P-115	**Encapsulation of a potential fish probiotic bacteria in Ca-alginate beads**	

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INTRODUCTION AND OBJECTIVE

Probiotics are live microorganisms that administered to the digestive tract of fish promote beneficial effects on their health and growth (Balcázar *et al*. 2006). The efficiency of probiotics depends on many factors, like their stability during feed processing and storage, the amount administered, as well as their viability in the fish gut (Gbassi *et al*. 2009). Colonization of the intestine by exogenous probiotics is influenced by many factors, including the capacity of the live cells to resist the acid and alkaline environment found in the fish digestive tract. Encapsulation is currently gaining attention as a method to increase the viability of probiotics in acid conditions. Among the available techniques for immobilizing living cells, entrapment in calcium alginate beads has been frequently used for the immobilization of probiotic lactic bacteria. Pdp 11 is a potential probiotic bacteria isolated from *Sparus aurata* skin identified as *Shewanella putrefaciens* (García de la Banda *et al*. 2009). However, in previous studies free cells were directly added to the feed and this fact generates doubts related to the final number of viable bacteria present in the lumen gut. Consequently, a key topic that needs improvement is related to the optimization of the probiotic administration procedure, with the purpose of guarantee effective bacterial concentrations into the fish gastro-intestinal tract.

Under this perspective, the aim of the present study was to evaluate the survival rate of Pdp 11 cells encapsulated in alginate, compared to that of free cells, during their exposure to *in vitro* digestive simulation.

MATERIAL AND METHODS

Pure culture of Pdp 11 cells were grown in tryptone soya agar supplemented with NaCl (TSA) at 22 ºC. Cells were encapsulated in sodium alginate matrix (2%). In brief, 1.5 mL of the alginate solution, 0.35 mL of sterile 0.85% NaCl and 0.15 mL of cell suspension containing 10.0 ~ 0.2 log10 CFU/mL were placed in a sterile syringe and added dropwise while stirring magnetically (100 rpm) through a 18-G needle into sterile 2% $CaCl_2$. After 10 min gelation, the beads were filtered, rinsed and kept in sterile NaCl solution until use. The weight and size of one hundred of beads were measured. In order to determine the viable counts of the entrapped cells into beads, quadruplicates of three capsules were re-suspended into 0.6 mL of sterile $NaHCO_3$ followed by homogenization and gentle shaking at room temperature. The homogenized samples were diluted to appropriate concentrations and the viable bacteria enumerated as log10 CFU/bead. For the preparation of digestive juices, *S. senegalensis* specimens ranging 15-20 g were sacrificed according to the requirements of the Council Directive 86/609/EEC and sterile enzyme extracts were obtained. The survival rate of encapsulated bacteria was evaluated after exposure to simulated fish digestive juice (1 mL of enzyme extract providing 100 U into 4 mL of sterile buffer). The simulated fish gastric conditions (SFGC) were performed from pH 2.0 to pH 7.0 whilst the simulated fish intestinal conditions (SFIC) in the range pH 7.0-12.0. For the assay, 50 beads or 0.15 mL of the free cell suspension containing similar CFU were incubated with the simulated digestive juices at 25 ºC for 120 min. The survival evolution of encapsulated or free cells was evaluated. All data were analysed by one-way ANOVA. Differences between means at $P<0.05$ were assessed using the LSD test.

RESULTS AND DISCUSSION

Beads formation consisted in a single-step process based on the gelation of alginate in the presence of calcium chloride solution. The stability of beads in marine water during 180 min was confirmed (data not shown). The cell loading in bead after encapsulation was found in the range of 7.4 to 8.1 log10 CFU/bead. The mean diameter and wheat weight of beads were 3.7 ± 2.3 mm and 14.8 ± 1.7 mg, respectively. The loss of viable cells during the encapsulation and coating procedures was lower than 3%.

No significant decrease in viable counts were observed neither in free nor in encapsulated cells during the SFGC at pH 7, 6 and 5 for up to 2 h of acidic exposition (Fig. 1). However, survival of free cells was drastically reduced after 5 min of incubation at more acidic pH (Fig. 1a). Results obtained show that the encapsulation significantly improved the survival of bacteria at pH 4, since them remained totally viable after 120 min of incubation (Fig. 1b). The protection of probiotics within alginate beads under simulated gastric conditions was also described by Chandramouli *et al.* (2004). By contrast at more acidic conditions, viable cells were noticed only during the first 5 min of incubation at pH 3, and no survival was recorded when beads were incubated at pH 2. Sultana *et al.* (2000) also described that encapsulation of probiotic bacteria did not effectively protect the cells at high acidic conditions.

It was no found significant differences in the survival of free cells after 120 min of SFIC incubation at pH 7, 8 and 9, which indicates that these pH values had no effect on

the probiotic viability (Fig. 2a). Survival of free cells decreased after 5 min of incubation at pH 10. On the contrary, alginate encapsulation improves the survival of Pdp 11 cells at pH 10. In this case, after 5, 60 and 180 min of incubation it was found 100, 71 and 46% of viable bacteria within the beads, respectively. At pH 11, only viable cells was recorded in beads during the first 5 min of incubation, and no alginate protection was recorded when capsules were incubated at pH 12 (Fig. 2b). The immobilization of *B. bifidum* and *L. acidophilus* in alginate beads is not effective to protect these cells under extreme values of pH (Trindade and Grosso 2000).

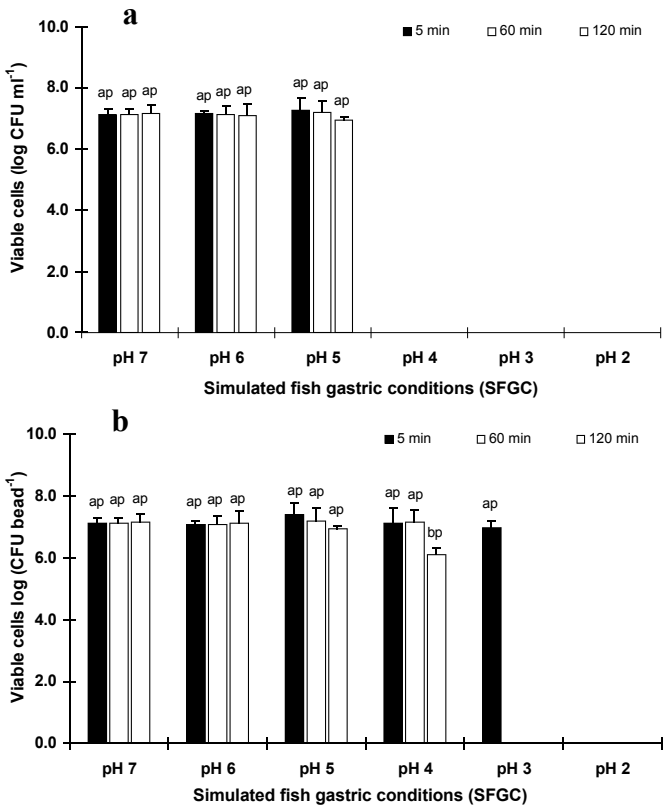

Fig. 1. Survival of free (a) and encapsulated (b) cells during exposure to SFGC.

Results obtained confirm that alginate encapsulation of probiotic cells improves their survival under simulated fish digestive conditions. However, encapsulation did not effectively protect the microorganisms at very extreme pH values. Despite this fact, most of aquacultured fish have a gastro-intestinal pH ranging from 3.5 to 9.5 (Chakrabarti *et al*. 1995). Taking this fact into account, it is reasonable to expect that under *in vivo* conditions, the encapsulated probiotic bacteria could keep their viability within the fish digestive tract.

CONCLUSIONS

Alginate encapsulation protects the bacterial cells during *in vitro* exposure at the gastro-intestinal conditions similar to those found in the digestive tract of fish. So, alginate beads could be a cheap, safe and protective delivery vehicle to administrate orally probiotic bacteria for fish species with interest in aquaculture.

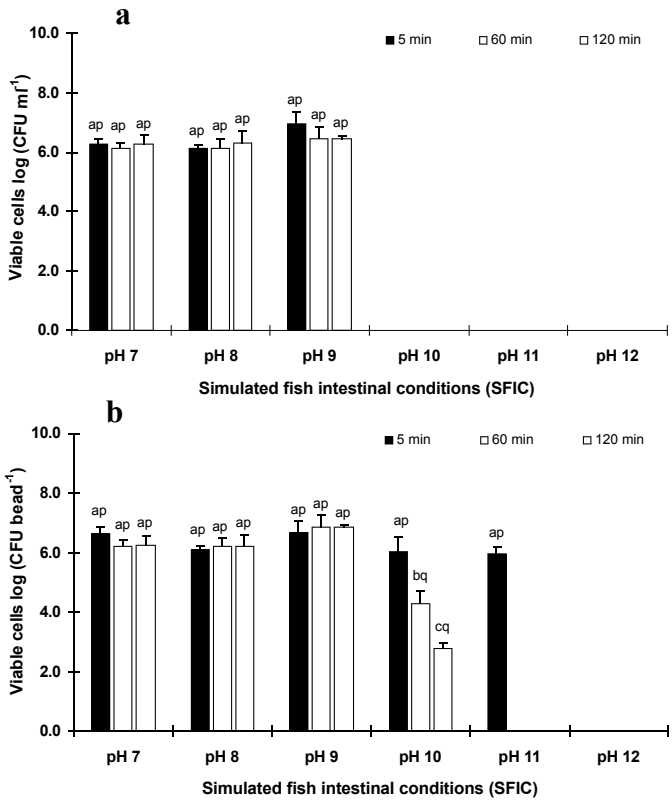

Fig. 2. Survival of free (a) and encapsulated (b) cells during exposure to SFIC.

REFERENCES

- Balcázar JL et al. (2006) *The role of probiotics in aquaculture*. Vet Microbiol 114: 173-186.
- Chakrabarti I et al. (1995) *Digestive enzymes in 11 freshwater teleost fish species in relation to food habits and niche segregation*. Comp Biochem Physiol 112A: 167-177.
- Chandramouli V et al. (2004) *An improved method of microencapsulation and its evaluation to protect Lactobacillus sp. in simulated gastric environments*. J Microbiol Methods 56: 27-35 (2004).
- García de La Banda, I. et al. (2010) *Influence of two closely related probiotics on juvenile Senegalese sole (Solea senegalensis, Kaup 1858) performance and protection against Photobacterium damselae subsp. Piscicida.* Aquaculture 206: 281-288.
- Gbassi GK et al. (2009) *Microencapsulation of Lactobacillus plantarum spp in an alginate matrix coated with whey protein*. Int J Food Microbiol 129: 103-105.
- Sultana K et al. (2000) *Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt.* Int J Food Microbiol 62: 47-55.
- Trindade CSF and Grosso CRF (2000) *The effect of the immobilization of Lactobacillus acidophilus and Bifidobacterium lactis in alginate on their tolerance to gastrointestinal secretions*. Milchwissenschaft 55: 496-499.