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Biological and chemical characterization of new isolated halophilic microorganisms from saltern ponds of Trapani, Sicily

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ABSTRACT

Halophilic microorganisms inhabiting hypersaline environments such as salt lakes, Dead Sea, or salt evaporation ponds, have acquired specific cell adaptation to grow within stressful conditions. In this study, we isolated heterotrophic and autotrophic microorganisms from several saltern ponds located at the Natural Reserve "Saline di Trapani e Paceco", Sicily, Italy. The aim of the study was to investigate the biotechnological potential of new microbial strains from saltern ponds, by capturing their biological and chemical diversity. After the isolation and identification of the sampled strains, their growth capacity was determined under low and high salinity conditions. The metabolomic profiles of heterotrophs and pigments production of photosynthetic organisms were analyzed. In parallel, antiproliferative tests on human cell lines were conducted with total extracts coming from the microorganism cultures, together with repair activity assessment of non-cytotoxic extracts. Some of the isolated strains were found to synthetize known bioactive molecules and to exert bioactivity on human cells. In particular, the high salinity increases cell repair activity, probably due to an higher production of antioxidants pigments (*e.g.* lutein and fucoxanthin) from photosynthetic microorganisms; same culture condition augment also concentration of molecules with interesting bioactivities, such as ectoine, betaine, trigonelline, amino acids and oxiglutathione from heterotrophic microorganisms. In conclusion, this work represents the first study on the isolation of halophilic microorganisms populating the 'Trapani-Paceco' saltern and shows how an interdisciplinary investigation based on marine microbiology, cell biology, and modern metabolomics can disclose their biotechnological potential.

1. Introduction

Living microorganisms have developed adaptive mechanisms to survive environmental changes [1]; however, only a few classes of microorganisms (*i.e.* extremophiles) can grow under extreme environmental conditions, such as high salinity, temperature and pH. Main strategies for surviving in such environments include the production of specialized secondary metabolites, which are of great interest for biotechnological research and industry [2].

Extremophiles that are able to grow in high salt concentrations are divided into halotolerant and halophiles. Halotolerant microorganisms can survive in a large range of salt concentrations up to saturation level. Halophiles instead require high salinity to grow, *i.e.* they grow optimally at 50 g/L of NaCl or higher, and can tolerate at least 100 g/L of NaCl [3]. All these microorganisms, which populate hypersaline environments such as solar salterns, salt lakes, and Dead Sea, have developed chemical strategies to protect themselves against osmotic shock [4]. In particular, a large number of halophilic organisms accumulate organic solutes such as glycine betaine, sugar, and ectoine under high salt concentration conditions [5]. Among these solutes, ectoine is commercialized in the cosmeceutical industry thanks to its ability to protect the skin from UVA rays [6]. Moreover, halophile organisms accumulate a large amount of

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carotenoids (*e.g.* β-carotene, lutein, fucoxanthin, neoxanthin, *etc.*) under high salinity and high light intensity conditions, to protect the cell against UV-related damage and oxidative stress. Due to the accumulation of these molecules (mainly carotenoids), salterns are frequently pink-red colored during the summer seasons [7]. Because carotenoids show beneficial effects on human health, the cultivation of halophilic microorganisms to harvest their bioactive pigments is of increasing interest for the blue economy.

Various taxa of halophilic microorganisms were found in such environments, belonging to all the three domains of living organisms: Archaea (*e.g.* haloarchaea), bacteria (*e.g.* cyanobacteria), and eukaryote (*e.g.* microalgae). Among these, the best-known and representative microalgae are two species of the flagellate green algae: *Dunaliella viridis* and *Dunaliella salina* [8]. The latter is one of the most studied microalgae due to its ability to accumulate a large amount (up to 14% of dried biomass) of β-carotene under extreme environmental conditions [9]; hence, it is one of the few microalgae that is employed for the industrial production of β-carotene [10,11]. *Dunaliella viridis* does not accumulate large amounts of β-carotene, but produces oxygenated carotenoids (*e.g.* lutein and neoxanthin) and grows faster than *Dunaliella salina* [8].

The microorganisms of the archaea domain most commonly found in hypersaline environments are the red-colored species haloarchaea of the genus *Halobacterium*. In particular, *Halobacterium salinarum* is the main inhabitant of solar salterns where the salt concentration exceeds the saturation level, together with *Dunaliella salina*.

Finally, among hypersaline bacteria, we can find both photosynthetic and heterotrophic bacteria. Examples of heterotrophic bacteria are the halophile red bacterium *Salinibacter ruber* and *Halomonas* sp., which were isolated from different solar salterns in Spain [12] and the south-eastern coast of India [13], respectively. Among photosynthetic bacteria, several strains of the *Cyanothece* group isolated from hypersaline habitats have been characterized for the production of exopolysaccharide (EPS) with biotechnological interest [14].

The objective of this work was to isolate and characterize halophilic microorganisms from the hypersaline pond environment to disclose their ability to grow under controlled laboratory conditions and to produce bioactive compounds, in order to highlight their potential for further biotechnological and industrial potential applications.

The natural area "Saline di Trapani e Paceco" was selected as the sampling site; it is formed by several pans dedicated to the extraction of sea salt in the province of Trapani, a city on the west coast of Sicily (Italy). The different salt concentration in these pans allows the proliferation of diverse halophilic and halotolerant microorganisms, whose pigments confer to the pans a color ranging from white to pink or red. The sampling led to the isolation and molecular identification of photosynthetic and heterotrophic halophilic strains. Total extracts of biomasses obtained with optimized culture conditions were tested for their potential bioactivities, such as cytotoxicity and cell viability recovery activity on a panel of human cell lines.

The photosynthetic halophile extracts showing significant cell viability recovery activity were further analyzed by HPLC for the determination of bioactive pigments. Heterotrophic halophiles extracts displaying cytotoxic activity on cancer cell lines were screened *via* liquid chromatography coupled to mass spectrometry (LC-MS/MS) to highlight variations of their metabolomes induced by low *vs.* high salinity growth conditions.

2. Material and methods

2.1. Sampling site

The sampling was carried out in the "Saline di Trapani e Paceco" during summer (September 2017), in two pinkish salt ponds characterized by different salinities (*i.e.* about 100 and 200 g/L of NaCl; site 1: GPS coordinates 37.966845, 12.505649 and site 2: GPS coordinates 37.996249, 12.532758). The location of ponds *Salinella* and *Galia* are shown in Fig. 1. Seawater from these ponds was collected in sterile 0.5 L flasks.

2.2. Isolation of microalgal strains

A combination of filtration and dilution methods was used for the isolation of individual microalgae species. The first phase of isolation involved the use of wide mesh filters (>200 μm) that allow removing the impurities from the initial sample (*i.e.* debris, sand, rock, macroalgae). The filtration method was coupled with serial dilutions in 24-well microplates to obtain a single strain per well. Once the individual strains were obtained, the liquid cultures were transferred into a solid medium to obtain individual colonies that can be used for molecular characterization by colony PCR. For serial dilutions, liquid and solid f/2 medium [15] with different concentrations of NaCl (ranging from 20 to 200 g/L) were used. In parallel, bacterial strains present in the initial cultures together with the microalgae were isolated by serial dilution methods in plates containing Difco™ Marine Broth + 2% agar. To obtain axenic cultures of microalgae, a mix of antibiotics (*i.e.* 100 μg/mL of Ampicillin, 50 μg/mL Streptomycin, 50 μg/mL Kanamycin and 10 μg/mL Gentamicin) was used for the removal of bacteria during the isolation phases.

2.3. Molecular identification of isolated microorganisms

Molecular characterization (starting from single colonies on a plate) was performed by amplification and sequencing of the 18S rDNA or ITS1 for microalgae and 16S rDNA for bacteria using Q5 ® high-fidelity DNA Polymerase (NEB). Colony PCR was performed picking one single colony in 25 μL of sterile TE (Tris-EDTA) buffer, which was incubated at 100 °C × 5 min followed by 5 min in ice. The samples were centrifuged at high speed and 1 μl of supernatant was used as a template for PCR reactions. 16S rRNA PCR amplifications were performed using the universal primers fD1 5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG and RD1 5′-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC and the following program: 95 °C (5 min), followed by 30 cycles at 95 °C (45 s), 50 °C (45 s) and 68 °C (90 s) and a final 10 min extension step at 68 °C. The identification of the cyanobacteria was done using a 16S-rDNA-ITS sequence established with the PacBio amplicon sequencing approach at the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig (unpublished data). 18S rRNA PCR amplifications were performed using the forward primer A (5′-ACC CTG GTT GAT CCT GCC AG-3′) and primer SSU-inR1 (5′-CAC CAG ACT TGC CCT CCA-3′) and the following program: 95 °C (5 min), followed by 32 cycles at 95 °C (30 s), 55 °C (30 s) and 68 °C (60 s) and a final 7 min extension step at 68 °C. PCR analysis for the species identification of *Dunaliella* strains was performed for the amplification of ITS1 region using the genus-specific primers [16] DITS_F 5′-AATCTATCAATAACCACA and DITS_R 5′-TTTCATTCGCCATTACTA and the following program: 95 °C (5 min), followed by 30 cycles at 95 °C (30 s), 52 °C (30 s) and 68 °C (60 s) and a final 5 min extension step at 68 °C. The PCR products were run on 1% of agarose gel (ethidium bromide 0.2 μg/mL) against Thermo Scientific™ Mas*Sr*uler™ DNA Ladder Mix. The PCR products were purified using the NucleoSpin® Gel and PCR Clean-up and sequenced by BMR service genomics (60 ng of DNA + 0.6 ng of each primer). The sequences obtained were then analyzed using online bioinformatics software BLAST for the alignment of sequences and the identification of the belonging species.

The 16S rRNA gene sequences from halophile bacteria *Halomonas* sp. (AN: MT258978), *Salinibacter ruber* (AN: MT258979), and *Brevibacterium* sp. (AN: MT258980) have been deposited in GenBank, as well as the ITS 1 gene sequence from *Dunaliella viridis* (AN: MT259044). The isolated strains were sent to the DSMZ culture collection with the code: 8464 *Brevibacterium* sp. *(Tp)*, 8465 *Salinibacter ruber (Tp)*, 8408 *Halomonas* sp. *(Tp)*, 8710 *Dactylococcopsis salina*, and 8719 *Dunaliella viridis (Tp)*.

Fig. 1. Aerial drone photo of Solar salterns of "Saline di Trapani e Paceco", Trapani (Italy); stars indicate the location of sampling, * = Salina Galia, ** = Salina Salinella.
Photo by Giovanni Nicolosi.

2.4. Cultivation of microalgal and bacterial halophilic strains

The isolated microalgae and cyanobacteria strains were maintained in the laboratory in liquid f/2 media + 50 g/L NaCl and solid media f/2 media + 50 g/L NaCl + 1,5 g/L agar (in Petri dishes). The heterotrophic halophilic bacteria were kept in the same medium with the addition of carbon sources (*i.e.* 3 g/L glucose, 8 g/L tryptone, 10 g/L yeast extract).

Two culture conditions for each strain (microalgae and bacteria) were set up, in biological triplicate (1 L), for salinity test: *i)* low salt (LS) concentration (*i.e.* 50 g/L NaCl); *ii)* high salt (HS) concentration (*i.e.* 100 g/L NaCl) and with a fixed light intensity of 100 μmol m^{-2} s^{-1}.

50 g/L NaCl represents the optimal growth condition for a large number of halotolerant and halophilic microorganisms, while 100 g/L was used as a stress condition to stimulate the production of secondary metabolites. Only the extreme halophile *Salinibacter ruber*, requesting higher salt concentration to survive, was grown in 150 g/L NaCl and 200 g/L as normal and stress conditions, respectively.

Cells were collected after 5 or 8 days, and the biomass was freeze-dried for further analysis. Cell growth of each strain was monitored daily by measuring the optical density at 550 nm for heterotrophic and 750 nm for phototrophic microorganisms, using a UV–Vis spectrophotometer and morphology observation at light microscope.

2.5. Metabolite extraction

2.5.1. Photosynthetic microorganisms

Freeze-dried biomass was extracted with a hydroalcoholic solution (ethanol/water: 3/1, v/v). Extraction was carried out by using vortex and pestle under dark conditions. Mixtures were centrifuged at 1500 ×*g* for 10 min at 4 °C. Supernatants were collected in clean tubes, and the extraction procedure was repeated for maximizing extract yield. Supernatants were dried-out by rotary evaporation and freeze-drying.

2.5.2. Heterotrophic microorganisms

Freeze-dried biomass was extracted with 70 mL of acetone (100%) and shook continuously for 3 h. After centrifugation, the organic phase was recovered and transferred in new pre-weighed Eppendorf tubes. Supernatants were dried by a speed vacuum. The dry mass of extracts was calculated by weighting the extract-containing Eppendorf.

2.6. Biological assays

2.6.1. Maintenance of human cell cultures

The human prostatic adenocarcinoma cell line (PC3) and the human prostatic epithelial cell line (PNT2) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. The human colorectal adenocarcinoma cell line (HT29) was maintained in McCoy's 5A medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The human lung epithelial-like cell line (BEAS-2B) was maintained in Bronchial Epithelial Cell Growth Medium (BEGM), containing all the recommended supplements. Cells were grown in a 5% CO_2 atmosphere at 37 °C.

2.6.2. Cytotoxicity and cell viability recovery assay

Cells (2×10^3 cells/well) were seeded in 96-well plates and kept overnight for attachment for cytotoxicity assessment. Dried extracts were dissolved in dimethyl sulfoxide (DMSO) and used for the treatment of cells. The final concentration of DMSO used was ≤0.5% (v/v) for each treatment. Cells were treated in biological triplicate with 1, 10, and 100 μg/mL of extracts for 48 h in complete cell medium. At the end of incubation with samples, cytotoxicity was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Extracts that did not show cytotoxicity on all cell lines were selected for the study of potential cell recovery activity on BEAS-2B. BEAS-2B cell lines were pre-injured with 10 mM of H_2O_2. After 1 h, the medium was removed and cells were recovered with a fresh medium containing 1, 10, and 100 μg/mL of selected extracts, to understand if extracts were able to activate repairing mechanisms and reduce oxidative damage,

restoring cell viability at the levels of the untreated control. Cells treated with only 10 mM of H_2O_2 for 1 h and then refreshed with only complete medium were used as positive control (*i.e.* injured cells). Untreated cells were used as negative control (100% of viability). Cell viability recovery activity was at the last evaluated by MTT assay.

2.6.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cell viability was evaluated using the MTT assay. Cells, after 48 h of treatments, were incubated with 10 μL (5 mg/mL) of MTT for 3 h at 37 °C in a 5% CO_2 atmosphere. Isopropanol (100 μL) was used to stop the incubation time and to solubilize purple crystals formed in each well. The absorbance was recorded on a microplate reader at a wavelength of 570 nm. The effect of the samples at different concentrations was reported as a percent of cell viability, calculated as the ratio between the mean absorbance of each treatment and mean absorbance of control cells (untreated cells that represent the 100% of viability).

2.7. Metabolome analysis

Untargeted metabolomics of the *endo*-metabolites was performed on three independent biological replicates of halophile bacteria grown in the two salinity conditions (*i.e.* LS and HS). Dry cell extracts were reconstituted in acetonitrile/water (1/1) to give a concentration of 5 mg/mL. 0.8 mg/L of naproxen (Sigma-Aldrich, Taufkirchen, Germany) and 0.1 mg/L of caffeine (Sigma-Aldrich, Taufkirchen, Germany) were added to the reconstituted samples as internal standards, since they are not expected to be produced by the investigated organisms. In addition, small aliquots of biological replicates were united into single "pool" samples, which were then processed together and in the same way as the biological samples.

Metabolome data were acquired in positive ionization mode with an Ultra High Performance Liquid Chromatography (UHPLC; UltiMate 3000 RS, Thermo Scientific Dionex, Waltham, MA, USA) coupled with electrospray ionization quadrupole time-of-flight (tandem) mass spectrometry (UHPLC-ESI-QTOF-MS(/MS); MaXis HD QToF, Bruker, Bremen, Germany). In particular, 5 μL of each sample was injected in the instrument and eluted through a Kinetex C18 reversed-phase column (1.7 μm, 150 × 2.1 mm from Phenomenex, Aschaffenburg, Germany) kept at 40 °C, with a flow rate of 300 μL/min. Gradient elution with water (+0.1% v/v formic acid) as eluent A, and acetonitrile (+0.1% v/v formic acid) as eluent B, was run as follows: 1% B for 0 to 2 min, linear gradient from 1% B to 100% B from 2 to 20 min, hold 100% B until 25 min and linear gradient from 100% B to 1% B from 25 to 30 min.

Pre-processing of LCMS spectral data for untargeted analytes profiling was performed in Rstudio [17]. The following steps were implemented to convert raw data into a data matrix suitable for analysis that contained so-called features, *i.e.* analytes. They are characterized by the *m/z* and retention time (RT), and their intensities in the different samples: feature detection (or peak picking) in individual samples followed by matching of features between samples (for chromatographic data), followed by retention time alignment and binning of the spectra into "buckets" (for spectroscopic data).

Normalization of obtained features intensity on the internal standards, together with filtering of data by retention time was done with Rstudio software (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria) in the Rstudio environment [18] (version 1.2.1578, PBC, Boston, USA).

Metabolite identification was performed by comparison to an in-house library of chemical standards measured under the same conditions as the analytes. A match was based on a correspondence of precursor mass, room temperature (RT), and MS/MS spectrum. When standards were not available, known metabolites annotation was carried out by searching their exact mass values and comparing their MS/MS spectra with online spectral databases (METLIN, https://metlin.scripps.edu, the human metabolome database, http://www.hmdb.ca, and GNPS, https://gnps.ucsd.edu/), to obtain putative metabolite identifications, these annotated metabolites are indicated with "put" (for putative).

2.8. Statistical analysis

Multivariate statistical analysis was used as a first step: A principal component analysis (PCA) was performed on the global dataset to provide an unbiased overview of individual samples and to reveal patterns in the data and relationships between groups.

To highlight the alterations in metabolite abundance between two different salt treatments (NaCl content of 50 g/L *vs.* 100 g/L), a univariate analysis was performed and depicted as a volcano plot, where statistical significance (*p*-value in a $\log_{10}$ scale) was plotted *vs.* the fold change (fc) in feature intensities in a $\log_2$ scale.

Finally, the differentially abundant metabolites were further analyzed. To explore the metabolic accumulation patterns, features with significant variations were subjected to hierarchical cluster analysis (HCA). The intensity of each feature was normalized and $\log_2$ transformed. Statistical analysis was carried out using Rstudio software.

2.9. High-Performance Liquid Chromatography (HPLC) analysis of bioactive pigments

Pigment measurements were conducted by High-Performance Liquid Chromatography (HPLC) on total extracts, according to the protocol described in Brunet et al. [19]. Pigment extraction from the freeze-dried biomass was carried out as in Orefice et al. [20], using methanol 100%. Before injection into the HPLC, 250 μL of an ion pairing agent (ammonium acetate 1 mol/L, with a final concentration of 0.33 mol/L) was added to 0.5 mL of the pigment extract and incubated for 5 min in the dark at 4 °C. This extract was then injected in the 50 μL loop of the Hewlett Packard series 1100 HPLC (Hewlett Packard, Wilmington, NC, USA), equipped with a reversed-phase column (C8 Kinetex column; 50 mm × 4.6 mm; 2.6 μm particle size, Phenomenex®, USA). The temperature of the column was steadily maintained at 20 °C, and the flow rate of the mobile phase was set up at 1.7 mL/min. The mobile phase was composed of two solvent mixtures: A, methanol/aqueous ammonium acetate (70/30, v/v) and B, methanol. During the 12-minute elution, the gradient between the solvents was programmed: 75% A (0 min), 50% A (1 min), 0% A (8 min) isocratic for 3 min. Chlorophylls and carotenoids were detected by diode-array spectroscopy (spectrum data collected in the range 350–750 nm) using a Hewlett Packard photodiode array detector, model DAD series 1100, and absorbance chromatogram was reported at 440 nm. Chlorophylls were also detected by fluorescence using a Hewlett Packard standard FLD cell series 1100 with excitation and emission wavelengths set at 407 nm and 665 nm, respectively. Identification and quantification of pigments were carried out using pigment standards from the D.H.I. Water & Environment (Horsholm, Denmark). From the calibration curves (absorbance or fluorescence signal *vs* pigment weight) using pure pigments, pigments detected in the samples were quantified.

3. Results and discussion

3.1. Identification of halophilic microorganisms

Different microorganisms (including heterotrophic bacteria and photosynthetic microalgae) have been isolated from saltern ponds through the combination of dilution and filtration techniques. Species identification of isolated strains was performed by amplification of specific molecular markers through PCR. All the microorganisms isolated, sampling sites, and reference genes used are listed in Table 1.

In the pond with elevated salt concentration (*i.e.* Salinella), the green alga *Dunaliella viridis* (*Dv*) and three associated heterotrophic bacteria were found: the ectoine producing strains *Halomonas* sp. (*Hsp*),

Table 1
Isolated microorganisms used in this study.

Species	Sampling sites	Organism type	Reference genes
Dunaliella viridis (Dv)	Pond Salinella	Phototrophic	ITS1
Salinibacter ruber (Sr)	Pond Salinella	Heterotrophic	16S
Brevibacterium sp. *(Bsp)*	Pond Salinella	Heterotrophic	16S
Halomonas sp. *(Hsp)*	Pond Salinella	Heterotrophic	16S
Dactylococcopsis salina (Ds)	Pond Galia	Phototrophic	16S-ITS
Navicula sp. *(Nsp)*	Pond Galia	Phototrophic	18S

Brevibacterium sp. (*Bsp*) and the extreme halophilic red-colored strain *Salinibacter ruber* (*Sr*). *Dv* are often associated with the more studied strain *Dunaliella salina* in extremely high salinity environments [21,22]. The same heterotrophic bacteria have been found in other salterns, such as in the south-eastern coast of India and in Bulgaria [13,23].

In the pond with lower salt concentration (*i.e.* Galia), we found the EPS producing cyanobacteria *Dactylococcopsis salina* (*Ds*) and the diatom *Navicula* sp. (*Nsp*). *Cyanothece* sp., closely related cyanobacterial species of *Ds*, has been found in the same place of our study [14]. Many diatoms can tolerate high salt concentration and have been found in both solar salterns during the first stage of salt evaporation [24] and in very saline and polluted lakes such as the Salton Sea in California [25]. The diatoms mainly found in hypersaline water belong to *Navicula*, *Nitzschia*, and *Amphora*.

3.2. Growth curve on different salinity

The growth under high and low salinity (*i.e.* HS and LS respectively) of the different isolated microorganisms was compared (Fig. 2). *Dv* and *Ds* grew better in LS condition, while *Nsp* showed similar growth curves in both tested conditions (Fig. 2A–C). Conversely, heterotrophic microorganisms did not display any difference in growth between the two conditions (Fig. 2D–F). Halophilic prokaryotes have developed different regulatory mechanisms to contrast high salt concentrations; they are able to modify plasma membrane fluidity, activate biosynthetic pathways for the production of antioxidants contrasting oxidative stress and can tightly regulate ion homeostasis [26].

The morphological changes of the photosynthetic microorganisms (*i.e. Nsp*, *Dv*, *Ds*) in LS and HS are shown in Fig. 3. *Nsp* is a pennate diatom with a length range of 10–20 μm; this unicellular alga in HS condition appeared with yellowish plastids (Fig. 3A and D), confirming previous findings [27]. *Dv* is a unicellular flagellate green alga of the genus *Dunaliella* with a smaller cell dimension (~12 × 8 mm) able to change its volume and shape in response to osmotic stress [28]. Here, under HS condition, they showed an increase of about 40% on their volume (~20 × 10 mm) (Fig. 3B and E). *Ds* cells are non-motile with a spherical or ovoid shape, 8–10 μm in length, and showed different coloration with salinity level: they were blue-green in LS and yellow-green in HS (Fig. 2C and F). Changes in salinity conditions are already known to induce physiological modifications in diatoms and benthic organisms [29].

3.3. Human cell viability

Extracts of photosynthetic microorganisms did not affect cancer cells viability (*i.e.* HT29 and PC3) at all concentrations tested, *i.e.* 1, 10, and 100 μg/mL (Fig. 4A and B). The only exception was *Dv* grown under low and high salinity (*i.e. Dv_LS* and *Dv_HS*, respectively) which exerted slight cytotoxicity on PC3 (73, 82 and 72 with Dv_LS, 95, 82 and 73 with Dv_HS, at 1, 10 and 100 μg/mL respectively).

Moreover, all extracts at 1 and 10 μg/mL did not induce a significant reduction of viability in normal cells, *i.e.* BEAS-2B and PNT2 (Fig. 4C and D). *Dv_LS* extracts showed a strong cytotoxic effect (35%) on BEAS-2B cells at the highest concentration of 100 μg/mL. On the contrary, BEAS-2B cells treated with extract of *Dv_HS* did not exhibit strong cell viability decrease (72%) (Fig. 4C). All extracts showed a slight or moderate cytotoxic effect on PNT2 cells (Fig. 4D) at 100 μg/mL, ranging from 80% of *Dv*_LS to 50% of viable cells of *Ds_HS*. The difference in cytotoxic effect found between *Dv_LS* and *Dv_HL* extracts confirms the influence of salinity on activation of specific biosynthetic pathways, supporting evidences already reported in literature on variation of secondary metabolites productions in diatoms and dinoflagellates [30,31]. Total extracts of *Dv_HS* and *Nsp* and *Ds* grown under low and high salinity (*i.e. Nsp_LS*, *Nsp_HS*, *Ds_LS*, and *Ds_HS*, respectively) were assayed for cell repair activity, since they did not exhibit cytotoxicity on the four human cell lines.

All heterotrophic bacteria extracts, except for *Sr* low salinity (*i.e. Sr_LS*) induced moderate cytotoxicity (50 to 70% of viable cells) at the highest concentration (100 μg/mL), on HT29 (Fig. 5A). In addition, the

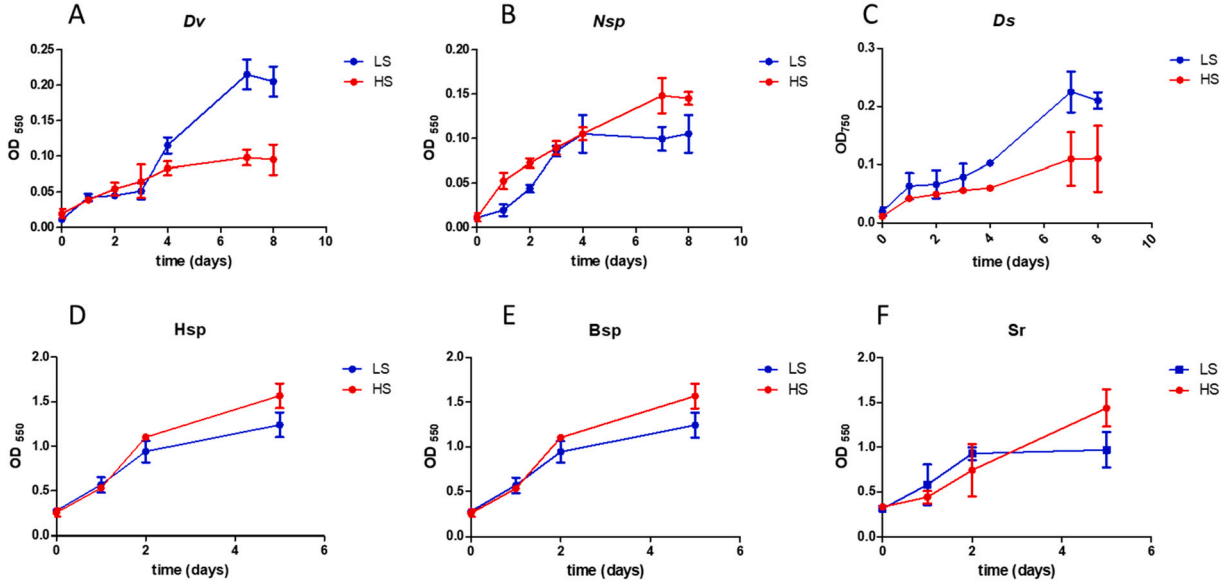

Fig. 2. Growth curves of the photosynthetic microorganisms Dunaliella viridis (A), Navicula sp. (B) and Dactylococcopsis salina (C), and of the heterotrophic microorganisms Halomonas sp. (D), Brevibacterium sp. (E), and Salinibacter ruber (F), under high salinity (HS, in red) and low salinity (LS, in blue) conditions. Measurements were done in triplicate ($n = 3$), obtained average and standard deviation values are shown in the graphs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

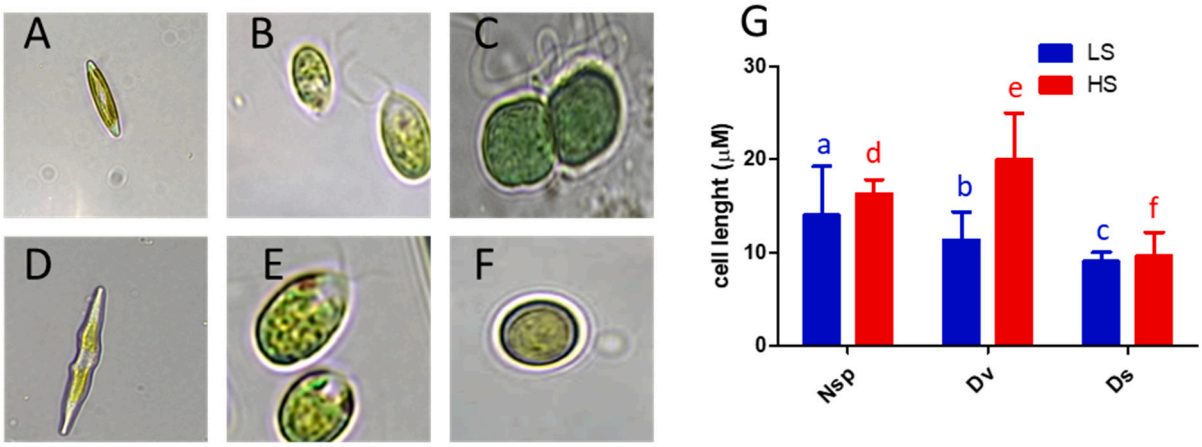

Fig. 3. Light microscopy pictures of Navicula sp. (Nsp, left panels), Dunaliella viridis (Dv, middle panels), and Dactylococcopsis salina (Ds, right panels) at the exponential growth phase in high salinity (HS) and low salinity (LS), (A–C) and (D–F) respectively. The cellular length in the different samples in the two conditions is shown in the bar graph (G). Measurements were done in triplicate (n = 3), obtained average and standard deviation values are shown in the graphs.

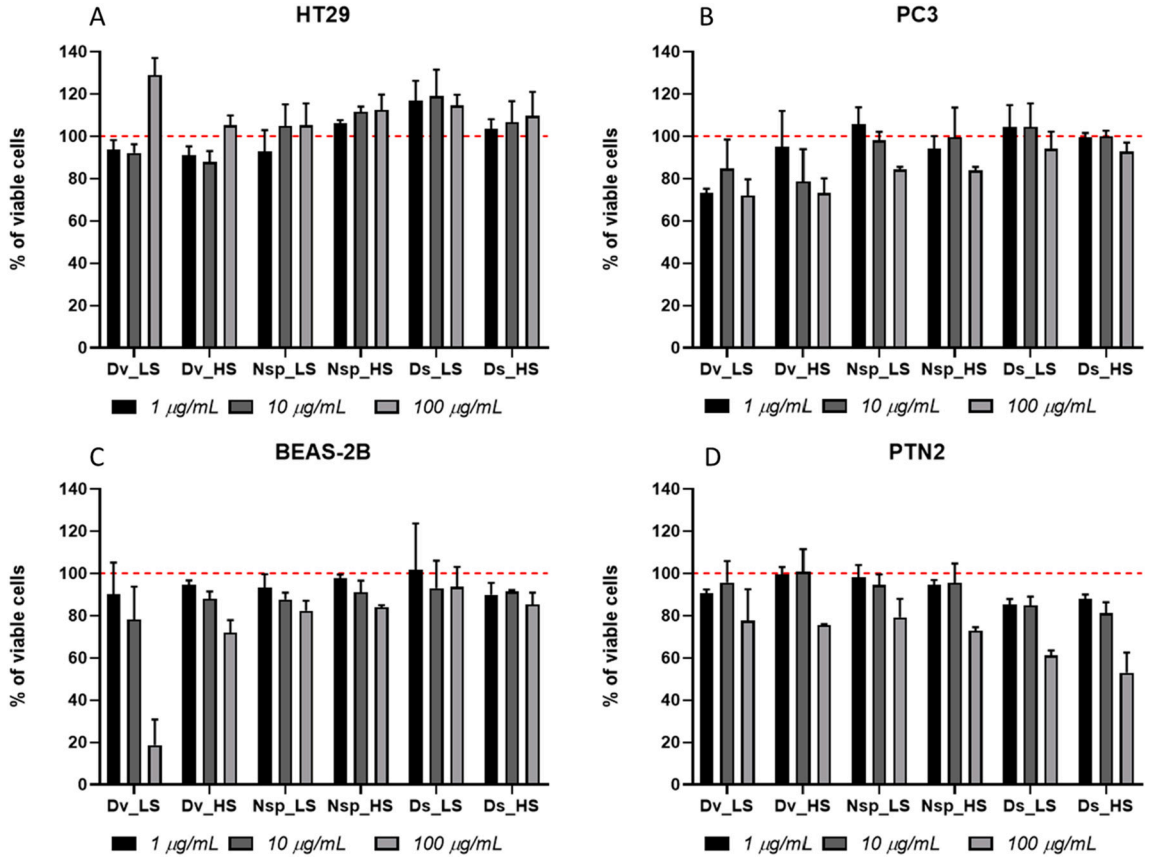

Fig. 4. Viability assay on HT29 and PC3 (A and B, cancer cell lines) and BEAS-2B and PNT2 (C and D, normal cell lines) of photosynthetic microorganisms. Dunaliella viridis (Dv), Navicula sp. (Nsp), Dactylococcopsis salina (Ds) under high salinity (HS) and low salinity (LS) growth conditions. Assays were performed in triplicate ($n = 3$), obtained average and standard deviation values are shown in the graphs. Untreated cells were used as control and corresponded to 100% of cell viability (dotted red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

same extracts induced a reduction of viability (from 80 to 50%) at 100 μg/mL on PC3 (Fig. 5B), except for *Hsp* grown in high salinity (*i.e. Hsp_HS*). *Hsp* was already investigated for its production of anti-proliferative compounds. In particular, biosurfactants produced by *Halomonas* sp. BS4 isolated in Thamaraikulam solar salt (India) exhibited an interesting *in vitro* antiproliferative activity against mammary epithelial carcinoma cells [32]. Thus, these species can be considered a potential marine source of bioactive compounds, and salinity an abiotic factor acting on regulation of its metabolism.

Contrarily to photosynthetic microorganisms extracts, all heterotrophic microorganisms showed slight or moderate cytotoxic effect (60 to 75% of viable cells) on normal cells, *i.e.* BEAS-2B and PNT2, at the highest concentration of 100 μg/mL (Fig. 5C and D) and hence were not selected for the cell viability recovery assay.

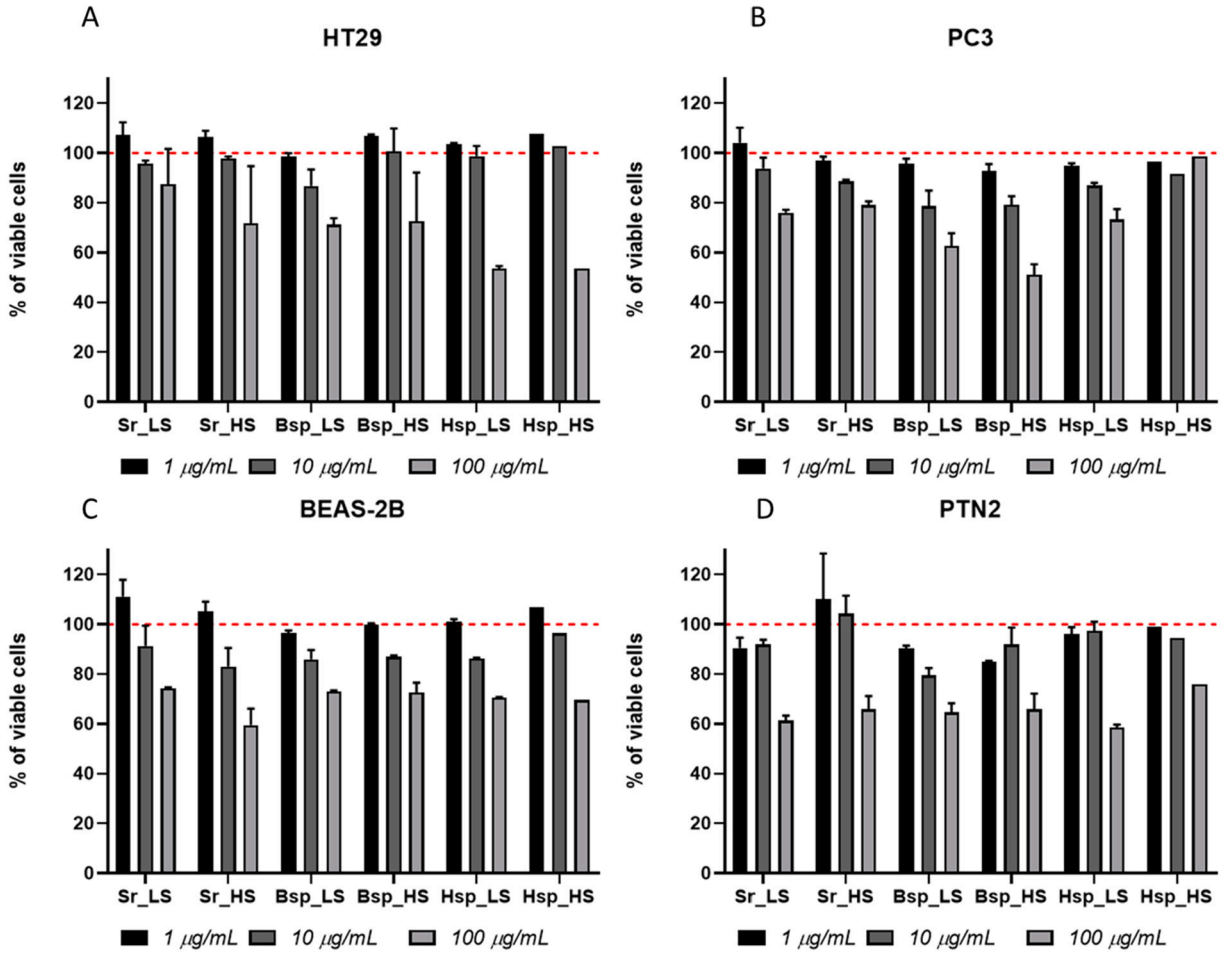

Fig. 5. Viability assay on HT29 and PC3 (A and B, cancer cell lines) and BEAS-2B and PNT2 (C and D, normal cell lines) of heterotrophic microorganisms. Salinibacter ruber (Sr), Halomonas sp. (Hsp), Brevibacterium sp. (Bsp) under high salinity (HS), and low salinity (LS). Assays were performed in triplicate ($n = 3$), obtained average and standard deviation values are shown in the graphs. Untreated cells were used as control and corresponded to 100% of cell viability (dotted red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Human cell viability recovery

Total extracts of *Dv_HS*, *Nsp_LS*, *Nsp_HS*, *Ds_LS*, and *Ds_HS* were tested for potential cell viability recovery activity on BEAS-2B. Cells treated with 10 mM of H_2O_2 for 1 h and then refreshed with only medium (positive control) showed a significant reduction of viability (57%, dotted red line in Fig. 6).

All extracts exerted moderate viability recovery activity, especially at 10 and 1 μg/mL. Treatments were able to restore cell viability up to a positive control level. The viability recovery activity assay highlighted how the increase of salinity seems to enhance production of molecules activating survival pathways in human cells (BEAS-2B injured with H_2O_2). This result is more evident with *Ds_HS* extract: 1 μg/mL can increase the viability of injured cells, at the same level of the untreated control. Our results confirm that high salinity growth condition can be employed as abiotic factor for the production of microalgal biomass with high beneficial properties against oxidative stress. Under high salinity, many microalgae (*e.g. Dunaliella salina*) accumulate large amounts of carotenoids that possess anti-oxidative activities [33].

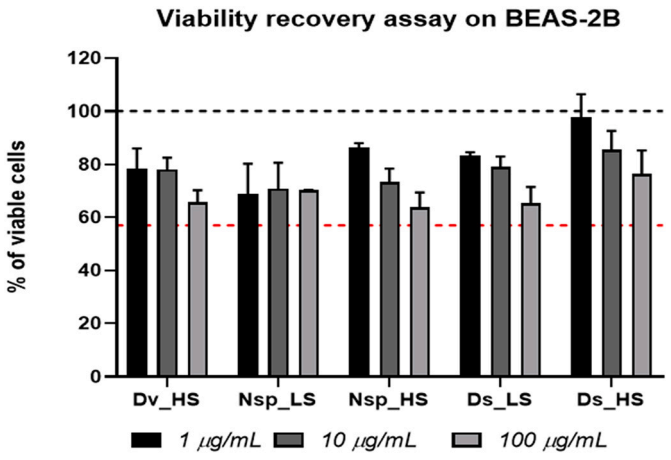

Fig. 6. Cell viability recovery assay on BEAS-2B cells with Dunaliella viridis high salinity (Dv_HS), Navicula sp. low salinity (Nsp_LS) and high salinity (Nsp_HS), Dactylococcopsis salina low salinity (Ds_LS) and high salinity (Ds_HS). The dotted black line indicates untreated control (100% of viable cells), and the dotted red line indicates cells injured with 10 mM of H_2O_2 for 1 h (57% of viable cells). Assays were performed in triplicate ($n = 3$), obtained average and standard deviation values are shown in the graphs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Carotenoids

A pool of bioactive pigments (*e.g.* carotenes and xanthophylls) could be responsible for the protective and antioxidant effect of natural extracts [34,35], acting with a synergistic effect on cells. For this reason, carotenoids analysis of samples that showed repair activity was

performed (*i.e. Dv_HS*, *Nsp_HS*, and *Ds_HS*), to characterize carotenoids content, potentially responsible for cell repair activation in H_2O_2-damaged cells.

Dv is a green alga that possesses lutein, chlorophyll *a* and b and a large number of mixed carotenoids. In our study, we identified other oxygenated carotenoids such as neoxanthin, violaxanthin, α- and β-carotene, and antheraxanthin (Fig. 7A). Moulton et al. found that lutein is the main carotenoid in this species confirming our findings [8]. Lutein is known to possess strong antioxidant activity, which can be even more effective than β-carotene [36]. The high concentration of lutein detected can be due to hypersaline culture condition, as already demonstrated for other *Dunaliella* species [37]; this carotenoid could be probably responsible, together with lower concentrated carotenoids, for repair activity found on BEAS-2B cells.

Navicula sp. is a diatom producing chlorophyll *a* and c2 as main photosynthetic pigments; it also produces fucoxanthin responsible for culture brown color. Other secondary carotenoids have been identified in our study such as neoxanthin, diadinoxanthin, cis-fucoxanthin, and diatoxanthin (Fig. 7B). Few studies are available in literature describing species of the genus *Navicula* (*e.g. N. clavata*) as producers of bioactive pigments [38]. Our results add information on carotenoids biosynthesis of this genus, supporting the hypothesis that total extracts possess antioxidant *in vitro* activity probably linked to the synergistic cooperation of fucoxanthin, chlorophylls and other accessory pigments.

Dactylococcopsis salina is a cyanobacterium that displayed chlorophyll *a* as main pigment and zeaxanthin and β-carotene at lower concentrations (Fig. 7C). However, *Ds* produced low concentration of these three carotenoids under HS condition; hence, this mix of pigments can partially account for the strong repair activity detected. No data are available in literature on carotenoids biosynthesis of *Dactylococcopsis salina* grown in high salinity condition. A recent study demonstrated that this halophilic cyanobacterium lacks genes encoding keto-carotenoid biosynthesis enzymes, such as two β-carotene ketolases crtW and crtO [39]; nonetheless, production of echinenone in this species has been found [40]. The same pigment mix identified in this study (chlorophyll *a*, zeaxanthin and β-carotene) has been identified also in *Cyanothece* sp. [41]. *Cyanothece* sp. is also able to produce large amounts of EPS under salt stress conditions; EPS from bacteria is known to possess antioxidant activities [42], which could explain our findings.

3.6. Non-targeted metabolomic profiling

To identify compounds of potential biotechnological interest, the metabolomes of heterotrophic halophiles extracts showing cytotoxicity were characterized. For this purpose, metabolites produced or transformed from *Bsp* and *Hsp* during growth under salinity stress conditions (LS and HS) were analyzed in a non-targeted manner by liquid chromatography coupled to mass spectrometry (LC-MS/MS). After data processing, 1714 high-quality features were obtained for *Bsp* samples, and 1030 features for *Hsp* (LS and HS conditions combined in each case).

The annotation of known metabolites resulted in a list of 68 features (for *Bsp*) and 51 features (for *Hsp*) representing identified or putatively identified metabolites (Tables S1, S2).

Metabolomics profiling showed the presence of ectoine, betaine, and trigonelline with high-intensity signals in *Bsp* as well as in *Hsp*, and for both LS and HS conditions. These metabolites possess interesting bioactivities. In particular, amino acids, betaine, and ectoine are known to be accumulated from some strains of *Brevibacterium*, together with other compatible solutes, to allow the survival of the organisms under osmotic stress caused by the hypersaline environment [43]. In addition, the plant hormone trigonelline has been claimed to have anticarcinogenic and hypocholesterolemic effects [44]. Choline was also detected in *Bsp* and *Hsp* strains in both salt treatments. This molecule not only has known neuro-active properties but is an essential component of phosphatidylcholine lipids.

3.7. Effect of low and high salinity (LS and HS) on metabolite accumulation

The influence of different salinity conditions on metabolite composition and abundance was then investigated. Both datasets were subjected to a principal component analysis (PCA), which showed for *Bsp* that samples belonging to the two salinity conditions were well separated in the first component (PC1), reflecting major differences in the metabolite levels between the two treatments (Fig. 8A). A similar behavior was observed for *Hsp*, with samples clustering by biological replicates in the PCA space (Fig. 8B); within group variance among LS replicates were slightly higher than for HS treatment.

The differences in metabolite accumulation driven by the two salinity treatments were visualized through volcano plots (Fig. 8C and D). In *Bsp* samples, a variation in metabolite features abundance (fold change fc > 2 and $p < 0.01$) was observed in 73 metabolites between LS and HS groups (Fig. 8C). While 30 of these features had higher abundance in the LS group compared to HS, 43 were found to be less abundant.

A total of 44 differentially expressed metabolites ($p < 0.01$ and fc $>$ 2) was found for *Hsp* strain, 6 of which were increased in intensity in HS when compared to LS treatment and 38 were decreased (Fig. 8D). Thus, while the overall fraction of regulated features was equal for *Hsp vs. Bsp* (73 out of 1714 = 4.25% *vs* 44 out of 1030 = 4.27%), the distribution between up- and downregulation was balanced for *Bsp* (30: 43), but asymmetric for *Hsp* (6: 38).

Dysregulated features for both strains *Bsp* and *Hsp* were identified wherever possible based on MS/MS analysis and spectral databases matching. However, the chemical complexity or low intensity of the majority of the features did not allow an entire annotation, reflecting a general, unresolved challenge of untargeted metabolomics.

To aid known metabolite identification, the R algorithm "CluMSID" [45] was applied: it assists annotation by clustering features based on MS/MS spectral similarity and unsupervised statistical methods. The

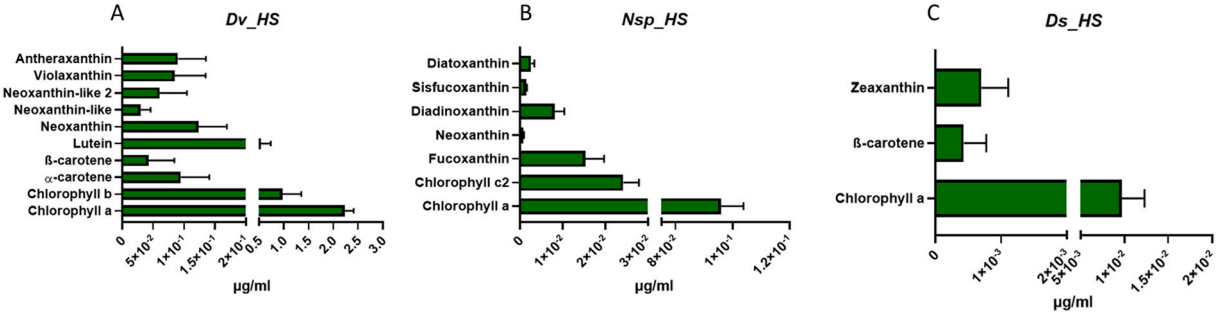

Fig. 7. Pigment analysis of selected microalgae strains under high salinity; Dunaliella viridis (Dv, A), Navicula sp. (Nsp, B), Dactylococcopsis salina (Ds, C). Analyses were performed in triplicate ($n = 3$), obtained average and standard deviation values are shown in the graphs.

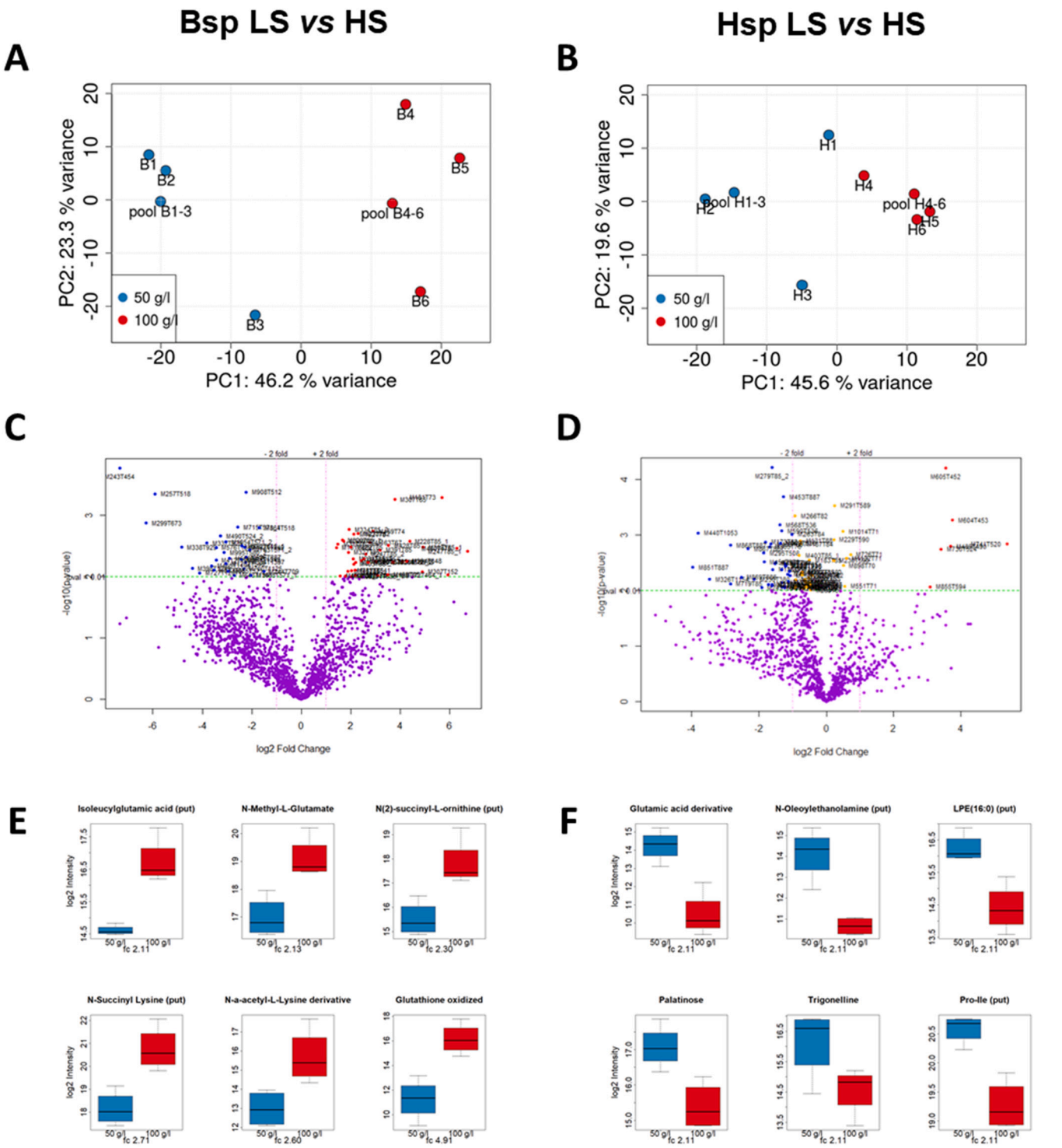

Fig. 8. Statistical analysis of metabolite accumulation under different salinity conditions: 50 g/l of NaCl (LS) in blue and 100 g/l of NaCl (HS) in red for Bsp (left: A,C, E) and for Hsp (right: B,D,F). Score plot of PCA of biological replicates and pool sample, clearly separating samples by salt treatment (A and B). Volcano plot of the differences in metabolites accumulation driven by the two different salt treatments (C and D). Data statistical significance (*p*-value in a log10 scale) is plotted in function of the fold change in a log2 scale, between NaCl content of 50 g/l and 100 g/l. The horizontal dashed line indicates a p-value of 0.01, and the two vertical dashed lines separate features having an absolute fold-change of 2. Dots towards the top of the plot in the Y-axis, represent features that have been affected with statistical significance (p-value<0.01). Dots on the left and right of the dashed vertical lines represent highly abundant features in the LS group (blue; fc < -2) and in the HS group (red; fc > 2), respectively. Purple dots are statistically insignificant features (p- value>0.01). Panels E and F show box plots of most interesting features with higher abundance in Bsp_HS treatment (E) and in Hsp_LS (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

similarity of MS/MS spectra can provide indeed valuable information on putative structural similarities between known and unknown analytes. Moreover, the SIRIUS 4 algorithm was applied. The method aims to identify molecular formulas in fragment spectra using exact mass and isotope pattern analysis and provides the structure that best explains the experimental data, based on the prediction of a molecular fingerprint of a query compound from its fragmentation tree and spectrum. The use of such tools allowed a broader classification of unknown dysregulated metabolites.

In *Bsp*, among features with higher abundance in HS treatment, all annotated metabolites resulted to be amino acids derivatives or short peptides (Fig. 8E and Table S2). The main ones are: *N*-methyl-L-glutamate ($log_2fc = 2.13$), Ile-Glu (putative annotation; $log_2fc = 2.11$), N(2)-succinyl-L-ornithine (putative annotation; $log_2fc = 2.30$), N-succinyl lysine (putative annotation; $log_2fc = 2.60$), oxidized glutathione ($log_2fc = 4.90$), together with other peptides derivatives for which a chemical structure was not predicted.

Lysine succinylation was discovered in the last decade as a new type of lysine acylation [46], present from eukaryotes to bacteria, which plays regulatory functions in various cellular processes. However, the role of this modification in bacteria remains largely unknown [47]. The succinylation reaction converts the overall charge of the molecule from positive to negative. The presence of this metabolite in *Bsp* and its increased intensity in HS treatment might therefore be explained as a response to higher local sodium concentrations in HS conditions, to maintain the structural integrity of cellular membranes.

In *Hsp*, the structural annotation of the most upregulated features in HS treatment was not possible. However, the features were recognized as peptidic based on MS/MS spectra similarity using the CluMSID algorithm and an isotope pattern analysis.

It is well known that halotolerant bacteria adopt various strategies to survive and grow in saline habitats. The accumulation of osmolytes, such as amino acids and small peptides is one of them [48]. We therefore postulate that the peptides and their derivatives found to have a higher concentration in the HS groups of both organisms could act as osmoprotectants. Due to their zwitterionic nature, they may exert a stabilizing effect on proteins and cellular membranes.

A nearly thirty-fold increase in oxidized glutathione ($log_2fc = 4.90$) was also observed in *Bsp* under HS compared to LS treatment. Glutathione is known to play an important role in the protection of bacterial cells against several environmental stresses, including osmotic stress. Its function might be related to oxidative stress response to maintain cell viability in high salinity environments [49,50], indicating an increased need for reducing power in such conditions. This finding can be interesting from an industrial perspective of Bsp, as glutathione is extensively used in the pharmaceutical and cosmetic production [51].

Among the identified metabolites found to be more abundant in *Bsp_LS* treatment, oleoyl glutamic acid ($log_2fc = -3.44$) was the only annotated compound, together with a cholesteryl ester derivative ($log2fc = -2.25$) and a presumed tyrosine metabolite (p–hydroxyphenyl acetic acid derivative; $log2fc = -2.40$), both of unknown exact chemical structure (Table S2).

Compounds with similar chemical structures were also found to be the most upregulated in the *Hsp_LS* treatment group (Fig. 8F): a glutamic acid derivative ($log_2fc = -3.80$), followed by N-oleoylethanolamine ($log_2fc = -3.46$). Other known metabolites displayed 2.5–3 fold higher intensities in the LS condition, these are: LPE (16:0) ($log_2fc = -1.84$), palatinose ($log_2fc = -1.68$), trigonelline ($log_2fc = -1.58$) and pro-Ile ($log_2fc = -1.32$).

4. Conclusions

Biotechnological exploitation of marine microorganisms from halophilic environment benefits from the identification of new species and an in-depth investigation of their eco-physiological characteristics. Modulation of salt content in laboratory cultures represents a fruitful strategy for the stimulation of biosynthetic pathways. Strains of hypersaline, photosynthetic, and heterotrophic microorganisms (bacteria and microalgae) were isolated from two saltern ponds at the natural reserve of "Saline di Trapani e Paceco". Samples coming from high salinity culture conditions showed a stronger viability recovery on human cells compared to the low salinity extracts, indicating high salinity as a potential strategy for the biomass production in cosmeceutical/nutraceutical applications. This activity can be connected to the production of a characteristic pool of carotenoids (*e.g.* lutein, fucoxanthin, neoxanthin) under high salinity condition. The cyanobacterium *Dactylococcopsis* showed the highest cell repair activity under high salinity that can be associated with the overexpression of the carotenoids zeaxanthin and β-carotene.

Heterotrophic cell extracts, *Brevibacterium* sp. in particular, showed anti-proliferative activity on cancer cell lines, reaching 50% of viable cells at the highest concentration on prostate cancer cells. The use of untargeted metabolomics for compound profiling and the detection of the response to salt stress enabled a straightforward identification of several bioactive metabolites of industrial interest such as ectoine, betaine, trigonelline, amino acids, and oxidized glutathione.

Although further investigations, for example by other omics techniques, are required to better understand the mechanisms in the salt adaptation of *Brevibacterium* sp. and *Halomonas* sp., the study demonstrates how a combination of marine microbiology, cell biology, and modern metabolomics can leverage the potential value of extremophile marine microorganisms.

CRediT authorship contribution statement

VV conceived and designed the study, collected and assembled data, drafted the article. CG performed the experiments of biological assay, and contributed to data interpretation, designing of the study and revision of the manuscript. FF performed the experiments of metabolomics and statistical analysis, and contributed to data interpretation, designing of the study and revision of the manuscript. SL participated in the acquisition, analysis, and interpretation of data, and revised the manuscript. MB, AB, CS, CB, FS participated in the interpretation of data and revised the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.algal.2021.102192.

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