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Nutritional and functional assessment of haloarchaea and microalgae from the Andalusian shoreline: Promising functional foods with a high nutritional value

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ABSTRACT

Marine microbiota is garnering increasing interest as a potential source of novel foods with enhanced nutritional and functional properties. In this study, the proximate composition and antioxidant capacity of five haloarchaea and microalgae species from the Andalusian Atlantic shoreline (*Halobacterium salinarum*, *Haloarcula hispanica*, *Dunaliella salina*, C5 and C13, and *Picochlorum spp*) were compared to two freshwater species (*Chlamydomonas reinhardtii* and *Chlorella sorokiniana*). All the species studied exhibited high protein content and antioxidant activity, with noticeable variations in total mineral content and fatty acid profile. Among the four extraction methodologies tested to prepare functional extracts, forced aqueous extraction followed by protein hydrolysis resulted in significantly greater yields and antioxidant activity, while microalgae demonstrated higher activity compared to haloarchaea tested in the present study. In conclusion, the diverse species examined displayed high nutritional and functional value, showing great potential to be part of the human diet as rich sources of readily available protein and antioxidant compounds.

1. Introduction

Microalgae comprise a vast, ubiquitous, and diverse group of microorganisms that have been hailed as a sustainable source of nutrients and functional ingredients (Martínez et al., 2018, Wu et al., 2021, Ferdous et al., 2021, Eilam et al., 2023), playing a pivotal role in aquatic ecosystems. Encompassing a wide range of phyla and families, they possess a remarkable capacity to utilize solar energy through photosynthesis, thus playing a significant role in the global carbon cycle (Borowitzka, 2018). Haloarchaea is a group of microorganisms classified within the domain of Archaea that exhibit significant genetic and physiological diversity, with great adaptability to various environments (Cavicchioli, 2011). They are extremely halophilic microorganisms that inhabit hypersaline environments where they must thrive with high temperatures or high UV radiation besides high salinity. These microorganisms have developed strategies to survive in such adverse conditions with unique compounds, such as C50-carotenoids, ether lipids, or extremozymes that can have health-related applications. Interestingly, extracts obtained from different species of haloarchaea have shown antimicrobial, anti-hemolytic, neuroprotective, antiviral, anti-

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inflammatory (Avila-Román et al., 2023), or antitumoral activities (Giani et al., 2023; Shahbazi et al., 2023), among others (Hou et al 2018; Hegazy et al., 2020, Lizama et al., 2021, Sahli et al., 2022).

Microalgae exhibit a remarkable nutritional profile, rich in essential nutrients for human health and development. Their nutrient composition includes proteins, carbohydrates, lipids, minerals, and vitamins. Of utmost importance are their high protein and omega-3 fatty acids content, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are pivotal for cardiovascular health and neurodevelopment. Although the nutritional composition of haloarchaea has been less studied, their fatty acid and bioactive components composition, as well as their potential applications in the food industry, have been described (Martinez-Checa et al., 2007, Desai et al., 2020, Gomez-Villegas et al., 2020, Ávila-Román et al., 2023). Beyond their nutritional composition, the bioavailability of nutrients from microalgae and haloarchaea can be significantly affected by their unique cellular structures, while their content of bioactive compounds with health-promoting effects confers them interesting bio-functional properties.

Microalgae and haloarchaea may find diverse applications in nutrition, industry, and aquaculture. In human nutrition and food industry, they have great potential as high-protein foods, dietary supplements, functional foods, and nutraceuticals (Morançais et al., 2018, Grivard et al., 2022, Kapravelou et al., 2023, Bhatnagar et al., 2024). Moreover, microalgae play a pivotal role in aquaculture, serving as a sustainable feed source for fish and other aquatic organisms. In addition, the potential applications of haloarchaea and microalgae extend beyond their nutritional content, since these microorganisms show promise in various environmental and industrial processes, including phosphate solubilizing to enhance P nutrition of vegetation growing in hypersaline soils (Yadav et al., 2015), bioremediation (León-Vaz et al., 2021), bioleaching, or the production of enzymes and bioactive molecules (Desai et al., 2020). Thus, their potential contributions to sustainable development have become increasingly evident.

The Andalusian Atlantic shoreline is a potential source of multiple microorganisms with great potential interest for the development of novel foods with high nutritional and functional value. Screening and bioprospecting campaigns in marine and freshwater environments allow the isolation of new strains and the identification of novel metabolites with bioactive properties and potential commercial applications. Once new microorganisms that can be cultivated at a laboratory or large scale are isolated and identified, nutritional characterization is the first necessary step, followed by the identification of bioactive compounds, appropriate extraction methods, and assessment of potentially beneficial biological activities like antioxidant, anti-inflammatory, anti-proliferative or antimicrobial capacity. Such analyses may be helpful to justify the potential of the newly isolated species to develop functional foods and ingredients. However, the study of new functional foods and extracts, as well as the bioactive compounds responsible for their beneficial claims, cannot be considered complete without the implementation of digestibility techniques that confirm the survival and potential bioaccessibility of the tested bioactive compounds along the digestive process. A necessary step to exert their beneficial effects in different tissues and organs.

In the present study, we have screened a collection of microalgae isolated from continental and marine waters of the Andalusian coast and a series of extremophiles microorganisms, including microalgae and archaea, that thrive at extremely high salinity in the crystallization ponds of the saline works located in the Odiel Marshlands in the southwest of Spain. Specifically, 5 different species of microalgae and haloarchaea and two well-known control freshwater microalgae have been selected with the aim of 1) studying their nutritional composition and the antioxidant activity of functional extracts prepared using different extraction methodologies, 2) selecting the species with the greatest nutritional and bio-functional potential to carry on an *in vitro* digestion process using conditions similar to those observed in the human gastrointestinal tract to study whether their protein content and antioxidant capacity are potentially absorbable to the bloodstream during intestinal digestion.

2. Material and methods

2.1. Chemicals and reagents

Folin–Ciocalteu reagent and gallic acid were purchased from Sigma-Aldrich, Madrid, Spain. Ethanol, diethylenetriaminepentaacetic acid (DETAPAC), trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), $FeCl_3$, and H_2O_2 were from Panreac/Applichem (Barcelona, Spain). Thiobarbituric acid (TBA) was from Merck (Darmstadt, Germany).

2.2. Haloarchaeal and microalgal biomass

Five different species of haloarchaea (*Halobacterium salinarum HM2* and *Haloarcula hispanica HM1*) and microalgae (*Dunaliella salina C5* and *C13*, *Picochlorum spp HM1*,) isolated from the Andalusian Atlantic shoreline and two control freshwater microalgae, *Chlamydomonas reinhardtii (704),* kindly provided by Dr. Emilio Fernández from the Univesity of Córdoba, and *Chlorella sorokiniana (*211–32), obtained from the algal collection of the Institute of Plant Biochemistry and Photosynthesis (IBVF-CSIC, Seville, Spain)*,* were cultured, harvested by centrifugation, freeze-dried and kept at 4°C in the dark for the following analysis.

Haloarchaea were cultured in hypersaline liquid medium that contained per liter: 10 g glucose, 156 g NaCl, 13 g $MgCl_2·6H_2O$, 20 g $MgSO_4·7H_2O$, 1 g $CaCl_2·6H_2O$, 4 g KCl, 0.2 g $NaHCO_3$, and 5 g yeast extract, pH 7, for 10 days at 37°C and 120 rpm. Microalgae were cultured in liquid media and bubbled with air enriched with CO_2 (5 % v/v). *Picochlorum* was cultured in F/2 medium (de la Vega et al., 2011), *Dunaliella* species were grown in Johnson's media (Johnson et al., 1968), and freshwater microalgae, *Chlamydomonas reinhardtii* and *Chlorella sorokinina*, were photomixotrophically cultured in liquid Tris-acetate phosphate (TAP) medium. All cultures were maintained in a thermostatic chamber at 25°C under continuous white light irradiation (100 μE m^2/s photosynthetically active radiation (PAR).

2.3. Proximal composition of haloarchaea and microalgae

The moisture content of haloarchaea and microalgae freeze-dried biomass was measured by drying to constant weight in an oven at 105 ± 1°C. Total N was determined according to Kjeldahl's method. Crude protein was calculated as N × 6.25. Total fat content was determined by gravimetry of the ether extract after acid hydrolysis of the sample using boiling 4 M HCl/zelite and reflux conditions during 45 min (Büchi Hydrolysis Unit B-411), followed by rinsing with type 2 water (40°C). Ash content was measured by calcination at 500°C to a constant weight. Fatty acid profile analysis was performed in aliquots of haloarchaea and microalgae (250 mg) that were methylated according to Lepage & Roy (1986) for gas chromatography analysis using an Agilent 7890A chromatograph equipped with a CTC Pal combi-xt model sampler and a Waters Quattro micro-GC mass spectrometer detector as previously described (Martinez et al., 2020). Fatty acid methyl esters (FAMEs) were separated using a 30 m × 0.25 mm ZB Fame capillary column (0.2 μm thickness) (Phenomenex, Torrance, CA, USA). The gas chromatography conditions were as follows: injector temperature 250°C, injection volume 2 μL Split (proportion 10:1), temperature gradient from 100°C to 210°C with a rate of 4 °C/min, hold time 5 min. The flow rate of the carrier gas (Helium) was 1 mL/min. Analysis time was 40 min and measurement range 45–450 uma (scan mode). Chromatographic data were recorded and integrated using Masslynx, version 4.1 software. FAMEs were identified using analytical standards and a mass spectral library. Peak areas were measured and used to calculate the percentage of each fatty acid. All the analyses were carried out at the Food Composition Analysis, and Structural Assessment Analysis Units (CIC, University of Granada).

2.4. Extraction procedure

To obtain functional extracts rich in bioactive compounds, the haloarchaea and microalgae underwent a series of processing steps. First, they were freeze-dried and the powder was stored at −20°C to preserve the plant material. Next, four different treatments were applied to obtain different extracts. Treatment 1 (T1) was a cold ethanolic extraction according to Martinez et al. (2023), treatment 2 (T2) was a forced cold ethanolic extraction process that combined a 40-second mechanical disruption with lysing beads (Precellys Tissue Homogenizer, Bertin Instruments) with the ethanolic extraction, treatment 3 (T3) was a forced aqueous extraction process (40 s mechanical disruption plus extraction process), and treatment 4 (T4) was a forced aqueous extraction process (40 s mechanical disruption) combined with protein hydrolysis using recombinant proteases according to Kapravelou et al (2013).

2.4.1. Ethanolic extracts

Ethanol was selected as the main solvent used in cold-non aqueous extraction process due to its optimum capacity to extract polyphenolic substances and its much lower toxicity compared to other widely used solvents such as methanol, acetone, or dimethyl sulfoxide (DMSO). Nevertheless, ethanol has proved itself a less efficient solvent to extract other valuable bioactive components like carotenoids when compared to others (Churio et al., 2022). The ethanolic extract process was developed by mixing 1 g dry weight of haloarchaeal or microalgal biomass with 15 mL of hydroalcoholic solution (Ethanol: type I water:12 N HCl; 50:50:0.25) at pH 2 and 4 °C using a reducing atmosphere (with N_2) for 30 min in a magnetic stirrer. After the first extraction, the extract was centrifuged at 3000 rpm for 5 min. The supernatant was stored, and the pellet was recovered to repeat the extraction process using 10 mL of hydroalcoholic solution. After the second extraction, all the supernatants were pooled together and stored at −20°C. To assess the ethanolic extract yield and extract concentration, aliquots (1 mL) were treated to evaporate ethanol using a vacuum evaporator (Savant DNA120 SpeedVac Concentrator, ThermoSci, Waltham, MA, USA). The evaporated extracts were frozen in liquid N and freeze-dried (Cryodos-50 lyophilizer, TELSTAR, Madrid, Spain) for 24 h. Then, the dry weight of the extract was calculated by subtracting the weight of the empty recipient containing each aliquot and referring it to a volume of 1 mL of initial extract, to the total volume of extract obtained, and, finally, to the grams of haloarchaea or microalgae used for the extraction. The process of forced extraction (T2) was done in the same way except for an initial mechanical grinding procedure in the ethanolic extraction solution using lysis beads that was carried on before the ethanol extraction. Specifically, 0.75 g of sample was placed in the lysis tube and 4 mL of extraction solution was added. The mixture underwent two rounds of lysis at 6500 rpm for 20 s followed by 15 min of ultrasound treatment at room temperature in an ultrasonic water bath (60–70 % intensity, 37 kHz frecuency, fisherbrand, Fisher Scientific). Then, 10 mL of cold extraction solution was added, the pH was adjusted to 2 and the extraction was carried out as previously described in two steps under N_2 atmosphere.

2.4.2. Protein extraction (T3)

To obtain an aqueous extract, 0.75 g of haloarchaea or microalgae were placed into a lysis tube and 4 mL of ice-cold type I water was added. The mixture underwent two rounds of lysis at 6500 rpm for 20 s followed by freezing at −80°C overnight. The frozen mixture was then thawed for 30 min at 23°C in combination with ultrasound treatment in an ultrasonic water bath (60–70 % intensity, 37 kHz frequency, fisher brand, Fisher Scientific). After thawing, 20 mL of type 1 water were added to the mixture; the pH of extraction was set at 9.0 using 3 M KOH, and protein was extracted by agitation in a magnetic stirrer for 45 min at room temperature. After this first extraction, the extract was centrifuged at 3000 rpm for 10 min and the supernatant was collected and stored on ice. The pellet was resuspended with 30 mL of type 1 water and the pH was checked and, if needed, adjusted to 9.0 with 3 M KOH. The pellet was then subjected to a second extraction process without lysis beads mechanical treatment under the same conditions as the first and the mixture was centrifuged. The two supernatants obtained were combined, the total volume measured, and three 500 μL aliquots were taken for further analysis and kept at −80°C. Additionally, three 500 μL aliquots were freeze-dried for 24 h using a Cryodos-50 lyophilizer (TELSTAR, Madrid, Spain) to assess the concentration of the aqueous extract. The remaining aqueous extract was used for protein hydrolysis.

2.4.3. Protein hydrolysis (T4)

To hydrolyze the protein in the aqueous extract from the previous extraction process, $MgSO_4$ and $CaCl_2$ were added to the solution at a concentration of 1 mM, the temperature of the protein solution was raised to 45°C, and the pH was set to 8.5 with KOH. Protease from *Bacillus licheniformis* (Sigma Aldrich, Madrid, Spain) was then added, and the mixture was stirred at 45°C for 30 min. Protease solution (2.88 Anson Units/mL) was added at a ratio of 0.3 AU/g protein in the aqueous extract. After the first hydrolysis process, the mixture was treated for an additional 30-min period with protease from *Aspergillus oryzae* (Sigma Aldrich, Madrid, Spain) using the same pH and temperature conditions. Protease solution (600 LAPU/mL) was added at a ratio of 100 LAPU/g protein in the aqueous extract. At the end of the hydrolysis process, the final volume was measured, six 1 mL aliquots were taken for further analysis, and three additional 500 μL aliquots were freeze-dried for 24 h using the Cryodos-50 lyophilizer (TELSTAR, Madrid, Spain) to assess the protein hydrolysate concentration. The remaining protein hydrolysate was also freeze-dried and stored at −80°C for future use in subsequent studies.

2.5. Characterization of antioxidant capacity

Antioxidant activity of the cold ethanolic extracts, aqueous protein extracts, and protein hydrolyzates was analyzed according to what has been described by Kapravelou et al., (2015,2020) and Martinez et al. (2023), using four different techniques. Briefly, Total polyphenol content (TPC) was assessed using the Folin-Ciocalteu method as described elsewhere (Dewanto et al. 2002). A calibration curve based on different concentrations of gallic acid (0–600 μg/mL) was used. The results were expressed as μg gallic acid equivalents (GAE). Reducing capacity of Fe^{3+} to Fe^{2+} (Duh et al., 1999) of functional extracts was analyzed spectrophotometrically according to what has been previously described by Kapravelou et al. (2015). The 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical uptake assay was performed based on the method of Miller et al. (1993) to measure the total antioxidant capacity of a fluid. For this, 6 μL of evaporated ethanolic extract or a standard solution of gallic acid (0–60 mg/L), was mixed with 294 μL of ABTS and incubated for 3 min. The optical density of the samples was then measured at 620 nm (Multiskan FC, Microplate Photometer, Thermo Fisher Scientific). The blank was made with 6 μL of water and 294 μL of ABTS. The results were expressed as μg of gallic acid equivalent (GAE) per mg of extract. Lipid peroxidation inhibition capacity (LPIC) was analyzed via thiobarbituric acid reactive substances (TBARS) formation (Ohkawa et al., 1979) in rat brain homogenate as a marker of lipid peroxidation after oxidative treatment with $H_2O_2/FeCl_3$. Brain tissue was mixed (1:10 w/v) with cold 1.15 % KCl/0.1 % Triton X-100 with the use of a homogenizer. The mixture was centrifuged at 810 × g, 4°C for 10 min, and the supernatant was aliquoted and stored at −20°C for lipid peroxidation assays. Brain homogenates were used to assess lipid peroxidation after treatment with a mixture of $FeCl_3/H_2O_2$ to induce lipid peroxidation, and the percentage of inhibition in TBARS formation caused by the different extracts as previously described by Kapravelou et al. (2015). Results were expressed as units (50 % inhibition of TBARs formation) per mg of extract or microorganism biomass.

2.6. *In vitro continuous dialysis digestion procedure*

To assess the digestibility and potential availability of protein and antioxidant potential from haloarchaea and microalgae, a continuous flow *in vitro* method was conducted according to Minihane et al. (1993) and Shen et al. (1994), following the INFOGEST recommendations for saliva, gastric and intestinal digestion solutions as well as food:digestive secretion ratios (Brodkorb et al., 2019) with some modifications. Specifically, 40 mL of a mixture of saliva solution and the selected haloarchaea or microalgae (amounting to 1 g) was used and stirred for 2 min at 37 °C. Then, 31 mL of gastric solution was added, and the pH was adjusted to 2 with HCl. Pepsin was subsequently added, and the solution was stirred for 2 h at 37 °C. Following gastric digestion, the resulting liquid was combined with 54 mL of intestinal solution and a dialysis membrane containing 2 mL of type 1 water and an amount of $NaHCO_3$ equivalent to the titratable acidity of the gastric solution to adjust the pH of intestinal digestion to 7.5. The mixture was stirred for 30 min at 37 °C, and the dialysis membrane was removed, emptying its contents into the digestion cup. Pancreatin and bile salts solution were then added, and the mixture was digested for 150 min, with fractions collected every 30 min.

2.7. *Statistical analysis*

We analyzed the effect of different haloarchaea and microalgae genera (*Halobacterium salinarum HM2*, *Haloarcula hispanica HM1*, *Dunaliella salina C5* and *C13*, *Picochlorum spp HM1*, *Chlamydomonas reinhardtii*, and *Chlorella sorokiniana*) and four extraction methods (T1, T2, T3, and T4) as the main treatments, on extraction yield, total polyphenol content, ABTS antioxidant capacity, ferric reducing capacity, and lipid peroxidation inhibition capacity. A mixed factorial analysis of variance (ANOVA) was conducted (Table 6). Post-hoc analyses were performed using the Bonferroni test. In addition, the influence of haloarchaea or microalgae genera on the digestibility of dry matter, protein, total polyphenol content, and reducing capacity, was analyzed by a one-way ANOVA. Duncan's test was used to detect differences between treatment means (Tables 7A-7C). Results are presented as mean values and standard error of the mean (SEM). Analysis of the data was done using SAS® OnDemand for Academics (SAS Institute, Inc., Cary, NC) and the level of significance was set at 0.05.

3. Results

3.1. *Proximal composition*

The nutrient content of microalgae and haloarchaea is presented in Table 1**.** Protein and total fat content of the different species studied ranged from 47.6 to 62.5 % and 3.88–6.95 %, respectively. *D. salina C13* and *C. sorokiniana* were the microalgae with the highest protein and total fat levels. Ash composition was markedly higher in *Halobacterium* and *Haloarcula* compared to the different microalgae, among which the highest ash content corresponded to *Dunaliella spp*. Regarding total-P expressed as mg per gram of ash, the highest concentration was found in *C. reinhardtii* and *C. sorokiniana*, followed by *Picochlorum* and *Dunaliella spp*. The amount of carbohydrates was highest in *Picochlorum spp*, *C. reinhardtii*, and *D. salina C5*, followed by *C. sorokiniana*, *D. salina C13*, and haloarchaea. A similar trend was observed for the caloric content of the different microalgae and haloarchaea calculated by the Atwater formula. Differences in nutrient composition between the two species studied of *D. salina* were apparent for total protein and ash that were higher in C13 *vs* C5, or in carbohydrate content that was higher in C5. Regarding the fatty acid profile (Fig. 1), differences were observed between haloarchaea and microalgae, the former characterized by a higher level of monounsaturated oleic acid in *Halobacterium* and *Haloarcula* compared to higher levels of polyunsaturated linolenic and linoleic acids in microalgae. Special mention must be made to *C. sorokiniana* in which the highest percentage corresponded to palmitic acid, followed by linolenic, linoleic, oleic, and stearic acids.

3.2. *Extraction yield and antioxidant capacity*

The influence of species and extraction procedure on the extraction yield of haloarchaea and microalgae is presented in Tables 2 and 6. There was a significant effect of species due to clear differences in extraction yield among the different samples. In this regard, the highest values corresponded to haloarchaea, followed by the microalga *Dunaliella salina*. Concerning the extraction treatment effect, aqueous extraction combined with lysis beads treatment gave rise to significantly higher extraction yields compared to ethanol extraction (with or without lysis beads treatment). Protein hydrolyzates showed higher extraction yields compared to aqueous protein extraction at basic pH due to the amount of $CaCl_2$, $MgSO_4$, and proteases added for hydrolysis treatment. These results were reflected in a significant treatment effect, the strength of which varied within the different genera tested, thus resulting in significant species × treatment interaction. Overall, the highest contribution to the total variance of the ANOVA model was exhibited by extraction treatment, followed by species and the interaction among both.

Different methodologies were tested to study the antioxidant capacity of functional extracts. Results of the antioxidant activity variables are expressed per milligram of extract or per gram of extracted sample to compensate differences in the extraction yield (Tables 3-6, Supplementary Table S1). The influence of different species and extraction methodologies on the total polyphenol content of haloarchaea and microalgae is presented in Tables 3 and 6. Antioxidant activity measured as total polyphenol content was influenced by two factors: first, species, with lower antioxidant capacity shown by haloarchaea compared to microalgae, and, second, extraction procedure, with significantly greater activity found in aqueous *vs* ethanol extracts. Specifically, the highest antioxidant capacity was exhibited by protein hydrolyzates. Among those, changes in antioxidant capacity were observed upon normalization of data to the quantity of departing product, with the highest total polyphenol content being found in *C. reinhardtii* and *C. sorokiniana*, followed by *D. salina* C5 and *H. salinarum*.

The effects of different genera and extraction processes on reducing capacity of haloarchaea and microalgae extracts are described in

Table 1
Proximal composition of haloarchaea and microalgae (g/100 g DM).

	H. salinarum	*H. hispanica*	*D. salina C5*	*D. salina C13*	*Picochlorum* spp. *HM1*	*C. reinhardtii*	*C. sorokiniana*
Protein	47.6	48.8	48.7	57.0	49.0	57.0	62.5
Fat	6.89	3.88	6.86	6.96	4.44	5.88	6.95
Carbohydrates	2.34	4.16	25.6	13.4	32.6	32.6	23.4
Ash	43.2	43.2	18.8	22.6	14.0	4.53	7.24
Total-P (mg/g ash)	28.6	31.5	44.6	37.1	70.4	229.9	178.6
Energy (Kcal/100 g)	261.7	246.6	359.2	344.3	366.3	411.3	405.8
Energy (Kjul/100 g)	1093.8	1030.7	1501.4	1439.0	1531.0	1719.1	1696.2

Results are duplicates of two independent analyses. DM, dry matter. Total carbohydrates are calculated by difference. Energy content is calculated based on the Atwater formula.

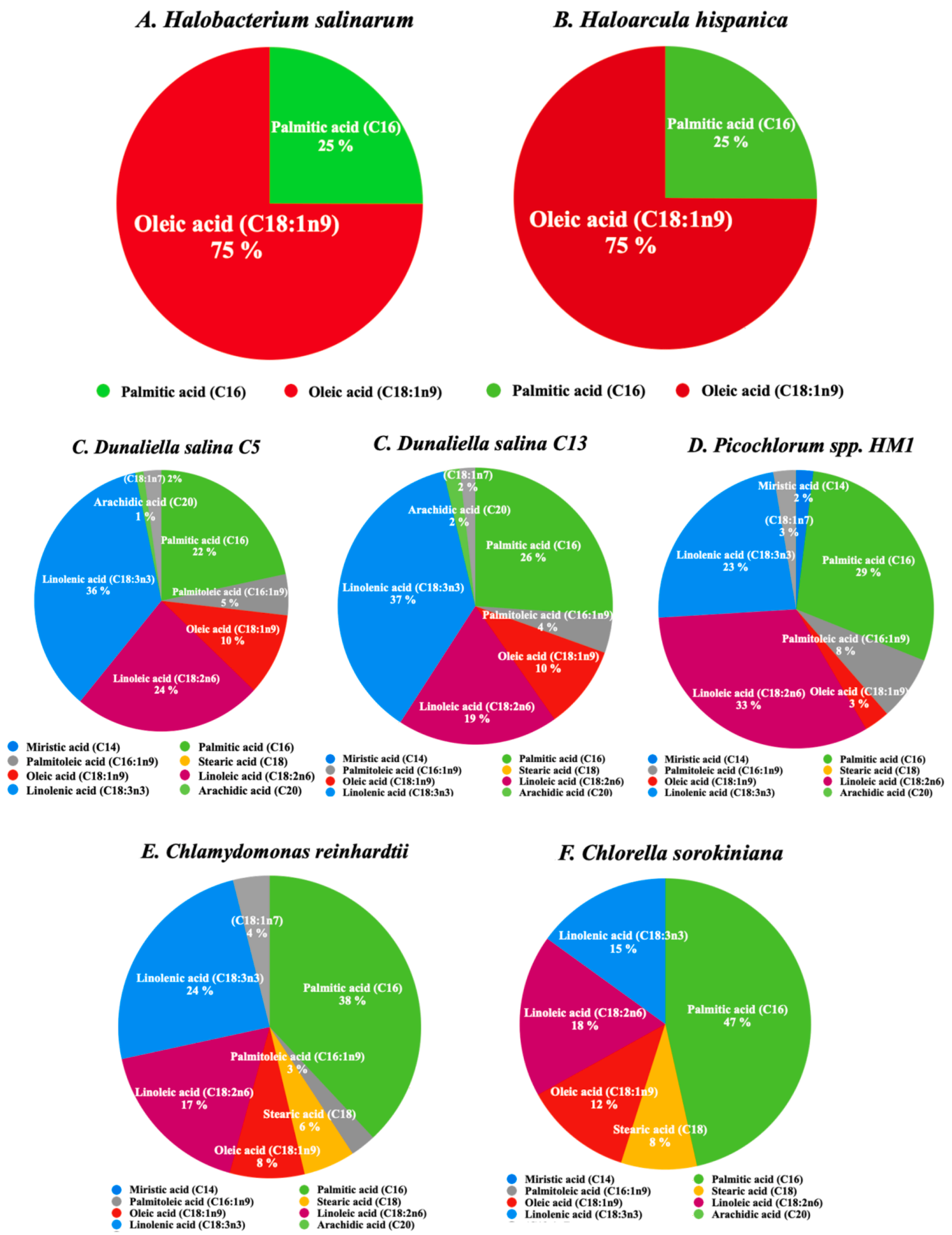

Fig. 1. Fatty acid profile of haloarchaea and microalgae. Results are expressed as percentage of the total area under the curve calculated by the sum of individual FAME.

Table 2
Extraction yield of functional extracts obtained using different extraction methodologies.

	H. salinarum	*H. hispanica*	*D. salina C5*	*D. salina C13*	*Picochlorum* spp. *HM1*	*C. reinhardtii*	*C. sorokiniana*
T1 (g/100 g)	29.1 ± 0.05A	36.7 ± 0.06B	32.6 ± 0.06C	34.4 ± 0.07BC	8.41 ± 0.03D	9.0 ± 0.02D	15.4 ± 0.02E
T2 (g/100 g)	35.9 ± 0.03A	37.1 ± 0.02B	40.2 ± 0.04C	38.0 ± 0.02B	16.8 ± 0.02D	13.3 ± 0.03E	14.2 ± 0.03E
T3 (g/100 g)	87.0 ± 0.05A	84.4 ± 0.04A	55.1 ± 0.06B	52.7 ± 0.03BE	59.0 ± 0.06C	36.9 ± 0.06D	50.8 ± 0.04E
T4 (g/100 g)	108.7 ± 0.07A	86.8 ± 0.04B	101.6 ± 0.07C	94.4 ± 0.07D	70.3 ± 0.07E	79.1 ± 0.06BD	90.1 ± 0.03F

Results are means ± SEM (n = 6). T1, cold ethanol extraction, T2, ethanol extraction with lysis beads, T3, aqueous extraction at basic pH with lysis beads, T4, aqueous extraction at basic pH with lysis beads followed by protein hydrolysis using recombinant proteases. A,B,C,D,E, means within the same row with different letters are significantly different ($P < 0.05$).

Table 3
Total polyphenol content of functional extracts obtained using different extraction methodologies.

	H. salinarum	*H. hispanica*	*D. salina C5*	*D. salina C13*	*Picochlorum* spp. *HM1*	*C. reinhardtii*	*C. sorokiniana*
T1 (µg GAE/mg extract)	2.50 ± 0.02A	2.01 ± 0.06A	3.46 ± 0.11B	4.08 ± 0.35C	3.55 ± 0.18B	10.3 ± 0.35D	6.16 ± 0.08E
T1 (µg GAE/g sample)	727.1 ± 4.5A	738.7 ± 23.7A	1129.1 ± 34.6B	1403.6 ± 121.5C	298.8 ± 15.1D	921.4 ± 31.1E	945.8 ± 12.7F
T2 (µg GAE/mg extract)	1.51 ± 0.01A	1.68 ± 0.01AB	1.82 ± 0.01B	2.50 ± 0.02C	4.28 ± 0.19D	11.6 ± 0.04E	6.50 ± 0.03F
T2 (µg GAE/g sample)	542.9 ± 4.8A	622.7 ± 21.7B	732.7 ± 2.17C	951.1 ± 9.14D	720.5 ± 32.5C	1534.2 ± 5.91E	920.5 ± 4.83D
T3 (µg GAE/mg extract)	5.32 ± 0.05A	6.67 ± 0.13B	14.8 ± 0.05C	14.9 ± 0.07C	12.1 ± 0.08D	26.8 ± 0.07E	18.9 ± 0.10F
T3 (µg GAE/g sample)	4629.8 ± 42.7A	5628.1 ± 111.4B	8163.2 ± 27.8C	7854.0 ± 36.7D	7159.2 ± 48.4E	9893.0 ± 25.1F	9609.8 ± 48.9G
T4 (µg GAE/mg extract)	10.6 ± 0.03A	10.9 ± 0.09B	11.6 ± 0.06C	10.3 ± 0.06A	13.7 ± 0.13D	16.1 ± 0.18E	14.9 ± 0.08F
T4 (µg GAE/g sample)	11495.1 ± 32.4A	9468.3 ± 77.0B	117851.5 ± 61.7C	9755.4 ± 59.4D	9650.4 ± 88.5BD	12694.3 ± 140.8E	13409.6 ± 74.3F

Results are means ± SEM (n = 6). GAE, gallic acid equivalents, T1, cold ethanol extraction, T2, ethanol extraction with lysis beads, T3, aqueous extraction at basic pH with lysis beads, T4, aqueous extraction at basic pH with lysis beads followed by protein hydrolysis using recombinant proteases. A,B,C,D,E,F,G, means within the same row with different letters are significantly different ($P < 0.05$).

Table 4
Reducing capacity of functional extracts obtained using different extraction methodologies.

	H. salinarum	*H. hispanica*	*D. salina C5*	*D. salina C13*	*Picochlorum* spp. *HM1*	*C. reinhardtii*	*C. sorokiniana*
T1 (µg GAE/mg extract)	0.32 ± 0.01A	0.65 ± 0.03B	0.92 ± 0.02B	0.70 ± 0.05B	6.00 ± 0.11C	3.80 ± 0.05D	2.32 ± 0.08E
T1 (µg GAE/g sample)	92.0 ± 2.20A	237.5 ± 9.64B	300.3 ± 7.72C	241.0 ± 18.8C	503.1 ± 9.00D	341.5 ± 4.72E	357.4 ± 12.7E
T2 (µg GAE/mg extract)	0.88 ± 0.02A	0.89 ± 0.01A	0.82 ± 0.02A	1.02 ± 0.02A	9.17 ± 0.11B	3.77 ± 0.25C	2.34 ± 0.03D
T2 (µg GAE/g sample)	294.5 ± 8.44A	331.7 ± 5.47A	331.1 ± 7.40A	387.2 ± 7.81B	1543.1 ± 18.5C	500.0 ± 32.9D	331.9 ± 4.44A
T3 (µg GAE/mg extract)	0.80 ± 0.03A	0.94 ± 0.02A	4.67 ± 0.02B	4.41 ± 0.01B	3.80 ± 0.06C	13.6 ± 0.22D	6.55 ± 0.12E
T3 (µg GAE/g sample)	699.1 ± 28.5A	794.0 ± 26.0A	2572.8 ± 9.65B	2325.6 ± 4.53C	2245.0 ± 34.1C	5026.5 ± 82.7D	3325.3 ± 59.8E
T4 (µg GAE/mg extract)	1.16 ± 0.01A	1.42 ± 0.01B	3.30 ± 0.01C	3.20 ± 0.03D	4.89 ± 0.03E	6.00 ± 0.10F	4.57 ± 0.04G
T4 (µg GAE/g sample)	1258.5 ± 9.04A	1230.4 ± 8.66A	3352.5 ± 15.2B	2980.3 ± 27.5C	3436.1 ± 19.7B	4746.7 ± 80.6D	4118.6 ± 3.94E

Results are means ± SEM (n = 6). GAE, gallic acid equivalents, T1, cold ethanol extraction, T2, ethanol extraction with lysis beads, T3, aqueous extraction at basic pH with lysis beads, T4, aqueous extraction at basic pH with lysis beads followed by protein hydrolysis using recombinant proteases. A,B,C,D,E,F,G, means within the same row with different letters are significantly different ($P < 0.05$).

Table 5
Antioxidant capacity (ABTS) of functional extracts obtained using different extraction methodologies.

	H. salinarum	*H. hispanica*	*D. salina C5*	*D. salina C13*	*Picochlorum* spp. *HM1*	*C. reinhardtii*	*C. sorokiniana*
T1 (µg GAE/mg extract)	0.10 ± 0.01A	0.12 ± 0.01AB	0.15 ± 0.01AB	0.16 ± 0.01B	0.32 ± 0.03C	1.27 ± 0.03D	0.90 ± 0.03E
T1 (µg GAE/g sample)	27.6 ± 1.58A	43.5 ± 1.30B	47.4 ± 1.69B	55.1 ± 4.15C	26.6 ± 2.55A	113.1 ± 1.77D	139.0 ± 4.1E
T2 (µg GAE/mg extract)	0.24 ± 0.01AB	0.29 ± 0.01AC	0.28 ± 0.02A	0.32 ± 0.07AC	0.15 ± 0.01B	0.53 ± 0.01C	1.65 ± 0.04D
T2 (µg GAE/g sample)	85.9 ± 2.36AD	106.9 ± 5.13AB	92.7 ± 15.4AB	86.4 ± 31.5B	25.8 ± 2.13C	70.5 ± 1.24D	234.3 ± 5.70E
T3 (µg GAE/mg extract)	1.01 ± 0.01A	2.85 ± 0.07B	0.99 ± 0.01A	1.01 ± 0.01A	1.68 ± 0.09C	4.92 ± 0.27D	1.00 ± 0.01A
T3 (µg GAE/g sample)	877.4 ± 12.0A	2409.1 ± 57.6B	545.4 ± 7.15C	532.2 ± 7.31C	992.1 ± 54.8D	1815.5 ± 98.1E	508.3 ± 4.45C
T4 (µg GAE/mg extract)	2.49 ± 0.02A	7.89 ± 0.21B	2.47 ± 0.05A	2.52 ± 0.03A	9.86 ± 0.20C	11.7 ± 0.14D	2.54 ± 0.05A
T4 (µg GAE/g sample)	2710.7 ± 27.0A	6847.9 ± 178.3B	2507.0 ± 53.5C	2377.8 ± 28.6CE	6928.4 ± 138.4B	9221.3 ± 111.2D	2288.7 ± 42.1E

Results are means ± SEM (n = 6). GAE, gallic acid equivalents, T1, cold ethanol extraction, T2, ethanol extraction with lysis beads, T3, aqueous extraction at basic pH with lysis beads, T4, aqueous extraction at basic pH with lysis beads followed by protein hydrolysis using recombinant proteases. A,B,C,D,E, means within the same row with different letters are significantly different ($P < 0.05$).

Tables 4 and 6. Results followed a similar trend to that observed for the total polyphenol content. Aqueous extracts, especially protein hydrolyzates, showed greater antioxidant capacity compared to ethanol extracts. Among the different extracts, there was also a clear species effect that was reflected in the highest value of antioxidant capacity for *C. reinhardtii*, *C. sorokiniana*, and *Picochlorum spp*, followed by *D. salina*. Among the ethanol extracts, *Picochlorum spp* showed the greatest activity followed by *C. reinhardtii* and *C. sorokiniana*.

The influence of different genera and extraction methodologies on antioxidant capacity measured by the ABTS assay is presented in Tables 5 and 6. Activity was again significantly higher in aqueous *vs* ethanol-extracted samples. In the later ones, a significant enhancing effect was achieved by the lysis beads forced extraction procedure. Regarding the aqueous extraction process, a significant improvement in antioxidant capacity was derived from the protein hydrolysis process (2 to 7-fold increments *vs* undigested protein extracts). Upon expressing

Table 6

Influence of species difference and extracting process on extraction yield and antioxidant capacity of haloarchaea and microalgae. ANOVA of species, treatment, and species × treatment interactions on extraction yield and antioxidant capacity of functional extracts from haloarchaea and microalgae.

	CV (%)	R^2	*Species Effect*	*Treatment Effect*	*Species × Treatment*
Extraction yield (g/100 g)	5.92	0.9911	$P < 0.0001$ (30.2 %)	$P < 0.0001$ (60.0 %)	$P < 0.0001$ (9.8 %)
Total-Polyphenol (µg GAE/mg extract)	3.96	0.9976	$P < 0.0001$ (33.5 %)	$P < 0.0001$ (55.5 %)	$P < 0.0001$ (11 %)
Total-Polyphenol (µg GAE/g sample)	2.55	0.9995	$P < 0.0001$ (2.7 %)	$P < 0.0001$ (93.9 %)	$P < 0.0001$ (3.4 %)
Reducing capacity (µg GAE/mg extract)	4.18	0.9984	$P < 0.0001$ (54.3 %)	$P < 0.0001$ (13.8 %)	$P < 0.0001$ (31.9 %)
Reducing capacity (µg GAE/g sample)	2.79	0.9994	$P < 0.0001$ (18.8 %)	$P < 0.0001$ (62.6 %)	$P < 0.0001$ (18.6 %)
ABTS (µg GAE/mg extract)	7.09	0.9981	$P < 0.0001$ (18.5 %)	$P < 0.0001$ (53.5 %)	$P < 0.0001$ (28.0 %)
ABTS (µg GAE/g sample)	6.62	0.9988	$P < 0.0001$ (10.6 %)	$P < 0.0001$ (66.5 %)	$P < 0.0001$ (22.9 %)
LPIC (UAA/mg extract)	17.4	0.9615	$P < 0.0001$ (92.0 %)	$P = 0.1974$	$P < 0.0001$ (7.9 %)
LPIC (UAA/g sample)	19.8	0.8595	$P < 0.0001$ (50.8 %)	$P < 0.0001$ (22.2 %)	$P < 0.0001$ (27.0 %)

The level of significance was set at $p < 0.05$. Data in parentheses represent the contribution to the total variance of the specific ANOVA component. GAE, gallic acid equivalents, LIPC, lipid peroxidation inhibition capacity.

Table 7A

In vitro dialyzability of protein from haloarchaea and microalgae after digestion simulating conditions present in the gastrointestinal tract.

	30′	**60′**	**90′**	**120′**	**150′**
H. salinarum	247.9 ± 6.37A	233.2 ± 4.36A	545.5 ± 7.15A	295.4 ± 5.76A	365.9 ± 7.29A
D. salina	327.2 ± 1.73B	904.8 ± 8.64B	1430.1 ± 22.7B	239.8 ± 2.17B	321.8 ± 8.32B
C. reinhardtii	319.3 ± 8.01B	1019.3 ± 10.4C	317.4 ± 26.2C	283.9 ± 16.3A	276.3 ± 4.73C
C. sorokiniana	280.0 ± 13.9C	319.7 ± 31.1D	315.7 ± 8.69C	296.6 ± 7.85A	278.2 ± 7.70C

Results are means ± SEM (n = 3) expressed as µg protein/mg dialyzate. A,B,C, means within the same column with different letters are significantly different (*P < 0.05*).

Table 7B

In vitro dialyzability of total polyphenol content from haloarchaea and microalgae after digestion simulating conditions present in the gastrointestinal tract.

	30′	**60′**	**90′**	**120′**	**150′**
H. salinarum	6.80 ± 0.33A	11.9 ± 0.06A	9.18 ± 0.10A	20.5 ± 0.10A	23.0 ± 0.08A
D. salina	5.70 ± 0.07B	24.7 ± 0.44B	52.3 ± 0.87B	8.03 ± 0.28B	13.3 ± 0.21B
C. reinhardtii	14.2 ± 0.09C	40.7 ± 0.10C	14.0 ± 0.08A	38.0 ± 0.14C	28.6 ± 0.02C
C. sorokiniana	9.39 ± 0.19D	16.3 ± 0.21D	13.0 ± 0.05A	11.3 ± 0.36D	12.0 ± 0.39D

Results are means ± SEM (n = 3) expressed as µg gallic acid equivalents (GAE)/mg dialyzate. A,B,C,D, means within the same column with different letters are significantly different (*P < 0.05*).

Table 7C

In vitro dialyzability of reducing capacity from haloarchaea and microalgae after digestion simulating conditions present in the gastrointestinal tract.

	30′	**60′**	**90′**	**120′**	**150′**
H. salinarum	0.53 ± 0.02A	0.60 ± 0.01A	0.54 ± 0.02A	0.61 ± 0.03A	0.47 ± 0.01A
D. salina	0.74 ± 0.02B	3.62 ± 0.34B	5.30 ± 0.09B	1.36 ± 0.01B	1.56 ± 0.02B
C. reinhardtii	0.52 ± 0.03A	2.76 ± 0.02C	1.38 ± 0.03C	1.83 ± 0.04C	1.47 ± 0.02B
C. sorokiniana	0.81 ± 0.03C	1.00 ± 0.15D	0.94 ± 0.07D	0.94 ± 0.04D	0.65 ± 0.05C

Results are means ± SEM (n = 3) expressed as µg gallic acid equivalents (GAE)/mg dialyzate. A,B,C,D, means within the same column with different letters are significantly different (*P < 0.05*).

the activity per gram of sample, the highest results for ethanol extracts were exhibited by *C. sorokiniana*, followed by *H. hispanica*. Among aqueous extracts, the highest activity was exhibited by protein hydrolyzates from *C. reinhardtii*, followed by *Picochlorum spp* and *H. hispanica*. Overall, antioxidant capacity measured by ABTS methodology presented some distinctive features compared with the previously described methodologies. Specifically, the significant increment in activity derived from the protein hydrolysis process and the high activity exhibited by *H. hispanica* samples (both ethanol and aqueous extracts).

The effects of species and extraction process on lipid peroxidation inhibition capacity (LPIC) of haloarchaea and microalgae extracts are described in Supplementary Table S1. LPIC could only be measured in ethanolic extracts, whereas no activity was found in aqueous extracts. When the activity was expressed as UAA per gram of departing sample, the highest activity of cold ethanol extracts without lysis beads corresponded to *C. sorokiniana* and *C. reinhardtii* followed by *H. hispanica*. However, when cold extraction was combined with the action of lysis beads, the highest activity corresponded to *D. salina C13* followed by *C. reinhardtii* and *H. hispanica*. Using either type of extraction procedure, the activity of *D. salina C13* was always higher than that of C5.

The previously described results on individual differences among the different species studied can be summarized in Table 6, where the two-way ANOVA procedure shows the general effects of species and extraction treatment on the yield and antioxidant capacity of the extracts studied. There were significant effects of species and extraction treatment and a significant species × treatment interaction on the different parameters studied. Nevertheless, the highest contribution to total variance (and thus, the higher weight of the specific ANOVA component) corresponded in general to treatment, followed by species effect and species × treatment interaction. The extraction yield was significantly higher in haloarchaea *vs* microalgae, whereas the extraction process with aqueous solution rendered higher yields compared to that with hydroalcoholic solution. Among the latter, it is worth mentioning the enhancing action of forced extraction with lysis beads. Antioxidant capacity was also significantly affected by species. However, in contrast to what has been described for extraction yield, higher activity was found in microalgae compared to haloarchaea. The extraction process also affected the antioxidant activity with higher values found in aqueous *vs* ethanol extracts. Concerning protein hydrolysis in aqueous extracts, it was significantly effective at improving ABTS antioxidant capacity, whereas its effect on other antioxidant analytical techniques varied. Nevertheless, due to its enhancing effect on extraction yield, the total antioxidant capacity expressed per gram of departing material was always higher than the results obtained with aqueous protein concentrate extracts.

3.3. *Bioaccessibility of nutrients and antioxidant capacity*

The potential bioaccessibility of nutrients and antioxidant capacity from the different haloarchaea and microalgae studied was assessed using an *in vitro* continuous dialyzability technique. Considering the nutrient composition, extraction yield, and antioxidant activity data obtained during the previous proximal composition and functional capacity screening stage, four of the seven species studied (*H. salinarum*, *D. salina C5*, *C. reinhardtii*, and *C. sorokiniana*) were selected for the *in vitro* digestion process. The effect of different genera on *in vitro* digestibility of dry matter, protein, and antioxidant capacity is presented in Fig. 2 and Tables 7A-C. Dry matter digestibility was highest in *H. salinarum* followed by *C. sorokiniana* and *D. salina*, whereas the lowest results were obtained for *C. reinhardtii* (Fig. 2). Regarding the amount of dialyzable protein expressed per mg of dialyzate (Table 7A), the highest content corresponded to *D. salina* followed by *C. reinhardtii* and *H. salinarum*. Specifically, *D. salina* peaked protein accessibility through the 10,000 MWCO membrane at 60 and 90 min of intestinal digestion, whereas *C. reinhardtii* did it at 60 min and *H. salinarum* at 90 min. Digestibility of total polyphenol content (TPC) and reducing capacity expressed as μg GAE/mg dialyzate (Tables 7B-C) was highest in *C. reinhardtii* and *D. salina*, followed by *C. sorokiniana*, and *H. salinarum*. Interestingly, digestibility of antioxidant capacity followed a similar pattern to that observed for protein accessibility in *C. reinhardtii* and *D. salina*, with peaks of antioxidant activity at 60–90 min of the intestinal digestion process, whereas for *C. sorokiniana* and *H. salinarum,* it was more evenly distributed along the five time-points of the intestinal digestion period.

4. Discussion

The sea is an extraordinarily promising source of new foods and functional ingredients rich in essential nutrients and bioactive compounds. To explore the nutritional and functional potential of newly developed foods and protein sources, a complete nutrient and bioactive profile analysis is needed that gives information regarding their potential health benefits achieved through antioxidant activity, modulation of gene expression, improvement of glucose and lipid metabolism or anti-inflammatory effects (Tejero Pérez et al., 2023). Furthermore, proximate composition and functional capacity may be significantly modified because of the different technological processes applied to a particular foodstuff. Likewise, the response of different foodstuffs to a digestion process in terms of nutrient or bioactive component release has been shown to differ greatly based on the structure of the food matrix or processing conditions. For that reason, *in vitro* or *in vivo* digestibility approaches can offer useful information on whether nutrients or bioactive compounds chemically present in a specific meal will result in adequate release and absorption of these components during the digestion process. In this study, all the included species were characterized by high protein content and by marked differences in total mineral content and fatty acid profile among microalgae and haloarchaea. In general, forced aqueous extraction led to considerably greater extraction yields and antioxidant capacity compared to ethanol extraction, while the later activity was markedly higher in microalgae compared to haloarchaea species. Specifically, *Chlamydomonas reinhardtii*, *Dunaliella salina,* and *Chlorella sorokiniana* gave rise to the functional extracts with the greatest antioxidant potential, whereas the highest bioaccessibility of protein and antioxidant capacity among the different species tested in this study was obtained from *Dunaliella salina*.

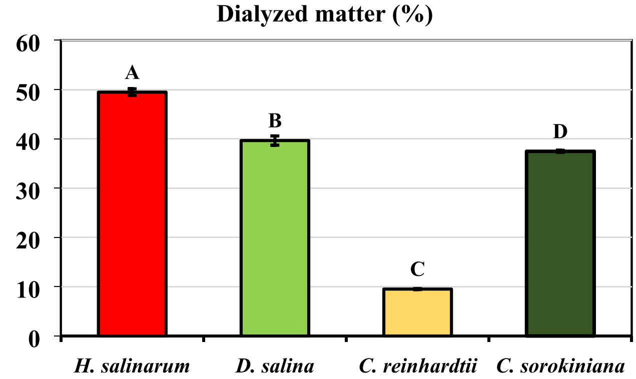

Fig. 2. *In vitro* dialyzability of dry matter from haloarchaea and microalgae after digestion simulating conditions present in the gastrointestinal tract. Results (expressed as % of dialyzed matter along the entire intestinal digestion process) are means (n = 3) ± standard error of the mean depicted by vertical bars. A,B,C,D, means with different letters are significantly different (ANOVA treatment, $P < 0.05$).

4.1. *Proximal composition*

Microalgae have been reported to provide a wide variety of essential nutrients like protein, minerals, vitamins, and lipids. Specifically, they are rich sources of n-3 PUFAs with great benefits for human health (Doughman et al., 2007). Our results agree with the published scientific literature regarding their protein content which is considerably higher than other plant-based protein-rich foods like cereals and legumes (Cáceres et al., 2014; Porres et al., 2007; Kapravelou et al., 2020). Furthermore, microalgae protein is considered of good quality, providing acceptable levels of most essential amino acids to meet the nutrient requirements except for threonine and sulfur-containing amino acids (Morançais et al., 2018; Wild et al., 2018). However, the biological value of microalgal proteins is usually lower compared to other protein-rich foodstuffs like casein or egg due to the lower digestibility of the former derived from the presence of a rigid and non-permeable thick wall (Wild et al., 2018; Martinez et al., 2022). Under our experimental conditions, protein bioaccessibility measured by an *in vitro* continuous dialyzability method showed that the haloarchaea and microalgae with the greatest digestibility matched those of species with a weakest cell wall structure like *Dunaliella salina* or *Halobacterium salinarum*, which also exhibited the greatest yields of functional extracts production. The total fat content of the genera studied in our study was lower than the values reported by Yao et al. (2015) for *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Schizochytrium limacinum*, *Nannochloropsis* or *Scenedesmus* sp. However, the lipid content of microalgae depends not only on species or family, but also on light exposure, salinity, culture conditions, or nutrient composition of the culture media. Their fatty acid profile is characterized by high levels of polyunsaturated fatty acids (PUFAs), especially n-3 and n-6, although they also showed considerable amounts of saturated and monounsaturated fatty acids, mainly palmitic and oleic. Polyunsaturated fatty acids, especially omega-3 like alpha-linolenic acid, and its derivatives EPA or DHA are of great interest due to their multiple health benefits. Thus, dietary sources of these fatty acids are constantly been sought. In a previous study, we demonstrated the potential of the microalga *Nannochloropsis gaditana* to provide a significant amount of bioaccessible omega-3 fatty acids that can be incorporated into the liver or erythrocytes after mid-term consumption of the microalgae by rats (Martinez et al., 2022). Regarding the lipid content and profile of haloarchaea in the present study, it is worth mentioning the high percentage of oleic acid that differs from data reported to other halophilic bacteria like *Halomonas almeriensis* or from the acetonic extract of *Haloarcula sp* that showed preferentially C16:0, C18:0 or C18:2n6 (Martínez Checa et al., 2005, Ávila-Román et al., 2023).

Ash and total-P content of the different microalgae studied were within the range of values reported in the literature (Mimouni et al., 2018), with considerably greater total mineral content in marine *vs* freshwater species. Haloarchaea and microalgae that exhibited the highest ash composition in the present study are known to thrive in high-salinity media, where mineral accumulation is part of their survival strategy. Regarding the mineral content of Haloarchaea, which showed the highest values of all studied species under our experimental conditions, Engel and Catchpole (2005) examined the cell of *H. salinarum* in a scanning electron microscope equipped with an X-ray spectrometer and reported that the intracellular concentration of K was 110 times over than of the medium, whereas it turned to 0.3 for Na and to 1.1 for Cl, thus suggesting a clear intracellular accumulation of K. In contrast, phosphorus accumulation was not observed since no major differences were found among all the species studied. Phosphorus can be distributed among phospholipids, proteins, nucleic acids, or storage compounds like phytic acid, with widely reported antinutrient or beneficial functions (Urbano et al., 2000), although the quantities of the latter in microalgae have been described to be lower than those of other plant foodstuffs like legumes (Wild et al., 2018; Porres et al., 2003; 2007). On the other hand, microalgae are promising dietary sources of essential minerals (Fox and Zimba, 2018) that can be incorporated into different foodstuffs like cookies in which they allow a greater accessibility of P, K, Ca, Mg, Fe, Zn, and Se (Uribe-Wandurraga et al., 2020), or breadsticks to increase their content of Fe and Se, improving color and texture stability of the final product (Uribe-Wandurraga et al., 2019).

Little is known about the nutritive value of haloarchaea, although this group of microorganisms and their related bio-products have been reported to exhibit health-related beneficial effects on human health such as their antioxidant capacity (Ávila-Román et al., 2023), antiviral, antihemolytic, anticancer, antiglycemic, and antilipidemic activities (Hou & Cui, 2018; Hegazy et al., 2020; Giani et al., 2022), or interesting functional properties (Martínez Checa et al., 2022) for their use in industrial applications (Moopantakath et al., 2023). *In vitro* bioaccessibility of total dry matter from *H. salinarum* was the highest among all the samples studied, a finding that points out to the good availability of nutrients and bioactive compounds from orally administered biomass or functional extracts of haloarchaea. Furthermore, the use of haloarchaea in agriculture has been recommended to improve the yield, nutritional composition, and available antioxidant capacity of leguminous *Medicago sativa* in saline soils acting in synergy with *Ensipher meliloti* (Martinez et al., 2015), thus showing clear environmental benefits.

4.2. Extraction yield and antioxidant capacity

Regarding the preparation and characterization of functional extracts from the different haloarchaea and microalgae tested, two factors have been analyzed in the ANOVA design implemented for this study, species and extraction treatment, which showed a good fit expressed as the correlation coefficient. Both factors had a significant effect on the different parameters studied, although the weight of each factor varied among the different antioxidant activity methodologies assayed. The extraction yield is not only related to the amount of functional extract that can be obtained from a specific material but also to the potential intestinal digestibility of nutrients and bioactive compounds. Three of the genera studied (*Haloarcula*, *Halobacterium*, and *Dunaliella*) have shown remarkable extraction yields matching the absence of cell wall or a more permeable cell wall structure made of protein layers outside the cell membrane that can disintegrate at low NaCl concentrations (Polle et al., 2020; Blaurock et al., 1976; Shiu et al., 2013). Specifically, Archaea possess cell walls that lack peptidoglycan in contrast to bacteria, whereas other different cell envelope components have been described. Among them stands a paracrystalline protein surface layer commonly mentioned as S-layer (Rodrigues-Oliveira et al., 2017). S-layers are composed of only one or two proteins and form different lattice structures. They are known to serve as protective coats, molecular sieves, molecule and ion traps, as well as playing important roles in surface recognition and cell shape maintenance. When compared to the thicker and non-permeable structure found in microalgae like *Nannochloropsis*, *Chlorella*, or *Chlamidomonas spp*, such structural differences contributed to a more efficient passage of nutrients and bioactive compounds from *Dunaliellla* and Haloarchaea to the extraction solution under our experimental conditions. This in turn prevented the implementation of harsh cell disruption treatments to ensure more efficient extraction of antioxidants, lipids and pigments (Martínez et al., 2022; Lee et al., 2017).

The extraction processes assayed have been selected based on the bioactive compounds specifically targeted. Whereas ethanol extraction was implemented aiming for polyphenolic and other ethanol-soluble components, aqueous extraction at basic pH has been carried out aimed to obtain preferentially protein and water-soluble compounds present in a bio-functional protein hydrolyzate with improved nutrient digestibility. Both extraction processes were combined with a forced physical treatment using lysis beads and ultrasound. Generally, the extraction process factor accounted for 60 % of the total variance in the ANOVA model, with aqueous extraction showing significantly greater yields compared to ethanol extraction. Extraction yields showed the same trend among the different species analyzed using either ethanol or aqueous extraction process, thus pointing out the fundamental aspect of cell wall structure. The contribution of species × treatment interaction to the total variance was minor compared to that of the species or treatment.

In the present study, the bio-functionality of the different species and extracts tested has been analyzed primarily by their antioxidant capacity due to its significant contribution to many of their potential beneficial actions together with their anti-inflammatory action or their glucose or lipid-lowering activities. To fully characterize the antioxidant activity of a functional extract, it is recommended that different tests are implemented to cover the distinctive mechanisms involved. We have implemented four assays based on the transfer of one electron like the ferric reducing antioxidant power or the Folin-Ciocalteau test, the transfer of both an electron and a hydrogen atom like the ABTS, and the assessment of quenching ability of a hydroxyl radical generated by the Fenton reaction to initiate a process of lipid peroxidation. With the exception of the total polyphenol content assayed by the Folin-Ciocalteau test, all the rest can be considered similar to other ROS-generating reactions that usually take place in the human body.

By and large, extraction methodology had a greater influence on antioxidant activity compared to the species effect, which further increased when the antioxidant data was normalized considering the extraction yield. The greater antioxidant capacity showed by the ethanol extracts when expressed per milligram of extract is due to the specific polyphenolic nature of their bioactive components, whereas upon expressing the activity per milligram of biomass, the higher extraction yields attained by the aqueous extraction procedure ended up in greater overall activity count. A specific differential effect could be observed in the ABTS assay that exhibited significant increments in antioxidant activity resulting from the protein hydrolysis process *vs* undigested aqueous protein extracts. Therefore, under our experimental conditions, the antioxidant activity was associated with a greater number of peptides or free amino acids released by the protein digestion process. Dryáková et al. (2010) reported a significant increase in the antioxidant capacity of whey protein hydrolyzates *vs* undigested protein and pointed out specifically to protease from *Bacillus licheniformis* leading to greater increments compared to other frequently used proteases. They also showed a greater correlation between the degree of protein hydrolysis using the above-mentioned protease and antioxidant activity, suggesting that greater antioxidant capacity was associated with smaller peptide chain length (<16 amino acids). Considering the above mentioned facts, the specific protease to accomplish protein hydrolysis as well as the amino acid sequence and structure of the target proteins are of outmost importance for the optimal functionality of the final product. In our study, we have used a broad specificity endoprotease from *Bacillus licheniformis* that shows ability to hydrolyze most peptide bonds, although it exhibited clear preference for sites containing hydrophobic residues either in P_2' or P_3' positions, particularly when Glu was in the P_1 position of casein (Adamson and Reynolds, 1996), and high specificity for aromatic, acidic, sulphur-containing, aliphatic, hydroxyl, and basic residues in P_1 position of whey protein (Doucet et al., 2003). We have also used an enzyme preparation (*Aspergillus oryzae*) consisting of a mixture of endoprotease and exopeptidase activities (Merz et al., 2015).

The sequential activity of this two protease preparations have ensured the generation of low molecular weight peptides with more efficient intestinal absorption and higher antioxidant capacity.

In addition, antioxidant activity would be inherent to specific amino acid sequences. Yan et al. (2015) have reported that peptides from rice residue protein containing amino acid residues like Asp, Pro, Trp, Tyr, Met, Cys, Leu, Arg, Ala, and His show higher antioxidant activity. Furthermore, they drew attention to the important role played by the molecular weight of peptides, with optimal activities within MW of 500–1500 Da, and the fact that no major differences in antioxidant capacity were apparent among the different proteases used in their study (alcalase, flavourzyme, protamex, pepsin, trypsin), with the exception of papain, and the combination of papain, protamex, and flavourzyme that led to greater antioxidant capacity. Likewise, Ambigaipalan et al. (2015) have hypothesized that protease activity may release some active amino acids like Cys, Trp, Tyr, and His during protein hydrolysis which would be responsible for a greater antioxidant capacity of the protein hydrolyzate.

4.3. Bioaccessibility of nutrients and antioxidant capacity

The total antioxidant activity present in a sample biomass or extract is an interesting parameter to characterize its bio-functional activity and potential health benefits. However, functional foods or ingredients must undergo a digestion process along the G.I. tract, where nutrients and bioactive compounds are released from the food matrix and cross the brush border membrane via paracellular or transcellular absorption into the bloodstream where they travel to the different organs and tissues to exert their beneficial effects. Furthermore, the effect of some bioactive compounds can be potentiated in response to their release during intestinal digestion. On the other hand, along the digestion process, many of the above-mentioned compounds can be inactivated and lose their antioxidant capacity or else remain unreleased from the food matrix and end up in the large intestine. Unabsorbed antioxidant capacity may be important at gut level since it can exert effective protection against oxidative stress conditions that are able to cause inflammation, dysregulation of cell cycle, or dysbiosis of intestinal microbiota, thus inducing significant benefits on colonic physiology (Martinez et al., 2023). Therefore, a study of the potential bioaccessibility appears to be of significant relevance and meaningful for this study, since it offers first-hand information about the real capability of the functional foodstuffs to render their full biological potential. In our study, *H. salinarum*, *D. salina C5*, *C. reinhardtii,* and *C. sorokiniana* were selected for *in vitro* digestion based on their nutrient composition (all are good sources of essential nutrients), extraction yield, and antioxidant activity. The species assayed exhibited a wide range of values within the different methodologies implemented to assess their antioxidant capacity combined with high (*Halobacterium*, *Dunaliella*) or low permeability (*Chlamydomonas*, *Chlorella*) of their cell wall structure. Although the four species tested exhibited a significantly greater amount of potentially accessible antioxidant capacity *vs* blank control, the combination of intermediate antioxidant capacity and permeable cell wall structure found in *D. salina C5* resulted in the highest content of activity per milligram of dialyzable matter at 60–90 min of intestinal digestion. Other samples with greater antioxidant capacity but lower permeability of their cell wall complex (*Chlamydomonas*, *Chlorella*), did not reach the amount of accessible antioxidant activity shown by *Dunaliella*, although *Chlamydomonas* was superior to *Chlorella* under our experimental conditions. In previous studies with *Nannochloropsis gaditana*, a microalga well-known for its rigid cell wall complex, a technological treatment of heat and pressure implemented to weaken the above-mentioned structure, led to a significant increase in extraction yield and *in vitro* availability of antioxidant capacity, as well as to enhanced *in vivo* protein digestibility and significant changes in fecal structure, with a markedly lower proportion of intact microalgae present (Martinez et al., 2022). Likewise, Wild et al. (2018) reported a higher *in vitro* protein digestibility (IVPD) of cell-disrupted *vs* undisrupted *Arthrospira*, *Chlorella*, *Nannochloropsis*, and *Phaeodactylum* using a stirred ball mill for cell wall disruption.

5. Conclusions

The different haloarchaea and microalgae studied exhibited interesting nutritional and bio-functional potential to become novel food components in the human diet. Due to its notable nutrient composition and efficient digestibility, *D. salina* appears the most eligible candidate, among those tested in the present study, to be included in the usual diet as a source of highly available protein, polyunsaturated fatty acids, and antioxidant compounds. *C. reinhardtii* and *C. sorokiniana* provide high levels of nutrients and antioxidants but their limited digestibility should be overcome through technological processing in a similar way to *Picochlorum spp*. The two haloarchaea tested are good potential sources of protein, minerals, and antioxidants with a high intestinal availability, showing great interest in the design of novel functional extracts for the treatment of different pathologies.

5.1. Strengths and limitations

Our results concerning the proximal composition, bio-functional value and potential nutrient and antioxidant accessibility from different microalgae and haloarchaea of the Andalusian Atlantic shoreline are of great relevance since they state very clear concepts concerning the nutritional and health-related benefits of consuming the former species or their functional extracts, making emphasis on the bioaccessible components provided. The study has certain limitations since we cannot assure that all bioactive components present in the different microalgae or haloarchaea studied have been available, or the extent of their availability, although the samples analyzed have clearly shown a greater *in vitro* accessibility *vs* blank control. Furthermore, the potential health-benefits of the species tested in the present experiment as well as the results obtained from the *in vitro* digestibility should be confirmed by an *in vivo* preclinical study using experimental animals before advancing to a clinical study.

CRediT authorship contribution statement

Rosario Martínez: Writing – original draft, Methodology, Investigation, Formal analysis. **Alejandro García Beltrán:** Methodology, Investigation, Formal analysis. **Garyfallia Kapravelou:** Writing – original draft, Methodology, Investigation, Formal analysis. **Ana Guzmán:** Methodology, Investigation, Formal analysis. **Aída Lozano:** Methodology, Investigation, Formal analysis. **Patricia Gómez-Villegas:** Writing – review & editing, Investigation. **Rosa León:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Javier Vigara:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Milagros Galisteo:** Writing – review & editing, Software, Data curation. **Pilar Aranda:** Supervision, Resources. **María López-Jurado:** Supervision, Resources. **José Prados:** Resources, Funding acquisition, Conceptualization. **Consolación Melguizo:** Resources, Funding acquisition, Conceptualization. **Jesus M. Porres:** Writing – original draft, Supervision, Software, Resources, Funding acquisition, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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