Aquaculture 438 (2015) 17–23

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Probiotic strains introduced through live feed and rearing water have low colonizing success in developing Atlantic cod larvae

Jorunn Skjermo [a,*,1], Ingrid Bakke [b,1], Stine Wiborg Dahle [a], Olav Vadstein [b]

[a] SINTEF Fisheries and Aquaculture, N-7465 Trondheim, Norway
[b] Department of Biotechnology, (NTNU) Norwegian University of Science and Technology, N-7491 Trondheim, Norway

ARTICLE INFO

Article history:
Received 30 October 2014
Received in revised form 15 December 2014
Accepted 16 December 2014
Available online 22 December 2014

Keywords:
Probiotic bacteria
Marine larvae
Microbial control
Real-time PCR
Microbacterium sp.
Ruegeria sp.

ABSTRACT

The use of probiotic bacteria is a strategy suggested to overcome microbial problems during early stages of farmed animals, including fish. However, few studies have investigated the efficiency in the establishment of probiotics in the fish and how lasting such treatments are. The objective of this study was to evaluate whether the microbiota of Atlantic cod larvae *Gadus morhua* L can be steered by introduction of selected probiotic candidates, and if there are particular developmental stages where this is more easily obtained. Cod larvae were given a mixture of 4 candidate probiotic strains (*Microbacterium* (ID3-10), *Ruegeria* (RA4-1), *Pseudoalteromonas* (RA7-14) and *Vibrio* (RD5-30)) through the live feed and the water during a treatment period of 24 h. The strains originated from reared cod larvae and are proven to have probiotic properties. The larvae were treated at seven different ages, namely days 0, 2, 4, 8, 16, 30 and 45 post-hatching, with one new experimental group started each time. A real-time PCR strategy was developed to quantify the relative amounts of the four added bacterial strains in live feed, and in water and cod larvae during 10 days after exposure. Only ID3-10 was measured to constitute considerable fractions of the larval microbiota for most of the treatments, despite the fact that all the probiotic candidates originated from cod larvae intestines. In most cases, the amounts of the added strains decreased to approximately background levels after a maximum of 4 days, indicating only a transient presence in the larvae. The results indicate that probiotic treatment aiming for colonization with new strains in the cod larvae is difficult, and challenges the probiotic concept for the larval stage unless continuous or repeated addition to the fish larvae is used.

1. Introduction

The use of probiotic bacteria is a suggested strategy to overcome microbial problems in early stages of farmed animals, including fish. However, few studies have documented the efficiency of incorporation and how lasting such treatments are. Atlantic cod *Gadus morhua* L. has for several decades been expected to become an important species in northern European aquaculture, but the production is still very low. The reason is a combination of low prices due to a revival in the cod fishery industry, competition with low price white fish species imported from Asia, and poor availability of juveniles of good quality. Adequate technology for intensive and predictable production of high quality juveniles is a prerequisite for cod culture to become a high volume industry. A major problem in the juvenile production of cod is high loss rates during the larval stage caused by infections. Except from a few cases the causative agent of the infection is not identified, and dysfunctional interaction between larvae and microbes is suggested as the underlying cause for the negative development (Olafsen, 2001; Vadstein et al., 2004). Opportunistic microbes may invade and infect stressed or immuno compromised larvae and cause high mortality. This is a management problem, and to improve the predictability and quality in the juvenile production and to avoid the development of an industry depending on the use of antibiotic agents, it is crucial to develop rearing technology for optimal microbial control (Skjermo and Vadstein, 1999).

The use of probiotic bacteria is one proposed tool for controlling the microbiota of marine fish larvae (Gatesoupe, 1999; Vadstein et al., 1993b; Vershuere et al., 2000), and some studies have documented that probionts can protect fish larvae against infections (Tinh et al., 2008). It has been suggested that the success of a strain as a probiotic is dependent on its ability to attach to the mucosa in the intestine (Vine et al., 2006) and to establish as a part of the commensal gastrointestinal microbiota by colonization (Nayak, 2010; Tinh et al., 2008). However, few studies have investigated the efficiency of incorporation into the fish and how lasting probiotic treatments are. Moreover, to what extent colonization success is dependent on age or developmental stage of the larvae has to our knowledge not been studied so far.

We have previously isolated bacteria from the intestine of cod larvae reared under different cultivation regimes and nominated several probiotic candidates depending on a set of screening criteria (Fjellheim et al., 2010). In the present study we used four of these probiotic

* Corresponding author. Tel.: +47 98 24 50 40.
E-mail address: Jorunn.Skjermo@sintef.no (J. Skjermo).
[1] J. Skjermo and I. Bakke contributed equally to this work.

http://dx.doi.org/10.1016/j.aquaculture.2014.12.027
0044-8486/

candidates to examine whether these bacteria were able to colonize the cod larvae and become a persistent part of the microbiota of the larval. We also aimed to evaluate whether the colonization success was dependent on the developmental stage of the larvae by repeating the experiment at seven different developmental stages from zero to 45 days post-hatching. The larvae were exposed to the selected probiotic strains for 24 h by adding them to the water and incorporating them into the live feed. A real-time PCR approach was used to quantify the relative abundance of the strains in the larvae during the 10 days following each of the seven treatments.

2. Materials and methods

2.1. Probiotic candidate strains

The four probiotic candidate strains (ID3-10, RA4-1, RA7-14, and RD5-30) used in this study were previously isolated from intestinal systems of reared cod larvae at the age of 109 (ID3-10) and 216 (RA4-1, RA7-14, and RD5-30) day degrees and were shown to improve the survival of yolk sac larvae of cod in small-scale in-vivo experiments (Fjellheim et al., 2010). Cultivation of the isolates was done at 12 °C, in 50% Marine broth (Difco) with salinity adjusted to 33 ppt. The OD_{660} was measured daily for all cultures and the volumes needed for the probiotic treatments, plus 10%, were harvested when the cultures were in late exponential or early stationary growth phase. The cultures were centrifuged at 7000 rpm for 10 min at 12 °C, and the bacterial cells were re-suspended in autoclaved seawater before use in the fish tanks. For use in live feed cultures the culture medium was not removed.

2.2. Larval rearing conditions

Cod eggs were held in incubators until hatching and were thereafter distributed in 3 production tanks of 160 l at densities of 100 larvae per litre, in a total of 16,000 larvae per tank. The water was microbial matured (Skjermo et al., 1997) and had a temperature of 7 °C until 6 days post-hatching (dph), thereafter gradually increased by 0.5 °C per day up to 12 °C on 14 dph. The tank water was aerated with bubble stones to secure distribution of live feed and larvae, and to reduce the risk for gas super-saturation. The water exchange in the tanks was 2 times per day from 0 to 10 dph, 3 times from day 11 to 20, 4 times from day 21 to 30, and 8 times from 31 to 57 dph.

The feeding regime comprised the addition of *Nannochloropsis* paste (Reed Mariculture Inc., US) corresponding to a final concentration of 1 mg carbon per litre from 1 to 20 dph, rotifers *Brachionus ibericus* Cayman batch cultivated on rotifer diet and *Pavlova* paste (both Reed Mariculture Inc., California) from 2 to 22 dph and *Artemia* nauplii (INVE, Dendermonde, Belgium) enriched with the oil emulsion MarolE (SINTEF Fisheries and Aquaculture, Trondheim, Norway) from day 18 to 34. The larvae were weaned to the formulated feed Gemma Micro 300 (Skretting, Stavanger, Norway) from day 30 to 34 and only fed the formulated feed for the rest of the experiment, to 57 dph. A robot was used for automated distribution of algae and feed in all the tanks in the experimental set-up. The tanks were cleaned by siphoning organic matters from the walls and bottom 3 times per week in the first feeding period and daily during and beyond weaning to formulated feed.

2.3. Design of probiotic treatments

The probiotic treatments were carried out in separate experimental tanks at 0, 2, 4, 8, 16, 30 and 45 dph. Each unit is denoted ExpD*n*, where *n* is the day post-hatching when the treatment was started. For each treatment day larvae were carefully collected randomly from the three production tanks and transferred to one experimental tank with a water volume of 100 l. For ExpD0 and ExpD2 1000 larvae were transferred and for the remaining days 1500 larvae.

After 2 h of acclimation the four candidate probiotic bacterial strains were added in equal numbers to the water to a final density of $5 \cdot 10^6$ CFU ml^{-1}. Live feed were also used for administration, using a protocol slightly modified after Makridis et al. (2000). Briefly, the rotifers or *Artemia* were washed and incubated at 2000 or 1000 individuals ml^{-1}, respectively, in an equal mixture of the four probiotic candidates with a final density of $4 \cdot 10^8$ CFU ml^{-1}. After 30 min the live feed organisms were harvested and washed carefully and fed to the larvae at normal densities for the actual developmental stage, from 4000 rotifers l^{-1} at day 2 up to 15,000 at day 16, and 5000 *Artemia* l^{-1} on day 30 and 45. Except from ExpD0 where the larvae were not fed, ExpD2 where the larvae were fed twice and in ExpD4 where the larvae were fed 3 times, the larvae were fed four meals during 22 h. The water exchange was turned on 24 h after the first treatment, adapted for the developmental stage of the larvae as described in Section 2.2, and the larvae were fed non-treated rotifers, *Artemia* or formulated feed for the rest of the experiment. The experimental tanks were terminated after 11 days and all the larvae in the tank counted.

2.4. Sampling scheme

From each of the three production tanks 35 larvae were sampled randomly for measurement of dry weights at day 56 ph. The larvae were killed with an overdose of Tricaine Methanesulphate (MS222), washed in autoclaved sea water and put in tin capsules and weighted after drying. For each of the experimental tanks larvae and water were sampled on day 0, immediately before the probiotic treatment started, and on days 1, 2, 4 and 11 after the start of the treatment. Larvae were picked randomly from the tank and killed with MS222 before they were washed in autoclaved seawater, pooled (10 individuals) and immediately frozen on dry ice. Tank water was sampled by filtering 50 ml directly on 0.2 μm hollow-fibre syringe filters (Dynagard, Microgon Inc., California). Rotifers and *Artemia* were sampled before and after the probiotic enrichments, counted and filtered directly on Dynagard filters. Both the larvae and the filters were stored at −20 °C until DNA extraction.

2.5. DNA extraction, PCR and DNA sequencing

DNA was extracted from larvae, water and live feed organisms using the Blood and Tissue Kit (Qiagen) as described by the manufacturers but with minor modifications as described in Bakke et al. (2011). Total DNA was extracted from samples with 10 pooled larvae. For determination of the 16S rRNA gene sequences, the SSU genes of the probiotic candidate strains were amplified using the primers Eub8F (5′-agagtttgatcmtggctcag-3′) and 1492R (5′-ggttaccttgttacgactt-3′) with the Amplitaq Gold polymerase (Applied Biosystems) as described by the producers. PCR products were purified with the Qiaquick PCR purification kit (Qiagen). DNA sequencing of PCR products were performed at Eurofins MWG Operon using the primers 518R (5′-attaccgcggctgctgg-3′), 1115R (5′-cgtaacgagcgcaaccct-3′) and 968 F (5′-aacgcgaagaaccttac-3′).

2.6. Primer design for real-time PCR

DNA sequences for the SSU rRNA gene were determined for the probiotic candidate strains, and used for taxonomic assignment and primer design. By using the classifier function of RDP (Wang et al., 2007) the strains were classified at the genus level as *Microbacterium* (ID3-10), *Ruegeria* (RA4-1), *Pseudoalteromonas* (RA7-14), and *Vibrio* (RD5-30). For each of the four probiotic candidates, SSU rRNA gene sequences for closely related strains were identified using the SeqMatch tool at the RDP (Cole et al., 2007; Cole et al., 2009). By aligning closely related sequences, the most variable regions of the SSU rRNA gene were identified for each of the 4 probiotic candidate strains. Real-time PCR primers targeting these strain specific sequences were designed for each of the strains using Primer Express (Applied Biosystems) and