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Pediococcus acidilactici in the culture of turbot (*Psetta maxima*) larvae: Administration pathways

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ABSTRACT

Probiotic administration to marine fish larvae and live prey has been shown to be a useful means to prevent bacterial infection and mass mortalities. Here, several experiments were carried out in order to determine the optimum protocol for *Pediococcus acidilactici* delivery to the turbot (*Psetta maxima*) gastrointestinal tract by its administration via rotifers supplemented for 1 or 24 h with the bacteria or directly via the water. The survival capacity of *P. acidilactici* in sterile seawater, in microalgae (*Isochrysis galbana*) culture and in rotifers (*Brachionus plicatilis*) was determined, with best results in rotifer culture.

The incorporation of *P. acidilactici* into the rotifer's gut was time-dependant, with *P. acidilactici* recovery from rotifer cultures supplemented with the lactic acid bacteria suspension for 24 h being significantly lower than from cultures supplemented for 1 h. Similarly, *P. acidilactici* recovery from the gastrointestinal tract of turbot larvae was higher when larvae were fed with 1-h supplemented rotifer cultures. However, significantly higher *P. acidilactaci* recovery from turbot larvae gastrointestinal tract was observed when the probiotic was directly administered in the rearing water.

Lastly, the bactericidal activity of the extratracellular products (ECPs) of *P. acidilactici* against a pathogenic bacterium for turbot, *Vibrio splendidus* (Hm 112) was assessed. The ECPs of *P. acidilactici* significantly inhibited the growth of the pathogenic bacteria *in vitro*. However, during *in vivo* experiments, the bacterial community, dominated mainly by *Vibrio* and *Pseudomonas*, was not apparently affected by *P. acidilactici* supplementation in rotifers or turbot larvae.

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1. Introduction

One of the most important difficulties encountered in commercial fish hatcheries has been larval mortalities attributed to opportunistic and pathogenic bacteria (Verschuere et al., 2000b). Traditionally, the impact of pathogens has been controlled or diminished using chemical agents such as antibiotics, resulting in the appearance of bacterial resistance that may have a negative impact on the final consumer (Huys et al., 2007; Smith et al., 1994). However, other management methods have emerged in recent years to overcome these problems. One alternative providing encouraging results is the administration of probiotics (Balcázar et al., 2006; Brunt and Austin, 2005; Gatesoupe, 2008; Vine et al., 2006), which are defined as "microbial cells that are administered in such a way as to enter the gastrointestinal tract to be kept alive, with the aim of improving health" (Gatesoupe, 1999). How probiotics work in fish is not completely understood; although it is widely accepted that selected probiotic bacteria can act as antagonistic agents to control populations of potential pathogens through competitive exclusion (Gatesoupe, 1999; Chabrillon et al., 2005; Villamil et al., 2003b; Vine et al., 2004) or are able to stimulate the host immune system (Kim and Austin, 2006; Díaz-Rosales et al., 2006; Picchietti et al., 2007; Panigrahi et al., 2004; Salinas et al., 2005; Villamil et al., 2002, 2003a). In addition, culture supplementation with probiotics has also been associated with a significant increase in growth rate of rotifers, an important prey species of fish which may have additional value when containing probiotics (Rombaut et al., 1999). Lactic acid bacteria (LAB) have been proposed as good probiotic candidates to be used in aquaculture because they have been extensively studied and are considered as GRAS (generally recognized as safe) microorganisms and therefore the need for further biosafety trials is reduced (Holzapfel et al., 1995). LABS are Gram positive, non-spore forming cocci or rods that produce lactic acid as their main metabolic product. Several studies have shown that LAB are able to colonize the stomach and intestines of fish (Ringø and Gatesoupe, 1998; Ringø and Strøm, 1994; Robertson et al., 2000). In some cases it has been demonstrated that their administration conferred protection to fish experimentally infected with

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bacteria (Gatesoupe, 1994; Gildberg et al., 1997; Robertson et al., 2000; Pirarat et al., 2006; Salinas et al., 2005) and that they are also able to inhibit pathogenic bacterial growth (Gildberg et al., 1995; Lewus et al., 1991). LAB are well known by their ability to produce bacteriocins, which are peptides commonly used in the food industry to prevent food spoilage and to inhibit the growth of pathogenic bacteria (Stiles, 1996). In the case of *P. acidilactici*, the main bacteriocin produced is pediocin, a small peptide with a broad inhibitory spectrum against microorganisms, including Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas*. This bacteriocin is stable over a wide pH range (3–8) and apparently most active at pH 4.0–5.0 (Jamuna and Jeevaratnam, 2004).

During the early stages of development, manipulation of the larval digestive system in aquaculture is an important tool to manage bacterial infections, by preventing the colonization and exclusion of pathogenic bacteria (Verschuere et al., 1999; Verschuere et al., 2000a) which could be achieved by the addition of probiotics either through the culture water or live food.

In this investigation, we looked at the bactericidal activity of the extracellular products (ECPs) of *P. acidilactici* against a pathogenic bacterium for turbot (*Psetta maxima*), *V. splendidus* (Hm 112) (Villamil et al., 2003c). Specifically, *P. acidilactici* was evaluated in the present study since it had previously been shown to increase both rotifer maximum density and specific maximum growth (Planas et al., 2004). In addition, this genus has been described as a probiotic for marine fish larvae (Gatesoupe, 2002) and juvenile fish (Aubin et al., 2005; Merrifield et al., 2010). In addition, the survival capacity of *P. acidilactici* in marine water as well as in *Isochrysis galbana* and in rotifer culture as well as its efficiency in the colonization of the gastrointestinal tract of turbot larvae was also evaluated using two main approaches; probiotic administration *via* water or *via* rotifer previously supplemented for 1 h and 24 h with *P. acidilacici*.

2. Materials and methods

2.1. Probiotic bacteria

Pediococcus acidilactici (NRRL B-5627) was obtained from the Northern Regional Research Laboratory, USA. It was grown overnight at 30 °C in Man Rogosa and Sharpe broth (MRS), washed and resuspended in sterile phosphate saline buffer (PBS 1×). *Vibrio splendidus (*Hm 112) (Villamil et al., 2003c), isolated from diseased turbot larvae, was cultured at room temperature on Marine Agar (MA). For long-term preservation, cultures were frozen at −80 °C in Tryptone Soy Broth supplemented with 1% of NaCl (TSB-1) or MRS with glycerol 15% (v v^{-1}).

2.2. Antibacterial activity of extracellular products (ECPs) from *Pediococcus acidilactici*

The ECPs extraction from *P. acidilactici* was performed according to Cabo et al. (1999). Briefly, *P. acidilactici* was grown overnight at 30 °C in MRS broth; after incubation, the pH was adjusted to 3.5 with 2 N HCl, then heated at 80 °C for 3 min and centrifuged at 500×*g* for 30 min. The supernatant was filtered through 0.45 µm, buffered at pH 6.0 and stored in aliquots at −80 °C. *Vibrio splendidus* (Hm112) was grown overnight at room temperature in TSB-1. The assay was performed in triplicate in a 96-well plate by dispensing 50 µl of the bacterial suspension (10^8 and 10^9 bacteria ml^{-1}) per well with 50 µl of the corresponding ECP treatment (undiluted or previously half diluted). Controls were included by incubating the bacteria with MRS instead of ECPs. After 15 h of incubation, changes in optical density (600 nm) were measured and the percentage of bacterial survival determined.

2.3. Rotifers and turbot larvae

Rotifers (*Brachionus plicatilis*) were cultured at 23 °C in 20-l conical tanks with seawater (20 ppt salinity) with gentle aeration. Rotifers were fed on baker's yeast (*Saccharomyces cerevisiae*) and subsequently enriched for 6 h with *I. galbana*.

Turbot eggs were provided by Stolt Sea Farm (Prodemar S.A, Spain), and incubated at 14 °C in flat-bottomed circular tanks with gentle aeration. Newly hatched larvae were transferred to 60-l tanks at a density of 40 larvae/l. The temperature was progressively increased up to 18 °C during the following 2 days. From mouth opening (day 3), the larvae were fed on rotifers. The level of rotifers was adjusted twice daily at 5 rotifers ml^{-1}.

2.4. Survival of *Pediococcus acidilactici* in seawater, microalgae and rotifer

In order to determine the survival capability of *P. acidilactici* under different environmental conditions, the bacterium was inoculated at 1×10^7 bacteria ml^{-1} in sterile flasks containing 100 ml of autoclaved and filtered seawater. Separately it was also inoculated in 100-ml flasks containing the microalgae *I. galbana* and rotifer cultures (50 rotifers ml^{-1}). Flasks were gently aerated and maintained at room temperature (RT) for 8 days. Samples of water of each source were daily taken and plated on MRS agar to determine the number of cultivable bacteria during the following 8 days.

2.5. Delivery of *Pediococcus acidilactici* to turbot larvae

In order to determine the most effective way of *P. acidilactici* administration to turbot larvae in terms of maximum LAB incorporation into the gastrointestinal tract of turbot larvae 3 days ph, three treatments were assayed. In the first treatment, *P. acidilactici* was administered to turbot larvae via rotifers which were previously maintained in a *P. acidilactici* suspension (1×10^8 CFU ml^{-1}) for 1 h. In the second treatment, the rotifers were maintained in the same conditions but during a longer time period of 24 h. In the third treatment, *P. acidilactici* was directly administered (1×10^8 CFU ml^{-1}) to seawater of the larval rearing tanks. In all cases larval survival and dry weight (60 °C during 48 h) was determined at 10 days ph.

2.6. Sample processing for bacterial count in rotifers and turbot larvae

Samples of rotifers were filtered (100-µm mesh), rinsed with 10 ml of sterile seawater (SSW), resuspended in 1 ml of SSW, aseptically homogenized in a glass homogenizer and aseptically transferred to sterile tubes. In order to determine the bacterial load in rotifers, samples were serially diluted and 100 µl of each dilution were plated in MRS agar and MA, plates were then incubated for 5 days at room temperature. After this time, the colonies were counted and the amount of bacteria was calculated as Colony Forming Units (CFU) per ml.

In the case of turbot larvae, bacterial recovery was conducted from the gastrointestinal tract according to Muroga et al. (1987). Larvae (10 fish per sample) were placed in sterile tubes and anaesthetised with MS-222. The seawater was completely removed and larvae were treated for 60 s with 0.1% (w v^{-1}) benzalkonium chloride in SSW. The larvae were subsequently washed in SSW to remove the chemical product, aseptically transferred to a sterile glass homogenizer, and homogenised in 1 ml of SSW. The suspension was then diluted and plated on MRS agar and on MA. In order to compare the efficiency of the benzalkonium chloride external disinfection, extra samples of 10 larvae were taken from each treatment, rinsed several times with SSW and plated as described before.

Because no bacterial growth was observed on MRS agar in rotifers nor in turbot larvae not treated with LAB, it was assumed that all

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