ASSESSMENT OF *IN VITRO* POSTBIOTIC CAPABILITIES OF THE PROBIOTIC *SHEWANELLA PUTREFACIENS* PDP11 GROWING UNDER DIFFERENT CULTIVATION CONDITIONS CONTAINING MICROALGAE DIETARY SUPPLEMENTS WIDELY USED IN AQUACULTURE

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Introduction

Probiotics have been established as potential tools to improve host health and environmental quality in aquaculture. For example,*Shewanella putrefaciens* Pdp11 (SpPdp11), a strain described as a probiotic for use in aquaculture (reviewedby Cámara et al. 2020). Despite the proven health benefits of probiotics, recent evidence suggests that bacterial viability is not necessary to attain effects. Thus, postbiotics have emerged as potential opportunities in the field of functional foods. The International Scientific Association for Probiotics and Prebiotics (ISAPP) convened a panel that defined postbiotics as a "preparation of inanimate microorganisms and/or their components that confer a health benefit to the host". Other natural dietary supplements, including microalgae, along with their extracted compounds, have also been studied as feed additives for several fish species, and have shown beneficial effects on fish health. Some of these important and well-characterized microalgae species are *Chlorella*, *Arthrospira*, *Microchloropsis*, and *Tisochrysis*, among others. These species are commonly used in fish diets and are known to improve health (Ansari et al., 2021).

Recent studies conducted by our group demonstrated how postbiotic production can be affected by different cultivation conditions of bacteria, especially the culture media (Domínguez-Maqueda et al., 2022). Information on this type of postbiotic activity is very scarce, especially in the case of aquaculture (Mora-Sánchez et al., 2020), and evaluation of the nutraceutical use of postbiotics to improve health management in fish and other cultivated aquatic organisms is an emerging area of research in aquaculture.

The present work evaluates the potential postbiotic, as extracellular products (ECPs), of SpPdp11 grown undern different cultivation conditions. These included different culture media composed of a blend of microalgae, to observe a possible synergistic effect. The ECPs obtained were evaluated for their cytotoxicity against different fish cell lines, enzymatic and antibacterial activity, and their effect on biofilm formation by several fish pathogens

Material and methods

SpPdp11 was grown on tryptic soy agar supplemented with NaCl (1.5 %) (TSAs) at 23º C for 24 h. Then, one to two colonies were transferred to 50 mL of tryptic soy broth supplemented with NaCl (1.5%) (TSBs) at 23ºC for 36h (10^9 CFU mL^{-1}, start of the stationary phase) with shaking at 80 rpm. Then, 1 mL of each culture was spread over sterile cellophane sheets placed on TSAs plates (T medium). Another 1 mL was spread on sterile cellophane sheets placed on plates containing an experimental aquafeed (160 g/L) agar (1.5 %) (F media), partial replacement of aquafeed with 25% of a blend of microalgae (*Chlorella fusca, Tisochrysis galbana, Microchloropsis gaditana* and *Arthrospira*

platensis) (160g/L) and agar (1.5 %) (medium FM) and total blend of microalgae (50 g/L) (medium M). The plates were incubated at 23 ºC and 15 ºC for 24 h and 48 h. Samples were collected by adding 2 ml sterile saline phosphate buffer (PBS). The obtained suspension was centrifuged (10,000 x g, 20 min, 4ºC) and the supernatant was filtered through membranes (0.22 µm, pore diameter), and kept at -80ºC until use. Internal controls (ICs) and culture media without bacterial inoculation, were obtained under the same conditions.

The cytotoxicity of ECPs on european sea bass brain (DLB-1), hepatocellular carcinoma of *Poeciliopsis lucida* (PLHC-1), *Fundulus heteroclitus* brain (FuB-1) and fibroblast cell line of *gilthead seabream* (SAF-1), cells was verified by exposing the cells to different doses of ECPs. After exposure, cell viability was determined using the MTT assay. Then, using the agar-well diffusion assay, the ECPs were screened for nutritional (protease, collagenase, lipase and amylase) and anti-nutritional (phytase, tannase and cellulose) activities. As virulence factors, hemolytic and DNAse activities was also determined. The antibacterial activities of the ECPs against pathogenic bacterial strains *Aeromonas hydrophila*, *Vibrio anguillarum* and *Tenacibaculum maritimum* were also evaluated. In all cases, 50 µL of ECPs was inoculated into 6 mm-diameter wells made in the plates and incubated at 23ºC for 24-48h. Plates were observed in the presence of a clear zone around the wells. Finally, the effect by ECPs on biofilm formation of *A. hydrophila, V. anguillarum* and *T. maritimum* was evaluated according crystal violet (CV) technique.

Results and discussion

Only four ECP conditions were not cytotoxic at the protein concentration tested in any case against the different fish cell lines assayed, T2324, FM2324, FM1548 and M2324. In addition, three of these four ECP conditions are included in the best six that are capable of hydrolyzing more than three nutritional compounds (casein, lipids and gelatin), but none of the ECP conditions hydrolyze antinutritional compounds in any case. Although no antibacterial activity wasobserved on ECPs, the biofilms of *V. anguillarum* and *T. maritimum* were significantly reduced by several conditions, especially those from FM media (FM2324 and FM1548).

The results obtained demonstrate the influence of culture conditions on the production of postbiotics, which has been widely reported (Aggarwal et al., 2022), suggesting that the activity, quantity and type of these derived products are mainly related to the type of bacterial strain and culture medium. Because of the wide target points of postbiotics, their application could be very extensive for use in many industries, such as food, healthcare products, cosmetics, and nutraceuticals (Sudhakaran et al., 2022). In terms of the aquaculture industry, optimized growth conditions can allow us to obtain promising postbiotics, which may be of interest for future *in vivo* experiments as supplemental additives for improving fish health.

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