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A protocol and cultivation system for gnotobiotic Atlantic cod larvae (*Gadus morhua* L.) as a tool to study host microbe interactions

Torunn Forberg [a,*], Augustine Arukwe [b], Olav Vadstein [a]

[a] Department of Biotechnology, Norwegian University of Science and Technology, N7491 Trondheim, Norway
[b] Department of Biology, Norwegian University of Science and Technology, N7491 Trondheim, Norway

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ABSTRACT

Commensal bacteria in the gut of all vertebrates play essential roles in the development and functionality of the host. Studying the host raised in the absence of bacteria, or under gnotobiotic conditions (with a known composition of bacteria) has become an important tool to unravel the complex host–microbe interactions. In this study we describe a protocol to generate bacteria-free Atlantic cod (*Gadus morhua* L.) larvae, independent on the continued addition of antibiotics. An experimental system allowing for feeding of the larvae was also developed, and used successfully during a gnotobiotic start feeding experiment.

1. Introduction

Commensal bacteria residing in the gut of vertebrates contribute to their hosts development, metabolism and immune system (Rakoff-Nahoum and Medzhitov, 2006). To unravel the host–microbe interactions, one successful strategy has been to study the host in the absence of bacteria, and under gnotobiotic conditions (with a known composition of bacteria) (Gordon and Pesti, 1971; Marques et al., 2006; Rawls et al., 2004). Gnotobiotic studies with mammals have revealed that bacteria-free animals often show metabolic deficiencies such as reduced digestion and vitamin synthesis (Rakoff-Nahoum and Medzhitov, 2006). Some of the differences observed may be due to the lack of bacterial enzymes, but the commensal microflora also directly affect host-regulation of metabolic enzymes (Backhed et al., 2004; Hooper et al., 1999 Hooper et al., 2001; Rawls et al., 2004). Bacteria-free mammals, as well as zebrafish (*Danio rerio* L.), have been shown to have a less developed intestine, and lower rates of intestinal cell proliferation (Rawls et al., 2004; Uribe et al., 1997). Recently, a study on bacteria-free sea-bass (*Dicentrarchus labrax* L.) larvae reported higher growth and more developed digestive tracts in the bacteria-free, compared to conventionally raised larvae (Rekecki et al., 2009). This is in contrast to the findings in mammals and zebrafish, and further studies are needed in order to elucidate the role host–microflora interactions may play in marine fish.

Gnotobiotic studies require a bacteria-free starting point, for mammals this is typically achieved by aseptic caesarian section to obtain the bacteria-free embryo (Smith et al., 2006; Wiseman, 1965). Oviparous fish species produce embryos that are sterile within the protective chorion (Pham et al., 2008), allowing for surface disinfection before hatching as a preliminary step to obtain bacteria-free larvae (Salvesen et al., 1997). A protocol to obtain bacteria-free zebrafish for use in gnotobiotic studies was established by Pham et al. (2008). Compared to most marine fish, zebrafish hatch at a fairly developed stage and they are phylogenetically quite distant. Gnotobiotic protocols do exist for some marine fish species (Dierckens et al., 2009; Munro et al., 1995; Verner-Jeffreys et al., 2003). However not all of these describe a system for feeding of the larvae, and the methods used to verify the gnotobiotic state were mainly culture-based. In their study on gnotobiotic sea bass (*Dicentrarchus labrax*) larvae Dierckens et al. (2009) investigated the presence of non-culturable bacteria, and also described a system for addition of live feed. However, their protocol relied on addition of antibiotics after hatching. This calls for an investigation of long-term effects of antibiotics on the development of the larvae. In addition, an antibiotics-based protocol may limit the types of bacteria that can be tested, as spontaneously resistant mutants need to be selected prior to their addition (Dierckens et al., 2009).

The aim of this study was to develop a protocol for generation of bacteria-free Atlantic cod (*Gadus morhua* L.) larvae, independent of continued addition of antibiotics, and to develop an experimental system that allows addition of live feed to the larvae without compromising the gnotobiotic state.

* Corresponding author at: Sem Sealandsveg 6/8, 7491 Trondheim, Norway. Tel.: +47 73591685.
E-mail address: torunn.forberg@biotech.ntnu.no (T. Forberg).

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2. Material and methods

2.1. Biological material

All manipulations of biological material were performed in a laminar flow hood, using pre-sterilized equipment. Cod eggs were delivered from Cod Culture Norway and Nofima Marin (Tromsø, Norway). Upon arrival the cod eggs (55–65 day degrees) were acclimatized in filtered (0.22 μm Micropore®) autoclaved (121 °C/20 min) seawater (FASW) at 6 ± 1 °C, in the dark. After hatching, the cod larvae were transferred to rearing bottles containing either bacteria-free (FASW) or bacteria-containing seawater. The bacteria-containing water was aged seawater, generated by filtering through a GF/F (Whatman®) filter to remove large particles, and stored for 2 weeks without aeration at room temperature (~20 °C) before use. Axenic *Isochrysis* sp. (CCAP 927/14) was purchased from "Culture collection of algae and protozoa" (http://www.ccap.ac.uk/index.htm), and used as feed for rotifers. Bacteria-free rotifers (*Brachionus* "Nevada") were obtained according to the protocol of Tinh et al. (2006), but modified by incubation of rotifer eggs in water containing 10 ppm of rifampicin and ampicillin until transfer to bottles containing axenic *Isochrysis* sp. Rotifer and algae cultures were grown in sterile 2 L Nalge™ bottles, in sterile F/2 media (Guillard and Ryther, 1962). The rotifers were fed *Isochrysis* sp. according to the calculated carbon requirements of the culture, using absorbance at 750 nm to estimate the carbon content of the algae culture (Reitan et al., 1994). Both algae and rotifer cultures were run semi-continuously (20 °C, constant light by fluorescent tubes), and were aerated by bubbling sterile filtered (Millex™ 0.22 μm filter) air into the culture through a glass rod.

Three different bacteria were used for mono-gnotobiotic rearing conditions; *Microbacterium* ND 2–7 and *Pseudoalteromonas* RA 7–14 both previously isolated from cod and identified as probiotic candidates (for details see Fjellheim et al., 2010), and the pathogenic bacteria *Vibrio anguillarum* HI610 (obtained from Inst. Marine Res., Bergen, Norway, courtesy of Prof. Øivind Bergh). All bacteria cultures were grown in light for 2–3 days at 20 °C in M-65 broth (0.5 g peptone, 0.5 g tryptone and 0.5 g yeast extract in 800 mL FASW and 200 mL MilliQ water), harvested by centrifugation and resuspended in FASW before addition to rearing bottles. The density of bacteria was determined by measuring absorbance (660 nm) and conversion to cells/mL by strain specific conversion factors. Plate counts on M-65 agar were performed at the end of experiments, to estimate the number of live bacteria in the rearing bottles.

2.2. Protocol for bacteria free cod larvae

The cod eggs were washed with FASW and disinfected by two rounds of 400 ppm glutaraldehyde treatment (Salvesen and Vadstein, 1995; Salvesen et al., 1997). The two disinfection rounds were performed with approximately one hour in-between. The disinfected eggs were transferred to sterile Petri dishes (140 mm, ~500 eggs/dish) filled with FASW containing 10 ppm ampicillin and rifampicin until hatching (Dierckens et al., 2009). When more than 50% of the eggs had hatched, larvae were transferred to new Petri dishes (~200 larvae/dish) with FASW to wash away residual antibiotics before stocking into rearing bottles. After stocking the temperature was increased by 1 °C/day until 12 °C was reached. Continuous light was used from day 3 after hatching.

A total of 10 experiments were performed to evaluate the reproducibility of the protocol with regards to successful achievement of bacteria-free larvae (Table 4). In one of these experiments, disinfection with glutaraldehyde was performed only once (experiment 8.)

To evaluate the effect of the four steps of this protocol on hatchability, 96 eggs from each step were placed individually into four 96-well plates containing 150 μL FASW per well (treatments are listed in Table 3). Hatching was registered the day after >50% of the eggs had hatched.

2.3. Short-term experiment with mono-gnotobiotic and bacteria-free conditions

A short-term experiment was performed to evaluate the protocol and the effects of different gnotobiotic conditions on survival of the cod larvae. Sterile Easyflasks™ from Nunclon™ were filled with 50 mL FASW, and stocked with 40 larvae per bottle. As a control, 3 bottles were filled with aged seawater. Bacteria or algae were added to the water at the day of stocking, with a final density of bacteria of about 10^6 cells/mL, or algae at 2 mg C/L (Table 1). No feed was added during the short term experiment. Dead larvae were counted from day 1 after stocking, without disturbing the bottles. After 5 days, 2 out of 3 replicates were discontinued, and the fish sacrificed using MS-222 (0.5 g/L, lethal dose). Survival in the remaining bottles with larvae (one from each condition), was registered until day 9.

2.4. Experimental system involving feeding

We developed an experimental system (Fig. 1) to enable first feeding experiments under gnotobiotic conditions. Sterile 2 L Nalgene® flasks were equipped with a glass rod to allow aeration with sterile filtered air (Millex™ 0.22 μm filters). The glass rod was surrounded by a plankton-net (35 μm) to avoid collisions between the larvae and air bubbles. The bottles were filled with approximately 2 L FASW, and stocked with 30 larvae/L. Axenic *Isochrysis* sp. (2 mg C/L) was added to all treatments, in accordance with the green-water technique (Skjermo and Vadstein, 1993). The larvae were fed gnotobiotic rotifers from day 3, until day 17 post-hatch. The amount of rotifers/larvae/day was added based on a feeding scheme developed by Prof. Yngvar Olsen (Supplementary material) and the approximate density of remaining rotifers in the rearing bottles. Dead larvae were removed and counted on days 3, 7, 10, 12 and 14. On day 17, the larvae were sacrificed using MS-222 (0.5 g/L, lethal dose) and photographed to enable later measurements. Four experimental conditions were chosen for the start feeding experiment, three gnotobiotic and one with a non-defined microbial community (aged seawater) (Table 2).

2.5. Evaluation of gnotobiotic conditions

To evaluate axenity, water samples were collected from the Petri dishes containing bacteria-free washed larvae, and from the 96-well plate with eggs treated twice with glutaraldehyde and incubated in antibiotics (Table 3). Water samples were also taken from the 50 mL rearing bottles on the final day of the short-term experiment. For the start feeding experiment, samples from the cod rearing water and from rotifer and algae cultures were taken every other day of the experiment. Liquid and solid M65 media and Marine Broth (Difco™) were used to check for bacterial contamination. Agar plates and tubes were incubated at 22 °C for up to one month. Serial dilution plating

Table 1
Conditions tested in the short-term experiment with unfed cod larvae.

	Replicates	Final concentration
Gnotobiotic condition		
Bacteria-free	3	–
Microbacterium ND 2–7	3	10^6 cells/mL
Pseudoalteromonas RA 7–14	3	
Vibrio anguillarum HI610	3	
Isochrysis sp.	3	2 mg C/L
Non-defined microbial conditions		
Aged water	3	10^6 cells/mL

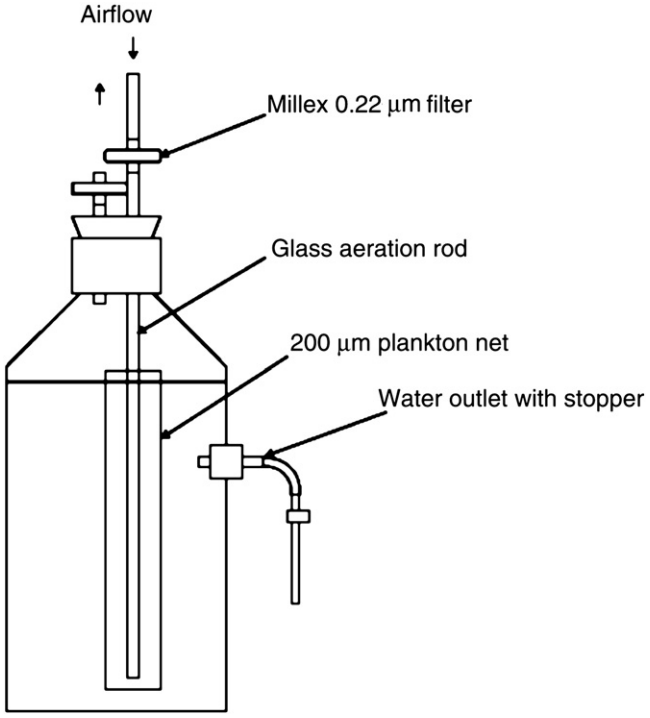

Fig. 1. Experimental system allowing for addition of live feed to the gnotobiotic cod larvae; 2 L Nalgene rearing bottle equipped with aeration glass rods and plankton netting.

was used to estimate the density of culturable bacteria in the rearing bottles containing aged seawater and mono-gnotobiotic conditions.

A FACSScan flow cytometer (Becton Dickinson, USA) was used to quantify the presence/density of bacteria in all cod rearing bottles from the start feeding experiment. SYBR Green (SYBR Green I, Molecular Probes) was used to stain DNA containing particles before detection and quantification using standard settings (Marie et al., 1999). Filtered autoclaved seawater (FASW) was used to quantify the number of background particles. Flow cytometry counts were performed on day 6, 10 and 17 after hatching.

3. Results

3.1. Evaluation of protocol for bacteria-free larvae

The hatching percentage was lowest for eggs that were not treated with glutaraldehyde, and highest for eggs treated once with glutaraldehyde (Table 3).

No bacterial growth was observed after dilution plating of samples taken from the Petri dishes containing washed, bacteria-free larvae, or from any of the 96 samples from well plate no. 4 (Table 3). Out of the 10 experiments performed, 9 were successful with regards to producing bacteria-free larvae (Table 4). The protocol for the unsuccessful experiment (expt. 8) differed however, as only one round of glutaraldehyde disinfection was performed.

Table 2
Conditions tested in the start feeding experiment.

	Replicates	Final concentration
Gnotobiotic condition		
Bacteria-free	2	–
Microbacterium ND 2–7	2	10^6 cells/mL
Pseudoalteromonas RA 7–14	2	
Non-defined microbial conditions		
Aged water	2	10^6 cells/mL

Table 3
Effects of different treatments on hatchability of cod eggs.

Plate no.	Treatment of eggs	# Eggs/plate	% hatched ± SD
1	None	90	82.2 ± 2.0
2	1 × glutaraldehyde	91	94.5 ± 2.4
3	2 × glutaraldehyde	88	85.2 ± 3.8
4	2 × glutaraldehyde + 1 day antibiotics	96	92.7 ± 2.7

SD = Standard deviation.

3.2. Axenity during first feeding

In two initial start feeding experiments, no bacterial contamination was observed in any of the rearing bottles (Table 4). Throughout the gnotobiotic first feeding experiment, the only bacterial contamination observed was from samples taken from bacteria-free bottle 1, on day 12 of the experiment (detected by plate count). No bacterial contamination was detected in the rotifer or algae cultures throughout the experiment. Flow cytometry was also used to evaluate axenity. Particles stained by SYBR Green were distinguished based on fluorescence intensity and light scattering properties (side scatter), and gated before counting. The gates were set to eliminate background noise. Flow cytometry results from the bacteria-free rearing bottles (samples taken on days 6, 10 and 17) are summarized in Table 5. The number of events found was either lower or very similar to those from FASW, indicating that there were no bacteria present in the bacteria-free treatments.

The gated events found in water from bacteria-free bottle 2 and in one of the gnotobiotic *Micrococcus* ND 2–7 bottles on day 17 are shown in Fig. 2.

3.3. Evaluation of gnotobiotic conditions

From the rearing bottles mono-associated with bacteria in the short-term experiment only the expected colony morphologies were observed on agar plates. Dilution plating of water samples showed that the initial concentration of bacteria (10^6/ml) was doubled on day 6 for the rearing bottles with *Pseudoalteromonas* RA 7–14. For the gnotobiotic condition with *Microbacterium* ND 2–7, the concentration of bacteria was reduced. The gnotobiotic condition with *Vibrio anguillarum* HI 610 also showed an increase in bacterial numbers (Supplementary material). Water samples from the bottles with *V.*

Table 4
Summary of experiments performed to evaluate the protocol for bacteria-free cod larvae. The bacteria-free flasks from the two experiments in bold font are part of the gnotobiotic short term and start feeding experiment described in this paper.

Experiment #	Duration (days)	# Flasks	# Larvae/rearing volume	# Bacteria-free flasks (final day of expt.)
Experiments without addition of live feed				
1	8	3	180/2 L	3
2	**5(9)[a]**	**3**	**40/50 mL**	**3**
3	9	3	30/50 mL	3
4	9	3	30/50 mL	3
5	9	4	30/50 mL	4
6	9	5	30/50 mL	5
Experiments with addition of live feed				
7	17	4	80/2 L	4
8[b]	12	3	80/2 L	0
9	**17**	**2**	**60/2 L**	**1**
10	11	5	60/2 L	5

[a] In experiment 2, two out of the three flasks were discontinued after day 5, while the third flask was discontinued on day 9.
[b] Experiment 8 was performed with only one round of glutaraldehyde disinfection.

Table 5
Number of gated events from water samples from bacteria-free cod larvae taken on different days, and for FASW (3 different batches) serving as a control for background "noise". Events recorded are per 47.3 μL sample.

	Gated events		
	Day 6	Day 10	Day 17
Bacterial-free 1	193	227	ND
Bacterial-free 2	176	238	42
FASW	123	17	23

ND = not determined.

anguillarum were taken on day 3, because these replicates were discontinued earlier as a result of high mortality of the cod larvae. The effect of the different gnotobiotic conditions on the survival of the cod larvae in the short-term experiment is shown in Fig. 3. On day 3, the replicates with the pathogenic *Vibrio anguillarum* HI 610 had significantly lower survival than the other conditions. On the final day of the experiment, day 9, more than 50% of the cod larvae were still alive for all conditions except for *V. anguillarum* HI 610 where mortality was 100%. In general, unfed cod larvae may be expected to live for 12 to 14 days (Fjellheim et al., 2010).

In the start feeding experiment, plating of water samples from the rearing bottles mono-associated with bacteria gave only one type of colony morphology, indicating that the gnotobiotic conditions were maintained throughout the study. On day 17, the density of bacteria as determined by dilution plating was around 10^6/ml for all treatments

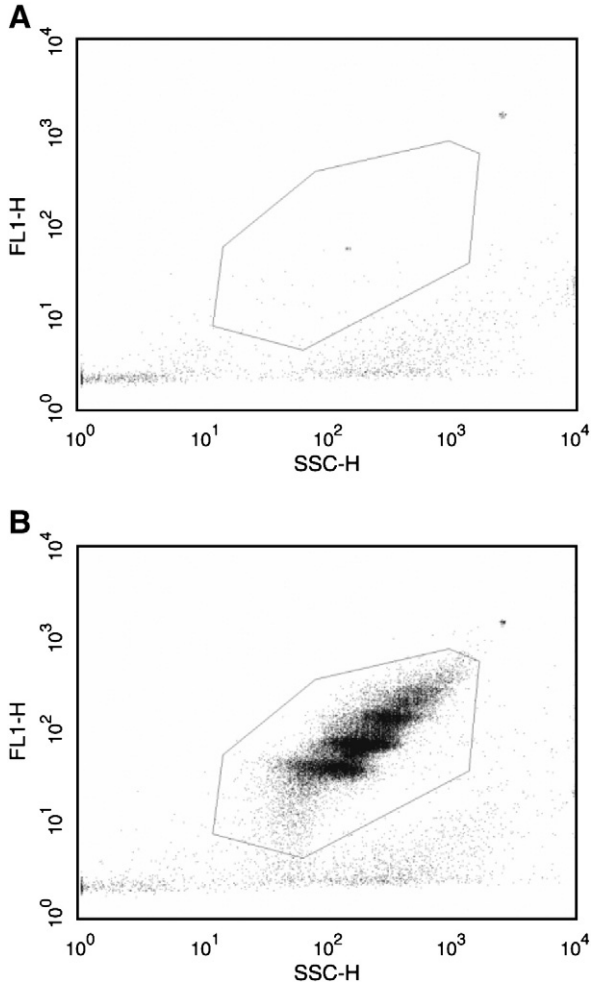

Fig. 2. Flow cytometry results with gated events (marked area). Green fluorescence (FL1-H) versus side scatter (SSC-H). A) Bacteria-free bottle 2, B) *Microbacterium* ND 2–7 treatment 2, both counted on day 17 (the cluster of events in upper right corner of the graph represent fluorescent mono-dispersed beads added to each sample).

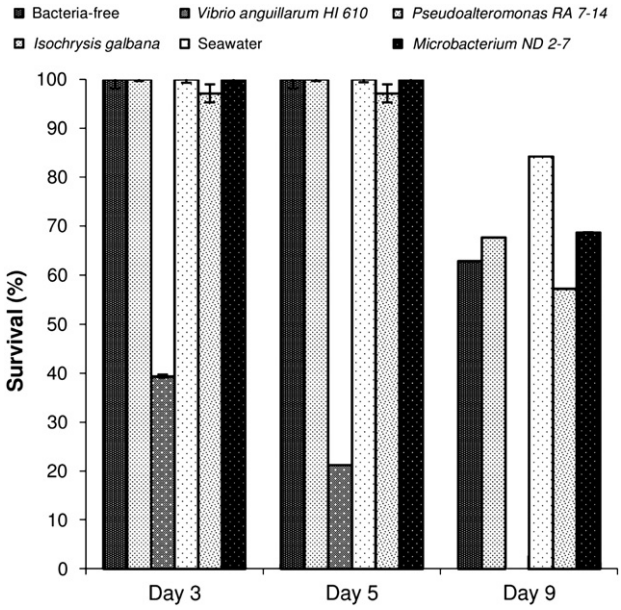

Fig. 3. Survival of cod larvae during the gnotobiotic short-term experiment (unfed larvae). The bars represent the average of the three biological replicates, and the error bars are 95% confidence intervals. No confidence interval is added on day 9, as only one bottle per condition remained.

(Supplementary material). Bacterial densities were also calculated from the flow cytometry results (Table 6). These densities were all higher than the densities from the dilution plating, which is expected, as dying and inactive cells are gated and counted along with the viable cells.

The survival of cod larvae during the start feeding experiment is shown in Fig. 4. On day 10, the survival in the bacteria-free bottles was significantly higher than the control (containing aged seawater) and the gnotobiotic condition with *Pseudoalteromonas* RA 7–14. Survival in bacteria-free rearing bottle 1 was only registered until day 12, as a result of detection of contaminant bacteria on this day. On day 14 and 17 the survival in the seawater and *Pseudoalteromonas* RA 7–14 treatments was significantly lower than for the *Microbacterium* ND 2–7 treatment. The remaining bacteria-free rearing bottle had the highest survival, with more than 60%.

Measurements of the length of individual larvae did not show any significant differences between larvae from the different conditions (Supplementary material), indicating no differences in growth.

4. Discussion

4.1. Protocol to generate bacteria-free larvae

In this study we developed a protocol to generate bacteria-free cod larvae, without continued exposure to antibiotics. In a previous study by Dierckens et al. (2009), as well as in the original disinfection-

Table 6
Calculated events/mL in water samples from the different microbial conditions in the start feeding experiment on days 6, 10 and 17.

	Calculated events (10^9/ml)		
	Day 6	Day 10	Day 17
Gnotobiotic condition:			
Microbacterium ND 2–7#1	0.19	18.0	8.55
Microbacterium ND 2–7#2	3.37	13.9	7.52
Pseudoalteromonas RA 7–14#1	3.38	12.8	3.49
Pseudoalteromonas RA 7–14#2	2.57	11.6	2.12
Non-defined condition:			
Aged water#1	3.39	21.4	13.4
Aged water#2	3.28	20.9	18.3

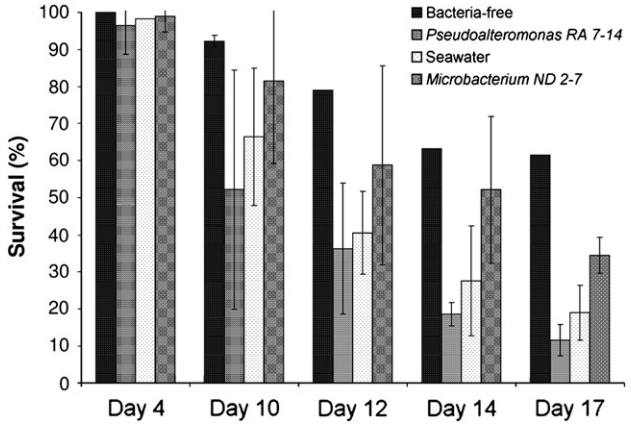

Fig. 4. Survival of cod larvae exposed to different microbial conditions throughout the start feeding experiment. The bars represent the average of the two biological replicates, and the error bars are 95% confidence intervals (on day 17 Bacteria-free n = 1).

protocol studies with glutaraldehyde, only one round of disinfection was used (Salvesen and Vadstein, 1995; Salvesen et al., 1997). One round of glutaraldehyde disinfection was found to be insufficient to achieve bacteria-free larvae in our study. The effect of glutaraldehyde disinfection is highly dependent on the bacterial load present on the eggs (Salvesen and Vadstein, 1995; Salvesen et al., 1997). Therefore, we investigated the effect of repeating the glutaraldehyde disinfection process. Two consecutive rounds of disinfection, followed by immersion in antibiotics did not induce negative effects on the hatchability of the cod eggs. This is in accordance with previous studies on Atlantic halibut (*Hippoglossus hippoglossus* L.) (unpublished results). The resulting disinfection protocol was highly efficient and reproducible, as no bacterial growth was observed from the hatched larvae in any of the 9 experiments with two disinfection treatments. It is important to notice that glutaraldehyde has a high temperature coefficient (Salvesen et al., 1997), thus adaptation of the developed protocol to other species must take this into consideration.

4.2. Maintenance of gnotobiotic conditions during start feeding

The described experimental system was used successfully in a gnotobiotic start feeding experiment, with only one out of six rearing bottles showing bacterial contamination. This contamination was probably introduced through one of the many manipulations necessary for feeding and cleaning (removal of dead larvae and debris) of the rearing bottles. Not all bacteria are culturable; therefore non-culture based methods should be used to evaluate axenity. We used flow cytometry combined with SYBR Green staining throughout the first feeding experiment to keep track of any contamination of non-culturable bacteria. Despite some variation in background noise and gated events from bacteria-free samples, the low level of events found in the beginning of the experiment did not increase significantly. Considering the stable, low level of gated events found in the water samples, we concluded that the axenity was kept throughout the 17 days. We observed that flow cytometry is a valuable tool to investigate the presence of bacteria in the rearing bottles, without the need for microscopy, or PCR and sequencing.

4.3. Effect of gnotobiotic conditions on growth and survival

In the short-term experiment, 5 different defined and 1 undefined (aged seawater) microbial conditions were tested with regards to their effect on survival of cod larvae. Aged seawater will theoretically be dominated by slow growing and non-opportunistic (K-selected) bacteria (Andrews and Harris, 1986; Skjermo et al., 1997). The selected bacterial strains had previously been tested *in vivo* with cod larvae (Fjellheim et al., 2010), but not under gnotobiotic conditions. All seawater used in the previously mentioned study had been aged before autoclaving, and probably contained a lot of dead bacteria. Dead as well as living bacteria may influence host responses (Caipang et al., 2009; Pan et al., 2008), which is why we sterile-filtered all seawater used for gnotobiotic conditions before autoclaving. Only one of the monognotobiotic conditions was clearly detrimental to the cod larvae, namely the pathogen *Vibrio anguillarum* HI 610.

In the start feeding experiment, the lowest survival was observed for cod larvae raised in aged seawater and in the gnotobiotic *Pseudoalteromonas* RA 7–14 bottles. On day 10, the bacteria-free bottles had significantly higher survival than the two previously mentioned conditions. This is in accordance with previous studies, where bacteria-free fish larvae have been shown to have high survival (Munro et al., 1995; Rekecki et al., 2009). At the last day of the experiment (day 17), the gnotobiotic *Micrococcus* ND 2–7 bottles had significantly higher survival than matured seawater and *Pseudoalteromonas* RA 7–14, indicating that this strain may be the best probiotic candidate out of the ones tested. The beneficial effect of this strain was not as clear in the study by Fjellheim et al. (2010), nor in our short-term experiment. This demonstrates the importance of extending experimental time by introducing feed to the cod larvae. In general, the variation in survival within the biological replicates was higher for conditions with bacteria than for the bacteria-free bottles. Monognotobiotic conditions are artificial with regards to the "real world" of larval rearing, and the effect of a bacterial strain added as pure culture may not be representative of the effect of microbes in a rearing tank. However, we now have the tools available to further investigate the effects of specific bacterial additives, without the complications of a pre-existing microflora. Further studies will be performed with increased complexity of gnotobiotic conditions combined with addition of disease-causing bacteria, such as the *Vibrio anguillarum* used here, as a challenge test.

4.4. Advantages of the gnotobiotic cod larvae model

To our knowledge this is the first published protocol to generate bacteria free cod larvae, and the first protocol for marine larvae that is independent on continued addition of antibiotics. This could alleviate the need for studies on potential long-term effects of the antibiotics on the host. In addition, we are not dependent on finding spontaneous resistant mutants for bacterial strains we want to use. The effect of adding a bacterium to the larvae is probably closely linked to its ability to compete in the gut, and factors such as generation time and metabolic activity of a strain can determine its establishment. When introducing antibiotic resistance to a strain, either via plasmids or by selecting spontaneous mutants, the acquired resistance represents an additional cost for the strain. This cost can result in a longer generation time and altered metabolic activity (Andersson and Levin, 1999; Feng and Rise, 2009; Levin et al., 2000; McDermott et al., 2006). This implies that a bacterial strain with introduced resistance may behave differently than the original strain, especially under competitive conditions.

The possibility of rearing gnotobiotic cod larvae opens up for more controlled studies on host–microbe interactions, including evaluation of the effects complex gnotobiotic conditions have. Moreover, by investigating the gene expression of bacteria-free larvae vs. larvae raised with bacteria, important host-responses to bacteria and potential functional consequences may be identified.

Supplementary materials related to this article can be found online at doi:10.1016/j.aquaculture.2011.02.047.

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References

Andersson, D.I., Levin, B.R., 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbiol. 2, 489–493.

Andrews, J.H., Harris, R.F., 1986. R-Selection and K-Selection and microbial ecology. Adv. Microb. Ecol. 9, 99–147.

Backhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A., Semenkovich, C.F., Gordon, J.I., 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. U. S. A. 101, 15718–15723.

Caipang, C.M.A., Brinchmann, M.F., Kiron, V., 2009. Profiling gene expression in the spleen of Atlantic cod, *Gadus morhua* upon vaccination with *Vibrio anguillarum* antigen. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 153, 261–267.

Dierckens, K., Rekecki, A., Laureau, S., Sorgeloos, P., Boon, N., Broeck, W.V.d., Bossier, P., 2009. Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. Environ. Microbiol. 11, 526–533.

Feng, C.Y., Rise, M.L., 2009. Characterization and expression analyses of anti-apoptotic Bcl-2-like genes NR-13, Mcl-1, Bcl-X1, and Bcl-X2 in Atlantic cod (*Gadus morhua*). Mol. Immunol. 47, 763–784.

Fjellheim, A.J., Klinkenberg, G., Skjermo, J., Aasen, I.M., Vadstein, O., 2010. Selection of candidate probionts by two different screening strategies from Atlantic cod (*Gadus morhua* L.) larvae. Vet. Microbiol. 144, 153–159.

Gordon, H.A., Pesti, L., 1971. Gnotobiotic animal as a tool in study of host microbial relationships. Bacteriol. Rev. 35, 390–429.

Guillard, R.R., Ryther, J.H., 1962. Studies of marine planktonic diatoms.1. Cyclotella Nana Hustedt, and Detonula Confervacea (Cleve) Gran. Can. J. Microbiol. 8, 229–239.

Hooper, L.V., Xu, J., Falk, P.G., Midtvedt, T., Gordon, J.I., 1999. A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc. Natl. Acad. Sci. U. S. A. 96, 9833–9838.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., Gordon, J.I., 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science. 291, 881–884.

Levin, B.R., Perrot, V., Walker, N., 2000. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics. 154, 985–997.

Marie, D., Brussaard, C.P.D., Thyrhaug, R., Bratbak, G., Vaulot, D., 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. Appl. Environ. Microbiol. 65, 45–52.

Marques, A., Ollevier, F., Verstraete, W., Sorgeloos, P., Bossier, P., 2006. Gnotobiotically grown aquatic animals: opportunities to investigate host–microbe interactions. J. Appl. Microbiol. 100, 903–918.

McDermott, J.R., Leslie, F.C., D'Amato, M., Thompson, D.G., Grencis, R.K., McLaughlin, J.T., 2006. Immune control of food intake: enteroendocrine cells are regulated by CD4+ T lymphocytes during small intestinal inflammation. Gut. 55, 492–497.

Munro, P.D., Barbour, A., Birkbeck, T.H., 1995. Comparison of the growth and survival of larval turbot in the absence of culturable bacteria with those in the presence of *Vibrio Anguillarum*, *Vibrio Alginolyticus*, or a marine *Aeromonas* sp. Appl. Environ. Microbiol. 61, 4425–4428.

Pan, X., Wu, T., Song, Z., Tang, H., Zhao, Z., 2008. Immune responses and enhanced disease resistance in Chinese drum, *Miichthys miiuy* (Basilewsky), after oral administration of live or dead cells of *Clostridium butyrium* CB2. J. Fish Dis. 31, 679–686.

Pham, L.N., Kanther, M., Semova, I., Rawls, J.F., 2008. Methods for generating and colonizing gnotobiotic zebrafish. Nat. Protocols. 3, 1862–1875.

Rakoff-Nahoum, S., Medzhitov, R., 2006. Role of the innate immune system and host-commensal mutualism. Current Topics in Microbiology and Immunology 1–18.

Rawls, J.F., Samuel, B.S., Gordon, J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc. Natl. Acad. Sci. U. S. A. 101, 4596–4601.

Reitan, K., Rainuzzo, J., Olsen, Y., 1994. Effect of nutrient limitation on fatty-acid and lipid-content of marine microalgae. J. Phycol. 30, 972–979.

Rekecki, A., Dierckens, K., Laureau, S., Boon, N., Bossier, P., Van den Broeck, W., 2009. Effect of germ-free rearing environment on gut development of larval sea bass (*Dicentrarchus labrax* L.). Aquaculture. 293, 8–15.

Salvesen, I., Vadstein, O., 1995. Surface disinfection of eggs from marine fish: evaluation of four chemicals. Aquacult. Int. 3, 155–171.

Salvesen, I., Øie, G., Vadstein, O., 1997. Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: Evaluation of concentrations and contact times. Aquacult. Int. 5, 249–258.

Skjermo, J., Vadstein, O., 1993. The effect of microalgae on skin and gut bacterial flora of halibut larvae. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinnereim, K. (Eds.), Proceedings of the First International Conference on Fish Farming Technology. A.A Balkema, Rotterdam, pp. 61–67.

Skjermo, J., Salvesen, I., Øie, G., Olsen, Y., Vadstein, O., 1997. Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquacult. Int. 5, 13–28.

Smith, K., McCoy, K.D., Macpherson, A.J., 2006. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin. Immunol. 19, 59–69.

Tinh, N.T.N., Phuoc, N.N., Dierckens, K., Sorgeloos, P., Bossier, P., 2006. Gnotobiotically grown rotifer *Brachionus plicatilis* sensu strictu as a tool for evaluation of microbial functions and nutritional value of different food types. Aquaculture. 253, 421–432.

Uribe, A., Alam, M., Midtvedt, T., Smedfors, B., Theodorsson, E., 1997. Endogenous prostaglandins and microflora modulate DNA synthesis and neuroendocrine peptides in the rat gastrointestinal tract. Scand. J. Gastroenterol. 32, 691–699.

Verner-Jeffreys, D.W., Shields, R.J., Birkbeck, T.H., 2003. Bacterial influences on Atlantic halibut *Hippoglossus hippoglossus* yolk-sac larval survival and start-feed response. Dis. Aquat. Org. 56, 105–113.

Wiseman, R., 1965. Gnotobiotics and the germ-free animal. Bioscience. 15, 187–189.