Letters in Applied Microbiology 2005, **40,** 274–277 doi:10.1111/j.1472-765X.2005.01676.x

Evaluation of candidate probiotic strains for gilthead sea bream larvae (*Sparus aurata*) using an *in vivo* approach

P. Makridis[1], S. Martins[1], T. Vercauteren[2], K. Van Driessche[3], O. Decamp[4] and M.T. Dinis[1]

[1]Center of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, Faro, Portugal, [2]Avecom NV, Bloemendalestraat, Beernem, Belgium, [3]Laboratory of Microbial Ecology and Technology (LabMET), Faculty of Agricultural and Applied Biological Sciences, Ghent University, Ghent, Belgium, and [4]INVE Technologies NV, Dendermonde, Belgium

2004/1365: received 29 November 2004 and accepted 7 December 2004

ABSTRACT

P. MAKRIDIS, S. MARTINS, T. VERCAUTEREN, K. VAN DRIESSCHE, O. DECAMP AND M.T. DINIS. 2005.

Aims: The aim of this study was to evaluate the effect of six bacterial strains on gilthead sea bream larvae (*Sparus aurata*).

Methods and Results: Six bacterial strains isolated from well-performing live food cultures were identified by sequencing fragments of their 16s rDNA genome to the genus level as *Cytophaga* sp., *Roseobacter* sp., *Ruergeria* sp., *Paracoccus* sp., *Aeromonas* sp. and *Shewanella* sp. Survival rates of gilthead sea bream larvae transferred to seawater added these bacterial strains at concentrations of $6 \pm 0{\cdot}3 \times 10^5$ bacteria ml^{-1} were similar to those of larvae transferred to sterilized seawater and showed an average of 86% at 9 days after hatching, whereas, survival rates of larvae transferred to filtered seawater were lower ($P < 0{\cdot}05$), and showed an average of 39%, 9 days after hatching.

Conclusion: Several bacterial strains isolated from well-performing live food cultures showed a positive effect for sea bream larvae when compared with filtered seawater.

Significance and Impact of the Study: The approach used in this study could be applied as an *in vivo* evaluation method of candidate probiotic strains used in the rearing of marine fish larvae.

Keywords: aquaculture, fish, live food, microbial control, rotifer cultures.

INTRODUCTION

In previous studies, evaluation of bacterial strains as potential probiotics for use in aquaculture has been based on the use of tests *in vitro*, as inhibition of pathogenic bacteria, growth in fish mucus, as well as the production of micronutrients, siderophores and enzymes (Olsson *et al.* 1992; Austin *et al.* 1995; Gatesoupe *et al.* 1997; Jöborn *et al.* 1997; Gatesoupe 1999; Gram *et al.* 1999). The characteristics *in vitro* of bacterial strains do not always reflect, however, the ability of the specific strains to have a positive effect on the fish during standard rearing conditions. In the case of marine fish larvae, heavy mortalities observed during the first feeding stage are seldom attributed to specific pathogens (Munro *et al.* 1994). Selection of probiotic bacteria for the larval stages based entirely on inhibition *in vitro* of fish pathogens would therefore be erroneous.

Correspondence to: Pavlos Makridis, Center of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8000-117 Faro, Portugal (e-mail: makridis@ualg.pt).

During the development of pelagic marine fish eggs the embryo is protected by the eggshell, so most bacteria are unable to infect it. Large numbers of bacteria can, however, colonize the egg surface (Hansen and Olafsen 1999). The nutrients released during hatching induce proliferation of opportunistic bacteria, which may cause problems for the larvae. The gut in newly hatched larvae is relatively devoid of bacteria. Therefore, there are favourable conditions for the establishment of opportunistic and possibly harmful bacteria on the epithelial surfaces of the gut. During the first feeding of larvae in intensive aquaculture conditions, large amounts of nutrients are added to the live food cultures, so the presence of opportunistic bacteria is commonly observed. Several studies related to marine fish larviculture have attempted to find an optimal mixture of bacteria that has a positive effect on the live food cultures, limits the

growth of opportunistic species and is not harmful to the fish larvae (Rombaut *et al.* 1999; Verschuere *et al.* 1999; Gomez-Gil *et al.* 2000).

The main object of this study was to determine whether specific bacterial strains with a possible beneficial effect on live food cultures, have any negative or positive effects on gilthead sea bream larvae.

MATERIAL AND METHODS

Six bacterial strains isolated from well-performing live food cultures were used in this study. The strains isolate 8, Mon2A, isolate 10, isolate 11 and I-strain were isolated from cultures of rotifers (*Brachionus plicatilis*), whereas LVS3 was isolated from *Artemia franciscana* cultures as described in Rombaut *et al.* 1999 and Verschuere *et al.* 1999 respectively. Briefly, the rotifers and *Artemia* were rinsed in autoclaved seawater, homogenized in sterile nine salt solution and serial dilutions were plated on marine agar under sterile conditions. The bacterial strains isolated were pure cultured, added 15% glycerol and stored at −80°C.

All bacterial strains were grown on marine agar 2216 dishes and 2–3 mg wet weight of each culture was added to 200 μl sterilized water and was boiled for 10 min. The universal 16S rDNA primers p63f (5′-CAG-GCC-TAA-CAC-ATG-CAA-GTC-3′) and p1378r (5′-CGG-TGT-GTA-CAA-GGC-CCG-GGA-ACG-3′) were used to amplify the 16S ribosomal genes, where 1 μl of the boiled product was added to 24 μl of the PCR master mixture. This master mixture was composed of the following reagents: 0·2 μM of each of the two primers, 200 μM of each deoxynucleotide triphosphate, 10x *Taq* DNA polymerase reaction buffer, 2·5 U 100 μl^{-1} of *Taq* DNA polymerase (Promega, Madison, WI, USA), 400 ng μl^{-1} bovine serum albumin (Hoffmann-La Roche, Basel, Switzerland), DNase- and RNase-free water (Sigma-Aldrich Chemie, Steinheim, Germany), and 1·5 mM $MgCl_2$.

The thermal cycler (Biozym Minicycler^{TM}, Landgraaf, The Netherlands) was programmed for 5 min at 94°C, followed by 30 cycles of 1 min at 95°C (denaturation), 1 min at 53°C (annealing) and 2 min at 72°C (elongation), followed by a final extension of 10 min at 72°C, and terminated with a decrease in temperature to 4°C to stop the PCR. The size of the PCR products was verified on a 1·2% agarose gel (Sambrook *et al*. 1989). The fragments of 16S rDNA of each RMBC strain were bidirectionally sequenced by IIT Universitaet Bielefeld (Germany). The results of the sequencing were used for homology searches by BLAST (Basic Local Alignment Search Tool; Altschul *et al.* 1990). Two databases were used: the NCBI database, http://www.ncbi.nlm.nih.gov, a large database containing sequences of different organisms and the ribosomal database project II, http://rdp.cme.msu.edu, a smaller database containing ribosomal DNA.

The effect of the six bacterial strains on unfed gilthead sea bream larvae was evaluated using a protocol similar to Bergh *et al.* (1992). All bacterial strains were cultured in tryptic soya broth supplemented with 2% NaCl (w/v) for 24 h. The concentration of bacterial cells in the cultures was determined by measuring OD_{600}. The bacterial cells were harvested by centrifugation, suspended in sterilized seawater of the same volume, and thereafter diluted in sterilized seawater. In a first preliminary experiment, two concentrations were tested, $\pm 5 \times 10^5$ cells ml^{-1} and $\pm 5 \times 10^6$ cells ml^{-1}, which will be referred as low concentration and high concentration respectively. Fertilized gilthead sea bream (*Sparus aurata*) eggs were obtained from a commercial hatchery (Timar, Portugal). The eggs were rinsed with filtered seawater and transferred to 24-well dishes, and two dishes were used for the testing of each bacterial strain. The bacteria were added at a high concentration to the first 12 wells of each dish, and at a low concentration to the other 12 wells. A single egg was transferred to each well in 1 ml of the diluted culture. Two dishes with sterilized seawater without bacteria were used as controls. The dishes were kept in the dark, dead eggs or larvae were removed from the dishes daily, and mortality and temperature were recorded.

In a second experiment, six-well polystyrene dishes were used and only the low concentration of bacteria was applied ($\pm 5 \times 10^5$ cells ml^{-1}). Three dishes were used as replicates for each bacterial strain tested, and 5 ml of the diluted cultures were added to each well. In addition, there were two different control treatments, where sterilized and filtered nonsterilized seawater were used, respectively, with three replicates each. The eggs were rinsed with filtered seawater, and about five eggs were transferred to each well. The eggs and the hatched larvae were kept in the dark. Dead eggs and larvae were removed from the eggs daily and the temperature was recorded. Samples were taken from the diluted cultures and the filtered seawater to determine the number of colony-forming units (CFU). Tenfold dilutions in sterilized 80% seawater were plated in Petri dishes with tryptic soya agar supplemented with 2% (w/v) NaCl.

RESULTS

The six bacterial strains used in this study were identified to the genus level as *Cytophaga* sp. (isolate 8), *Roseobacter* sp. (isolate 10), *Ruergeria* sp. (isolate 11), *Paracoccus* sp. (Mon2A), *Aeromonas* sp. (LVS3) and *Shewanella* sp. (I-strain). These bacterial strains will further be referred to by their genus name only.

During the first experiment, the hatching percentage was similar in all treatments (76%), indicating that the addition of bacteria in the wells had no influence on the hatching rate.

The survival of the gilthead sea bream larvae was calculated as a percentage of the yolk sac larvae present at the end of the day of hatching (Figs 1 and 2). High mortalities occurred on day 6 after hatching and there onwards. Larvae in the presence of bacterial strains *Paracoccus* and *Roseobacter* at low and high concentration showed a higher survival rate than in sterilized water during this period, but the differences from the other treatments were not significant ($P > 0{\cdot}05$). Larvae in seawater added *Cytophaga* at low and high concentrations showed increased mortalities compared with the control, but again this differences in mortalities were not significant ($P > 0{\cdot}05$). In general, the concentration of bacteria used had no influence on the mortality rates. However, aggregates of bacteria were observed in the wells of the high concentration treatments, so only the low concentration treatment was used in the second experiment.

The hatching rate was higher in the second experiment (94%), which indicated that the egg batch was of better quality than in the first experiment. The hatching rate was nevertheless lower in the filtered seawater treatment (80%) ($P < 0{\cdot}05$). Mortality rates of sea bream larvae transferred to seawater added bacteria followed a similar pattern as in the first experiment, although there was a delay compared with the first experiment, as the highest mortalities were observed from day 8 after hatching and onwards (Fig. 3). Survival rates of gilthead sea bream larvae transferred to seawater added bacteria were similar to those of larvae transferred to sterilized seawater and showed an average of 86% at 9 days after hatching, whereas, survival rates of larvae transferred to filtered seawater were lower than in the other treatments ($P < 0{\cdot}05$), and showed an average of 39%, 9 days after hatching. The initial bacterial density in filtered seawater was $3{\cdot}5 \pm 0{\cdot}3 \times 10^3$ bacteria ml^{-1}, so it was much lower compared with the treatments added bacteria, where the bacterial concentration was on average $6 \pm 0{\cdot}3 \times 10^5$ CFU ml^{-1}.

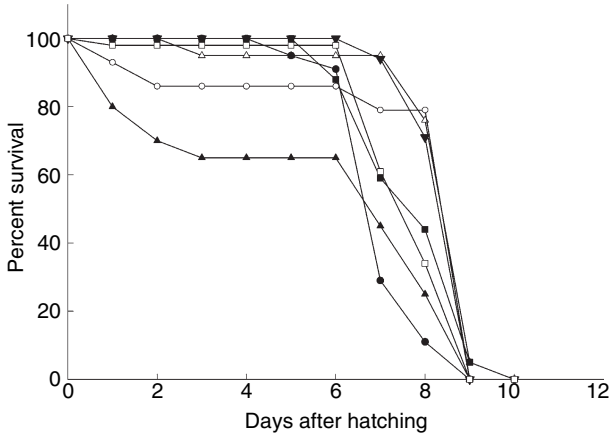

Fig. 1 Survival of starving gilthead sea bream larvae in the presence of different bacteria at a high concentration during the first experiment. (△) *Paracoccus*; (■) *Aeromonas*; (○) *Shewanella*; (●) *Cytophaga*; (▼) *Roseobacter*; (▲) *Ruergeria*; (□) control

DISCUSSION

The bacterial strains used in this study have been earlier evaluated as candidate probiotic bacteria for live food cultures. Even if such probiotic bacteria have a positive effect on the live food cultures, they may have some deleterious effect on fish larvae. The simple approach used in this study made possible a quick assessment of their effect on gilthead sea bream larvae. The larvae used in this study were unfed and as they depleted their energy reserves, they all eventually died. However, the

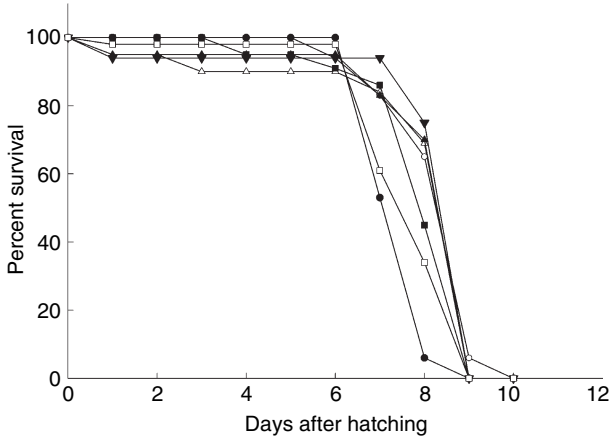

Fig. 2 Survival of starving gilthead sea bream larvae in the presence of different bacteria at a low concentration during the first experiment. (△) *Paracoccus*; (■) *Aeromonas*; (○) *Shewanella*; (●) *Cytophaga*; (▼) *Roseobacter*; (▲) *Ruergeria*; (□) control

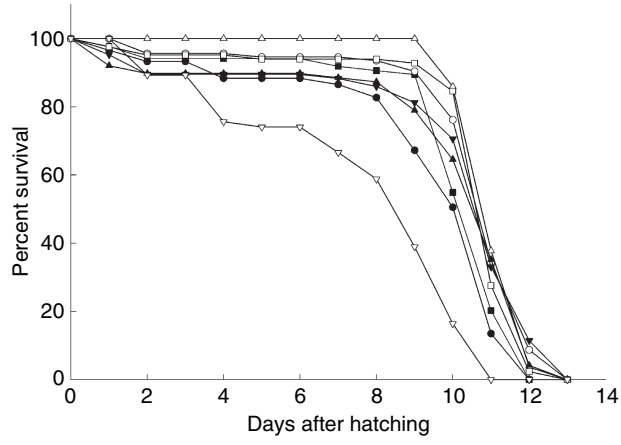

Fig. 3 Survival of starving gilthead sea bream larvae in the presence of different bacteria at a low concentration during the second experiment. (△) *Paracoccus*; (■) *Aeromonas*; (○) *Shewanella*; (●) *Cytophaga*; ▼ *Roseobacter*; (▲) *Ruergeria*; (□) control 1; (△) control 2

effect of the different treatments could be assessed by the differences in the rate of mortality during the experimental period. The use of sterilized water in the control treatment was not equivalent to sterile conditions, because a number of bacterial probably adhered to the gilthead sea bream eggs (Hansen and Olafsen 1999) when the eggs were transferred to the dishes. These bacteria could have proliferated in the wells in the presence of the nutrients released by the larvae. The presence of the added bacteria might have limited the growth of these opportunistic bacteria (Jöborn *et al.* 1997; Gatesoupe 1999). In the treatment with filtered seawater the bacteria present upon the transfer of the larvae probably proliferated in high numbers and caused the increased mortalities shown in this treatment. The numbers of bacteria present in the treatments with added bacteria were much higher than in the dishes with added filtered seawater, but the mortalities were lower in the treatments added bacteria. This indicated that the species composition of the bacterial communities present may be the most important issue and not merely the total numbers of bacteria (Makridis *et al.* 2000). Normal practice in commercial hatcheries never includes use of sterilized seawater, so the more realistic control treatment in this experiment was the filtered seawater treatment. The addition of bacteria significantly improved the survival of sea bream larvae compared with this control treatment. This method, after the necessary adaptations, could be used for the evaluation of probiotics bacteria in other species as well.

ACKNOWLEDGEMENTS

The study was part of a research project titled 'The use of revolving multifunctional bacterial communities for bacterial management in marine fish larvae' with reference CRAFT-Q5CR-2002-72221, which was financed by the European Community. We thank Peter C. Hubbard for reviewing the manuscript.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic logical alignment search tool. *J Mol Biol* **215**, 403–410.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I. and Griffith, D.R.W. (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *J Fish Dis* **18**, 93–96.

Bergh, Ø., Hansen, G.H. and Taxt, R.E. (1992) Experimental infection of eggs and yolk sac larvae of halibut, *Hippoglossus hippoglossus* L. *J Fish Dis* **15**, 379–391.

Gatesoupe, F.J. (1999) The use of probiotics in aquaculture. *Aquaculture* **180**, 147–165.

Gatesoupe, F.J., Zambonino Infante, J.L., Cahu, C. and Quazuguel, P. (1997) Early weaning of seabass larvae, *Dicentrarchus labrax*: the effect on microbiota, with particular attention to iron supply and exoenzymes. *Aquaculture* **158**, 117–127.

Gomez-Gil, B., Roque, A. and Turnbull, J.F. (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* **191**, 259–270.

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I. and Nielsen, T.F. (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Appl Environ Microbiol* **65**, 969–973.

Hansen, G.H. and Olafsen, J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. *Microb Ecol* **38**, 1–26.

Jöborn, A., Olsson, J.C., Westerdahl, A., Conway, P.L. and Kjelleberg, S. (1997) Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. *J Fish Dis* **20**, 383–392.

Makridis, P., Fjellheim, A.J., Skjermo, J. and Vadstein, O. (2000) Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. *Aquacult Int* **8**, 376–380.

Munro, P.D., Barbour, A. and Birkbeck, T.H. 1994. Comparison of the gut bacterial flora of start-feeding larval turbot reared under different conditions. *J Appl Bacteriol* **77**, 560–566.

Olsson, J.C., Westerdahl, A., Conway, P.L. and Kjelleberg, S. (1992) Intestinal colonization potential of turbot (*Scophthalmus maximus*) – and dab (*LImanda limanda*) – associated bacteria with inhibitory effects against *Vibrio anguillarum*. *Appl Environ Microbiol* **58**, 551–556.

Rombaut, G., Dhert, P., Vanderberghe, J., Verschuere, L., Sorgeloos, P. and Verstraete, W. (1999) Selection of bacteria enhancing the growth of axenically hatched rotifers (*Brachionus plicatilis*). *Aquaculture* **176**, 195–207.

Sambrook, J., Fritsch, E.F and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Plainview, New York.

Verschuere, L., Rombaut, G., Huys, G., Dhont, J., Sorgeloos, P. and Verstraete, W. (1999) Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. *Appl Environ Microbiol* **61**, 29–35.