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

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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 turbot larvae survival under the experimental conditions, *i.e.* when the survival of turbot 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 turbot larval survival might be caused by AHL-induced *in situ* production of virulence factors by uncharacterised opportunistic bacteria, while EC5 can counteract this effect. 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.

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 turbot larvae coincides with the start of feeding (Munro et al., 1994). Most bacterial species isolated from the intestinal tract of larval turbot belong to Vibrionaceae (Nicolas 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 turbot 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 turbot larvae, by rinsing of rotifers (Keskin and Rosenthal, 1994) or exposure of rotifers to ultraviolet radiation (Munro et al., 1999). The microbial environment of turbot 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 microbially-matured water containing microalgae could result in higher percentage of viable and fast-growing larval turbot. An alternative approach for microbial management of turbot larviculture involved the selection of beneficial bacteria (probiotic bacteria). These bacteria were isolated from the rearing environment of turbot 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 turbot 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 turbot 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

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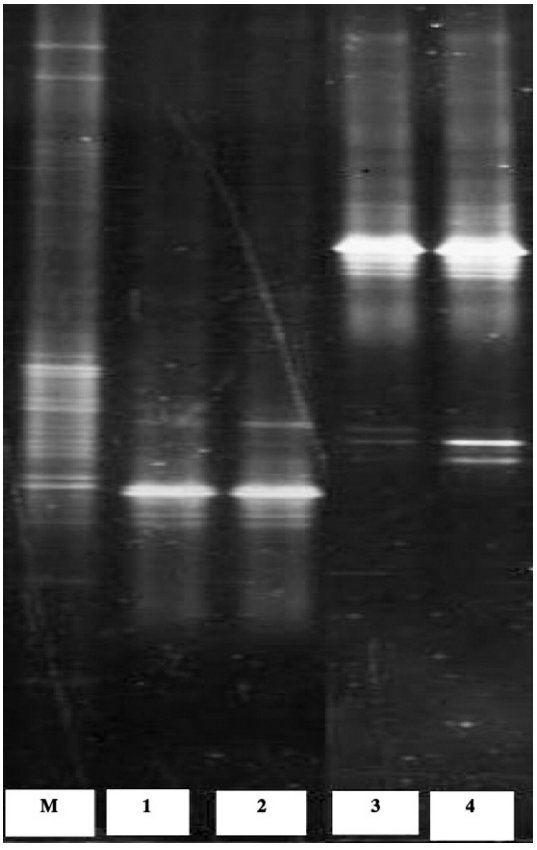

Fig. 1. PCR-DGGE profile of the enrichment cultures used in this study. M: marker; 1: EC5-rifampicin-resistant; 2: EC5-rifampicin-sensitive; 3: EC3-rifampicin-resistant; 4: EC3-rifampicin-sensitive.

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 profiles (Tinh et al., 2007) show that the rifampicin-sensitive and rifampicin-resistant enrichment cultures have the same dominant bands (Fig. 1). Before the experiment started, 200 µl of the stock cultures were inoculated into Nine Salt Solution (NSS, see composition in Tinh et al., 2006) supplemented with 113 mg l^{-1} of sodium acetate and 4 mg l^{-1} of ammonium chloride (C/N ratio=20). The enrichment cultures were grown in this medium for two weeks. Every two days, fresh medium 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., 1997) 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.

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

Table 1
Outline of the experiments conducted in this study

Exp.	Treatment					
	1	2	3	4	5	6
1	Control	AHL addition[a]	Rifampicin addition[b]	Rifampicin+AHLs		
2	Control	AHL addition[a]	EC5 (added to water)[c]	EC5 (added to water)+AHLs	EC5 (added to water+ bioencapsulated in rotifers)	EC5 (added to water+ bioencapsulated in rotifers)+AHLs
3	Control	AHL addition[a]	EC3 (added to water+ bioencapsulated in rotifers)[c]	EC3 (added to water+ bioencapsulated in rotifers)+AHLs	EC5 (added to water+ bioencapsulated in rotifers)	EC5 (added to water+ bioencapsulated in rotifers)+AHLs

Eight replicates were performed for each treatment in each experiment.
The control treatments had no addition of bacteria and AHL.
[a] AHL (*N*-acyl homoserine lactone) mixture (Table 2) was added daily to the water at 1 mg l^{-1}.
[b] Rifampicin was added on the first day at 10 mg l^{-1}.
[c] EC3 and EC5 (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 10^6 CFU ml^{-1}, and/or bioencapsulated in the rotifers for 60 min before feeding.