An acyl homoserine lactone-degrading microbial community improves the survival of first-feeding turbot larvae (**Scophthalmus maximus** L.)

Nguyen Thi Ngoc Tinh a,b, Vu Hong Nhu Yen b, Kristof Dierckens a, Patrick Sorgeloos a, Peter Bossier a,*

a Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, 9000 Gent, Belgium
b Research Institute for Aquaculture No.2, 116 Nguyen Dinh Chieu, Hochiminh City, Vietnam

**Abstract**

Two N-acyl homoserine lactone (AHL) degrading enrichment cultures (EC3 and EC5), originating from the microbial community of the **Penaeus vannamei** shrimp gut, were incorporated into first-feeding turbot larvae through addition to the rearing water and/or bio-encapsulation in rotifers, prior to their feeding to the turbot larvae. Both ECs were able to colonize the larval gut and to persist up to five days after their addition was discontinued. However, only EC5 was effective in improving turbort larvae survival under the experimental conditions, i.e. when the survival of turbort larvae was compromised through the daily addition of AHL molecules (1 mg l⁻¹). The latter treatment reduced the survival to 5.9% or 10.4% dependent on the experiment (while in the control treatment, the survival was 35% and 92.1%, respectively). Through the addition of EC5, the effect of AHL could be nullified. There was a strong negative correlation between the residual AHL concentration in the water and the larval survival on the last day. The negative effect on turbort larval survival might be caused by AHL-induced interference. These results suggest that quorum sensing interference might become part of novel non-antibiotic based strategies to overcome high mortalities in the industrial larval production of marine fish.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Turbot (**Scophthalmus maximus** L.) is an important aquaculture species in Southern Europe and in Shandong province in China. One of the most critical aspects in turbot farming is the highly variable survival during the larval rearing phase. Research on this critical phase has focused on the nutritional requirements of larvae during early exogenous feeding, together with the microbial characteristics of the intensive rearing environment (Shields, 2001). Major bacterial colonization of the gut of turbort larvae coincides with the start of feeding ( Munro et al., 1994). Most bacterial species isolated from the intestinal tract of larval turbort belong to Vibrionaceae (Nicolás et al., 1989; Gatesoupe, 1990; Munro et al., 1994; Blanch et al., 1997). Live food organisms, especially rotifers, were shown to be the main source of bacterial colonization of turbort larval gut (Gatesoupe, 1990; Keskin and Rosenthal, 1994; Munro et al., 1994). Efforts have been made to reduce the bacterial load associated with rotifers before feeding to turbort larvae, by rinsing of rotifers (Keskin and Rosenthal, 1994) or exposure of rotifers to ultraviolet radiation (Munro et al., 1999). The microbial environment of turbort larviculture can be controlled by manipulating the r/K-strategists proportion of the bacterial community. Salvesen et al. (1999) reported that, a lower proportion of r-selected bacteria in tanks with microbiologically-matured water containing microalgae could result in higher percentage of viable and fast-growing turbort larvae. An alternative approach for microbial management of turbort larviculture involved the selection of beneficial bacteria (probiotic bacteria). These bacteria were isolated from the rearing environment of turbort larvae (Huys et al., 2001; Hjelm et al., 2004a,b) or from rotifer cultures (Gatesoupe, 1994). Several bacterial strains, when introduced to the rearing water or bioencapsulated in the rotifers, were retrieved in high numbers in the larval gut, and were able to improve the survival rates of the first-feeding turbort larvae (Gatesoupe, 1994, 1997; Makridis et al., 2000). Recently, disruption of quorum sensing was suggested as a new strategy for microbial control in aquaculture (Defoirdt et al., 2004). In the present study, we investigated the use of two enrichment cultures of N-acyl homoserine lactone (AHL)-degrading bacteria in controlling the overall microbial activity in fish larvae, thus, aiming at improving the survival of turbort larvae in their first-feeding period.

2. Materials and methods

2.1. Source of bacteria and growth conditions

Microbial communities were collected from the digestive tract of healthy Pacific white shrimp juveniles **Penaeus vannamei**, maintained in culture on formulated feeds at Ghent University, Belgium. The
digestive tract was removed from the shrimp body after dissection and was homogenized by means of a stomacher blender (Seward, UK). After homogenizing, the suspensions were centrifuged at 1600 g for 5 min, and the supernatant was preserved at −80 °C in 20% glycerol.

These microbial communities were used as seed material for isolating AHL-degrading bacteria (Tinh et al., 2007). Two enrichment cultures, originating from two different shrimp individuals, were obtained. They were made resistant to 100 mg l⁻¹ rifampicin and were preserved in 20% glycerol at −80 °C. The PCR-DGGE profile of the enrichment cultures used in this study. M: marker; 1: ECS-rifampicin-resistant; 2: ECS-rifampicin-sensitive; 3: EC3-rifampicin-resistant; 4: EC3-rifampicin-sensitive. was obtained. They were made resistant to 100 mg l⁻¹ rifampicin and were preserved in 20% glycerol at −80 °C.

These microbial communities were used as seed material for isolating AHL-degrading bacteria (Tinh et al., 2007). Two enrichment cultures, originating from two different shrimp individuals, were obtained. They were made resistant to 100 mg l⁻¹ rifampicin and were preserved in 20% glycerol at −80 °C. The PCR-DGGE profile of the enrichment cultures used in this study. M: marker; 1: ECS-rifampicin-resistant; 2: ECS-rifampicin-sensitive; 3: EC3-rifampicin-resistant; 4: EC3-rifampicin-sensitive.

Before hatching, the turbot larvae were distributed to 1000-ml beakers containing 500 ml of autoclaved seawater, at a density of 30 larvae per beaker. Neither aeration nor water exchange was provided during the entire experimental period. The water temperature was maintained at 16 °C.

2.2. Turbot eggs

Eggs of turbot (S. maximus L.) were obtained from a commercial hatchery (France Turbot, France) by artificial stripping and fertilization. Upon arrival, the eggs were first acclimatized to the temperature of the rearing water (16 °C). Afterwards, they were conditioned for 6 h in 5-l of UV-treated seawater. During the conditioning process, the water was exchanged 500% using UV-treated seawater.

After acclimatization, a calculated amount of eggs was collected on a 300-µm nylon sieve, and then washed two times with 4-l of autoclaved seawater to remove the residual organic matter, which may affect the efficacy of the disinfectants. Subsequently, the eggs were disinfected in 1-l solution containing 50 ppm of glutaraldehyde and 50 mg l⁻¹ of rifampicin, for 5 min. After disinfection, the eggs were rinsed two times with 4-l of autoclaved seawater to remove the residual disinfectants. Three sub-samples of 10 ml were withdrawn from the egg suspension to check the effectiveness of disinfection. The eggs samples were homogenized by means of a stomacher blender (Seward, UK) for 6 min and spread on Marine Agar (MA) (Difco, Detroit, USA) plates, which were subsequently incubated at 25 °C for 48 h.

After disinfection, the eggs were distributed to the glass cones which contained 800 ml of autoclaved seawater, at a density of 750 eggs l⁻¹. A mild aeration was provided, which passed through a 0.22-µm filter. At 16 °C, the eggs hatched approximately after 72 h.

2.3. Experiment setup

After hatching, the turbot larvae were distributed to 1000-ml beakers containing 500 ml of autoclaved seawater, at a density of 30 larvae per beaker. Neither aeration nor water exchange was provided during the entire experimental period. The water temperature was maintained at 16 °C. The light was kept at a very low intensity (0.070 mW cm⁻²). Once a day, the dead larvae were counted and removed from the beakers. On day 3 post-hatch, the larvae were fed was added (fed-batch culture) and the optical density of the culture was determined using a spectrophotometer (Thermo Spectronic). Two days before the experiment started, the enrichment cultures were acclimatized to 16 °C, which is the temperature of the rearing water during the experiment.

CV026 strain, a mini-Tn5 mutant derived from a Chromobacterium violaceum strain (Mcclean et al., 1987) was used as an AHL-reporter to detect the residual AHL concentration in the rearing water. This mutant cannot produce AHL, but it can detect and respond to a range of AHL molecules (with acyl chain of four to eight carbons), by inducing the synthesis of the purple pigment violacein. CV026 was grown in Luria–Bertani (LB) medium, containing tryptone (BD, France, 1% w/v), yeast extract (Sigma, Germany, 0.5% w/v) and NaCl (BD, France, 0.4% w/v). This medium was supplemented with 20 mg l⁻¹ of kanamycin.

### Table 1

Outline of the experiments conducted in this study

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>AHL addition&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rifampicin addition&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ECS (added to water)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Rifampicin + AHLs</td>
<td>ECS (added to water + AHLs)</td>
<td>EFS (added to water + bioencapsulated in rotifers)</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>AHL addition&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rifampicin addition&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ECS (added to water)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Rifampicin + AHLs</td>
<td>ECS (added to water + AHLs)</td>
<td>EFS (added to water + bioencapsulated in rotifers)</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>AHL addition&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ECS (added to water + bioencapsulated in rotifers)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ECS (added to water + bioencapsulated in rotifers)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ECS (added to water + AHLs)</td>
<td>EFS (added to water + bioencapsulated in rotifers)</td>
<td>EFS (added to water + bioencapsulated in rotifers)</td>
</tr>
</tbody>
</table>

Eight replicates were performed for each treatment in each experiment.

The control treatments had no addition of bacteria and AHL.

<sup>a</sup> AHL (N-acyl homoserine lactone) mixture (Table 2) was added daily to the water at 1 mg l⁻¹.

<sup>b</sup> Rifampicin was added on the first day at 10 mg l⁻¹.

<sup>c</sup> ECS and ECS (enrichment cultures containing a mixture of AHL-degrading bacteria obtained after 6 cycles of selective growth on AHL) were added daily to the water at 10⁶ CFU ml⁻¹, and/or bioencapsulated in the rotifiers for 60 min before feeding.
with rotifers at 3 rotifers ml\(^{-1}\). Subsequently, the feeding rate increased with 1 rotifer ml\(^{-1}\) on each following day. From day 7 onwards, the feeding rate was kept at 7 rotifers ml\(^{-1}\). To bioencapsulate ECs in the rotifers, the latter were immersed in an EC suspension (circa 5×10⁸ CFU ml\(^{-1}\)) for 60 min prior to feeding. After immersion, the rotifer-associated EC number was about 140 CFU rotifer\(^{-1}\).

In total, three experiments were conducted (Table 1). Eight replicates were performed for each treatment. No bacteria were added in the control treatment. One mg l\(^{-1}\) rifampicin was added on the first day at 10 mg l\(^{-1}\) of AHL mixture (Table 2) was added daily to the rearing water in the AHL treatment. Rifampicin was added once at 10 mg l\(^{-1}\), both to the egg incubating water and to the larval rearing water (experiment 1). ECs were added once to the egg incubating water, and daily to the rearing water at 10⁶ CFU ml\(^{-1}\), and/or bioencapsulated in the rotifers. The experiments were stopped as soon as the fish survival in one of the treatments dropped below 10%.

The residual AHL concentration in the rearing water was determined in the last experiment. Once a day, three samples of 10 ml of rearing water were taken from treatments 2, 4 and 6. Ten microvolumes from each sample was dropped on a LB agar plate, on which 50 μl of an overnight-grown CV026 culture had been spread plated. After incubation of LB plates at 28 °C for 24 h, the diameters of the purple-pigmented halos produced by CV026 were measured. The residual AHL concentration in the rearing water at each sampling time was extrapolated based on a standard curve relating the AHL concentration with the diameter of the violacein producing halo (Diameter of purple-pigmented halo=6.529 ln[AHL]+10.355).

### 2.4. Collection of water and fish samples for bacterial counts

Water samples (3×10 ml) were collected twice, i.e. one day after the first-feeding and on the last day of each experiment. Fish samples were collected on the last day of each experiment. In the last experiment, the fish larvae from treatments 3 and 5 were sampled further for five days after the experiment had been stopped, to determine the ability of EC3 and EC5 to colonize the fish gut. Three samples of ten larvae were taken randomly from all the replicates of each treatment. The fish larvae were collected on a sterile 250-μm sieve. Surface bacteria were removed according to a procedure described by Huys et al. (2001). The fish sample was first immersed in a benzocaïne solution (Sigma, 0.1% w/v) for 10 s, subsequently transferred to benzalkonium chloride solution (Sigma, 0.1% w/v) for another 10 s, and rinsed three times in autoclaved NSS for 5 s each time. Subsequently, the larvae were transferred to a sterile plastic bag containing 10 ml of NSS and homogenized by means of a stomacher blender for 6 min.

The water samples and the fish homogenates were serially diluted in NSS. Fifty microvolumes from each dilution was plated (spiral plater™, Spiral Systems Inc, USA) on Marine Agar and on Marine Agar supplemented with 100 mg l\(^{-1}\) of rifampicin, for enumerating the total culturable bacteria and enrichment cultures’ density, respectively. The plates were incubated at 16 °C for 48 h, then at 25 °C for another 48 h. The numbers of colonies were counted afterwards.

### 2.5. Data analysis

Parametric assumptions were tested using Shapiro–Wilk’s test for normality and Levene’s test for homogeneity of variances. In some cases, the survival data were arcsine-transformed to meet the assumptions. In the second step, the data were compared between treatments using one-way ANOVA, followed by Tukey test for homoscedastic data or a non-parametric test for non-homoscedastic data, at a significance level of 0.05. Two-way ANOVA was performed (at a significance level of 0.01) to determine the interaction between the experimental factors. The correlation between the residual AHL

### Table 2

<table>
<thead>
<tr>
<th>AHL molecule</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Butanoyl-DL-homoserine lactone</td>
<td>C₄-HSL</td>
</tr>
<tr>
<td>N-Butanoyl-DL-homoserine thiolactone</td>
<td>C₄-HST</td>
</tr>
<tr>
<td>N-Hexanoyl-DL-homoserine lactone</td>
<td>C₆-HSL</td>
</tr>
<tr>
<td>N-Heptanoyl-DL-homoserine lactone</td>
<td>C₇-HSL</td>
</tr>
<tr>
<td>N-Octanoyl-DL-homoserine lactone</td>
<td>C₈-HSL</td>
</tr>
</tbody>
</table>

### Table 3

Survival of turbot larvae on day 5 post-hatch, experiment 1 (mean±SE, n=8)

<table>
<thead>
<tr>
<th>Factor</th>
<th>AHL addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin addition</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>(Treatment 1) 15.5±5.0⁰</td>
</tr>
<tr>
<td>Rifampicin addition × AHL addition¹</td>
<td>(Treatment 2) 16.1±2⁰</td>
</tr>
<tr>
<td>+</td>
<td>(Treatment 3) 98.8±0.9⁰</td>
</tr>
<tr>
<td>P=0.005</td>
<td>(Treatment 4) 97.1±1.3⁰</td>
</tr>
</tbody>
</table>

Treatments with different superscripts are significant different from each other (P<0.05).

AHL: N-acyl homoserine lactone.

The AHL mixture was added daily to the water at 1 mg l\(^{-1}\).

¹Interaction between two factors “rifampicin addition” and “AHL addition”.

### Table 4

Survival of turbot larvae on day 8 post-hatch, experiment 2 (mean±SE, n=8)

<table>
<thead>
<tr>
<th>Factor</th>
<th>AHL addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECS addition</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>(Treatment 1) 35.0±12.2⁰</td>
</tr>
<tr>
<td>ECS addition × ECS addition to water (¹)</td>
<td>(Treatment 2) 5.9±1.8⁰</td>
</tr>
<tr>
<td>Only to water</td>
<td>(Treatment 3) 25.4±7.7⁰</td>
</tr>
<tr>
<td>To water and through bioencapsulated rotifers</td>
<td>(Treatment 4) 12.5±4.2⁰</td>
</tr>
<tr>
<td>P=0.867</td>
<td>(Treatment 5) 54.1±14.1⁰</td>
</tr>
<tr>
<td></td>
<td>(Treatment 6) 28.4±9.5⁰</td>
</tr>
</tbody>
</table>

Treatments with different superscripts are significant different from each other (P<0.05).

AHL: N-acyl homoserine lactone.

ECS: Enrichment culture containing a mixture of AHL-degrading bacteria obtained after 6 cycles of selective growth on AHL. The AHL mixture was added daily to the water at 1 mg l\(^{-1}\).

¹Interaction between two factors “AHL addition” and “ECS addition to water”.

ⅡInteraction between two factors “AHL addition” and “ECS addition in to water and through bioencapsulated rotifers”.

<Author's personal copy>
Table 5
Survival of turbot larvae on day 7 post-hatch, experiment 3 (mean±SE, n=8)

<table>
<thead>
<tr>
<th>Factor</th>
<th>AHL addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Enrichment culture addition</td>
<td>–</td>
</tr>
<tr>
<td>EC3</td>
<td>(Treatment 1) 93.1±2.9abc</td>
</tr>
<tr>
<td>EC5</td>
<td>(Treatment 3) 62.1±8.6b</td>
</tr>
<tr>
<td>AHL addition×EC3 addition1</td>
<td>(Treatment 5) 96.7±1.4a</td>
</tr>
<tr>
<td>AHL addition×EC5 addition2</td>
<td>(Treatment 6) 94.3±2.5abc</td>
</tr>
</tbody>
</table>

Treatments with different superscripts are significant different from each other (P<0.05).
AHL: N-acyl homoserine lactone.
EC1 and EC3: Enrichment cultures containing mixtures of AHL-degrading bacteria obtained after 6 cycles of selective growth on AHL.

In the second experiment, the effectiveness of the microbial enrichment culture EC3, added in different ways either in the presence or absence of AHL mixture, was verified. As shown in Table 4, in the absence of added AHLs (treatments 1, 3 and 5), no significant difference in larval survival on day 8 post-hatch was observed (P>0.05). In contrast, in the presence of AHLs, EC5 (added to the water and additionally bioencapsulated in the rotifers) (treatment 6) could significantly improve the survival of turbot larvae, as the survival in treatment 6 (94.3%) was significantly different (P<0.001) from that in treatment 2 (10.4%). However, a large variability within each treatment was noticed. There was no interaction (P>0.05) between the “AHL addition” and “EC5 addition” factors.

3. Results

3.1. Survival of turbot larvae

In total three experiments were conducted. The aim of the first experiment was to verify the effect of an AHL mixture added to the larval rearing water. In subsequent experiments, AHL-degrading microbial enrichment cultures, added to the water or supplied through rotifers, were investigated for their putative probiotic effect.

The proliferation of bacteria present in the turbot culture in treatments 3 and 4 (experiment 1) was restricted using the antibiotic rifampycin (no bacterial growth was detected in these treatments). Table 3 shows a relatively low survival of turbot larvae in the presence of AHLs and in the absence of rifampycin (1.67%) on day 5 post-hatch, compared to that in the control treatment (15.5%) (P<0.05). On the other hand, in the presence of rifampycin and AHLs (treatment 4), the survival was exceptionally high (97.1%) and was comparable to that in the absence of AHLs (98.8%). Hence in the presence of rifampycin, AHL addition had no effect. There was an interaction (P<0.01) between two factors, AHL addition and antibiotic addition.

In the second experiment, the effectiveness of the microbial enrichment culture EC3, added in different ways either in the presence or absence of AHL mixture, was verified. As shown in Table 4, in the absence of added AHLs (treatments 1, 3 and 5), no significant difference in larval survival on day 8 post-hatch was observed (P>0.05). In contrast, in the presence of AHLs, EC5 (added to the water and additionally bioencapsulated in the rotifers) (treatment 6) could significantly improve the survival of turbot larvae with respect to the control (only AHL addition, treatment 2) (P<0.05). However, a large variability within each treatment was noticed. There was no interaction (P>0.05) between the “AHL addition” and “EC5 addition” factors.

In the third experiment, we attempted to compare the effect of two enrichment cultures, EC3 and EC5, added to the turbot culture in both ways (Table 5). It is obvious that EC3 could not protect the turbot larvae from the detrimental effect of AHL addition, as the survival in treatment 2 (10.4%) and treatment 4 (2.1%) were not significantly different from each other (P>0.05). In contrast, EC5 was shown to be effective in protecting turbot larvae, as the survival in treatment 6 (94.3%) was significantly different (P<0.001) from that in treatment 2. In addition, there was a strong interaction (P<0.001) between “AHL addition” factor and “EC5 addition” factor, as the addition of EC5 could nullify the negative effect of AHL addition (treatment 5 and 6).

3.2. Bacterial counts

The EC numbers and the numbers of total culturable bacteria in rearing water and in fish gut were determined on the last day (day 7 post-hatch) of experiment 3 (Figs. 2 and 3). The addition of AHLs did
not stimulate the growth of enrichment cultures, as the EC numbers in the water and in the fish gut were similar in the last four treatments (circa 10^2 CFU larva^{-1} and 10^3 CFU ml^{-1}) in the fish gut and in the water, respectively. The numbers of total culturable bacteria in the water in the last four treatments increased considerably and reached approximately 5 x 10^6 CFU ml^{-1}. The EC numbers in the fish gut in treatments 3 and 5 were determined until day 12 post-hatch, to verify the ability of the ECs to colonize the fish gut. Therefore, during the post EC-feeding period (from day 8 to day 12), ECs were not added to the culture and the larvae were fed with non-bioencapsulated rotifers. Although the EC numbers decreased slightly after the EC addition had discontinued, both enrichment cultures were able to colonize the fish gut up to day 12, whereas EC3 was found at slightly higher numbers (Fig. 4).

### 3.3. Residual AHL concentration

The residual AHL concentration in the rearing water was also monitored during experiment 3. In all treatments, the residual AHL concentration before day 5 was below the detection limit (0.1 mg l^{-1}) by an AHL-reporter, C. violaceum CV026 strain (Fig. 5). This might be facilitated by chemical degradation of AHL molecules, known to happen at high pH (Yates et al., 2002) (pH value of the water was 7.95 on day 2). Microbial degradation in the absence of ECs can be excluded, since the residual AHL concentration value after 24 h of AHL addition in non-autoclaved seawater was the same as that for autoclaved seawater (data not shown). From day 5 post-hatch, the cumulative residual AHL concentration in treatment 2 (AHL addition) and treatment 4 (EC3 + AHL) started to increase, whereas the residual AHL concentration in treatment 6 (EC5 + AHL) was still below the detection limit up to day 6, indicating a strong biological degradation by EC5. On the other hand, the biological degradation by EC3 was considerably weaker, compared to that by EC5. It was noticed that the pH value of the water decreased with time and reached 7.50 on day 5. At this pH value, the chemical degradation is significantly reduced (Yates et al., 2002).

### 4. Discussion

For a stable production of marine larvae in intensive rearing systems, control of the proportion of r/K-strategists has been discussed as being more important than overall microbial control, since a proliferation of opportunistic (r-selected) bacteria under unfavourable environmental conditions may have an adverse effect on the survival of the larvae (Nicolas et al., 1989; Skjermo and Vadstein, 1999). On the other hand, it has been proposed that an r-selected probiotic bacterium with a short lag phase and a fast growth rate in intestinal mucus will be the most competitive in vivo (Vine et al., 2004). However, it was found that turbot larvae grew better when the proportion of fast-growing bacteria in the rearing water was low (Salvesen et al., 1999).

EC3 and EC5, which were used in our study, might be considered as K-strategists as they are slow-growing bacteria in in vitro conditions (unpublished data), however, their growth in intestinal mucus of turbot larvae was not tested.

A strong evidence for the involvement of bacteria in turbot larvae mortality was provided by Munro et al. (1995), who obtained very high turbot survival values in the absence of culturable bacteria (>55% after 14 days feeding on axenic rotifers). Our study confirmed the negative effect of the standing microbial community on the survival of turbots since the fish survival in the presence of an antibiotic was significantly higher than in the other treatments (experiment 1). However, according to Munro et al. (1994), there was no correlation between the bacterial load in the gut and the turbot larval survival, moreover, incidences of high mortality were not associated with high numbers of recognizable pathogens. In our study, similar numbers of total culturable bacteria were observed in the gut, although the fish survival varied between treatments. This further indicates that the number of bacteria is less important. Rather the type of microbes or the microbial activity in the fish gut might be the determining factor. In relation to the first possibility, Blanch et al. (1997) observed a clear succession of the bacterial communities that colonized the gut of turbot larvae from day 1 to day 90 post-hatching. A high heterogeneity of the Vibrio group was observed in the intermediate period when the highest mortalities of turbots larvae occurred. Overall, the composition of the gut microbiota, and not the number, directly affected the survival rate of turbot larvae.

In relation to the possible importance of microbial activity, we used a novel approach to control the microbial community and its activity in turbot larviculture. The enrichment cultures of AHL-degrading bacteria were used with the purpose of controlling the opportunistic bacteria, whose virulence might be mediated by the AHL-dependent quorum-sensing system. It is known that AHL-mediated quorum sensing systems regulate a range of important biological functions, including virulence, in many gram-negative bacteria (Ebert, 1999; Dong and Zhang, 2005; Julus et al., 2005). The mode of action of AHL molecules was verified in experiment 1, by using an exogenous source of AHL in combination with antibiotic treatment. The survival of turbots larvae in the presence of both AHL and rifampicin was comparable to that in the presence of rifampicin alone. This excluded the possibility that the AHL molecules are toxic to the fish larvae. On the other hand, the AHL treatment alone caused a remarkable reduction in the larvae survival. This is an indirect indication of the fact that, AHL molecules may stimulate the production of virulence factor of some opportunistic pathogenic bacteria present in the culture water and in the fish gut. Buch et al. (2003) found that AHL molecules were produced by most of the Vibrio anguillarum strains isolated from rainbow trout infected with vibriosis. However, no direct relation was found between the AHL production and the virulence of the strains. Our data also demonstrated that there was no significant difference in total bacterial count between the treatments with and without AHL.
addition (experiment 3). Thus, the introduction/secretion of AHL molecules (at the used concentration) does not stimulate the growth, but rather the virulence of opportunistic pathogenic bacteria.

The EC5 mixture used in this study was among the enrichment cultures which were isolated from P. vannamei shrimp gut and showed a strong AHL-degrading property in vitro (Tinh et al., 2007). EC5 was made resistant to rifampicin [for experimental purpose], so that its presence in turbot culture can be detected by plating the water/fish samples on a growth medium containing the respective antibiotic. It should be emphasized that, EC5 was only able to exhibit a probiotic effect when an exogenous source of AHL molecules was added, presumably stimulating the virulence of opportunistic pathogenic bacteria.

In the last experiment, we compared the effect of two enrichment cultures, EC3 and EC5. A strong negative correlation (P<0.001) was found between the residual AHL concentration in the water and the larval survival on the last day of the experiment (treatments 2, 4 and 6; Fig. 5 and Table 5) (it should be emphasized that the C. violaceum strain can detect all the added AHL molecules with more or less the same sensitivity). This indicates that an increase in fish survival due to the presence of an enrichment culture is related to its capacity to degrade the added AHIls. For instance, EC3 was shown not to be as effective in degrading AHIls in vivo, resulting in high larval mortality, despite the fact that it was detected in equal concentration in the gut as EC5 was.

We also investigated the capability of the ECs to colonize the fish gut. Since many fish pathogens are able to adhere to the mucosal surface of the fish gut (Olafsen and Hansen, 1992), mucosal adhesion was chosen as one of the five criteria for selection of probiotics in fish (Nikoskelainen et al., 2001). Moreover, long residence time of probiotics in the intestinal tract can prolong their potential benefit (Nikoskelainen et al., 2001). Moreover, long residence time of probiotics in the intestinal tract can prolong their potential benefit against the presence did not improve the survival of turbot larvae. The adhesion property of EC5 as a potential probiotic needs to be studied in more depth, using specific techniques such as immunohistochecmistry.

Most of the beneficial bacteria administered in turbot larviculture are autochthonous (Huys et al., 2001; Hjelm et al., 2004a,b). However, a probiotic strain isolated from another organism might perform as well as the native-born one. Similarly, Ringø (1999) found that a strain of Carnobacterium divergens, isolated from Atlantic salmon, was able to colonize the gut of newly-hatched turbot larvae. This characteristic of EC5 may favor its possible application in different fish/shrimp species. On the other hand, there are several features that may restrict the application of EC5 as a probiont. First, the addition of EC5 may alter the phenotype expression of the AHL-producing resident (and eventually advantageous) microbiota. Secondly, EC5 may not be effective against those fish pathogens, for which virulence is not regulated by an AHL-mediated quorum sensing system. Hence, it will be interesting to investigate the effectiveness of EC5 in the absence of AHL addition, under lab-scale as well as hatchery conditions. Due to the inherent large variability in larval survival between replicates, this type of experiments will need to be set up with a sufficient number of replicates.

Acknowledgements

This study was supported by a doctoral grant of the Research Fund BOF of Ghent University, Belgium (grant number B/03663-011D5502).

We thank France Turbot for kindly providing turbot eggs, and the technicians from the ARC for their assistance in providing algae and rotifer stock and in setting up the experiments.

References


Huys, L., Dhert, P., Rolles, R., Olevier, F., Sorgeloos, P., Swings, J., 2001. Search for beneficial bacteria in the intestinal tract can prolong their potential benefit against the presence did not improve the survival of turbot larvae. The adhesion property of EC5 as a potential probiotic needs to be studied in more depth, using specific techniques such as immunohistochecmistry.

Most of the beneficial bacteria administered in turbot larviculture are autochthonous (Huys et al., 2001; Hjelm et al., 2004a,b). However, a probiotic strain isolated from another organism might perform as well as the native-born one. Similarly, Ringø (1999) found that a strain of Carnobacterium divergens, isolated from Atlantic salmon, was able to colonize the gut of newly-hatched turbot larvae. This characteristic of EC5 may favor its possible application in different fish/shrimp species. On the other hand, there are several features that may restrict the application of EC5 as a probiont. First, the addition of EC5 may alter the phenotype expression of the AHL-producing resident (and eventually advantageous) microbiota. Secondly, EC5 may not be effective against those fish pathogens, for which virulence is not regulated by an AHL-mediated quorum sensing system. Hence, it will be interesting to investigate the effectiveness of EC5 in the absence of AHL addition, under lab-scale as well as hatchery con-


