# EXPERIMENTAL COLONIZATION OF *ULVA* SPP. WITH ALGAL-EPIPHYTIC ANTAGONISTIC BACTERIA AS A STRATEGY FOR PATHOGEN CONTROL IN INTEGRATED MULTI-TROPHIC AQUACULTURE RECIRCULATING SYSTEMS

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## Introduction

Probiotics are a potential tool for bacterial control in aquaculture (Pintado et al. 2011), decreasing the use of disinfectants and antibiotics and contributing to an ecosystem approach, which is more sustainable and respectful to the environment.

Bacteria of the *Roseobacter* clade (α-proteobacteria), such as *Phaeobacter gallaeciensis* or *Ph. inhibens*, can reduce growth and kill fish pathogens, such as *Vibrio anguillarum*, by producing the antibiotic tropodithietic acid (Brinkhoff et al., 2004). These bacteria have also demonstrated their effectiveness as probiotic in aquaculture by reducing the mortality of fish larvae (Planas et al. 2006). Moreover, *Phaeobacter* bacteria form biofilms and can be grown on biofilters, constituting a new strategy for the control of pathogenic vibrios in the water of aquaculture systems (Prol et al. 2014).

Marine macroalgae, e.g. *Ulva* species, provide an important niche for biofilm-forming bacteria, including those from the Roseobacter clade, that possess antagonistic activities (Rao et al. 2007). Cultures of *Ulva* spp. are being used in Integrated Multitrophic Aquaculture (IMTA) as biofiltration systems in fish farms, both in open and recirculating systems (IMTA-RAS) (Neori et al. 2004; Msuya and Neori, 2010). The aim of this work was to study the experimental colonisation of Ulva sp. algae with selected probiotic bacteria of the *Roseobacter* clade as a possible strategy to control pathogenic bacteria in IMTA-RAS systems.

## Materials and Methods

Experiments were conducted with two species of *Ulva*: *U. rigida* and *U. australis*, and two algae-epiphytic bacteria strains with antagonistic activity against *V. anguillarum*: *Phaeobacter inhibens* 5URC3 and *Phaeobacter gallaeciensis* 4UAC3, which were previously isolated from wild *U. rigida* and *U. australis*, respectively.

Algae thallus discs of 2 cm diameter, obtained from the same cultured clone, were placed in 6 well plates with 10 ml of synthetic seawater (SSW) supplemented with Guillard's F/2 medium adjusted to a concentration of 20 mg.L$^{-1}$ of N (from nitrate). Medium was sterilised by membrane filtration (0,22 $\mu$m). Algae cultures were inoculated with $10^7$ CFU ml$^{-1}$ bacteria by adding 0,1 ml of a three-day culture of the bacteria in Marine Broth (MB) at 20°C. Controls were conducted in parallel without addition of bacteria. The plates were cultured in an temperature-controlled benchtop shaker (New Brunswick), at 18ºC and 80 rpm orbital agitation, with a daylight-type LED panel and a 12:12 photoperiod.

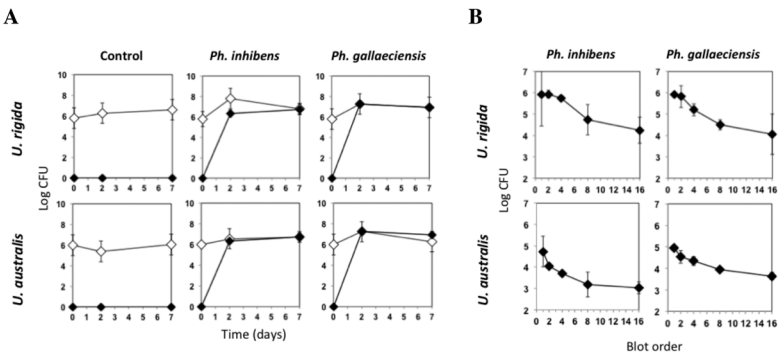


**Figure 1.** Colonization (A) and Detachment (B) kinetics of the antagonistic bacteria *Ph. inhibens* and *Ph. gallaeciensis* on *U. rigida* and *U. australis* surface. (◊) Total bacteria Log CFU disc$^{-1}$; (□) Brown-pigmented colony forming bacteria Log CFU disc$^{-1}$, corresponding to the introduced *Phaeobacter* strain.

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Colonization was estimated taking samples in duplicate at 0, 2, 7 and 14 days. Algae discs were rinsed three times sterile seawater (SSW) to eliminate non-adhered bacteria. Adhered bacteria were collected from washed alga by swabbing with sterile swabs, and swab heads were transferred into a sterile 2 mL microcentrifuge tubes with 1 ml of SSW. Tubes were vortexed for 5 min at a maximal speed to re-suspend the bacteria. A volume of 0,1 ml of the bacterial suspension was used to perform serials dilutions were spread in Marine Agar (MA) plates which were cultured at 20ºC and total colony forming units (CFUs) counted at 14 days. *Phaeobacter* sp. CFUs were identified by the characteristic brown pigmentation of the colonies. The rest of the bacterial suspension (0,9 ml) was centrifuged at 12.000 x g, 15 min and the bacterial pellet kept at -20ºC for DNA extraction and PCR-DGGE analysis. Algae discs samples were also taken for scanning electron microscopy (SEM).

Detachment of the bacteria from the algae was evaluated at day 5 using a method proposed by Herrera et al. (2007). Briefly, washed *Ulva* spp. discs were placed on a MA plate and a 500 g weight was place con top. After 1min, the disc was removed and placed onto a second MA plate. This blotting step was repeated through a succession of 16 MA plates. The number of detached cells in the plates with order number 1, 2, 4, 8, and 16 was determined by transferring the agar from each plate to 10ml of SSW and subsequently blending in a Stomacher (400 Seward, England). Mixes were serially diluted and spread out on MA plates and detached CFU (DCFU) were estimated.

**Results and discussion**

Both *Phaeobacter* strains were able to colonise and to maintain in the algae surface at concentration over $10^6$ CFU on the disc (Figure 1A), constituting the 100 % of total bacteria. Ph. gallaeciensis showed slightly higher concentrations on both *Ulva* species. Detachment kinetics (Figure 1B) showed similar results for both *Phaeobacter* in *U. rigida*, but differences were observed on *U. australis*. Ph. inhibens showed a higher detachment rate and a lower persistence than for *Ph. gallaeciensis*. Similar experiments are being conducted with *U. ohnoi*, to select the combination with the highest persistence.

The modification of the bacterial epiphytic microbiota will be analysed by PCR-DGGE. Also SEM analysis are on progress to observe how the bacterial biofilm is formed on the algae thallus.

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