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RESEARCH ARTICLE

Microalgae and cyanobacteria as microbial substrate and their influence on the potential postbiotic capability of a bacterial probiotic

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Abstract

Postbiotics are metabolic by-products from microorganisms that provide health benefits to the host. Their secretion can be influenced by various conditions affecting bacterial metabolism. This study presents a novel approach for producing potential postbiotics, specifically extracellular products (ECPs), from the probiotic strain *Shewanella putrefaciens* SpPdp11, grown under different culture conditions. These conditions include aquafeed media, with partial or total microalgae/cyanobacteria replacement as the microbial substrate, as well as variations in temperature and growth phase. The use of microalgae/cyanobacteria as substrates may represent a valuable strategy for generating novel postbiotics with unique properties. The ECPs assessed were evaluated for their in vitro cytotoxic, hydrolytic and antimicrobial activities. Three conditions (ECPs derived from aquafeed media with partial (FM2324 and FM1548) or total (M2324) microalgae/cyanobacteria replacement) were non-cytotoxic to various fish cell lines and hydrolysed key nutritional compounds (casein, lipids, amylase and gelatin). Proteomic analysis of these ECP conditions revealed common structural and regulatory DNA-associated proteins, while differentially expressed proteins were associated with amino acid metabolism and antioxidant system (FM2324 and FM1548) and chemotaxis system (M2324). The results highlight the potential of the selected postbiotics as feed additives for future in vivo studies, aligning with sustainable development for aquaculture.

INTRODUCTION

The aquaculture industry plays a vital role in providing a sustainable source of protein for human consumption (Ahmad et al., 2021). However, the intensification of aquaculture practices has led to an increased incidence of pathogens and diseases, and promoting efforts have been made to mitigate their impact (Abdel-Latif et al., 2020; Pérez-Sánchez et al., 2018). Diseases management in aquaculture mainly relies on the use of antibiotics (Assefa & Abunna, 2018), which are often administered via prolonged immersion or by incorporation into the fish diet (Yasin et al., 2023). However, the overuse of antibiotics in aquaculture raises concerns

about the risk of transferring pathogen resistance to humans (Pepi & Focardi, 2021) and poses significant threats to the health of aquatic ecosystems (Lulijwa et al., 2020). These concerns have led to a growing interest in alternative options, including natural dietary supplements, probiotics, prebiotics, marine algae and herbs, which have shown beneficial effects on fish health while promoting environmentally sustainable aquaculture practices (Bahi et al., 2023; Idenyi et al., 2022; Liang et al., 2022).

Probiotics are defined as live microbial cells that provide health benefits to the host when administered in adequate amounts (Merrifield et al., 2010). Although its viability is considered a crucial factor in determining its effectiveness, there are some concerns regarding the safety, stability and standardization of live probiotic cells (Choudhury & Kamilya, 2019). As a result, there is increasing interest in exploring alternatives that involve the use of non-viable microbes as recent evidence suggests that bacterial viability may not be essential for promoting beneficial effects on the host (Cuevas-González et al., 2020; Moradi et al., 2021; Sudhakaran et al., 2022). Postbiotics defined as non-viable bacterial products or metabolic by-products, including bacteriocins, organic acids and extracellular products (ECPs), among others, are gaining attention for their beneficial biological activities (Salminen et al., 2021). The potential advantages of postbiotics over probiotics include reduced interaction with food components (Vera-Santander et al., 2023), simplified processing that allows incorporation before heat treatment (Li & Tran, 2022), easier storage and transport (Rafique et al., 2023), improved safety by reducing the risk of infection or pathogen contamination (Ho et al., 2023) and their potential as a more sustainable alternative in the field of functional foods (Quintanilla-Pineda et al., 2023).

The production and bioactivity of postbiotics are influenced by various factors, including the choice of bacterial strains, culture media, bacterial treatment methods and the growth phase (Domínguez-Maqueda, García-Márquez, et al., 2024). Therefore, optimizing the production of postbiotics offers significant potential for their application in various biotechnological fields, including the aquaculture and aquafeed industries. In this context, microalgae have emerged as a highly promising substrate to be included in bacterial growth media. For example, studies have shown that microalgae substrates used in bacterial probiotics culture can enhance or maximize the production of ECPs with diverse activities by affecting the fermentation process, the biochemical composition and functionality of the secreted metabolites (Pagnini et al., 2023; Ricós-Munoz et al., 2023). This approach not only broadens the scope of postbiotic applications in aquaculture and aquafeed but also aligns with the industry's shift towards more sustainable and eco-friendly practices (Ma & Hu, 2023).

The probiotic strain *Shewanella putrefaciens* Pdp11 (SpPdp11), originally isolated from healthy gilthead seabream (*Sparus aurata*) specimens (Chabrillón, Rico, Arijo, et al., 2005), has demonstrated beneficial effects both in vitro and/or in vivo for farmed fish species such as Senegalese sole (*Solea senegalensis*) and *S. aurata* (Cámara-Ruiz et al., 2020; Cordero et al., 2015; Tapia-Paniagua et al., 2015). Building upon our previous research, this study explores the use of a blend of microalgae (*Chlorella fusca, Tysochrysis lutea* and *Microchloropsis gaditana*) and cyanobacteria (*Arthrospira platensis*) as a nutrient source in probiotic culture media for obtaining ECPs as potential postbiotics. In addition, aquafeed media were used to compare with previous results and evaluate the effects of the microalgae blend, both alone and in combination with aquafeed. Afterwards, the study assesses the in vitro effects of the SpPdp11 ECPs on the viability of different fish cell lines, the hydrolytic activity on nutritional and antinutritional compounds and their antagonistic activity against several fish pathogens. Finally, the ECPs with the most promising bioactivities were further characterized at proteomic level to elucidate their proteomic profile.

EXPERIMENTAL PROCEDURES

Bacterial strains, media and culture conditions

SpPdp11 (CECT 7627) was cultured on tryptic soy agar (TSA) supplemented with NaCl (1.5%) (TSAs) at 23°C for 24 h. Then, one to two colonies were cultured on 50 mL of tryptic soy broth (TSB, Oxoid Ltd. Basingstoke, UK) supplemented with NaCl (1.5%) (TSBs) at 23°C for 36 h (10^9 CFU/mL, $O.D._{600nm} = 1.5$, onset of stationary phase) on shaking (80 r.p.m.).

Extracellular product extraction under different culture conditions

Extracellular products (ECPs) were obtained from a solid medium using the cellophane plate technique (Liu, 1957). In brief, aliquots of 1 mL of SpPdp11 cultures were spread over sterile cellophane sheets placed on plates containing a partial replacement of experimental aquafeed with 25% of a blend of microalgae (*C. fusca*, *T. lutea* and *M. gaditana*) and cyanobacteria (*A. platensis*) in a ratio of 1:1:1:1 (160 g/L) and agar (1.5%) (FM medium). A second medium (M) consisted of a blend of microalgae and cyanobacteria (50 g/L) with agar (1.5%) (medium M). The experimental

aquafeed was formulated to mimic commercial diets, with the microalgae blend replacing 25% of the feed. TSA was used as control media, while aquafeed media served as a culture condition to study the ECPs secreted by the probiotics grown on farmed fish feed (Table S1). Both media (FM and M) were compared to previously described T and F media (Domínguez-Maqueda, García-Márquez, et al., 2024) with cultures incubated at 23°C for 24 h and at 15°C for 48 h. These temperatures were chosen to reflect conditions close to those found in aquaculture environments as described by Domínguez-Maqueda, García-Márquez, et al., 2024; Domínguez-Maqueda, Espinosa-Ruíz, et al., 2024. Media without bacterial inoculation referred to as internal controls (ICs) were also incubated under the same conditions of temperature and time to check for possible background from the media. The different conditions tested are summarized in Figure 1.

Bacterial cells from different culture conditions and ICs were harvested with 2 mL of sterile phosphate-buffered saline (PBS, pH 7.2) after 24 h and 48 h incubation. Cells were centrifuged (10,000 × g, 20 min, 4°C) and the supernatants were filtered through 0.45- and 0.2-μm-pore-size membrane filters (Merck Millipore) to obtain only the ECPs. ICs were harvested similarly from non-inoculated media. ECPs were also concentrated using Amicon Ultra centrifugal filters (10 K) (Merck Millipore). Protein concentration was determined using Qubit Protein assay kits and the Qubit 2.0 fluorometer (Thermo Fisher Scientific). To ensure the absence of bacterial growth, aliquots of the ECPs were cultured on TSA plates and incubated for 24–48 h at 23°C. ECPs were kept at −80°C until use.

Characterization of ECP activities

Cytotoxicity

Cell culture

The established SAF-1 cell line (ECACC n°00122301) obtained from fibroblast cells of gilthead seabream (*S. aurata*) was seeded in 25 cm^2 plastic tissue culture flasks (Nunc, Germany) using L-15 Leibowitz medium, supplemented with 10% Fetal Bovine Serum (FBS), 2 mM/L L-glutamine, 100 i.u./mL penicillin and 100 μg/L streptomycin. The cells were incubated at 25°C in a humidified atmosphere (85% humidity, 5% CO_2). The DLB-1 cell line (CVCL_HG31) obtained from European sea bass (*Dicentrarchus labrax*) brain (Morcillo et al., 2017) was also cultured under similar conditions in L-15 Leibovitz medium containing 0.16% NaCl, 15% FBS, 20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid or chemical formule $C_8H_{18}N_2O_4S$ (HEPES), Thermo Fisher Scientific), 2 mM/L glutamine, 100 i.u./mL penicillin and 100 μg/mL streptomycin. The cells were cultured at 25°C in an incubator (85% humidity and 5% CO_2). The FuB-1 (CVCL_YJ47) cell line obtained from mummichog (*Fundulus heteroclitus*) brain was also grown at 25°C (85% humidity, 5% CO_2) in L-15 Leibowitz medium supplemented with 15% FBS, 2 mM/L glutamine, 100 μg/mL streptomycin, 100 i.u./mL penicillin and 10 mM HEPES (Ruiz-Palacios et al., 2020). Lastly, the established cell line PLHC-1 (ATCC® CRL2406™) derived from a hepatocellular carcinoma of the clearfin livebearer (*Poeciliopsis lucida*) was seeded into 25 cm^2 plastic tissue culture flasks in Eagle's minimum essential medium (EMEM) (Sigma)

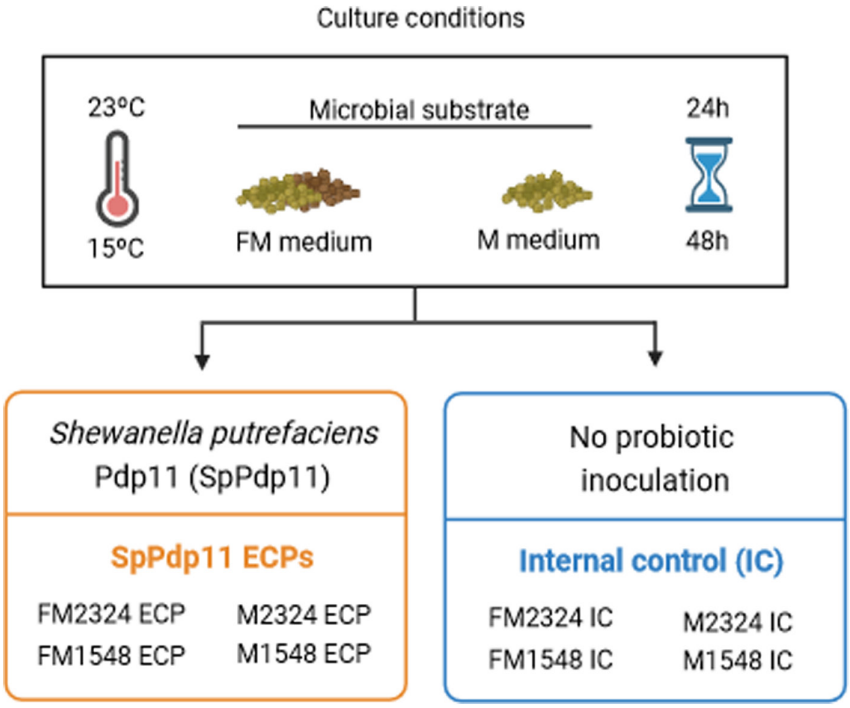

FIGURE 1 Culture conditions for SpPdp11 for ECPs extraction and nomenclature assigned. FM: Partial replacement of aquafeed with a 25% blend of microalgae (*Chlorella fusca, Tisochrysis lutea* and *Microchloropsis gaditana*) and cyanobacteria (*Arthrospira platensis*) (1:1:1:1) (160 g/L) and agar (1.5%); M: Blend of the microalgae and cyanobacteria (50 g/L) and agar (1.5%) (medium M). Internal controls (ICs) were named according to their respective conditions with the addition of 'Internal Control; IC'.

supplemented with 2 mM/L L-glutamine and Eagle salts adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 5% FBS, 100 i.u./mL penicillin and 100 mg/mL streptomycin. The cells were grown at 30°C in a humidified atmosphere (85% humidity and 5% CO_2).

MTT assay

The cytotoxicity of the ECPs was tested on each cell type in five replicates. When the cell lines reached approximately 80% confluence, they were detached from the flasks using trypsin according to the standard trypsinization methods (0.25% trypsin for SAF-1, DLB-1 and FuB-1 cells, and 0.05% trypsin for PLHC-1 cells) with some modifications (Mayor et al., 2023; Ruiz-Palacios et al., 2020). Aliquots of 100 μL containing 50,000 cells/well were seeded into 96-well tissue culture plates and incubated for 24 h at the optimal temperature for each cell line. This cell concentration was previously determined so that satisfactory absorbance values would be obtained in the cytotoxic assay, and to avoid cell overgrowth. After that, the culture medium was replaced with 100 μL/well of the ECP extracts containing protein concentrations of 0.75, 1 and 1.5 mg/mL. Control wells received the same volume of culture medium. Cells were incubated for 24 h and then their viability was determined using the MTT assay, which is based on the reduction of the yellow soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, Sigma-Aldrich) to a blue, insoluble formazan product by mitochondrial succinate dehydrogenase (Stevens et al., 1991). Cells were washed with PBS and 200 μL of MTT (1 mg/mL) were added per well. After 4 h incubation, the cells were washed again and the formazan crystals were solubilized with 100 μL/well of dimethyl sulphoxide (DMSO). The plates were shaken (5 min, 100 rpm) in dark conditions, and the absorbance was determined at 570 nm and 690 nm in a microplate reader.

Hydrolytic enzyme production

Phytase, tannase and cellulase activities, known for its antinutritional properties, mostly associated with vegetal compounds, were assayed according to Kumar et al. (2010) on agar plates (1.5% agar) containing 1% w/v of Na-phytate (P-8810, Sigma), 2% w/v of tannic acid (P-403040, Sigma) and 1% w/v of carboxymethyl cellulose (CMC) (C-5678, Sigma) respectively. Furthermore, protease, gelatinase, lipase and amylase activities were assayed according to Chabrillón, Rico, Balebona, and Moriñigo (2005) on agar plates containing 2% w/v of skim milk (Pirinea, Spain), 1% w/v of gelatine (Oxoid, UK), 1% w/v of Tween-80 (Panreac, EEUU) and 4% w/v of starch (Labkem) respectively. The haemolysis and DNase activity of the ECPs were determined using agar test plates (Oxoid) and Columbia agar plates containing 5% (w/v) sheep blood respectively. In all cases, 50 μL ECP samples (0.5 μg protein/μL) and ICs were inoculated into 6-mm-diameter wells made in the plates and incubated at 23°C for 24–48 h. The plates were observed for the presence of a clear zone around the wells. Hydrolytic activities against starch, CMC and DNase were indicated by a clear zone around the colonies after flooding the plates with Lugol, Congo red solution 0.1% w/v and Hydrochloric acid (HCL) 1 M for starch and CMC respectively. The haemolytic activity was determined according to the signs of α-haemolysis (green zones around colonies or wells), β-haemolysis (clear zones) or γ-haemolysis (no zones) on the plates (Pieniz et al., 2014). Aliquots of 50 μL of PBS and *Vibrio harveyi* cells (10^8 CFU/mL, grown as explained below) were used as negative and positive controls respectively. The absence of a clear zone was interpreted as the absence of activity. The lowest ECP concentration with a clear zone around the well was designed as the minimum concentration of each activity. Each ECP condition was tested in triplicate, and each experiment was repeated twice.

Antagonistic effect

Antimicrobial activity

Fish pathogenic bacterial strains *Aeromonas hydrophila* (Arijo et al., 2005), *Vibrio harveyi* 16/00 (Arijo et al., 2005), *Vibrio anguillarum* (CECT 522), *Photobacterium damselae* subsp. *damselae* (CECT 626) and *P damselae* subsp. *piscicida* (Díaz-Rosales et al., 2003) were cultured on TSA plates at 23°C for 24 h. In addition, *Tenacibaculum maritimum* (CECT 4276), *T. soleae* (CECT 7292) and *T. gallaicum* (CECT 7122) were cultured on *Flexibacter maritimus* medium (FMM) (Pazos et al., 1996) plates supplemented with agar (1.5%) at 28°C for 48 h.

The plates were examined for the presence of inhibition of bacterial growth, indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity was expressed in terms of the average diameter of the zone inhibition in centimetres. Growth across the entire area was interpreted as an absence of antibacterial activity. Each ECP condition was tested in triplicate and each experiment was repeated twice.

Proteomic analysis

Sample preparation, in-gel digestion and peptide extraction

The ECPs were obtained and conserved as described above (Section 2.2) until use. Then, a gel-assisted

proteolysis was carried out (Marrero et al., 2023). Briefly, the protein solution was entrapped in a polyacrylamide gel matrix reduced with dithiothreitol and cysteine residues were carbamidomethylated with iodoacetamide. Afterwards, the proteins were digested by trypsin (Promega), and peptides were extracted from the gel using an acetonitrile/formic acid solution. After extraction, the peptides were purified and concentrated using a C18 ZipTip (Merck Millipore) according to the manufacturer's instructions.

Liquid chromatography high-resolution mass spectrometry

Samples were injected into an Easy nLC 1200 UHPLC system coupled to a Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher). Software versions used for the data acquisition and operation were Tune 2.9 and Xcalibur 4.1.31.9. HPLC solvents were as follows: solvent A consisted of 0.1% formic acid in water and solvent B consisted of 0.1% formic acid in 80% acetonitrile. From a thermostatic autosampler, 2 μL (equivalent to 100 ng of the peptide mixture) was automatically loaded onto a trap column (Acclaim PepMap 100, 75 μm × 2 cm, C18, 3 um, 100 A, Thermo Fisher Scientific) at a flow rate of 20 μL/min and eluted onto a 50 cm analytical column (PepMap RSLC C18, 2 μm, 100 A, 75 μm × 50 cm, Thermo Fisher Scientific). The peptides were eluted from the analytical column with a 120 min gradient ranging from 2% to 20% solvent B, followed by a 30 min gradient from 20% to 35% solvent B and finally, to 95% solvent B for 15 min before re-equilibration to 2% solvent B at a constant flow rate of 300 nL/min. The LTQ Velos ESI Positive Ion Calibration Solution was used to externally calibrate the instrument prior to sample analysis, and an internal calibration was performed using the polysiloxane ion signal at m/z 445.120024 from ambient air. MS1 scans were conducted from m/z 300 to 1750 at a resolution of 120,000. Using a data-dependent acquisition mode, the 20 most intense precursor ions of all precursor ions with +2–+5 charge were isolated within a 1.2 m/z window and fragmented to obtain the corresponding MS/MS spectra. The fragment ions were generated in a higher energy collisional dissociation (HCD) cell with a fixed first mass at 110 m/z and detected in an Orbitrap mass analyser at a resolution of 30,000. The dynamic exclusion for the selected ions was 30 s. Maximal ion accumulation time allowed in MS and MS2 mode was 50 ms. Automatic gain control was used to prevent overfilling of the ion trap and was set to 3×10^6 ions and 10^5 ions for a full MS and MS2 scan respectively.

Data analysis for protein identification

The acquired raw data were analysed using Proteome Discoverer™ 2.5 (Thermo Fisher Scientific) platform with the SEQUEST® HT engine using mass tolerances of 10 ppm and 0.02 Da for precursor and fragment ions respectively. For the identification of the MS2 spectra, the UniProt/Swiss-Prot and Gene Ontology (GO) annotation databases for *Shewanella baltica* BA175 were used as reference proteome for being phylogenetically and genomic closest to SpPdp11. Up to two missed tryptic cleavage sites were allowed. Oxidation of methionine and N-terminal acetylation were set as variable modifications, while carbamidomethylation of cysteine residues was set as fixed modification. Peptide spectral matches (PSM) and consecutive protein assignments were validated using the Percolator® algorithm (Käll et al., 2007) based on a target-decoy approach using a reversed protein database as the decoy by imposing a strict cut-off of 1% false discovery rate (FDR). Peptide identifications were grouped into proteins according to the law of parsimony and results were filtered to contain only proteins with at least two unique peptide sequences.

Label-free relative quantification analysis

Label-free quantitation was carried out using the Minora feature (Palomba et al., 2021) in Proteome Discoverer™ 2.5. The following parameters were set: a maximum retention time alignment of 10 min with minimum of S/N of 5 for feature linking mapping. Abundances were based on precursor intensities. Normalization was performed based on total peptide amount, and samples were scaled on all averages (for every protein and peptide, the average of all samples is 100). The normalized and scaled relative abundance of each protein was expressed as mean ± standard deviation (SD) of three biological replicates. Protein abundance ratios were directly calculated from the grouped protein abundances. Abundance ratio p-values were calculated by Analysis of Variance (ANOVA) based on the abundances of individual proteins or peptides.

Functional analysis and interaction of proteins

The protein output ID for *S. baltica* BA175 was used for Cluster of Orthologous Group (COGs) annotation. The protein raw data files and metadata are available in the public Mass Spectrometry Interactive Virtual Environment (MASSIVE) database (Choi et al., 2020) (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp).

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 22.0. Normality and homogeneity of variance of the data were determined by using Shapiro–Wilk and Levene's tests respectively. Differences were statistically analysed by one-way analysis of variance (ANOVA) with Tukey and Games–Howell post hoc tests when statistical requirements were fulfilled. Non-normally distributed data were analysed by the non-parametric Kruskal–Wallis test, followed by a multiple-comparison test. Statistical significance was set for $p \leq 0.05$.

RESULTS

Cytotoxicity of ECPs

The cytotoxic activity of SpPdp11-derived ECPs, together with their respective ICs, was investigated using different cell lines. SpPdp11 ECPs showed no cytotoxic effects on the DLB-1 cell line, except under specific conditions such as T1548, both at 1 and 1.5 μg protein/mL, and F2324 at all concentrations tested (Figure 2). In contrast, ICs F23, FM23 (0.75 μg protein/mL) and M23 (0.75 and 1 μg protein/mL) significantly increased cell viability at lower concentrations tested. Apart from ICs, some of the ECP conditions significantly increased cell viability, especially with FM and M media. Such is the case of FM2324 at 1 and 1.5 μg protein/mL, FM1548 at 0.75 μg protein/mL, M2324 at 0.75 μg protein/mL and M1548 at 1 μg protein/mL. On the contrary, no effects were observed with ECPs obtained from F and T media (Figure 2).

Regarding the FuB-1 cell line (Figure 3), the ECPs from SpPdp11 grown on FM and M media exhibited a proliferative effect on these cells at all concentrations tested, whereas ICs did not affect cell viability. With respect to T and F media, only T23 control and T2324 at the highest concentration were tested, F23 control at 0.75 and 1 μg protein/mL and F1548 at 0.75 μg protein/mL exerted proliferative effects on FuB-1 cell line. On the other hand, T1548 and F2324, at 1 and 1.5 μg protein/mL, respectively, as well as the IC F23 at 1.5 μg protein/mL, demonstrated cytotoxic activity on this cell line.

Overall, SpPdp11 ECPs were not cytotoxic to the PLHC-1 cell line, except for T1548 at 0.75 μg protein/mL, and the ICs FM23 and M23, both at 1.5 μg protein/

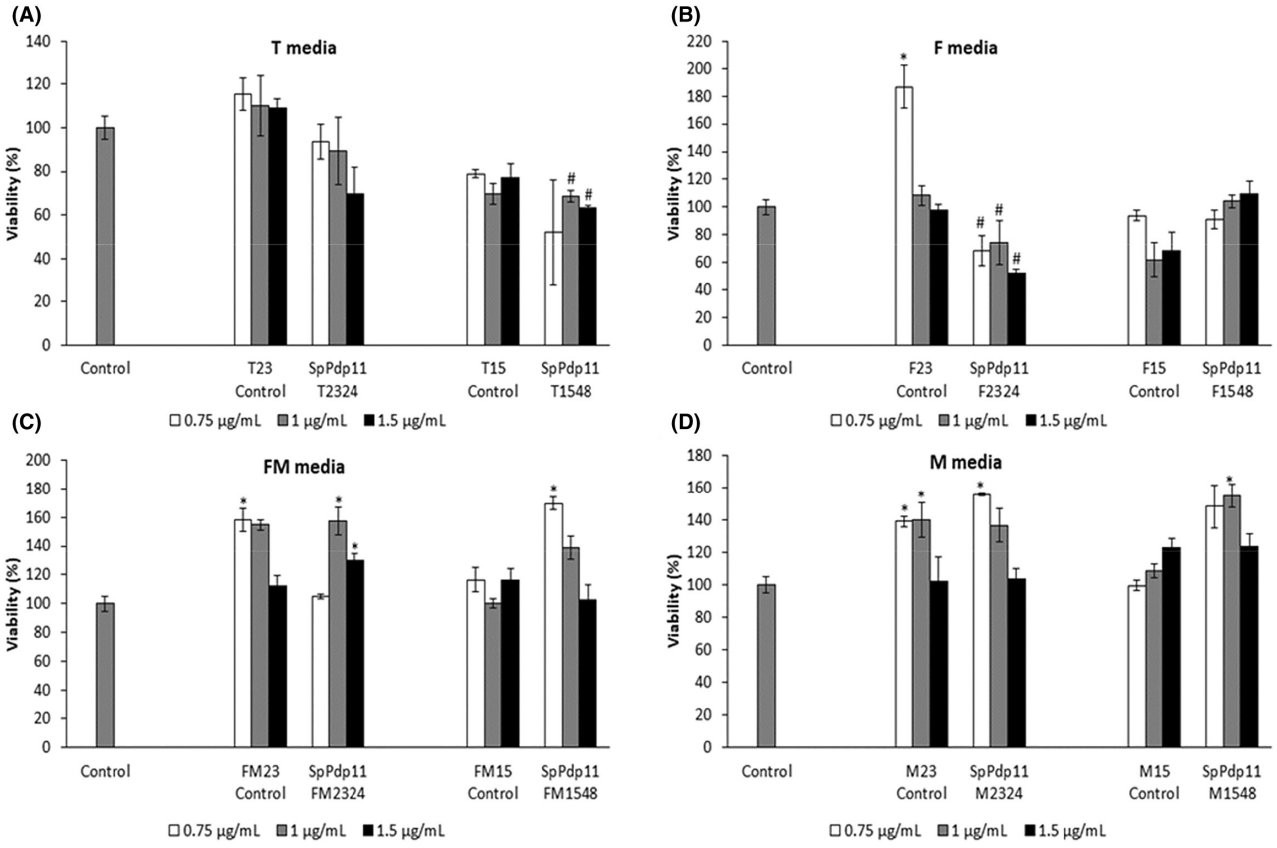

FIGURE 2 Cytotoxic effect produced by extracellular products (ECPs) from SpPdp11 and its internal controls on brain cell line of European sea bass (DLB-1). Graphics are distributed according to the different culture media; (A) T media; (B) F media; (C) FM media and (D) M media. The cell viability was determined after 24 h of incubation. The concentration of ECPs tested on all cells were 0.75, 1 and 1.5 μg protein/mL. Values represent the mean ± SD of three replicates. Hash mark (#) and asterisks (*) indicate decreased and increased cell viability, respectively, compared to the different ECPs conditions and the control ($p < 0.05$).

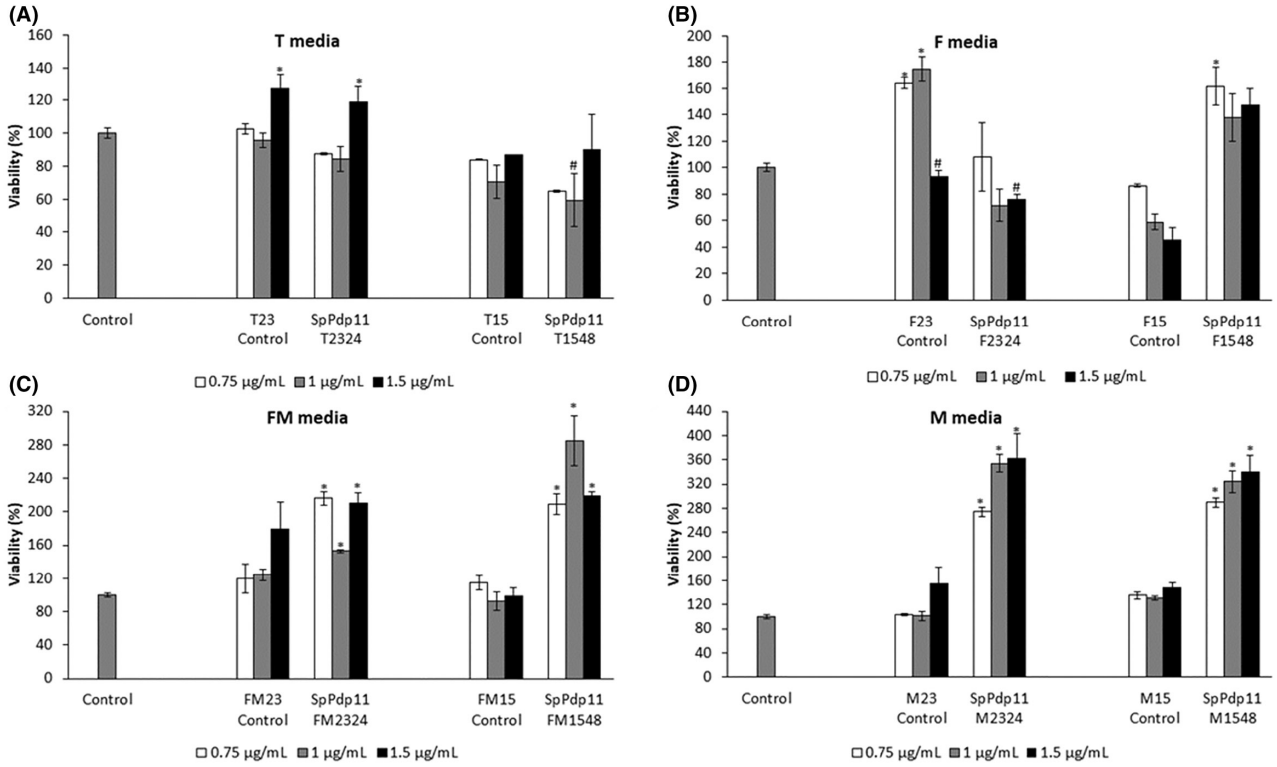

FIGURE 3 Cytotoxic effect produced by extracellular products (ECPs) from SpPdp11 and its internal controls on brain cell line of mummichogs (FuB-1). Graphics are distributed according to the different culture media; (A) T media; (B) F media; (C) FM media and (D) M media. The cell viability was determined after 24 h of incubation. The concentrations of ECPs tested on all cells were 0.75, 1 and 1.5 µg protein/mL. Values represent the mean ± SD of three replicates. Hash mark (#) and asterisks (*) indicate decreased and increased cell viability, respectively, compared to the different ECP conditions and the control ($p < 0.05$).

mL. In addition, F1548, M2324 and M23 control promoted cell viability at the lowest concentration tested, whereas F2324 and FM1548 did it at the highest (Figure 4).

Finally, when the effects of ECPs on SAF-1 cell viability were analysed, it was observed that the highest ECP concentration tested (1.5 µg protein/mL) of F2348, F1548 and M1548, as well as the lowest concentration of T1548 (0.75 µg protein/mL), significantly reduced cell viability. On the other hand, the ECP conditions FM2324 (0.75 µg protein/mL), FM1548 (1 and 1.5 µg protein/mL) and M2324 (0.75, 1 and 1.5 µg protein/mL) significantly increased SAF-1 cell viability. Moreover, regarding the ICs, all of them significantly increased cell viability at 23°C at least in one of the concentrations tested. Thus, a proliferative effect was observed with T23 and FM23 controls at the highest concentration, while this effect was observed with M23 control at the lowest, and F23 control at all concentrations tested (Figure 5).

Hydrolytic activities of ECPs

The hydrolytic activities of the SpPdp11 ECPs were evaluated, and results are summarized in Table 1. All ECP conditions demonstrated gelatin hydrolysis. Starch was hydrolysed only in the FM1548 condition, while casein and lipid were hydrolysed by all ECP conditions except for those obtained from M media. On the other hand, SpPdp11 ECPs did not hydrolyse any antinutritional compound (phytate, tannins or cellulose). The ICs did not exhibit any hydrolytic activity (data not shown).

Regarding DNase activity, the highest activity was observed in ECPs from F, FM and M media, when incubated at 23°C for 24 h, except for FM1548, which also showed DNase activity.

Antagonistic effect

The results obtained demonstrated no antimicrobial activity against the tested pathogens in any of the ECP conditions assayed.

Three ECP conditions, from partial (FM2324 and FM1548) or total (M2324) replacement of microalgae and cyanobacteria-enriched culture media, exerted the most promising activities. These ECP conditions were not cytotoxic in any case and showed higher hydrolytic capabilities. For this purpose, these three ECP conditions were further selected for proteomic analysis in order to delve into specific composition and functional characterization. The ECP sample T2324 was

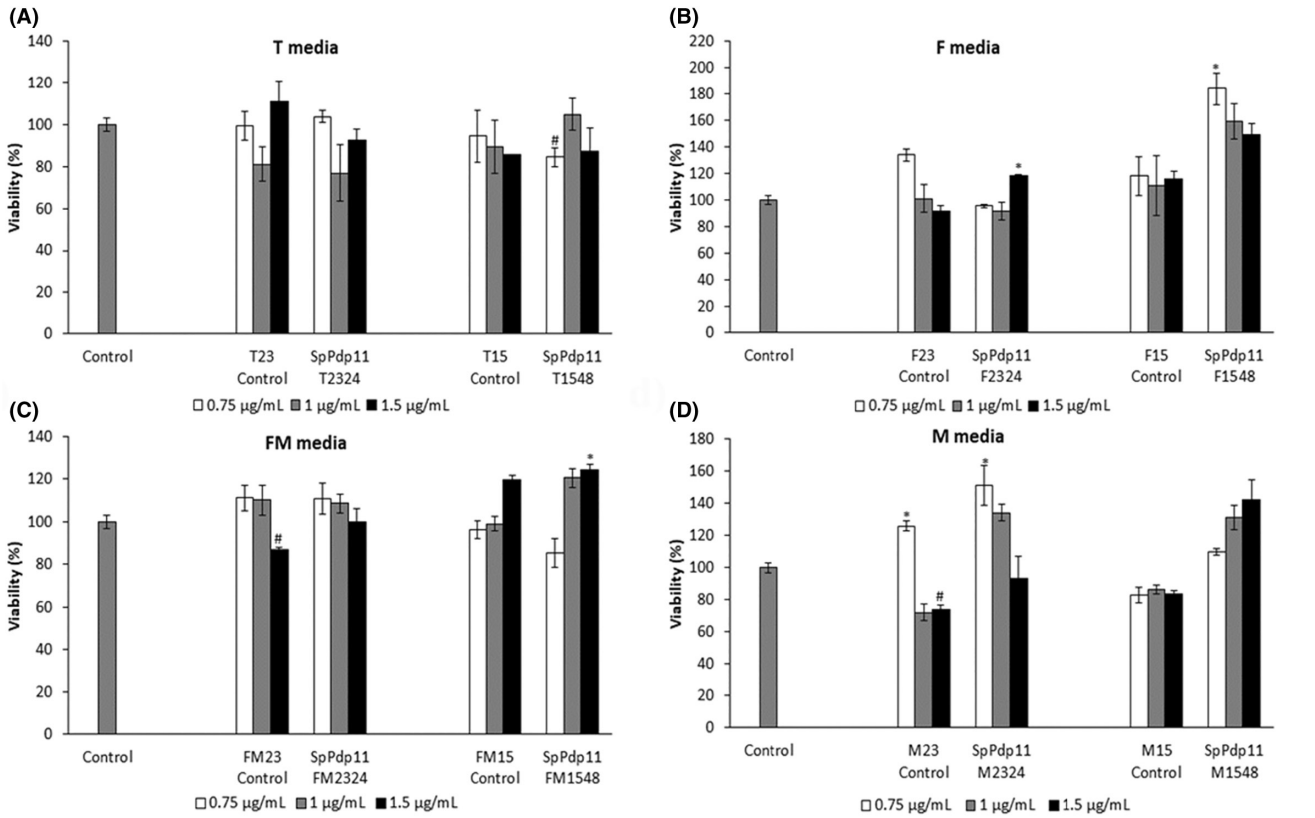

FIGURE 4 Cytotoxic effect produced by extracellular products (ECPs from SpPdp11 and its internal controls on clearfin livebearer hepatoma cell line (PLHC-1)). Graphics are distributed according to the different culture media; (A) T media; (B) F media; (C) FM media and (D) M media. The cell viability was determined after 24 h of incubation. The concentration of ECPs tested on all cells were 0.75, 1 and 1.5 µg protein/mL. Values represent the mean ± SD of three replicates. Hash mark (#) and asterisks (*) indicate decreased and increased cell viability, respectively, compared to the different ECP conditions and the control ($p < 0.05$).

maintained as control for proteomic analysis for being the optimal growth condition of SpPdp11.

Proteomic analysis

Three ECP conditions, derived from partial (FM2324 and FM1548) or total (M2324) replacement of microalgae and cyanobacteria-enriched culture media, showed the most promising activities. These ECP conditions were not cytotoxic and exhibited the highest hydrolytic capabilities. Therefore, these three ECP conditions were selected for proteomic analysis in order to delve into specific composition and functional characterization. The ECP sample T2324 was maintained as a control for proteomic analysis because it represented the optimal growth condition of SpPdp11.

Volcano plots displayed the differentially expressed proteins (DEPs) in each ECP condition using a threshold of ≤1.3 (Figure 6). By comparing the DEP profile of SpPdp11 ECP conditions, a total of 229 DEPs in FM1548 were identified. Among these DEPs, 150 DEPs were found to be downregulated, while the other 79 DEPs were upregulated compared to the control (Figure 6A). On the other hand, for the ECP condition FM2324, 197 DEPs were identified, with 88 downregulated and 109 upregulated proteins (Figure 6B). Lastly, in the M2324 condition, 165 DEPs were identified, including 92 downregulated and 73 upregulated proteins (Figure 6C). ICs from each ECP condition did not show a proteomic profile.

A Venn diagram analysis revealed 85 overlapping proteins common to all ECP conditions, conforming to the core proteome (Figure 7). Additionally, each ECP condition displayed unique DEPs, with M2324 having 41, FM1548 having 63 and FM2324 having 30 unique proteins (Figure 7).

Owing to the high number of DEPs identified in each ECP condition, the cluster of orthologous groups (COG) annotation of the DEPs detected in each SpPdp11 ECP condition was carried out and is summarized in Table S2. In this sense, FM2324, FM1548 and M2324 had five common proteins: FtsA (COG0849), DnaC (COG1484), XRE (COG1396), LysR family (COG0583) and XerD (COG4974). Additionally, FM2324 and FM1548 shared five overexpressed proteins, including chorismate synthase (COG0082), threonine deaminase (COG1171), NADH:ubiquinone oxidoreductase (NqRE) (COG2209), acyl-CoA-transferase CaiB (COG1804) and PilB ATPase (COG2804). According to

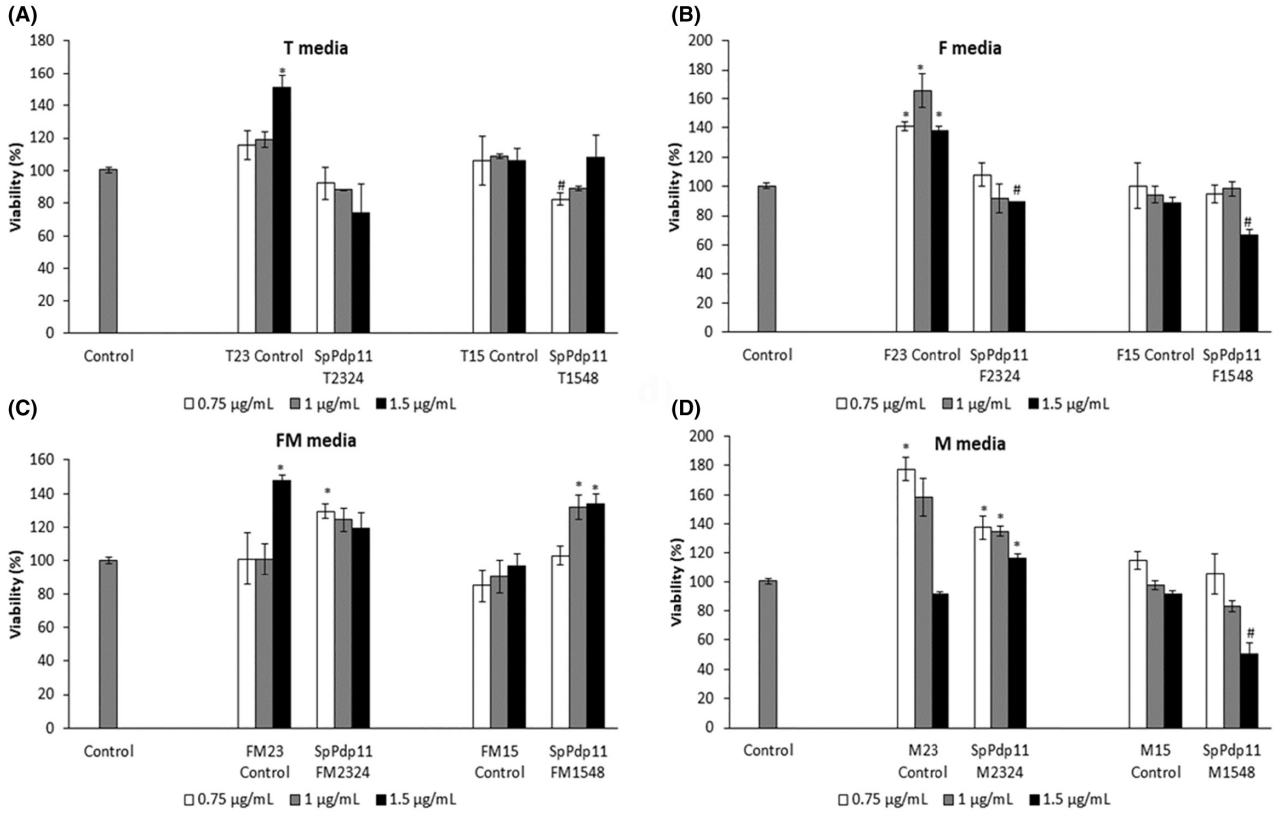

FIGURE 5 Cytotoxic effect produced by extracellular products (ECPs) from SpPdp11 and its internal controls on fibroblast cell line of gilthead seabream (SAF-1). Graphics are distributed according to the different culture media; (A) T media; (B) F media; (C) FM media and (D) M media. The cell viability was determined after 24 h of incubation. The concentration of ECPs tested on all cells were 0.75, 1 and 1.5 µg protein/mL. Values represent the mean ± SD of three replicates. Hash mark (#) and asterisks (*) indicate decreased and increased cell viability, respectively, compared to the different ECP conditions and the control ($p < 0.05$).

TABLE 1 Hydrolytic activities of SpPdp11 ECPs extracted under different culture conditions.

	ECPs conditions							
	T2324	**F2324**	**FM2324**	**M2324**	**T1548**	**F1548**	**FM1548**	**M1548**
Hydrolytic activity								
Amylase	ND	ND	ND	ND	ND	ND	756	ND
Gelatinase	200	14.06	400	862	12.50	28.13	189	1050
Caseinase	25	28.13	1600	ND	100	12.50	378	ND
Lipase	14.06	7.80	400	ND	12.50	15.63	47.25	ND
Phytate	ND	ND	ND	ND	ND	ND	ND	ND
Cellulase	ND	ND	ND	ND	ND	ND	ND	ND
Tannins	ND	ND	ND	ND	ND	ND	ND	ND
DNase	ND	148	234	147	ND	ND	358	ND
Haemolysis	γ	γ	γ	γ	γ	γ	γ	γ

Note: Values represent the minimum protein concentration (µg protein/µL) of the samples exhibiting enzymatic activity.
Abbreviation: ND, not detected.

the particular proteins overexpressed in each ECP condition, FM2324 was characterized by overexpression of thioredoxin (COG0526), FM1548 overexpressed HemN oxidase (COG0635) and cytochrome c (COG4235), and M2324 overexpressed methyl-accepting chemotaxis proteins (MCP) (COG0840), chemotaxis receptor CheD (COG1871) flavin reductase (COG0346) and catechol 2,3-dioxigenase (COG0369) proteins.

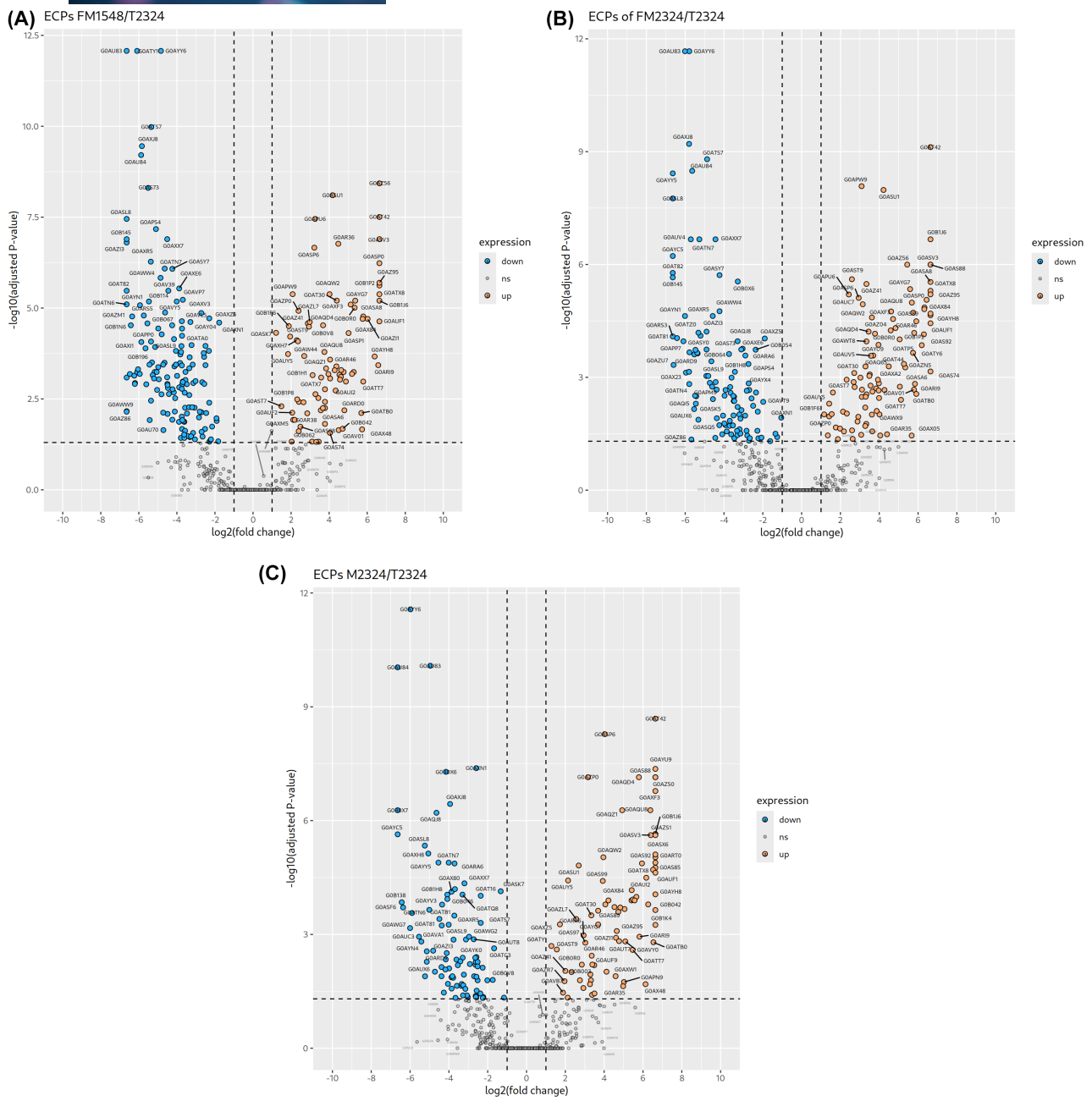

FIGURE 6 Volcano plots showing the distribution of identified proteins for SpPdp11 ECP conditions: (A) FM1548, (B) FM2324 and (C) M2324. The x-axis shows log2/fold change), and the y-axis shows −log10 (adjusted *p*-values). The two dotted black vertical lines represent a −log2 of 0.5–2 for the fold change, while the dotted horizontal line denotes −log 10 of our significance threshold ($p \leq 0.05$). Blue dots represent upregulated proteins (up); orange dots represent downregulated proteins (down); and grey dots represent non-differentially expressed proteins (ns).

DISCUSSION

Cytotoxicity analysis

The cytotoxicity profile of SpPdp11 ECPs varied depending on the media used and the cell lines tested. Specifically, ECPs produced in FM and M media–enriched with microalgae and cyanobacteria–generally exhibited lower cytotoxicity compared to those produced in T and F media. This could suggest that the inclusion of microalgae and cyanobacteria as a nutritional source may contribute to the production of bioactive molecules that enhance cell health, as opposed to the cytotoxic effects observed with traditional feed (F) or culture (T) media. For instance, Hussein et al. (2020) reported that microalgae metabolites exhibit antioxidant properties, reducing oxidative stress in cells. Thus, obtaining SpPdp11 ECPs from microalgae/cyanobacteria-enriched media could have a synergistic effect, as SpPdp11 has also demonstrated antioxidant

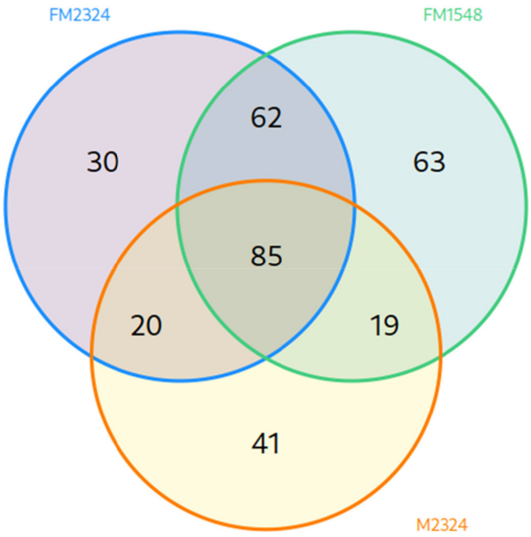

FIGURE 7 Venn diagram illustrating the distribution (unique or common) of all identified DEPs across different ECP conditions (FM2324 in blue, FM1548 in green and M2324 in yellow) compared to the control.

properties (Vidal et al., 2016). Culturing the probiotic in such enrich media could positively influence postbiotic production and modulate its effect on different fish cell lines tested.

For example, in DLB-1, FuB-1 and PLHC-1 cells, none of the ECPs obtained from FM and M media exhibited cytotoxicity. However, in SAF-1 cells, only one ECP condition (M1548) exhibited cytotoxicity at the highest concentration (1.5 μg protein/mL). This cytotoxic effect could be due to the release of toxic contaminants or metabolites, such as mycotoxins, biogenic amines or cyanogenic glycosides, among others, which can occur during fermentation processes (Sivamaruthi et al., 2018) or as a result of microbial metabolism of different substrates (Kim et al., 2020). In addition, different fish cell lines can exhibit different sensitivities in in vitro cytotoxicity assays (Rodrigues de Souza et al., 2023). Specifically, the four fish cell lines used in this study have been shown to respond differently to toxicants, stressors and media, and they exhibit different mitochondrial metabolism (Espinosa-Ruiz et al., 2022). Moreover, cytotoxic responses in these cell lines appear to be influenced by a dose-dependent effect (Domínguez-Maqueda, García-Márquez, et al., 2024).

On the other hand, FM23 control and M23 control conditions were cytotoxic at the highest concentration (1.5 μg protein/mL) in PLHC-1 cells, suggesting that the cytotoxicity could be attributed to the media itself. Microalgae can also be a rich source of cytotoxic bioactive compounds (Hassan et al., 2022). Some metabolites, while described as antioxidants, can also exhibit cytotoxic effects, as observed with carotenoids (Pereira et al., 2021) or phenolic compounds (Lopez-Corona et al., 2022), which may extend to other potential metabolites as well. This may suggest that SpPdp11 ECPs may have a mitigating effect on the cytotoxicity of the media, particularly in FM and M media.

Although FM and M media seem to be more favourable for producing postbiotics with safer and desirable profiles, further studies will be necessary to elucidate the mechanisms of action between the probiotic and the media. Nonetheless, these findings allow us to preliminary select the best postbiotics for future in vivo experiments.

Hydrolytic and haemolysis activities

Hydrolytic activities of ECPs were strongly dependent on the media in which they were produced. ECPs obtained from FM media hydrolysed gelatin, casein and Tween-80, similar to what was reported for T and F media (Domínguez-Maqueda, García-Márquez, et al., 2024). The variation in the composition of individual nutrients in the growth media of a microorganism is an important factor, which can modulate its postbiotic capabilities (Domínguez-Maqueda, García-Márquez et al., 2024). The F media exerted the highest hydrolytic activity, followed by FM and M media (F >FM >M). This hierarchy could be related to the varying proportions of the experimental aquafeed in the media, with F media being composed entirely of aquafeed, FM media containing 75% and M media lacking experimental aquafeed. The high lipid, carbohydrate, pigment and protein content of microalgae (González-González, & de-Bashan, L.E., 2021) in the M media may be sufficient for supporting probiotic growth requirements, reducing the need for increased metabolite secretion and consequently lowering its hydrolytic capability. All these findings may suggest that FM media as a promising media to obtain postbiotics with enhanced hydrolytic activity.

Proteases, such as caseinase and gelatinase, which have been associated with helping the bacteria augment tissue invasion and colonization (Mekasha & Linke, 2021), were found in the ECPs. These proteases may play a role in gut colonization and nutrient absorption, as suggested by the ability of SpPdp11 to encode proteins necessary for gut colonization (Cámara-Ruiz et al., 2020; Seoane et al., 2019). This is particularly relevant in carnivorous fish, such as *S. aurata* or *S. senegalensis*, where SpPdp11 has been reported to improve digestibility (Lobo et al., 2014; Varela et al., 2010). Additionally, amylase activity, only detected in FM1548, could facilitate the digestion of complex carbohydrates in fish (Nolasco-Soria, 2021). Lipase activity was also found in ECPs from T, F and FM media. Lipase activity was also observed in ECPs from T, F and FM media, potentially supporting lipid digestion, as has been previously demonstrated by SpPdp11 in *S. senegalensis* (de la Banda et al., 2010).

Several ECP conditions also demonstrated DNase activity, especially when they were obtained at 23°C and 24 h of incubation, regardless of the culture media. However, this activity was absent at 15°C, except for FM1548. In this way, modifying the cultivation conditions of the probiotic strain could affect this activity. Although the production of DNase enzymes has been considered a virulence factor, nucleases secreted by probiotics could also be considered useful (Hasegawa et al., 2010). For example, *Lactobacillus* spp. has demonstrated activity against Gram-negative bacteria and bacteriophages and may be associated with nutritional functions Liévin-Le Moal et al., 2014. The production of DNase enzymes by SpPdp11 may serve a protective role by degrading extracellular DNA in biofilm matrices, inhibiting or dispersing biofilm formation of pathogens like *Staphylococcus aureus*, *Pseudomonas aeruginosa* (Deng et al., 2023) or *Burkholderia pseudomallei* (Pakkulnan et al., 2023). This ability to degrade extracellular DNA could also confer advantages in terms of pathogen adhesion and/or inhibition, further suggesting the potential safety and efficacy of SpPdp11 ECPs for use in aquaculture.

The haemolytic activity has been reported to be culture and/or temperature dependent (Yi et al., 2022). In our study, no β-haemolysis activity was detected in any of the ECPs tested. This finding supports the safety of the ECPs, as haemolytic activity can indicate virulence and its absence is crucial for probiotics and their derivatives in industrial applications (Cizeikiene & Jagelaviciute, 2021).

Antibacterial activity

SpPdp11 ECPs did not exert any antagonistic effect against all fish pathogens assayed, which agrees with previous results (Domínguez-Maqueda, García-Márquez, et al., 2024). However, this contrasts with the antibacterial activity previously observed for SpPdp11 cells (Chabrillón, Rico, Balebona, & Moriñigo, 2005). The lack of antibacterial activity of the ECPs, in comparison with the cells, is in line with other studies reporting that postbiotics from different bacterial strains reduced or abolished their antagonistic effect in comparison to their bacterial viable cells. For example, Păcularu-Burada et al. (2020) described that postbiotics from wild lactic acid bacteria (LAB) did not exert an antagonistic effect against *Aspergillus niger*, in contrast with their viable cells. In addition, Quintanilla-Pineda et al. (2023) demonstrated how postbiotics obtained from some bacterial strains isolated from fish natural microbiota showed less antagonistic effects against *Aeromonas salmonicida* subsp. *salmonicida* and *Yersinia ruckeri*, compared to viable cells. Thus, a possible explanation for the lack of antibacterial effect in the ECPs could be that they were too diluted to exhibit noticeable effects. It is also possible that the lack of activity was due to the absence of contact-dependent growth inhibition (CDI) system, which requires direct physical contact between bacterial cells (Ikryannikova et al., 2020). Despite this, SpPdp11 may influence virulence factors, biofilm formation or quorum-sensing mechanisms in pathogens (Azami et al., 2022; Ishikawa et al., 2021), among others. These hypotheses related to the antagonistic potential of SpPdp11 deserve further investigation.

Proteomic analysis

Based on the previously studied features of the SpPdp11 ECPs, proteomic analysis was conducted on ECPs obtained from different culture conditions, FM1548 and FM2324 from FM media and M2324 from M media. The aim of the study was to uncover the bacterium's adaptive metabolic and regulatory responses. This analysis revealed both common and unique proteins across the different cultivation conditions, offering significant insights into the bacterium's adaptation mechanisms.

A core set of 85 proteins was commonly present across all ECP conditions, highlighting their essential role in the bacterium's survival and adaptability. Among these core proteins were transporters, efflux systems, proton and porin channels, and binding proteins were among the core proteins observed. These proteins not only facilitate the exchange of nutrients and signal molecules but also play crucial roles in extruding xenobiotics, aiding in cell adhesion and maintaining structural cell integrity (Prajapati et al., 2021).

Among the commonly overexpressed proteins, the transcriptional regulator with an XRE family HTH domain (COG1396) was particularly notable. Its consistent upregulation across all conditions underscores its critical role in modulating gene expression in response to environmental changes or nutrient availability (Lu et al., 2019). Additionally, proteins associated with energy metabolism and electron transport, such as DNA replication protein DnaC (COG1484) and CoA-transferase (COG1804), were consistently overexpressed. This suggests an increase in metabolic activity and higher energy demand, possibly driven by elevated rates of cellular replication and growth under nutrient-rich conditions (Ali et al., 2020; Juliana et al., 2013; Röttig & Steinbüchel, 2013). The presence of these proteins indicates a significant upregulation of metabolic pathways necessary for survival and growth, and energy production and utilization, which is critical for the bacterium's adaptation to efficiently exploit the available resources in the media.

In addition to the common proteins, each ECP condition exhibited a notable number of unique proteins, reflecting specific adaptations to the distinct culture conditions. In the FM2324 and FM1548 conditions,

which were derived from FM media, an increased abundance of proteins involved in amino acid metabolism and antioxidant systems was observed. For instance, chorismate synthase (COG0082), a key enzyme in the shikimate pathway, was overexpressed. This pathway is crucial for the biosynthesis of aromatic amino acids, folates and other essential metabolites needed for bacterial growth (Neetu et al., 2020). The enzyme is also an important antimicrobial target in Gram-positive bacteria such as *Listeria monocytogenes* (Vanajothi et al., 2023). Additionally, another prominent enzyme, threonine deaminase (COG1171), which catalyses the conversion of threonine to α-ketobutyrate and ammonia as a key step in isoleucine biosynthesis (Yeo et al., 2023), was also overexpressed. This enzyme's activity can be downregulated by L-isoleucine in *Escherichia coli*, demonstrating a mechanism of metabolic control through negative feedback (Jia et al., 2024).

Moreover, proteins such as cytochrome c (COG4235), involved in superoxide removal, and thioredoxins (COG0526), which maintain the thiol/disulphide balance, were also overrepresented. Cytochrome c plays a crucial role in regenerating oxygen and utilizing electrons in oxygen reduction without sustaining damage, unlike many other antioxidants (Pereverzev et al., 2003). Thioredoxins are essential for repairing oxidative damage by reducing oxidized disulphide bonds, which is vital for helping cells manage oxidative stress (Arnér & Holmgren, 2000). The overexpression of thioredoxin reductase in the probiotic *Lactobacillus plantarum* WCFS1 has been shown to enhance tolerance to oxidative stress (Yu et al., 2020), suggesting that a similar protective mechanism might be at play in SpPdp11 ECPs. These findings are consistent with previous studies indicating the antioxidant capabilities of SpPdp11 when included in fish diets, potentially linked to these overexpressed proteins (Vidal et al., 2016). Additionally, ECPs from SpPdp11 demonstrated antioxidant effects in ex vivo assays using head kidney leukocytes from *D. labrax* challenged with the pathogen *P. damselae* subsp. *piscicida* (Domínguez-Maqueda, Espinosa-Ruíz, et al., 2024). This suggests that ECP's antioxidant activity could improve the cellular health of various cell lines assayed in this study, playing a critical in defending against oxidative stress.

Furthermore, both FM2324 and FM1548 conditions showed overexpression of proteins involved in primary responses, such as components of the type II secretion system (T2SS) and T4 pilus assembly pathways (COG2804). The T2SS can secrete a range of proteins and effectors, often associated with pathogenic bacteria but also found in non-pathogenic species, including those in *Shewanella* genus (Cianciotto & White, 2017). For instance, in *Shewanella oneidensis*, for example, T2SS is linked to iron reduction and extracellular respiration (Gralnick et al., 2006). In non-pathogenic contexts, T2SS can facilitate symbioses or secrete degradative enzymes like proteases, lipases or DNases (Korotkov & Sandkvist, 2019), which aligns with the hydrolytic activities observed in the ECPs. Additionally, pili are essential for bacterial functions such as adhesion, locomotion and gene transfer, physically connecting bacteria to their environment (Pelicic, 2023). For example, the pili gene cluster in *Bifidobacterium breve* UCC2003 is essential for colonization and persistence in the murine gut (O'Connell Motherway et al., 2011). Similarly, enhanced expression of Tad pili *in B. lactis* A6 during stationary phase aids in intestinal colonization and persistence (Wang et al., 2022). These findings may correlate with earlier studies where SpPdp11 demonstrated in vitro adhesion capabilities to the intestinal mucus of *S. senegalensis*, competing with fish pathogens for adhesion sites (Chabrillón, Rico, Arijo, et al., 2005; Chabrillón, Rico, Balebona, & Moriñigo, 2005; Seoane et al., 2019).

In the ECPs obtained from M media (M2324), a distinct set of unique proteins was identified, notably including two methyl-accepting chemotaxis proteins (MCPs) (COG0840 and COG1871). MCPs play a crucial role in chemotaxis, enabling motile bacteria to navigate environmental gradients of attractants and repellents. This ability helps bacteria regulate their movement in response to factors like temperature, pH and nutrient levels, thereby avoiding unfavourable environments and adapting to more suitable niches (Feng et al., 2021). Additionally, MCPs are well studied for their role in plant–microbe interactions (Karmakar, 2021), as they are involved in the biodegradation of aromatic compounds (ACs) and polycyclic aromatic hydrocarbons (PAHs), which are commonly found in the structure of plant cell walls (Colin et al., 2021). This is consistent with the presence of catechol 2,3-dioxygenase protein (COG0369), a key enzyme involved in phenolic degradation (Murphy et al., 2023), and flavin reductase protein (COG0346), which provides reducing power for the electron transport chain during fatty acid oxidation and amino acid degradation (Henriques et al., 2021).

There are many proteins identified in the different ECP conditions which are not expected to be present extracellularly. In this way, it is also plausible that certain intracellular proteins may have been secreted through non-classical secretion pathways or released in response to cellular stress (Rabouille, 2017). Non-classical secretion mechanisms, as observed in other bacterial proteins, do not require a signal peptide and have been documented to transport intracellular proteins to the extracellular environment (Maricchiolo et al., 2022). The majority of these proteins play different functions when they are in the intra- and extracellular environments, and several of their functions are related to survival (Kang & Zhang, 2020). Therefore,

although the DEPs identified have been associated with roles according to their GO category, some of the identified proteins may have different functions, which they perform as secondary roles in the extracellular space.

In summary, the proteomic analysis reflects the metabolic versatility of SpPdp11 in response to different culture conditions. The findings emphasize the importance of transcriptional regulation, energy metabolism and stress response mechanisms in facilitating bacterial adaptation to diverse environments. This suggests that the strategic use of postbiotics, particularly in aquaculture, may support more effective and sustainable management strategies. However, the body of research on postbiotics remains somewhat constrained, and further empirical investigations and in vivo challenges will be essential to fully understand their potential benefits and to foster broader acceptance within the aquaculture, aquafeed and biotechnological industry.

AUTHOR CONTRIBUTIONS

Marta Domínguez-Maqueda: Methodology; formal analysis; data curation; writing – original draft; writing – review and editing; investigation. **Olivia Pérez-Gómez:** Methodology; software; formal analysis. **Jorge García-Márquez:** Methodology; writing – review and editing; formal analysis; investigation. **Cristóbal Espinosa-Ruíz:** Investigation; methodology; formal analysis. **Alberto Cuesta:** Investigation; supervision. **Mª. Ángeles Esteban:** Supervision; project administration. **Francisco Javier Alarcón-López:** Resources. **Casimiro Cárdenas:** Formal analysis; software; methodology. **Silvana T. Tapia-Paniagua:** Writing – review and editing; supervision; validation. **Mª. Carmen Balebona:** Writing – review and editing; project administration; supervision; funding acquisition; visualization. **Miguel Ángel Moriñigo:** Writing – review and editing; project administration; supervision; funding acquisition; visualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data are available in the manuscript.

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