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Cultivation and biochemical characterization of isolated Sicilian microalgal species in salt and temperature stress conditions

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ABSTRACT

In the last years, the possibility to exploit autochthone microalgae in regional applications has been explored. The regional-based microalgal industry may bring several benefits, as autochthone microalgae are already adapted to the biotic and abiotic stresses of their environment. In this work, this concept was applied to Sicily, in which three microalgal strains were collected from the coastline. Monoalgal strains were then isolated and molecular characterization was performed for the species determination. Three of them, two strains of *Chlorella* and one of *Dunaliella*, were cultivated in lab-scale in four different conditions: Low Temperature-Low Salt (LT-LS), High-Temperature-Low Salt (HT-LS), Low Temperature-High Salt (LT-HS) and High Temperature-High Salt (HT-HS) to investigate the role of each condition on the growth performance, the productivity and the biochemical composition of the microalgal biomass. In particular, lipid, fatty acid composition and antioxidant capacity were assessed. Results indicated that one of the Sicilian strains of *Chlorella* has a better growth performance at a higher temperature while the *Dunaliella* strain is tolerant to high-salt stress.

Moreover, the biochemical composition appears to be strongly influenced by temperature and salt stresses: the lipid content decreased in all the strains and a significant shift in fatty acid composition was observed, with an increase in the content of *n*-3 PUFAs in some cases. Results indicated that also the carotenoids content decreased in some of the tested stress conditions. The results obtained in this research represent a first step for developing a regional-based microalgal industry in Sicily by exploiting the natural biodiversity of the Sicilian environment.

KEYWORDS

Microalgal agriculture, biomass, high salinity, heat, PUFAs, carotenoids

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1. INTRODUCTION

Microalgae have nowadays gained considerable attention because of their biotechnological and industrial interest as a result of their wide range of applications such as food, pharmaceuticals, cosmetics, energetics and phycoremediation, among others [1–3]. Albeit their promising application, the microalgal industry is not fully matured as microalgae production is still not economically convenient because of low biomass yields [4]. However, several strategies can be used for the optimization of productivity. With this purpose, some of the most recent researches rely on the optimization of algal reactor efficiency [5] or the combination of nutrient availability and lightning strategies [6]. Another possible strategy is the "phycoprospecting", *i.e.* the exploitation of selected indigenous microalgae with favourable characteristics for applications in the regional-based production [7]. The cultivation of autochthone microalgae in their environment offers several advantages, being already adapted to both biotic and abiotic stresses [8]. This allows reducing the acclimation process of the strains to a new cultivation condition, leading to economic and time savings. Indeed, native strains are the best candidates for outdoor cultivation [9], which is the cheapest alternative in microalgae industrial production. However, the weakness of microalgae outdoor cultivation is the lower productivity comparing the indoor cultivation where optimal growth condition can be used [10]. For this reason, exploiting the diversity of "Nature's culture collection" [7] and selecting the best microalgae strains for outdoor cultivation can help to pave the way toward industrial algal cultivation. Indeed, several studies focus on the characterisation and isolation of local strains and the investigation of their industrial potential [9,11,12] and some of them have been based in Sicily [13–15]. However, to the authors' knowledge, none of these studies included microalgae biochemical characterization and production rate, combining two stress conditions

such as high temperature and salinity. The selection of microalgal strains resistant to both high salinity and temperature is crucial for their industrial cultivation in the outdoor system in Sicily, where the diurnal temperature during summertime may reach 40°C. Moreover, the use of high-salinity wastewater (e.g. deriving from an aquaculture system) could reduce the dependence on freshwater and limit contamination [16].

This work focuses on autochthonous microalgae selection and their characterization for a possible biotechnological application in both aquaculture and food industry. Three microalgal strains, *Chlorella* sp *Pozzillo*, *Chlorella* sp *Barcarello* and *Dunaliella viridis,* isolated during the summer from the Sicilian coastline and a salter pond were tested for their tolerance to heat and salt stress by cultivating them for eight days at two different temperatures and salinities. The objective of this work was to verify if these strains can grow in extreme conditions and if salinity and temperature stresses may trigger the production of high-value biomolecules such as lipids, PUFAs and carotenoids.

2. MATERIALS AND METHODS

2.1. Microalgal strains, isolation, and molecular characterisation

In this study three microalgal strains isolated from different locations on the coastline of Sicily characterised by green or red summer blooms were selected: a) *Dunaliella viridis* (*D. viridis*), previously isolated from Natural Reserve of Saltern ponds, Trapani (TP) [15], b) *Chlorella* sp *Pozzillo* previously isolated from Pozzillo beach, Palermo (PA) [14], c) *Chlorella* sp *Barcarello* isolated as *Chlorella* sp *Pozzillo* [14] from Barcarello beach, Palermo (PA) (GPS coordinates 38°12'28.1"N 13°16'55.6"E). *Chlorella* sp strains were isolated from beaches close to the city with a standard salinity (approx. 21 g L^{-1} NaCl), while *D. viridis* from a salter pond with salinity above the saturation level (>200 g L^{-1} NaCl). *Chlorella* sp *Barcarello* was isolated by the combination of filtration and dilution methods. The first phase of isolation involved the

filtration through wide mesh filters (>200 μm) for removing the impurities from the initial sample (i.e. debris, sand, rock, macroalgae). The filtration was coupled with serial dilutions in 24-well microplates to obtain a single strain per well. Once the individual strain was obtained, the liquid cultures were transferred into a solid medium to obtain individual colonies that were used for molecular characterization by colony PCR. For serial dilutions, liquid and solid f/2 medium with a concentrations of 20 g L^{-1} NaCl was used. The molecular characterization of *Chlorella* sp *Barcarello* was done as for *Chlorella* sp *Pozzillo* [14]**.** Molecular characterization was performed by colony polymerase chain reaction (PCR) using Q5 ® high-fidelity DNA Polymerase (NEB), the forward primer A (5′-ACC CTG GTT GAT CCT GCC AG-3′) and primerSSU-inR1 (5′-CAC CAG ACT TGC CCT CCA-3′). For the Colony PCR the following program was used: 95 °C (5 min), 32 cycles of 95 °C (30 s), 55 °C (30 s) and 68 °C (60 s) and a final 7 min extension step of 68 °C. The PCR products were run on 1% of agarose gel (ethidium bromide 0.2 μg/mL) against Jena Bioscience Low Range DNA Ladder, Linear Scale DNA Ladders. 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 analysed using online bioinformatics software BLAST for the alignment of sequences and the identification of the belonging species. The sampling site, type of bloom and the reference gene used for the species identification in the selected strains are summarized in Table 1.

Microalgal Species	**Sampling Site**	**GPS coordinates**	**Reference gene**	**Algal bloom**
***Chlorella* sp**	Pozzillo beach	38°11'01.0"N 13°08'39.0"E	18S rDNA [14]	Green
***Chlorella* sp**	Barcarello beach	38°12'28.1"N 13°16'55.6"E	18S rDNA	Green
Dunaliella viridis	Saltern pond of Trapani	38°00'51.2"N 12°32'07.4"E	18S rDNA, ITS [15]	Red

Table 1: Sampling site, reference genes for species identification, and type of bloom of *Chlorella sp Pozzillo*, *Chlorella sp Barcarello* and *Dunaliella viridis*.

2.2. Microalgal growth

Microalgal pre-cultures were set up by inoculating 10 mL of a back-up culture in 100 mL of the same liquid medium used for the main experiment. When the cells were in the late exponential phase (around 10 days of cultivation), they were used as inoculum for the main experiment. When the pH was above 8.0, pure CO_2 was supplied until it reached the value of 7.0.

Three microalgal strains, *Chlorella* sp *Pozzillo*, *Chlorella* sp *Barcarello* and *D. viridis,* were grown in 1 L Erlenmeyer flasks placed in an oscillating incubator (Corning Lse) under a 127 $\mu E/m^2$ s photon flux in three cultivation conditions: Low Temperature-Low Salt (LT-LS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (HT-HS). Light intensity was measured with a Delta Ohm-HD 9021 quantometer equipped with a Photosynthetic Active Radiation (PAR) probe (Delta Ohm LP 9021 PAR). The medium for the cultivation of *Chlorella* strains in LS conditions is made in artificial seawater (6.3 mM KCl, 2.0 mM $NaHCO_3$, 7.1 mM KBr, 0.36 mM H_3BO_3, 0.024 M Na_2SO_4, 9 mM $CaCl_2$ $2H_2O$, 0.046 M $MgCl_2$ $6H_2O$, 0.35 M NaCl). It is a modified F/2 medium [17] with the following final composition: 3.5 mM $NaNO_3$, 0.036 mM NaH_2PO_4 H_20, 0,12 µM $FeCl_3$ $6H_2O$, 0,12 µM Na_2EDTA, 0.04 µM $CuSO_4$ $5H_2O$, 0.076 µM $ZnSO_4$ $7H_2O$, 0.042 µM $CoCl_2$ $6H_2O$, 0,91 µM $MnCl_2$ $4H_2O$, 0.025 µM Na_2MoO_4 $2H_2O$. The medium for the cultivation of *Dunaliella*, instead, is a modified Ramaraj medium (R.M.) [18] with the following composition: 2.68 mM KCl, 1.5 M NaCl, 0.025 M $NaHCO_3$, 0.15 mM H_3BO_3, 8.07 10^{-4} mM $CaCl_2$ $2H_2O$, 5 M $MgSO_4$ 7 H_2O, 5 mM $NaNO_3$, 0.1 mM NaH_2PO_4, 3.08 10^{-3} mM $FeCl_3$, 8.07 10^{-4} $ZnCl_2$, 3.85 10^{-4} mM $CoCl_2$ 6 H_2O, 10^{-2} $MnCl_2$ 4 H_2O, 3.72 10^{-4} mM $CuSO_4$ 5 H_2O, 2.38 10^{-3} mM Na_2MoO_4 $2H_2O$. The HS conditions consisted of

the double concentration of NaCl, resulting in 0.70 M NaCl (about 40 g L^{-1} against 20 g L^{-1} of LS conditions) for *Chlorella* species and 3 M (about 180 g L^{-1} against 90 g L^{-1} of the LS) for *Dunaliella*. All cultures were grown at 24°C in LT conditions and 34°C in HT conditions. In Table 2 there is a clarifying scheme of the employed conditions.

The pH of the F/2 media (both low and high salinity) was around 7, while the one of the R.M. around 9. Electrical conductivity was 45.7 mS/cm in F/2 LS and 73.3 mS/cm in F/2 HS, while it was 113.5 mS/cm in R.M. LS and 192.2 mS/cm in HS condition. Each experiment was carried out for 8 days. The concentration of the microalgal suspension was monitored by reading the absorbance at 750 nm. Then, the values were converted in concentration [g L^{-1}] through calibration curves obtained by filtering a known volume of algal suspension with glass fibre filters (pore size ø = 0.7 µm), washed twice with 10 mL ammonium bicarbonate (0.5 M) and dried at 105°C for 24 h. The dry weight was determined gravimetrically. For each condition, a biological triplicate (n=3) was performed. The value of pH of the cell suspensions was measured at the same moment as optical density by employing a pHmeter (Crison MM 41). Values are reported as mean together with the standard deviation.

Condition	*Species*	*T [°C]*	C_{Nacl} *[g* L^{-1}*]*
LS LT	*Chlorella* sp *Pozzillo*	24	20
LS LT	*Chlorella* sp *Barcarello*	24	20
LS LT	*Dunaliella viridis*	24	90
HS LT	*Chlorella* sp *Pozzillo*	24	40
HS LT	*Chlorella* sp *Barcarello*	24	40
HS LT	*Dunaliella viridis*	24	180
LS HT	*Chlorella* sp *Pozzillo*	34	20
LS HT	*Chlorella* sp *Barcarello*	34	20
LS HT	*Dunaliella viridis*	34	90
HS HT	*Chlorella* sp *Pozzillo*	34	40
HS HT	*Chlorella* sp *Barcarello*	34	40
HS HT	*Dunaliella viridis*	34	180

Table 2: Scheme of the employed experimental conditions.

2.3. Determination of productivity in batch

Batch productivity was calculated by employing the following equation:

$$P = \frac{X - X_o}{t - t_0} \quad (1)$$

where X_0 is the initial biomass concentration (g L^{-1}) at the initial time t_0 and X the biomass concentration at the final time t. For convenience purposes, a final time of 8 days was adopted for all the cultivation series, which averagely corresponded to the end of the exponential phase. Data are expressed as an average of three cultivation replicates (n=3) and error bar is reported.

2.4. Sample preparation

After 8 days of cultivation, the cell suspension was harvested by centrifugation and the obtained biomass was frozen in liquid nitrogen and freeze-dried for 48 h in a bench lyophilizator (FreeZone 2.5L, LABCONCO, US). The biomass was then stored at -20°C for further analysis.

2.5. Total lipids quantification and analysis of total fatty acids

Total lipids were determined gravimetrically according to Folch et al. [19]. Total fatty acid (FA) methyl esters were determined from the total lipid content of dried microalgae, according to the method described by Lepage and Roy [20] and were analysed as described by Messina et al. [21].

2.6. Total phenolic content

Folin-Ciocalteu reagent assay [22] was carried out to determine the total phenolic content of samples [23] of microalgae. Gallic acid was used as the standard for calibration (5–500 mg mL^{-1}) and results were expressed as mg of gallic acid equivalents (GAE) per g of microalgae dry weight

(DW) [10, 20–22]. The absorbance of each dilution was recorded at 725 nm, using a microplate reader (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA). Each sample was analysed in triplicate.

2.7. DPPH radical scavenging activity

The free radical scavenging ability of microalgae extracts was determined through the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as reported previously [13,24,25]. The dried samples were re-suspended in methanol and analysed at various concentrations (from 0.1 to 2 mg mL^{-1}). An aliquot (40 mL) of each extract was mixed with 160 µL of DPPH methanolic solution (0.1 mM). The mixture was vigorously mixed and incubated in darkness for 30 min. Absorbance was recorded at 517 nm. Each sample was measured in triplicate (n=3). The results were reported as IC 50 (i.e., Inhibitory Concentration to cause a 50% decrease of the absorbance). A lower IC 50 value indicates higher antiradical activity.

2.8. Beta carotene and chlorophyll HPLC analysis

Beta carotene and chlorophyll were analysed as previously showed [27]. Briefly, ~6 mg of freeze-dried biomass was disrupted together with 50 µL deionized water, 3 mL acetone and 0.7 g glass beads (0.1 mm) by vortex mixing for 2 minutes (Maxi Mixer, Asal). The extraction with acetone was repeated twice. The samples were evaporated and resuspended in methanol before HPLC injection. Carotenoid extracts were analysed with an Infinity 1220 LC (Agilent Technologies, US) equipped with a photodiode array detector and column oven at room temperature. Separation of the compounds was achieved using a Synergi Hydro-RP 18C (5 µm, 250x4 mm, Phenomenex) column with a mobile phase consisting of acetonitrile: water (9:1; v/v) as solvent A and ethyl acetate as solvent B and a constant flow of 1 mL min^{-1}. The gradient program applied was: (i) 0–16 min, 0–60% B; (ii) 16–30 min, 60% B; (iii) 30–32 min 100% B

and (iv) 32-35 min 100% A. Method adapted from Couso et al. [28]. The injection volume was 100 µL; chromatograms were recorded at 450 nm. Quantification of chlorophyll *a* and *β*-carotene was performed through calibration curves. Calibration curves were obtained with Chlorophyll *a* (analytical standard, Sigma-Aldrich) in the concentration range between 8 and 500 µg/mL and with β-carotene (synthetic, ≥ 95%, Sigma-Aldrich) in the concentration range between 2 and 500 µg/mL Each sample was analysed in triplicate.

2.9. Statistical analysis

All tests were carried out in triplicate and expressed as a mean ± standard deviation (SD). Data were analysed by a one-way variance analysis (ANOVA) using the SPSS 20.0 statistical software program and deviations were considered significant at $P < 0.05$. Different letters were used to indicate significant differences among various treatment in each species at $p<0.05$.

Non-metric multidimensional scaling (nMDS) [29] was used to evaluate patterns in polyphenols, DPPH, lipid content, SFA, MUFA, PUFA, PUFA *n*-3, PUFA *n*-6, *n*-3/*n*-6 chlorophyll *a* and *β*-carotene, as a function of salinity and temperature variables. In the nMDS representation, increasing distances among points in the ordination plot, represent decreasing similarity among the same points. The Euclidean distance on the normalized data was used as a distance measure. Pearson product correlations for the relative proportions of the variables are shown in the vector loading plot. The length and direction of the vectors indicate the strength of the correlation, while the direction and size of each vector indicate the relative abundance of that variable in the samples.

Multivariate data analysis was conducted using the PRIMER 6 v.6.1 software package (PRIMER-E Ltd., Plymouth).

3. RESULTS AND DISCUSSION

3.1. Molecular identification of *Chlorella* sp *Barcarello*

The Colony PCR using the universal primers for the amplification of 18S gave the single product corresponding to the expected size, as shown in Figure S1 of supplementary material. The obtained sequence was deposited in Genbank database with the name *Chlorella* sp (Barcarello) and the code MT259187.1. Table 3 shows the percentage of identity of the DNA sequence of 18S rRNA gene from *Chlorella* sp Barcarello with other known *Chlorella* strains.

Isolate species	***Target***	***Identity***	***Accession***
Chlorella sp Barcarello	*18S* ribosomal RNA gene	100%	MT259187.1
Chlorella sp Pozzillo	*18S* ribosomal RNA gene	99.6%	MT259188.1
Chlorella vulgaris isolate UMT-M1	*18S* ribosomal RNA gene	99.6%	KJ561358.1
Chlorella kessleri strain SAG 211-11g	*18S* ribosomal RNA gene	98.8%	X56105.1

Table 3: Identity of the DNA sequence of 18S rRNA gene from *Chlorella* sp Barcarello with other known *Chlorella* strains.

3.2. Microalgal growth and productivity

Chlorella sp *Pozzillo*, *Chlorella* sp *Barcarello* and *D. viridis* were grown in four different conditions: LT-LS, LT-HS, HT-LS, HT-HS. Growth curves are reported in Figure 1. At the end of the cultivation, *Chlorella* strains reached a final biomass concentration ranging between 0.3 and 1 g L^{-1}, similar values were observed by other authors using the same genus [30,31]. The final biomass concentration of *D. viridis* was, instead, around 0.3 g L^{-1}, as observed also by other authors [32].

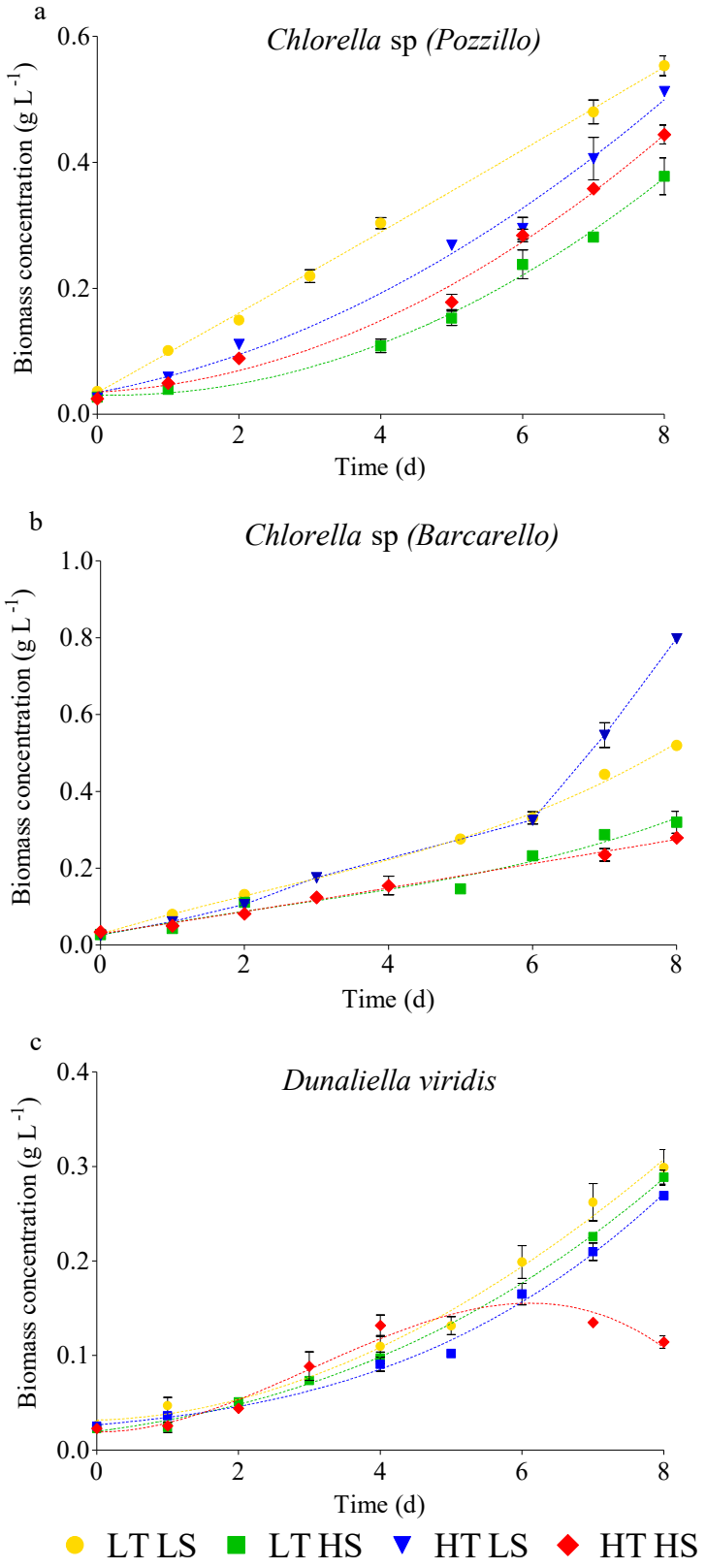

Figure 1: Growth curves of *Chlorella* sp *Pozzillo* (a), *Chlorella* sp *Barcarello* (b) and *Dunaliella viridis* (c) at Low Temperature-Low Salt (LT-LS), Low Temperature-High Salt (LT-HS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (HT-HS). Data are reported as means (n=3) with s.d. as error bars.

As shown in Figure 1 a, *Chlorella* sp *Pozzillo* displayed the best growth performance when cultured in LT-LS condition, while it progressively decreased in HT-LS, in HT-HS and LT-HS. On the other hand, *Chlorella* sp *Barcarello* (Figure 1 b), showed an improved growth compared to the others in the HT-LS condition, while the LT-LS condition was similar to the other *Chlorella* strain. The same strain showed lower growth performances (and similar to each other) in LT-HS and HT-HS conditions. *D. viridis* (Figure 1c) showed similar initial growths in all the tested conditions, while a decrease of growth was observed in the last days of the HT-HS one. Batch productivities were also calculated from the data for all 8 days, and the results are reported in Figure 2. In *Chlorella* genus the productivity ranged from 0.04 to 0.15 g $L^{-1}d^{-1}$, and the only condition in which there was an evident increase was in *Chlorella* sp *Barcarello* HT-LS condition, while *D. viridis* showed productivity around 0.03 g L^{-1} in almost all the tested conditions except for HT-HS, case in which the combination of the two stresses made the productivity to decrease. These results are in line with other studies [33,34]. It is worth noting that the reported productivity is calculated on the base of batch data after 8 days of growth (averagely the duration of the exponential phase), while industrial cultivation is usually based on continuous mode. Furthermore, the productivity may significantly vary when scaling up on industrial systems. Even if the data do not yet indicate a commercially viable scenario, they may be compared with literature data obtained in outdoor photobioreactors [35–37] in similar conditions. *Chlorella* genus was reported to respond differently when exposed to different temperatures. For example, Lu et al. found that the optimum growth temperature for a strain of *Chlorella* sp was 25 °C [38] while for *Chlorella vulgaris* it was found at about 30°C [39]. The variety is highlighted when considering the high range of optimum temperatures: Zhang et al. found the optimum growth for a strain of *Chlorella* sp at 18 °C [40] while Turon et al. at 35°C for *Chlorella sorokiniana* [41].

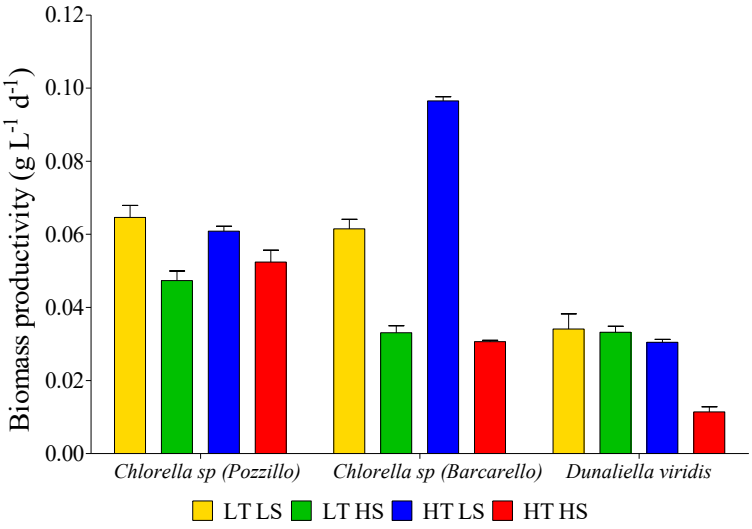

Figure 1: Batch productivities of *Chlorella* sp *Pozzillo*, *Chlorella* sp *Barcarello* and *Dunaliella viridis* grown at Low Temperature-Low Salt (LT-LS), Low Temperature- High Salt (LT-HS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (HT-HS). Data are reported as mean (n=3) and error bars report the standard deviations.

The response of *Chlorella* strains shown in this study at different temperatures is, therefore, well inserted in the context of the abovementioned studies, in both cases of *Chlorella* sp *Pozzillo* and *Barcarello.* Furthermore, a *Chlorella* strain isolated from the Mediterranean Sea was found to have a better growth response at the highest tested temperature compared to lower temperatures by Pulina et al. [42]. Even if the maximum temperature of the cited study was 17°C, far from the 34°C tested in the present study, we may assume that the trend is similar and that increasing the temperature leads to an advantage in terms of growth performance. This may be related also to the location from which the strains were harvested, in both cases the Mediterranean Sea. *D. viridis*, on the other hand, was reported to have a similar growth performance at 25°C and 35°C also by other authors [43] confirming our findings. The increase of the growth performance of *Chlorella* sp *Barcarello* with temperature is an advantage if considering the high costs associated with temperature control system in outdoor facilities and that in Sicily external temperature during summer may reach 40°C.

The effect of salinity on *Chlorella* sp growth was also previously reported by Asem et al. [44], who observed an optimal growth at a salinity of 30 g L^{-1} NaCl. This concentration is comparable with the LS condition of the present study, and the decrease of growth performance of both *Chlorella* strains HS condition found in this study is therefore in line with the abovementioned research. *D. viridis* is known as a halotolerant strain, following the locations where it was selected, a salter pond in Sicily. Byrd et al. reported, for several *Dunaliella* strains, the optimum salinity [45]. The optimum salinity for *D. viridis* is 60 g L^{-1} NaCl, close to the concentration of the LS condition in this study (90 g L^{-1}) (Table 1). We may conclude that this specific strain of *D. viridis*, isolated from a pond where NaCl was above 200 g L^{-1}, has a wide range of salt tolerance and has a similar response at very high salt concentrations. This trait may result in an advantage if considering that it may be cultivated also in brines coming from wastes from industry or natural sources.

3.3. Total lipids quantification

After the growth, microalgal biomass was harvested and freeze-dried and the biomass was then employed to perform total lipids quantification. The lipid content of the analysed algal strain is shown in Figure 3.

Chlorella strains showed a lipid content between 12.03±0.15% (*Chlorella* sp *Pozzillo* HT-HS) and 17.53±2.24% (*Chlorella* sp *Barcarello* LT-HS), while *D. viridis,* lipid content ranged between 13.51±1.14% (HT-HS) and 20.74±1.42% (HT-LS) accordingly to the literature [46,47].

D. viridis grown in low salinity conditions, both at low and high temperature, showed the highest lipid content (20.41±2.63% and 20.74±1.42%, respectively), while the lowest lipid content was observed when both high temperature and high salinity (HT-HS) are present.

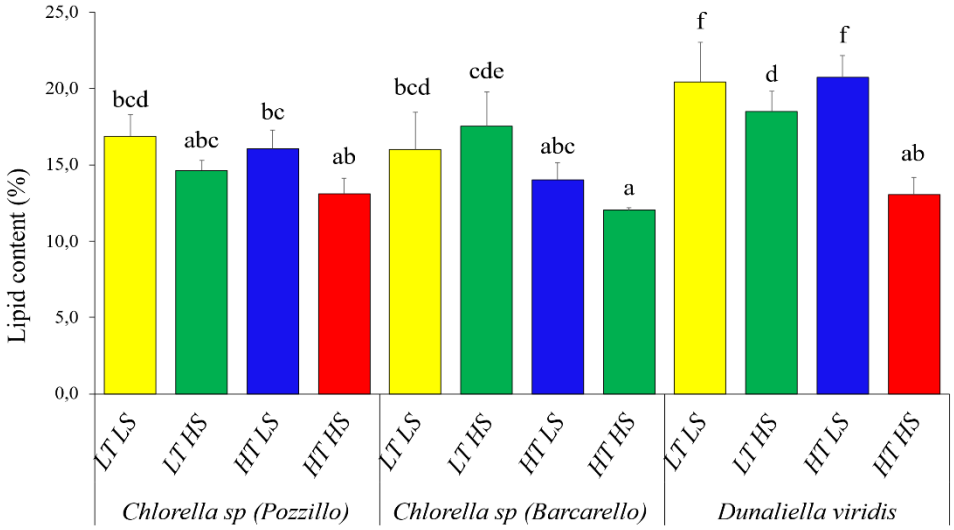

Figure 3: Total lipid content on dry weight (DW) in the strains *Chlorella* sp *Pozzillo* in *Chlorella* sp *Barcarello* and *Dunaliella viridis*. Values are reported as means (n=3) and error bars report the standard deviations. Different letters indicate significant differences among various treatment in each species at $p<0.05$

In *Chlorella* strains, at low salinity conditions (LS, 0.35 M) (Table 1), the lipid content was 16.86±1.42% (LT-LS) and 16.06±1.24% (HT-LS) in *Chlorella* sp *Pozzillo*, and 15.98±2.48% (LT-LS) and 14.00±1.14% (HT-LS) in *Chlorella* sp *Barcarello*. The values observed are comparable to the values obtained by Rismani and Shariati [48] in *Chlorella vulgaris* at the salinity of 0.2 M. At high salinity conditions (HS, 0.7 M) *Chlorella* sp *Pozzillo* always showed a decrease in lipid content compared to the relevant low salinity condition. Similar results were obtained by Campennì et al. [49], who found that the lipid content in *Chlorella protothecoides* tends to decrease when increasing the salinity above 0.35 M. A different result was found in *Chlorella* sp *Barcarello,* for which, at LT-HS conditions, a slight increase in lipid content was observed. Similar results were obtained by other authors [50,51] who observed an increase in lipid content in *Chlorella vulgaris* and *Chlorella sorokiniana* respectively. Finally, at HT-HS conditions, *Chlorella* sp *Barcarello* showed a decrease in the lipid content as observed in all the other species.

As regards temperature, in the present work the stress caused by the high temperature tended to decrease the lipid content in all the *Chlorella* strains; this result is confirmed by several studies showing that the cultivation of *Chlorella vulgaris* at a temperature of 25° C led to a lipid content larger than those cultivated at 30° C. [46,52,53]. A slightly different behaviour was observed in *D. viridis*, where, at LS conditions, similar lipid contents were obtained at low and high temperature. Finally, similarly to *Chlorella* strains, when the two stressing conditions are simultaneously present (HT-HS), the lowest value in lipid content is observed. This result is in line with the ones detected by Rismani and Shariati [48] on the same species in high salinity conditions (NaCl 3 M).

In this work, all species showed a tendency to reduce lipid content as stress increased and salinity seemed to have the most discriminating effect.

As for industrial applications, both growth performances and lipid production need to be considered. If we look at the obtained results, the best parameters were observed in the strains *Chlorella* sp Barcarello HT-LS (18.47±0.23 mg L^{-1} d^{-1}) and in *Chlorella* sp Pozzillo LT-LS (11.97±0.09 mg L^{-1} d^{-1}) (Table S1 Supplementary Material). Also in this case, the lipid productivity may be compared with the one obtained from outdoor photobioreactors [35–37].

3.4. Analysis of fatty acids

Fatty acids were analysed starting from total lipid extract. Results are shown in Table S2 of Supplementary Material and Figure 4. In *Chlorella* strains, saturated fatty acids (SFAs) ranged from 23.57±1.73% total fatty acids (TFAs) (*Chlorella* sp *Barcarello* LT-LS) to 29.98±1.31% TFAs (*Chlorella* sp Barcarello LT-LS) while in *D. viridis* they ranged from 17.07±1.42% TFAs (LT-LS) to 41.56±0.16% TFAs (HT-HS) (Table S2 of Supplementary Material and Figure 4). Monounsaturated fatty acids (MUFAs) in *Chlorella* strains varied from 2.57±1.14% TFAs

(*Chlorella* sp Barcarello HT LS) to 5.97±0.12% TFAs (*Chlorella* sp *Barcarello* HT-HS) and in *D. viridis* from 5.85±0.05% TFAs (LT-LS) to 9.13±0.07% TFAs (LT HS) (Table S2 of Supplementary Material and Figure 4). Lastly, polyunsaturated fatty acids (PUFAs) in *Chlorella* strains ranged from 65.78±0.57% TFAs (*Chlorella* sp Pozzillo HT-HS) to 73.12±1.92% TFAs (*Chlorella* sp Barcarello LT-LS), while in *D. viridis* from 46.77±0.14% TFAs (HT-HS) to 64.91±0.22% TFAs (HT-LS) (Table S2 of Supplementary Material and Figure 4). These results are in line with other works in literature [48,54,55].

Microalgae are a source of *n*-3 fatty acids. In recent years, many studies have been undertaken to evaluate the effect of various treatments on the quantity and quality of lipids obtained from microalgae, in particular, the omega-3 fatty acids including alpha-linolenic acid (ALA) (C18:3 *n*-3), eicosapentaenoic acid (EPA) (C20:5 *n*-3), docosahexaenoic acid (DHA) (C22:6 *n*-3) [56]. This interest is justified by their industrial importance caused by the significant health benefits they provide [57] and consequently their high market value [58].

In general, it can be observed that *Chlorella* strains (both Pozzillo and Barcarello) showed a higher PUFA *n*-6 content and the predominant fatty acid is linoleic acid, 18:2 *n*-6; while in *D. viridis* the PUFA *n*-3 were the most representative PUFA class, with the highest concentration of 18:3*n*-3 (Table S2 of Supplementary Material).

As demonstrated by Pandit et al., the fatty acid profile of *C. vulgaris* varies with saline stress [59]. Our results confirmed their findings: in particular, we observed that the combination of HS and HT factors caused a significant decrease of the PUFA class in all the analysed strains: from 72.75±0.28 to 65.78±0.58 *in Chlorella* sp *Pozzillo*, from 73.12±1.92 to 68.94±0.23 in *Chlorella* sp *Barcarello* and from 63.08±3.50 to 46.77±0.14 in *D. viridis*. In particular, in *D. viridis* the decrease of PUFA is linked to an increase of SFA, which reached 41.54±0.71%

(HT-HS) with an increase of 16:0 (36.51±0.21). Also, a reduction in the ratio *n*-3/*n*-6 is observed for PUFA, as it significantly 18:3n3 decreases (9.14±0.24) and 18:2*n*-6 increases (25.74±0.28). This condition demonstrated how the synergistic effect of the two factors (HT-HS) resulted in a significant shift in fatty acid composition.

It is important to underline that the content of *n*-3 PUFA shows the same trend of the total lipids in *D. viridis* (Figure 4). On the other hand, the strains of *Chlorella* showed in both the analysed strains a different behaviour as *n*-3 PUFA increased when the cells were cultured in high salt condition (LT-HS) (Figure 4 and Table S2 of Supplementary Material). Furthermore, the production of some classes of significant fatty acids (EPA and DHA) were triggered in temperature stress conditions in *Chlorella* sp *Pozzillo* and *D. viridis* (Table S2 of Supplementary Material), although in both *Chlorella* strains total *n*-3 PUFA class decreased in HT conditions.

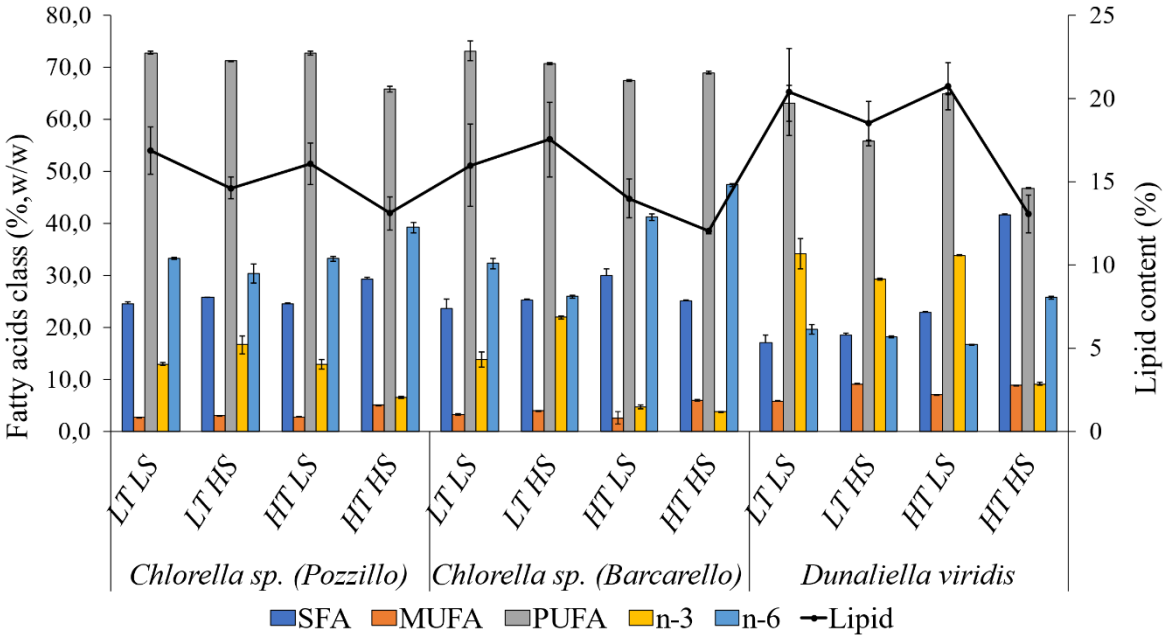

Figure 4: Fatty acids class composition (%,w/w) and total lipids *of Chlorella* sp *Pozzillo, Chlorella* sp *Barcarello* and *Dunaliella viridis* grown at Low Temperature-Low Salt (LT-LS), Low-Temperature-High Salt (LT-HS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (LT-HS). Data are reported as means (n=3) with standard deviation.

3.5. Total phenolic content and DPPH radical scavenging activity

The total phenolic content and anti-oxidative activity of microalgae, the latter evaluated through the DPPH assay, are shown in Figure 5. The total phenolic content (Figure 5a) varied significantly amongst the microalgae species examined ($p<0.05$), with *D. viridis* HT LS accumulating the highest concentration (6.32±0.04 mg GAE g^{-1} DW) and *Chlorella* sp *Pozzillo* LT HS and *D. viridis* HT HS the lowest (2.96±0.05 and 1.62±0.04 mg GAE g^{-1} DW, respectively).

As regards the anti-oxidative activity of microalgae, it is expressed through the IC_{50} parameter and results are shown in Figure 5b. The anti-oxidative activity is expressed as the quantity of antioxidant (biomass) needed to have the inhibition of 50% of radical DPPH activity, consequently, low values of IC_{50} correspond to a higher antioxidative activity of microalgal biomass. In general, in the strains of *Chlorella* values of IC_{50} ranged between 2.46±0.01 and 4.96±0.01 mg DW mL^{-1} (*Chlorella* sp *Barcarello* HT-LS and HT-HS, respectively), while in *D. viridis* between 3.00±0.07 and 8.41±0.65 mg DW mL^{-1}.

The different concentration of polyphenols is an intrinsic peculiarity of each species, in fact Maadane et al. [60] showed that different species of microalgae have different polyphenolic content and that the genus *Dunaliella* presents a higher polyphenolic content than *Chlorella* sp [60], confirming the result of this work.

Studies on the simultaneous effect of temperature and salinity have barely been performed [61] and the results on the polyphenol content further highlight the combined effect of the two factors salinity and temperature (HT-HS). This condition caused in *D. viridis* a significant decrease in polyphenols and also the lowest antioxidant activity (Figure 5b), with the values of IC 50 at 8.41±0.65 mg DW mL^{-1}.

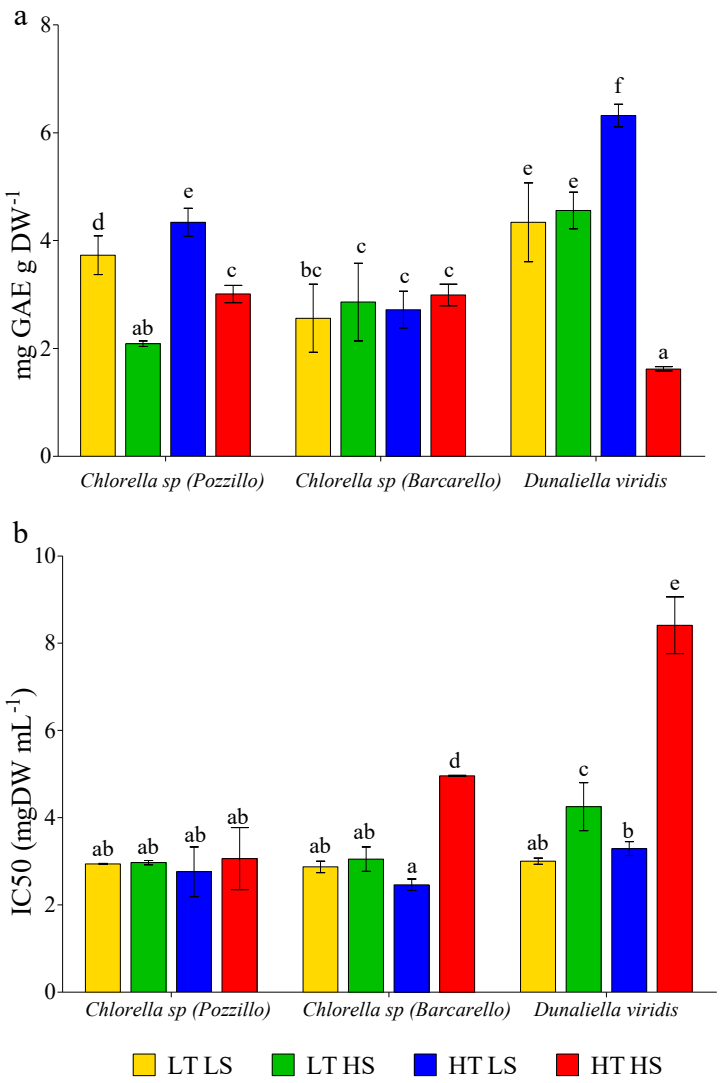

Figure 5: Total polyphenol content (mg GAE g^{-1} DW) (a) and DPPH radical scavenging activity (IC 50, mg DW mL^{-1}) (b) in the strains *Chlorella* sp *Pozzillo* in *Chlorella* sp *Barcarello* and in *Dunaliella viridis*. Values are reported as means (n=3) and error bars report the standard deviations. Different letters indicate significant differences among various treatment in each species at $p<0.05$

Studies conducted by Singh et al. [62] on the congeneric *Dunaliella salina*, have shown an increase in antioxidant activity under different stress conditions (increased temperature or salinity) [62]. Our data showed that the increase in temperature under conditions of low salinity maintains a high antioxidant activity (IC 50 3.29±0.16 mg DW mL^{-1}) and that the combination of the two factors is the trigger that significantly reduces antioxidant activity.

This synergistic effect is also observed in *Chlorella* sp *Barcarello* where the combination of HT and HS led to a lower antioxidant activity (IC 50 4.96±0.01 mg DW mL^{-1}), while HT LS showed the best antioxidant activity (IC 50 2.46±0.13 mg DW mL^{-1}).

3.6. Beta carotene and chlorophyll analysis

Figure 6 shows the total content of chlorophyll *a* and *β*-carotene.

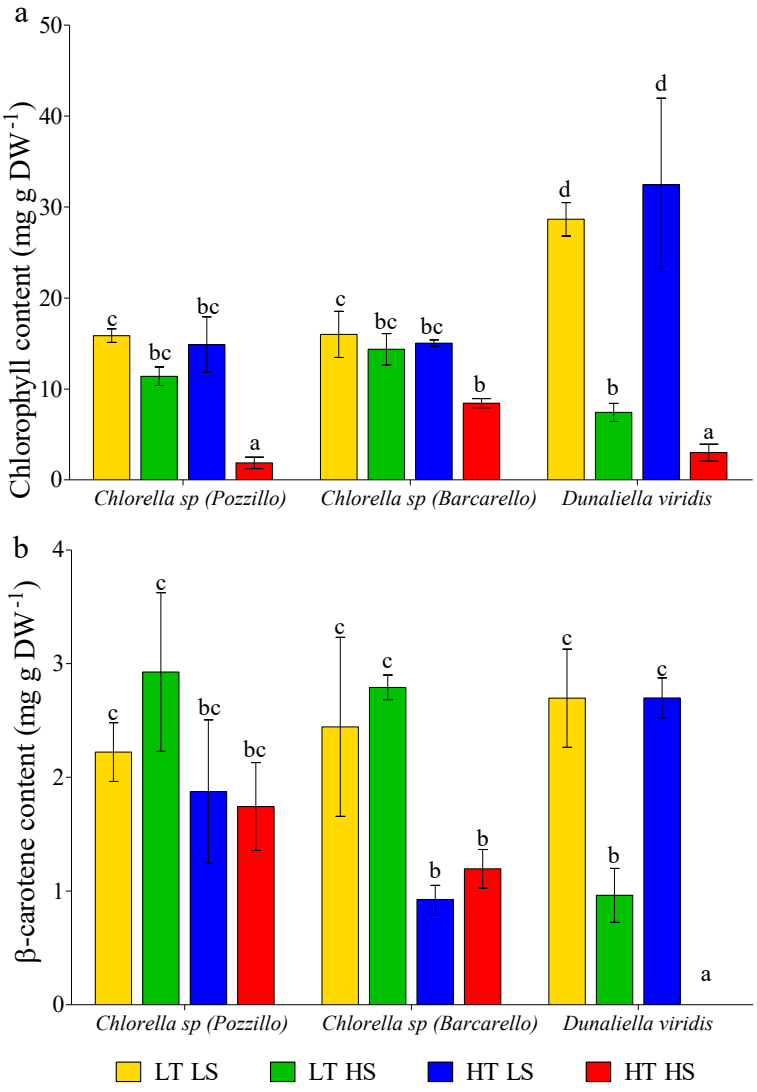

Figure 6 Chlorophyll *a* (mg g^{-1} DW) (a) and β-carotene (mg g^{-1} DW) (b) in the strains *Chlorella* sp *Pozzillo* in *Chlorella* sp *Barcarello* and in *Dunaliella viridis*. Values are reported as means (n=3) and error bars report the standard deviations. Different letters indicate significant differences among various treatment in each species at $p<0.05$

In general, chlorophyll *a* in *Chlorella* strains ranged between 1.88±0.63 and 16.01±2.55 mg g^{-1} DW respectively in *Chlorella* sp *Pozzillo* HT-HS and *Chlorella* sp *Barcarello* LT-LS, while in *D. viridis* from 3.00± 0.94 and 32.45± 9.54 mg g^{-1} DW in HT-HS and HT-LS conditions (Figure 6a). These values are in line with other works in literature regardless of temperature variations; for example, Ani Azaman et al.[63] have obtained values of 17.93 (mg g^{-1} DW) and 15.69 (mg g^{-1} DW) of chlorophyll *a* in *C. sorokiniana* and *C. zofingiensis* respectively [63]. A significant decrease is observed in *Chlorella sp Pozzillo* HT-HS and in *Chlorella* sp *Barcarello* HT-HS (Figure 6a).

In *D. viridis* a significant increase in chlorophyll *a* content was observed with temperature stress (Figure 6a), as also observed by Srirangan et al. [43] which observed that in *D. viridis* the chlorophyll content was positively influenced by the increase in temperature from 25 to 35°C [43].

As demonstrated for both lipids (Figure 3) and antioxidant activity (Figure 5), the increase of salinity in *D. viridis* induced a significant decrease in chlorophyll *a* production; a similar result was observed by Jiménez and Niell [64] who detected that the content of chlorophyll *a* and *b* significantly decreased with increasing salinity [64].

As reported in Figure 6b, the β-carotene content in *Chlorella* strains varied between 0.93±0.12 and 2.79±0.11 mg g^{-1} DW in *Chlorella* sp *Barcarello* HT-LS and LT-HS, respectively, while in *D. viridis* it ranged between 0.00, 2.70±0.43 and 2.70±0.17 mg g^{-1} DW in HT-HS, LT-LS and HT-LS respectively. The values of β-carotene determined are in line with values obtained by other authors [44].

As shown in Figure 6 b, *β*-carotene content in *Chlorella* strains was negatively affected by the temperature increase. The *β*-carotene content increased from 2.22±0.26 (*Chlorella* sp *Pozzillo*

LT-LS) to 1.88±0.63 mg g^{-1} DW (*Chlorella* sp *Pozzillo* HT-LS) and from 2.44±0.79 (*Chlorella* sp. *Barcarello* LT-LS) to 0.93±0.12 mg g^{-1} DW (*Chlorella* sp *Barcarello* HT-LS). The production of β-carotene seems not to have been influenced by the salinity factor for both *Chlorella* strains, as also observed by Ko et al. [61].

On the contrary, in *D. viridis* salinity caused a significant reduction in β-carotene content. While there are no significant differences when temperature varied, high salinity led to a drastic decrease in *β*-carotene content, which combined with high temperatures, leads to an inability of the microalgae to produce β-carotene (Figure 6b).

We can further state that the combination of temperature and salinity stresses (HT-HS condition) resulted in a drastic reduction of chlorophyll *a* and β-carotene content in all the microalgal strains, as showed also by Ko et al. [61]. In all the stress conditions a correlation between antioxidant activity and *β*-carotene production was observed as demonstrated by Singh et al. [62] in *D. salina*; these results suggest that under stress conditions the antioxidant effect may be due to an increase in *β*-carotene production [62]. There is no correlation between the antioxidant capacity and phenolic content, indicating that these compounds may not contribute significantly to the antioxidant activity of these microalgae [60].

Considering their properties, carotenoids have various industrial applications in food, nutraceutical and pharmacological industries [65]. Therefore, possible use of these microalgal strains in the industrial sector should consider that the highest productivity of chlorophyll *a* was obtained in *Chlorella* sp *Barcarello* HT-LS (Table S1 supplementary materials). As regards *β*-carotene the optimum productivity was obtained in *Chlorella* sp *Pozzillo* LT-LS and LT-HS (0.16±0.03 and 0.47± 0.03 mg L^{-1} d^{-1}) (Table S1 supplementary materials).

3.7. nMDS and Pearson's correlation

The nMDS and Pearson's correlation studies (Figure 7) revealed that the assembling of the three microalgae strains is affected both by every single abiotic variable (salinity and temperature) and by the combination of the two abiotic variables. When *D. viridis* is subjected to high temperatures (HT) and high salinity (HS), it shows a different behaviour compared to the same species not subjected to the synergistic effect of the two stresses (LT-LS, LT-HS and HT-LS).

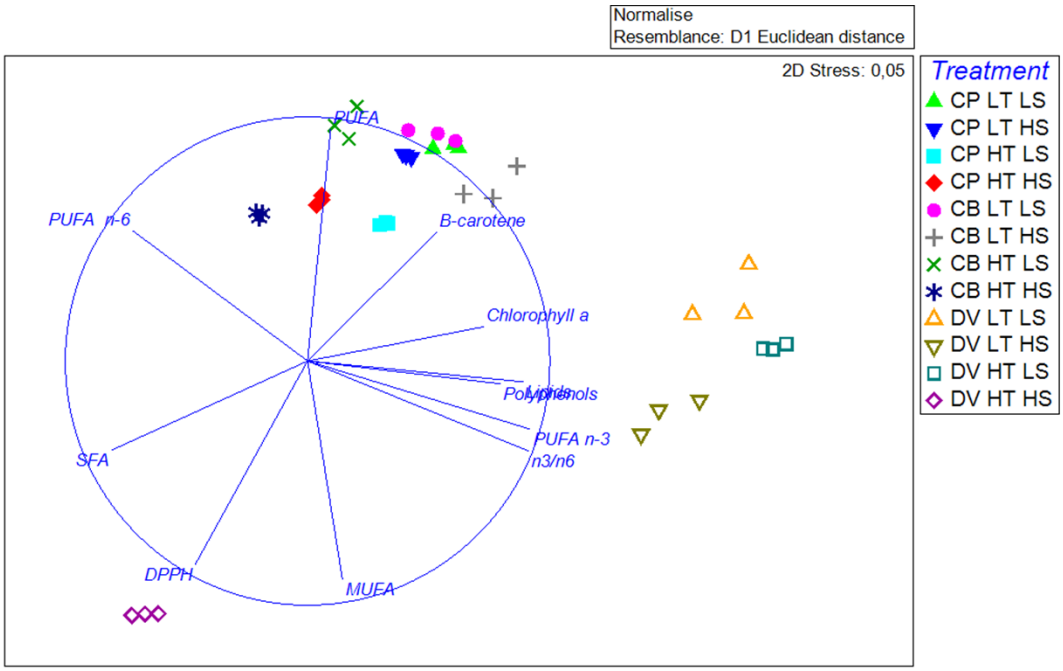

Figure 7: nMDS and Pearson's correlation used to evaluate patterns in polyphenols, DPPH, lipid content, SFA, MUFA, PUFA, PUFA *n*-3, PUFA *n*-6, *n*-3/*n*-6 chlorophyll *a* and *β*-carotene, in relation to salinity and temperature variables in the strains *Chlorella* sp *Pozzillo* (CP) in *Chlorella* sp *Barcarello* (CB) and in *Dunaliella viridis* (DV). LT: low temperature, LS: low salinity, HT: high temperature, HS: high salinity.

D. viridis and both *Chlorella* sp strains are clearly separated from each other while *Chlorella* sp *Pozzillo* and *Barcarello* show a similar trend between themselves. This proves that the three strains are not only influenced by the intrinsic characteristics of the species but even by the abiotic stressors.

3.8. CONCLUSIONS

In this work, two strains of *Chlorella* and one of *Dunaliella* were cultivated on a lab scale in four different conditions: Low Temperature-Low Salt (LT-LS), High-Temperature-Low Salt (HT-LS), Low Temperature-High Salt (LT-HS) and High Temperature-High Salt (HT-HS). Kinetic parameters were estimated together with biochemical parameters.

Results indicated that high salinity decreased the productivity of both *Chlorella* strains and *D. viridis*. The increase of temperature enhanced the productivity in *Chlorella* sp *Barcarello*, while the combination of HT and HS worsened the growth performance in all strains.

The increase of temperature and salinity decreased the lipid content in all the strains and caused a significant shift in fatty acid composition, leading, in *Chlorella* strains cultivated in LT-HS condition, to an interesting increase in the production of *n*-3 PUFAs. Results indicated that also the carotenoids composition is influenced by the stress environmental conditions in which microalgae are cultivated, with a decrease in chlorophyll *a* and β-carotene in many of the stressed conditions.

Although microalgae resulted able to tolerate high temperatures and high salinity, microalgal growth was significantly affected by those environmental stressors. For this reason, the kind of application is a major factor to consider when choosing the growth parameter from an industrial perspective. For example, in Sicily, the diurnal temperature during summer may reach 40°C and in an outdoor facility, one of the main costs is the cooling system, not necessary when employing heat-tolerant strains with the appropriate application. To this respect, the microalgae *Chlorella* sp *Barcarello* resulted in markedly suitable for being cultivated at high temperature. It is worth noting the relevant shift in lipid content and its composition, together with the

changes in carotenoid's composition, that may be interpreted as an adaption to the stress condition in which the cells grew.

The data obtained from this preliminary research lay the foundation for the development of a regional-based algal industry that exploits a local resource, the microalgal strains, to produce biomass and other high-value compounds.

DECLARATION OF COMPETING INTERESTS

Authors have no competing interest to declare.

AUTHOR CONTRIBUTION

Rosaria Arena (RS) (rosaria.arena@unipa.it) and Serena Lima (SL) (serena.lima@unipa.it) contributed equally to this study. SL concepted and designed the study, collected and assembled data, drafted the article, participated to the obtaining of funding and to the final approval of the article. RS performed lab analysis, collected and assembled data and gave a precious contribute in writing the article.

Valeria Villanova (VV) (valeria.villanova@bioenv.gu.se) revised the article critically for important intellectual content, participated to the obtaining of funding and to the final approval of the article.

Nadia Moukri (NM) (nadia.moukri@unipa.it) performed the experiments on microalgae cultivation, collected and assembled data.

Eleonora Curcuraci (EC) (eleonora.curcuraci@unipa.it) performed lab analysis, collected and assembled data and gave a precious contribute in writing the article.

Concetta Maria Messina (CM) (concetta.messina@unipa.it) and Andrea Santulli (AS) (andrea.santulli@unipa.it) participated to the interpretation of data, gave a precious contribute in revising the manuscript critically for important intellectual content and participated to the obtaining of funding and to the final approval of the article.

Francesca Scargiali (FS) (francesca.scargiali@unipa.it) participated in the analysis and interpretation of data, made a critical revision of the article for important intellectual content, participated in the obtaining of funding and the final approval of the article.

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