CO-CULTING *Ulva ohnoi* WITH ANTAGONISTIC *Phaeobacter* BACTERIA AS A STRATEGY TO PROTECT FISH-ALGAE IMTA-RAS CULTURES FROM VIBRIOSIS

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Cultures of *Ulva* spp. are currently being used in Integrated Multitrophic Aquaculture (IMTA) as biofiltration systems in fish farms, both in open and recirculating systems (IMTA-RAS). *Ulva ohnoi* has been identified as ideal candidate for filtering fish effluents due to their high growth rates and capacity to absorb and metabolize nitrogen (Oca et al. 2016).

Ulva species, provide an important niche for biofilm-forming bacteria, including those from the *Phaeobacter* genus, that possess antagonistic activities against pathogenic *Vibrio* species (Brinkhoff et al 2004). These bacteria have 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).

The aim of this work was to study the experimental colonisation of *U. ohnoi* by co-culturing the algae with selected vibrio-antagonistic *Phaeobacter* bacteria as a possible strategy to control pathogenic vibriosis in fish-algae IMTA-RAS systems.

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

Algae thallus discs of 2 cm diameter, obtained from the same *U. ohnoi* 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) (F/2-N medium). Medium was sterilised by membrane filtration (0,22 μm). 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 a temperature-controlled benchtop shaker (New Brunswick), at 18ºC and 80 rpm orbital agitation, with a daylight-type LED panel and a 12:12 photoperiod.

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. *Phaeobacter* 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 blotting method proposed by Herrera et al. (2007). Briefly, washed *Ulva* 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.

Both *Phaeobacter* strains were able to colonise *U. ohnoi* surface at concentration over 10^6 CFU on the disc after two days, constituting the 100 % of total bacteria, and maintaining those levels for seven days. Detachment kinetics showed similar results for both *Phaeobacter* in *U. ohnoi* but a slightly higher persistence was observed for *Ph. gallaeciensis*, so this strain was selected.

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Sacale-up of the selected combination *U. ohnoi – Ph. gallaeciensis* was done transferring 7-days *Ulva* disks from the place cultures to 5 l flasks with 4 l of sterile F/2-N medium, and re-inoculating with inoculated with 10^7 CFU ml^{-1} of *Ph. gallaeciensis*. After seven days in the flasks, the cultures were transferred to vertical cylindrical tanks with 40 of non-sterile F/2-N medium and bottom aeration to tumble the seaweeds. Experimental conditions (irradiances and seaweed stocking densities) were similar as the ones used by Oca et al. (2016). The F/2-N medium was renewed weekly, seaweeds weighted and the amount of biomass adjusted to the initial stocking density. Samples for microbial analysis were taken weekly and analysed as described previously.

The antagonistic activity of the *Ph. gallaeciensis* grown on the *U. ohnoi* surface will be analysed *in vitro* against a *Vibrio anguillarum* strain pathogenic for fish (Skov et al. 1995), in presence and absence of nutrients, following the procedure described in Prol et al (2012).

Acknowledgments
This work is funded by Spanish Ministerio de Economia y Competitividad (AGL2013-41868-R).

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