at approximately weekly intervals throughout each experiment.

The bacterial community of eggs and larvae was analysed by cultivation at 15 °C for 5–7 days on TSA-sw and presumptive *Vibrio* bacteria on TCBS agar. Results are expressed as mean numbers of CFU larvae^{-1} and in each g wet weight of eggs and samples of live prey. The bacterial profiles of the homogenates of each sample and from the growth collected of TSA-sw agar plates containing 200–250 CFU plate^{-1}, were analysed using PCR and the denaturing gradient gel electrophoresis (PCR-DGGE) as previously described (Bjornsdottir et al., 2009). Briefly, 254 bp fragments of the V4 part of the bacterial 16S rRNA gene was amplified using the primers 533F-GC and 787R (TAC Copenhagen). The amplified products (30 µL) were loaded onto a 30–60% denaturing gradient of urea-formamide in an 8% acrylamide-bis gel and electrophoresed on a Dcode DGGE system (Dcode, BioRad) at 60 °C under 60 V and 20 mA for 14 h. The two *Vibrio* strains selected for bacterial treatment (*Vibrio* sp. and *V. splendidus*) appeared at the same location on each gel with this method (product B in the relative mobility standard). The relative mobility standard run in each gel also contained *Pseudoalteromonas elyakovii* used for bacterial treatments (product A), *Marinovum algicola* (product C, DSM 10251,) and *Shewanella baltica* (product D, 99% similarity to GenBank accession number CP000891). The gels were stained for 15 min in SYBR Gold nucleic acid stain (Invitrogen) and visualised using UV light with an InGenius LHR gel imaging system (Syngene). Images were recorded with GeneSnap software and analysed with Gene Tools software (Syngene). A sterile pipette tip was then used to excise ~0.5 mm core from the centre of bands of interest and the products identified by sequence analysis, as previously described (Bjornsdottir et al., 2009). The 254 bp products excised from the PCR-DGGE gels generally proved too short for a decisive species identification using 16S rRNA sequencing. Hence, the products are identified to the family or genus level and only occasionally to the species level, using ≥97% cut-off limits. Furthermore, a definite identification occasionally proved impossible due to the presence of more than one sequence in some of the excised products.

Table 1
Experimental design. Bacterial treatment of halibut eggs and larvae was carried out in three distinct experiments. Eggs were treated at weekly intervals post fertilization (dpf) and larvae through offering bacteria-treated live feed at selected days post onset of first feeding (dpff). Groups labelled with identical superscript letters ([a, b]) denote sibling tank units, containing larvae of a common silo origin.

Experiment	Sample	Group	Treatment day
1	Eggs	Control	No treatment
		Treated	0, 6 and 13 dpf (10^7 CFU L^{-1} day^{-1}), 14 dpf (10^9 CFU L^{-1})
2	Larvae	Control[a]	No treatment
		Treated[a]	0 dpff†, 1 dpff†, 17 dpff†, 18 dpff†
3	Larvae	Control[b]	No treatment
		Treated[b]	0 dpff†, 1 dpff†, 17 dpff†, 18 dpff†

†*Artemia* nauplii bathed in the bacterial mixture for 30 min prior to offering to larvae in the first (10^9 CFU L^{-1}) and second (10^7 CFU L^{-1}) daily feedings.

2.5. Statistical analysis

The data were analysed using SigmaStat® release 3.5 (Systat Software, Inc. CA 94804-2028 USA). The normality of the data distribution was analysed using the Kolmogorov–Smirnov test. Survival of eggs in the two groups was compared using a *t*-test. Survival of first feeding larvae in individual incubators was compared using a *t*-test and by comparing the number of surviving, dead and total number of larvae in individual incubators using the Chi-square test. A *t*-test was used to analyse the growth and success of larvae in individual incubators compared to mean values of success in all production units of each period ($n = 15$ and 23 in the two experiments, respectively). The numbers of cultivable bacteria are expressed as mean ± S.D. of a minimum of two samples with each sample analysed in duplicate. Differences were considered statistically significant when $p < 0.05$. A Pearson's correlation was used to analyse the relationship between bacterial numbers and larval growth, survival and metamorphosis characteristics used for evaluation of larval success.

3. Results

3.1. Selection of isolates for bacterial treatment

Of the 384 isolates collected from overall successful first feeding larvae, 13 isolates were selected based on growth inhibiting activity against selected test strains. Presumptive identification using partial 16S rDNA sequencing of the isolates revealed six groups; *Vibrio* sp. V798 (99% similarity to GenBank accession number DQ146994), *P. elyakovii* (99% similarity to GenBank accession number AB000389) and *Vibrio splendidus* (100% similarity to GenBank accession number AJ874364) in addition to *Marinomonas* sp. (99% similarity to GenBank accession number DQ191961.1), *Pseudoalteromonas* sp. (98% similarity to GenBank accession number PSU85859) and *Pseudoalteromonas* sp. (99% similarity to GenBank accession number AY573037.1). Representative isolates belonging to the first three groups were selected for bacterial treatment, based on identification only in overall successful larvae and not in samples from any of the incubators resulting in overall poor larval success. The growth inhibition properties of the three selected isolates against test isolates are shown in Table 2.

Freeze-dried preparations of the three isolates contained highly different numbers of CFU, with the highest numbers observed for *V. splendidus* (10^{11} CFU g^{-1}) and lower numbers for *Vibrio* sp. and *P. elyakovii* (10^7 and 10^8 CFU g^{-1}, respectively), indicative of high ratio of dead cells. Freeze-dried preparations of all three isolates were, however, found to be highly reproducible after storing in a freeze-dried form at −80 °C for three months.

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Table 2
Growth inhibition activity of the three strains selected for bacterial treatment. Shown is the zone of clearing in mm when screening for *in vitro* inhibitory activity of the three strains against two fish pathogens. Also shown is the number of isolates from selected test samples (A–F) where growth inhibition was observed around discs containing cultures of the three strains selected for bacterial treatment (>2 mm zone of clearing). The test samples are represented by 10–12 randomly selected isolates from the dominating cultivable community of surface sterilized larvae collected at various days post onset of first feeding (dpff) from incubators resulting in an overall poor larval success (Bjornsdottir et al., 2009).

	Vibrio sp.	*Pseudoalteromonas elyakovii*	*Vibrio splendidus*
Aeromonas salmonicida subsp. *salmonicida* (NCIMB 1102)	<2 mm	8–10 mm	2 mm
Vibrio anguillarum (F-139-03)	<2 mm	<2 mm	10 mm
A-incubator 2–5, at 37 dpff (10 isolates)	1 of 10	0 of 10	2 of 10
B-incubator 11–2, at 37 dpff (11 isolates)	2 of 11	0 of 11	0 of 11
C-incubator 2–13, at 37 dpff (12 isolates)	10 of 12	9 of 12	9 of 12
D-incubator 11, at 0 dpff (12 isolates)	0 of 12	1 of 12	2 of 12
E-incubator 2–13, at 17 dpff (12 isolates)	9 of 12	4 of 12	4 of 12
F-incubator 2–13 at 7 dpff (12 isolates)	11 of 12	5 of 12	5 of 12

3.2. Survival and quality of eggs and larvae

Bathing fertilized eggs in the bacterial mixture immediately post fertilization and repeatedly at weekly intervals throughout the 14 days incubation period did not affect the survival of fertilized eggs ($p=0.458$) (Table 3). Fertilization of eggs in incubators selected for the bacterial treatment ($n=8$) was 39.5% ± 19.9 compared with 30.9% ± 12.0 in the control group ($n=7$), but the difference between the groups was not found to be significant ($p=0.130$). The success of fertilization was not found to affect the survival of successfully fertilized eggs that was 31.5% ± 16.5 in the treated compared to 34.4% ± 14.6 in the untreated group ($R^2=0.05$–0.06).

Larvae originating from treated as well as untreated eggs in experiment 1 (Table 1) collapsed late during first feeding, indicative of poor larval quality in both groups. Hence, the survival and overall success of larvae from these groups could not be calculated.

Compared with the groups receiving no treatment, offering bacteria-treated *Artemia* to larvae resulted in significantly improved larval survival ($p<0.001$) in the two experiments that were carried out (Table 4, experiments 2 and 3). Significantly improved growth of larvae ($p<0.001$) was furthermore observed in experiment 2 compared with the group receiving no treatment, but not in experiment 3 ($p=0.06$). No significant relationship was observed between larval growth and the overall quality of larvae at the end of the first feeding period ($p=0.05$–0.78).

3.3. Analysis of the bacterial community

The CFU numbers in eggs increased by 2 to 3 log-units during the first week following fertilization, with ~1 log-unit higher numbers observed in treated compared to untreated eggs (Table 3). Significantly higher numbers of presumptive *Vibrio* bacteria on TCBS agar were detected in treated compared to untreated eggs at this time point ($p=0.005$). Surface sterilization of eggs prior to transfer to yolk sac incubators at 14 dpf resulted in ~1 log-unit reduction in the numbers of CFU and presumptive *Vibrio* in treated eggs, whereas a significant ~3 log-unit reduction in the CFU numbers was observed in the control group ($p=0.047$), however, without a significant difference in CFU numbers observed between the two groups ($p=0.167$). A negative relationship was furthermore observed between egg survival and CFU numbers prior to surface sterilization at 14 dpf in the treated group only ($R^2=0.80$ and 0.1 for the treated and untreated group, respectively).

The numbers of CFU in surface sterilized first feeding larvae increased by ~2 log-units during the first week in feeding, with ~10^5 CFU larvae^{-1} and ~10^4 *Vibrio* larvae^{-1} remaining throughout the first feeding period. Bacterial treatment did not result in increased numbers of CFU ($p=0.069$) or presumptive *Vibrio* bacteria on TCBS ($p=0.131$) in samples of *Artemia* collected throughout both periods ($n=20$).

A PCR-DGGE analysis of the bacterial community of fertilized eggs revealed variable numbers and localization of amplified products visible in the gels, with analysis of eggs from some of the incubators shown in Fig. 1. One week post treatment, products identical to the *P. elyakovii* (product A in the standard) and the *Vibrio* sp. and *V. splendidus* isolates used for bacterial treatment (product B in the standard) were detected only in samples of treated eggs but in no samples collected from the control incubators. A product identical to the *P. elyakovii* (product 1) was found following surface disinfection of eggs prior to hatching, whereas a product identical to the *Vibrio* isolates used for bacterial treatment (product 5) was observed in treated eggs from only a part of the incubators included in the study and may no longer be detected following surface sterilization of eggs prior to hatching. A product identified as *Psychroserpens* sp. was

Table 3
Numbers of cultivable bacteria (CFU) in fertilized eggs sampled immediately post fertilization (0 dpf), at 7 dpf and prior (†) as well as post (††) surface sterilization at transfer to yolk sac incubators at 14 dpf. Shown are mean numbers ± S.D. of CFU in each g wet weight of untreated eggs from seven incubators (Control) and eggs from eight incubators that were bathed repeatedly in a suspension of 10^7 CFU L^{-1} of the bacterial mixture (Treated). Also shown are mean survival values ± S.D. of eggs from the incubators included in each group. Calculated *p*-values when comparing bacterial numbers in the two groups are also shown, with statistically significant difference between the groups denoted with an asterisk (*).

Experiment 1	Control ($n=7$)	Treated ($n=8$)	*p*-values
CFU on MA[§]			
0 dpf	$0.5\times10^4\pm10^4$	$4.6\times10^3\pm10^3$	0.690
7 dpf	$1.9\times10^6\pm10^6$	$1.8\times10^7\pm10^7$	0.093
14 dpf†	$1.7\times10^7\pm10^7$	$2.8\times10^7\pm10^7$	0.569
14 dpf††	$4.8\times10^4\pm10^4$	$4.1\times10^6\pm10^6$	0.167
CFU on TCBS[§]			
0 dpf	$1.9\times10^3\pm10^3$	$0.2\times10^3\pm10^2$	0.841
7 dpf	$0.9\times10^4\pm10^4$	$0.9\times10^6\pm10^6$	0.005*
14 dpf†	$2.2\times10^3\pm10^3$	$0.6\times10^4\pm10^4$	0.229
14 dpf††	$0.1\times10^2\pm10^0$	$1.6\times10^3\pm10^3$	0.183
Survival (%)	34.4 × 14.6	31.5 ± 16.5	0.458

[§] Marine Agar (MA) and Thiosulphate Citrate Bile Salts Sucrose Agar (TCBS).

Table 4
Dry weight (g) and success (%) of first feeding larvae, estimated at ~60 days post onset of first feeding in experiments 2 and 3. Shown is the resulting success of untreated larvae (Control) and larvae offered bacteria-treated *Artemia* nauplii (Treated). Groups labelled with identical superscript letters ([a, b]) denote sibling tank units, containing larvae of a common silo origin. Statistically significant differences between the treatments are denoted with an asterisk (*).

Experiment 2	Control[a]	Treated[a]	*p*-values
Dry weight (g larvae^{-1})	152.0	167.4	0.01*
Survival (%)	47	67	<0.001*
Malpigmentation (%)	2	10	0.004*
Incomplete eye migration (%)	12	12	–
Ratio normal fry ~57 dpff (%)	75	80	0.4
Experiment 3	**Control[b]**	**Treated[b]**	***p*-values**
Dry weight (g larvae^{-1})	83.2	85.7	0.06
Survival (%)	26	47	<0.001*
Malpigmentation (%)	0	0	–
Incomplete eye migration (%)	10	12	0.87
Ratio normal fry ~57 dpff (%)	81	80	0.19

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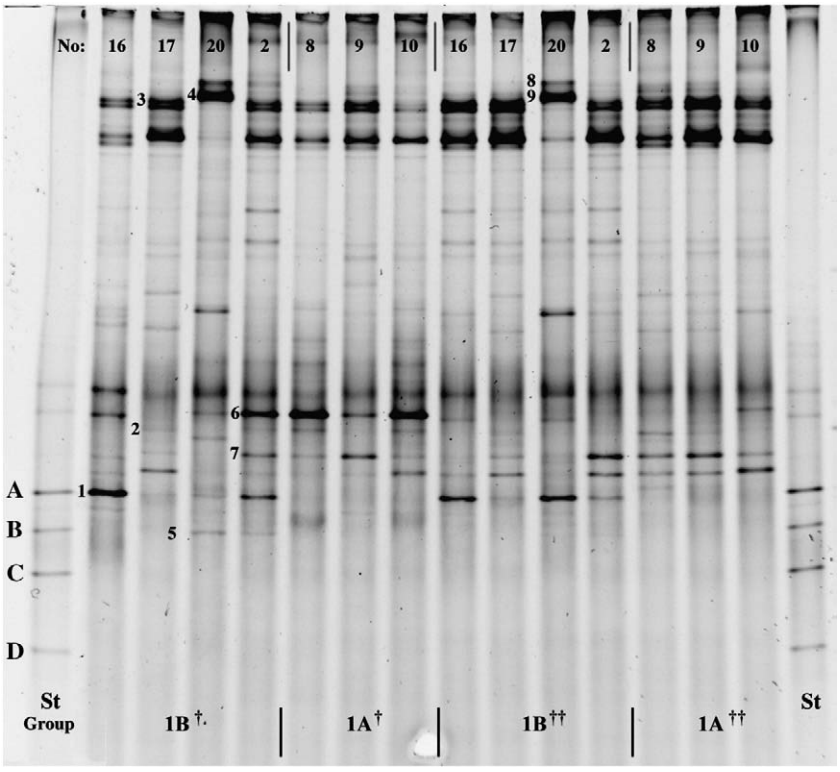

Fig. 1. DGGE profiles in pools of fertilized eggs collected from selected incubators pre (†) and post (††) surface sterilization of eggs at transfer to yolk sac incubators. Shown are the DGGE profiles of untreated eggs (1A) compared with eggs treated repeatedly with the bacterial mixture during the egg incubation period (1B) in experiment 1. Also shown are relative mobility standards A–D (St), with "A" representing the *Pseudoalteromonas elyakovii* and "B" representing the *Vibrio* sp. and the *V. splendidus* isolates used for bacterial treatment. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787). Labelling indicates products that were excised from the gel and identified by sequence analysis (Table 5).

furthermore identified only in treated eggs from one of the incubators, prior as well as post surface sterilization (products 4, 8 and 9). *Tenacibaculum ovolyticum* was identified as a part of the dominating bacterial community of eggs in both groups (product 3) and *Pseudoalteromonas*/*Lacinutrix* co-dominated the bacterial community in most samples (product 7). A product identified as *Marinomonas* sp. was furthermore identified in eggs from most incubators but may have been removed following surface disinfection at 14 dpf (product 6).

After one week in feeding, products appearing at the same localization within the gel as the isolates used for bacterial treatment were observed in surface sterilized larvae from both groups (Fig. 2, products 11, 12, 14 and 19). Identical products were furthermore

Table 5
Groups identified as a part of the bacterial community of halibut larvae. The marks indicate bands excised from gels in samples of fertilized eggs (◊) and larvae after one (□) and three (■) weeks in feeding. Also shown are products excised from samples of the live feed (♠). The products identified are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787).

Product	BLAST identification	Division (% similarity)	GenBank
1 ◊	***Pseudoalteromonas* sp.**	**γ-Proteobacteria (100%)**	**EU935099.1**
2 ◊	*Pseudoalteromonas* sp.	γ-Proteobacteria (100%)	EU935099.1
3 ◊	*Tenacibaculum ovolyticum*	Flavobacteria (100%)	AY771741.|
4 ◊	*Psychroserpens* sp.	Flavobacteria (100%)	DQ167236.1
5 ◊	***Vibrio* sp.**	**γ-Proteobacteria (99%)**	**AM941184.1**
6 ◊	*Marinomonas* sp.	γ-Proteobacteria (100%)	DQ681162.1
7 ◊	*Pseudoalteromonas* sp.	γ-Proteobacteria (100%)	DQ985065.1
	Lacinutrix sp.	Flavobacteria (100%)	EU581705.1
8 ◊	*Psychroserpens*	Flavobacteria (100%)	DQ167236.1
	Lacinutrix sp.	Flavobacteria (100%)	EU581705.1
9 ◊	*Psychroserpens* sp.	Flavobacteria (100%)	DQ167236.1
10 □	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
11 □	***Pseudoalteromonas* sp.**	**γ-Proteobacteria (100%)**	**EU935099.1**
12 □	***Vibrio* sp.**	**γ-Proteobacteria (100%)**	**EU655423.1**
13 □	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
14 □	***Vibrio* sp.**	**γ-Proteobacteria (100%)**	**EU655423.1**
15 □	*Shewanella* sp.	γ-Proteobacteria (97%)	EU617351.1
16 □	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
17 □	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
18 □	*Vibrio* sp.	γ-Proteobacteria (99%)	AM941184.1

(continued on next page)

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Table 5 (*continued*)

Product	BLAST identification	Division (% similarity)	GenBank
19 □	***Vibrio* sp.**	**γ-Proteobacteria (100%)**	**EU655423.1**
20 □	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
21 ♠	*Pseudoalteromonas* sp.	γ-Proteobacteria (100%)	EU935099.1
22 ♠	*Pseudoalteromonas* sp.	γ-Proteobacteria (100%)	EU935099.1
23 ♠	*Acinetobacter* sp.	γ-Proteobacteria (99%)	EU073105.1
24 ♠	*Pseudoalteromonas* sp.	γ-Proteobacteria (100%)	EU935099.1
25 ♠	*Moraxella* sp.	γ-Proteobacteria (100%)	EF409330.1
26 ♠	*Pseudomonas* sp.	γ-Proteobacteria (100%)	EU935094.1
27 ♠	*Corynebacterium* sp.	Actinobacteridae (99%)	EU071498.1
28 ♠	*Corynebacterium* sp.	Actinobacteridae (99%)	EU071498.1
29 ♠	*Vibrio* sp.	γ-Proteobacteria (99%)	AM941184.1
30 ♠	*Vibrio* sp.	γ-Proteobacteria (99%)	AM941184.1
31 ■	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
32 ■	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
33 ■	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
34 ■	***Vibrio* sp.**	**γ-Proteobacteria (100%)**	**EU655423.1**
35 ■	*Acinetobacter* sp.	γ-Proteobacteria (99%)	EU794195.1
36 ■	Uncultured *Sphingobacteriales*	Sphingobacteria (98%)	EU361312.1
	Tenacibaculum sp.	Flavobacteria (98%)	AB274770.1
37 ■	*Flexibacter aurantiacus*	Flavobacteria (98%)	AB078044.1
38 ■	*Marinomonas* sp.	γ-Proteobacteria (100%)	EU052766.1
39 ■	*Stenotrophomonas* sp.	γ-Proteobacteria (100%)	EU054384.1
40 ■	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
41 ■	*Vibrio* sp.	γ-Proteobacteria (99%)	AM941184.1
42 ■	***Vibrio* sp.**	**γ-Proteobacteria (99%)**	**AM941184.1**
43 ■	*Pseudoalteromonas* sp.	γ-Proteobacteria (100%)	EU935099.1
44 ■	*Pseudoalteromonas* sp.	γ-Proteobacteria (100%)	EU935099.1
45 ■	***Pseudoalteromonas* sp.**	**γ-Proteobacteria (100%)**	**EU935099.1**
46 ■	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1
47 ■	*Vibrio* sp.	γ-Proteobacteria (100%)	EU655423.1

Marked in bold are products appearing at the same localization within the gel as the isolates used for bacterial treatment.

observed in most samples of both treated and untreated *Artemia* (Fig. 2). Distinct products identified as *Pseudoalteromonas* sp. were, however, observed in samples of treated *Artemia* only (products 21, 22 and 24). Similarly, products identified as *Corynebacterium* sp. and *Moraxella* sp. were only detected in samples of untreated *Artemia* (products 25, 27 and 28). Products were only excised from a restricted number of samples of the live prey but the overall results indicate the relative dominance of *Vibrio*, *Pseudomonas* and *Pseudoalteromonas* within the bacterial community of treated as well as untreated live prey.

After three weeks in feeding, highly diverse PCR-DGGE profiles were observed in larvae sampled from individual incubators, with only a part of the total bacterial community being cultivable (Fig. 3). Products identical to the *Vibrio* isolates used for bacterial treatment were detected as a part of the total as well as the cultivable bacterial community of surface sterilized larvae from both groups (products 34 and 42), while products identical to the *P. elyakovii* used for bacterial treatment may only be detected as a part of the cultivable bacterial community (product 45). Various *Vibrio* groups dominated the total bacterial community but were not observed as a part of the cultivable bacterial community of surface sterilized larvae. Other *Vibrio* groups and *Pseudoalteromonas* sp. were furthermore identified as a part of the cultivable bacterial community but were not found as a part of the total bacterial community of surface sterilized larvae.

4. Discussion

The main finding of this study is improved survival and growth of halibut larvae as a result of live prey treatment using selected autochthonous bacteria. A mixture of three bacterial isolates was used for the treatments. All three isolates were isolated from the dominating cultivable gut community of normally developed larvae in tanks resulting in high larval survival and overall success.

The three isolates that were selected for bacterial treatment of halibut eggs and larvae, *Vibrio* sp., *V. splendidus* and *P. elyakovii*, all belong to genera commonly isolated from fish and their environment and have been tested as probionts in fish culture (Fjellheim et al., 2007; Gomez-Gil et al., 2000; Hjelm et al., 2004; Makridis et al., 2005). *V. splendidus* has been identified in association with hatchery-reared halibut (Verner-Jeffreys et al., 2003a) and some isolates have been implicated as potential pathogens for turbot larvae (Gatesoupe et al., 1999; Thomson et al., 2005). Avirulent isolates have, however, commonly been grouped together with virulent *V. splendidus* (Garnier et al., 2007; Thomson et al., 2005) and the relative dominance of the selected isolate within the intestinal community of healthy and overall successful halibut larvae and to the improved larval survival obtained in the present study may therefore implicate inadequate definition of the various organisms belonging to this group, as previously suggested (Gatesoupe et al., 1999; Verner-Jeffreys et al., 2003a). In agreement with previous findings (Gatesoupe, 2002; Gomez-Gil et al., 2000; Suzer et al., 2008), the present results show improved larval survival and possibly also improved larval growth as a result of the bacterial treatment. Improved larval growth is important with respect to recent findings indicative of improved growth during early production stages persisting during the on-growing phase (Imsland et al., 2007), further emphasizing the importance of a healthy gut community of larvae during early production stages.

The lack of relationship between CFU numbers and the overall success of first feeding larvae observed in the present study suggests that the bacterial community structure should be considered rather than bacterial numbers, as previously pointed out by other authors (Makridis et al., 2000b; Zhou et al., 2009). The PCR-DGGE method has been widely used for successful analysis of the bacterial diversity of environmental samples, including various species of fish (Brunvold et al., 2007; Griffiths et al., 2001; Jensen et al., 2004). Only a small part of the 16S rRNA gene is, however, analysed using this method and a poor separation of closely related species may therefore be expected as was observed with the two *Vibrio* isolates used for bacterial treatment in the present study. Other closely related groups were, however,

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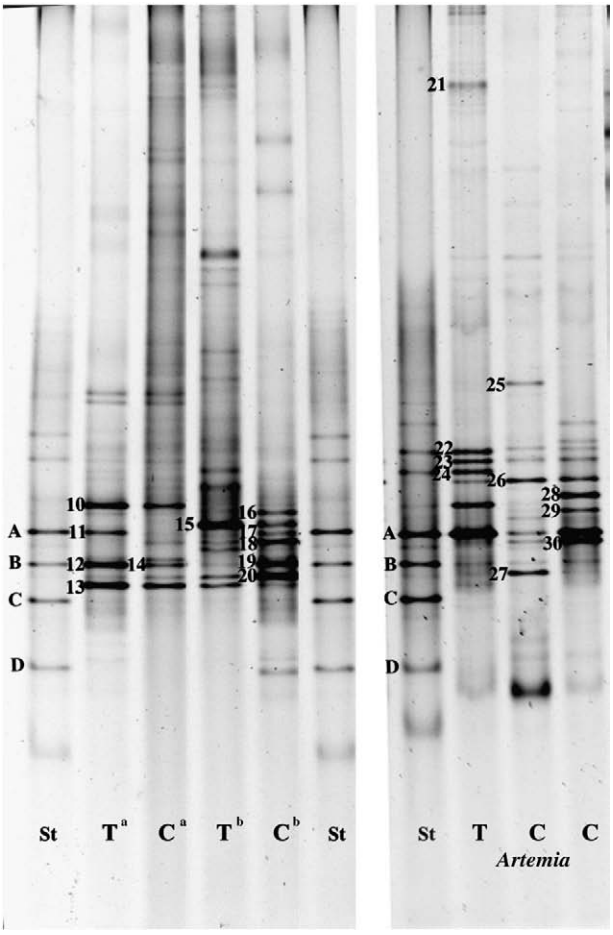

Fig. 2. DGGE profiles in pools of ~100 surface sterilized larvae collected from individual incubators 7–8 days post onset of exogenous feeding. Shown are the profiles of larvae offered untreated *Artemia* (C) and *Artemia* after grazing in the bacterial mixture for 30 min prior to offering to larvae (T). Also shown are the profiles of *Artemia* (C) and bacteria-treated *Artemia* (T). Groups labelled with identical superscript letters ($^{a, b}$) denote sibling tank units, containing larvae of a common silo origin. Included in the figure are relative mobility standards A–D (St), with "A" representing the *Pseudoalteromonas elyakovii* and "B" representing the *Vibrio* sp. and the *V. splendidus* isolates used for bacterial treatment. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787). Labelling indicates products that were excised from the gel and identified by sequence analysis (Table 5).

successfully separated and the method has proved successful for analysis of the bacterial community of fish larvae (Bjornsdottir et al., 2009; Brunvold et al., 2007; Jensen et al., 2004).

Products identical to the isolates selected for bacterial treatment were detected as a part of the dominating bacterial community of treated eggs but not in eggs from any of the control incubators. Only a restricted number of bacterial species have been found to be able to penetrate the protective outer layers of fish eggs, but bacteria have commonly been found attached to the egg surface where heavy on-growth may lead to elevated mortality (Hansen and Olafsen, 1999). Bacterial treatment of eggs did not affect the survival of fertilized eggs in the present study, indicating the impartiality of the selected isolates during the egg stage. The variable CFU numbers and PCR-DGGE pattern of eggs revealed no differences that could be related to the highly variable survival rates of eggs observed in individual incubators included in the study. Surface disinfection of eggs prior to hatching resulted in a significant reduction in CFU numbers in the control group only, indicating a well established bacterial community of treated eggs and that was not easily removed by surface sterilization. A product identical to the *P. elyakovii* used for bacterial treatment was furthermore detected following surface disinfection of eggs from all incubators, indicating a successful colonization of the isolate amongst the dominating bacterial community of eggs. All together, the results suggest the possibility to successfully manipulate the bacterial community of halibut eggs and thereby creating a favourable bacterial environment already prior to hatching of larvae. This is of importance with respect to the observations that the intestinal bacterial community of marine larvae is established by the ingestion of bacteria by drinking long before the larvae actually start feeding (Olafsen, 2001).

The microbial community of feeding larvae has commonly been found to reflect the microbial composition of the live prey (Korsnes et al., 2006; Verner-Jeffreys et al., 2003b). A considerable increase in CFU numbers was observed in the larval gut during the first two weeks of feeding live *Artemia*, however, without the previously reported increase in CFU numbers following bacterial treatment of the live prey (Makridis et al., 2000a). Products identical to the *Vibrio* isolates used for bacterial treatment were found within the gut community of surface sterilized larvae from all groups, indicating the live prey origin and a successful colonization of members of this group as a part of the indigenous bacterial community of larvae, as previously suggested (Verner-Jeffreys et al., 2003b). Also, products identical to the *P. elyakovii* used for bacterial treatment were observed in most larval samples, however, only as a part of the cultivable bacterial community. In agreement with previous observations (Makridis et al., 2002), the present results indicate that offering bacteria-treated live prey to larvae during the first two days of exogenous feeding may prove insufficient for influencing the species composition of the microbial community of larvae. The authors furthermore point out that the larval gut has already been colonized by bacteria during this

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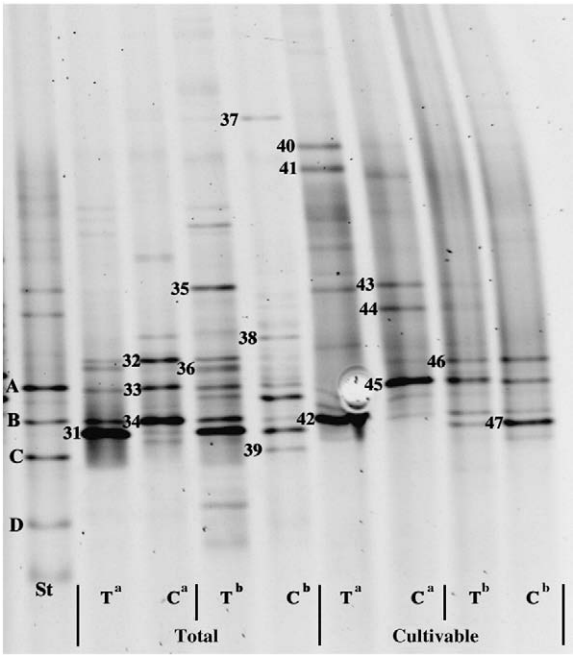

Fig. 3. DGGE profiles in pools of ~75 surface sterilized larvae sampled from individual incubators at 19–21 days post onset of exogenous feeding. Shown are the profiles of larvae offered untreated *Artemia* (C) and *Artemia* after grazing in the bacterial mixture for 30 min prior to offering to larvae (T). Also shown are the profiles of the cultivable bacterial community in the same samples (cultivable). Groups labelled with identical superscript letters ($^{a, b}$) denote sibling tank units, containing larvae of a common silo origin. Included in the figure are relative mobility standards A–D (St), with "A" representing the *Pseudoalteromonas elyakovii* and "B" representing the *Vibrio* sp. and the *V. splendidus* isolates used for bacterial treatment. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787). Labelling indicates products that were excised from the gel and identified by sequence analysis (Table 5).

stage and repeated treatments may therefore be needed in order to maintain the probionts and their effects in the gastrointestinal tract of larvae. The present results, however, indicate that a well established gut community may not have developed until after ~3 weeks in feeding. The bacterial community structure of the live prey was only partly reflected in larvae after the first week in feeding, with a number of products observed in samples of the live prey not detected in larvae. After three weeks in feeding, however, the bacterial community structure of surface sterilized larvae reflected the community structure observed in most samples of the live prey. Elevated bacterial numbers and an unfavourable bacterial community of the live prey may therefore affect larvae to a various extent, depending on the number of days spent in grazing on the live prey. Hence, the variable bacterial profiles of first feeding larvae observed in the present study may be explained by the highly variable CFU numbers and bacterial community structure commonly observed in samples of the live prey, also in samples of enriched *Artemia* prepared from the same egg batches. The bacterial quality of the live prey should therefore be carefully monitored, especially during the first 3 weeks of offering exogenous feed to larvae.

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