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Functional characterisation of *Euglena gracilis* following growth medium enrichment

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ABSTRACT

In recent years, microalgae, particularly *Euglena gracilis*, have been candidates for the food/feed industry thanks to their nutritional and functional properties. However, the inclusion of *E. gracilis* in the diet of livestock animals is little studied, especially regarding its antioxidant activity. Furthermore, microalgae are known for their variability in nutritional quality and functional properties, mainly due to cultivation conditions. For this reason, the aim of the present work was to investigate the nutritional and functional aspects (total phenolic content TPC, Folin-Ciocalteu assay); antioxidant activity (ABTS and FRAP assays) of *E. gracilis*, grown in three different media, following chemical extraction (H_2O:EtOH) and *ex vivo* digestion method. The microalgae growth media were characterised as follows: EgM (standard medium); ETX (standard medium + aminoacidic-extract); DOE-ETX (ETX + microelements). The results showed an interesting nutritional profile for all the microalgae analysed, although the values were modulated by media nutrients. Although EgM (6.94 ± 0.25 g/L) was characterised by a significant higher growth yield than ETX (5.75 ± 0.14 g/L) and DOE-ETX (4.72 ± 0.17 g/L), results also confirmed by the paramylon content (4.35 ± 0.13; 3.16 ± 0.08; 2.25 ± 0.05 g/L, respectively) ($p < 0.05$), it did not show a high functional profile. More specifically, DOE-ETX showed higher values for TPC, ABTS, and FRAP, following chemical extraction, in particular 50 and 75% EtOH and *ex vivo* digestion. These results confirmed the potential of *E. gracilis* as a valuable source of functional feed ingredients. Further investigations will be crucial to optimise the formulation of the culture medium to obtain a high yield of algae with improved functional characteristics.

HIGHLIGHTS

- Constant population growth has prompted scientific research to investigate new protein alternatives. *Euglena gracilis* represents a valid candidate.
- The type of growth medium influences the nutritional and functional profile (total phenolic content and antioxidant activity) of *E. gracilis*.
- The addition of microelements to the *E. gracilis* medium resulted in a higher phenolic content and more marked antioxidant activity.

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Introduction

In recent years, microalgae and cyanobacteria, part of the broader group of algae, thanks to the wide range of bioactive compounds with functional properties they possess, have emerged as attractive candidates for research applications and industrial purposes, such as pharmaceutical, cosmetic, industrial, agricultural, and nutritional ones (Alam et al. 2020). In particular, food and feed research is investigating the role of microalgae as valid alternatives to protein sources, given the forecasted growing demand. More specifically, according to the United Nations report, the global population will reach ~8.5 billion by 2030, 9.7 billion by 2050, and will peak at 10.4 billion by 2100 (United Nations Department of Economic and Social Affairs PD 2022), a phenomenon that is expected to cause increasing difficulties in meeting demand for food by negatively impacting the environmental health (Röös et al. 2017). As the highest demand products will be of animal origin, mainly meat and dairy products, the

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increase in feed requirements for farm animals is significant (Bellet and Rushton 2019).

In addition to the most studied and commercialise algae, such as *Chlorella vulgaris* and *Spirulina platensis* (Frazzini et al. 2022), *Euglena gracilis* has drawn a great deal of interest thanks to its potential for several types of applications.

Euglena gracilis is an ubiquitous and unicellular photosynthetic eukaryotic flagellate of the Discoba supergroup, phylum Euglenozoa, often included in the eukaryotic microalgae's group, commonly found in freshwater habitats (Zoltner and Field 2022). It is characterised by the absence of cell walls, thus making the nutrients stored inside the cells easily available for absorption (Zeng et al. 2016). Due to this peculiar morphology, the green protozoan can grow under photoautotrophic (using direct sunlight), heterotrophic (using an external carbon source), or mixotrophic (using both kinds of sources) conditions (Blum and Buetow 1963; Šantek et al. 2010).

Furthermore, due to its nutritional and functional composition, *E. gracilis* represents a very promising source of food and feed. Specifically, Aemiro et al. (2019) studied the chemical composition of *E. gracilis*, reporting the presence of up to 30.9% dry matter (DM) of crude protein (CP) [containing all 20 amino acids (AAs)], 20% DM of lipid content [rich of polyunsaturated fatty acids (PUFAs)], 5.8% DM of ash and 0.1% of neutral detergent fibre (NDF). In parallel, several scientific studies have reported that *E. gracilis* is able to produce functional compounds, such as provitamin A (β-carotene), vitamin C (ascorbate), vitamin E (α-Tocopherol) and, in particular, paramylon. The latter is a high-molecular-weight polysaccharide (β-1,3-glucan), found exclusively in euglenoids and more specifically in *Euglena* species, whose role is to store energy and carbon (Gissibl et al. 2019). In particular, paramylon is characterised by immunomodulatory properties (Skov et al. 2012; Watanabe et al. 2017; Barsanti et al. 2022), finding its own market space in the nutraceutical sector, especially after Food and Drug Administration (FDA) released the certification as a food additive in 2017 (GRAS Notice No. 513, FDA 2014). For this reason, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) approved *E. gracilis* dried whole cells as a novel food (EFSA Panel on Nutrition NF and FA et al. 2020).

It has been widely reported that the composition of *E. gracilis* varies depending on several factors, mainly environmental (temperature, CO2, presence of stress-inducing elements, presence of light and aerobic or anaerobic conditions) (Constantopoulos and Bloch 1967; Wang et al. 2018). Furthermore, as stated in the literature, the nutritional profile of *E. gracilis* is easily modulated by acting directly on the composition of the culture medium, particularly on the sources of nitrogen and carbon, the main nutrients for this microalgae (Regnault et al. 1990; Ivušić and Šantek 2015; Schwarzhans et al. 2015). At the same time, modulation of culture conditions, as a strategy to increase the functional value of *E. gracilis*, is only described to optimise paramylon production, but without considering secondary compounds, such as phenols and antioxidant molecules.

Therefore, in light of the above, the aim of this study was to characterise not only the nutritional but also the functional profile of *E. gracilis* to assess its potential as an animal feed ingredient. Specifically, the total phenolic content (TPC) and antioxidant activity of *E. gracilis* were analysed by 2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays, after chemical extraction and *ex vivo* digestion (a faster digestion method), in standard medium and after supplementation with AAs extract and microelements.

Materials and methods

Materials

Axenic *E. gracilis* (UTEX 753) was purchased from the Culture Collection of Algae at the University of Texas at Austin (UTEX). Cells were maintained in defined organic medium (EgM) at a predetermined temperature (22 ± 2 °C) in unshaken flasks (500 mL) in the dark and subcultured weekly.

The three media tested, whose formulations were based on preliminary data for the modulation of growth rate and nutritional and functional profile, are listed below.

- EgM medium (1 L): 2 g of trypticasein soy broth, 3 g of yeast extract, 0.01 g of calcium chloride ($CaCl_2$), 20 g of glucose, 0.1 mg of vitamin B1, and 0.05 mg of vitamin B12.
- Aminoacidic-extract (ETX) medium (1 L): 5 g aminoacidic extract (characterised by the presence of all AAs), 0.01 g of $CaCl_2$, 20 g of glucose, 0.1 mg of vitamin B1, and 0.05 mg of vitamin B12.
- DOE-ETX medium (1 L): same constituent of ETX, with the addition of 0.3 g of magnesium sulphate ($MgSO_4$), 1.5 g of potassium-dihydrogen-phosphate (KH_2PO_4), 0.05 g of tetrasodium-ethylenediaminetetraacetic acid (Na_4EDTA), and 0.45 mg of microelement mix consisting of 10% of iron (Fe), 5% of

cupper (Cu), 5% of manganese (Mn), 1% of zinc (Zn), 0.5% of boron (B), and 0.5% of molybdenum (Mo).

All media were sterilised at 121 °C for 20 min and then cooled before inoculation. Vitamins and glucose were sterilised separately and added before inoculation to avoid degradation or unwanted reactions.

Methods

Growth yield and paramylon content

The growth period lasted 7 days. Every day for 1 week, 4 ml of each sample was taken under sterile conditions to monitor growth yield and paramylon content ($n = 3$). More specifically, the cell biomass was measured gravimetrically, whereby 2 ml of the sample was centrifuged, washed with distilled water (to remove salts and impurities), dried in an oven overnight, and finally measured with an analytical balance.

Paramylon was quantified following the method of Barsanti et al. (2001). Specifically, 2 mL of the sample were centrifuged and resuspended in a solution containing 1% (w/v) of Sodium Dodecyl Sulphate (SDS) and 5% (w/v) of disodium-EDTA (Na_2EDTA) and then incubated at 37 °C for 30 min. The resulting paramylon granules were recovered by centrifugation (10 min at 1000 g). After centrifugation, treatment with SDS-NA_2EDTA was repeated and the paramylon was washed ($n = 2$) with hot distilled water (70 °C). After the second washing, the granules were dried overnight at 60 °C for total weight determination.

Chemical analysis

The chemical analysis was performed following the *Official Methods of Analysis* according to AOAC (2005). In particular, DM was obtained by drying the samples in a forced-air oven at 65 °C for 24 h (AOAC method 942.05). Ashes were determined by placing the samples in a muffle at 550 °C for 3 h (AOAC method 942.05). CP content was evaluated by the Kjeldahl method (AOAC method 2001.11), while ethereal extract (EE) by ether extraction in Soxtec system (DM 21/12/1998). Finally, crude fibre (CF) was determined following AOCS (2009) (method Ba 6a-05).

Chemical extractions

Different solvent concentrations of water:ethanol (H_2O:EtOH) were prepared as follows: 0% EtOH; 25% EtOH; 50% EtOH; 75% EtOH; 100% EtOH. Subsequently, the samples were extracted for three times ($n = 3$), following the protocol of Brighenti et al. (2017) with minor adaptations. More precisely, (0.150 ± 0.05 g) of samples, previously ground (diameter of 1 mm, rotor mill Retsch Mod. zm 200, Hann, Germany), were combined with 5 ml of solvent (H_2O:EtOH) and incubated for 1 h, at room temperature (RT), in the dark and under shaking conditions. At the end of the incubation, the samples were centrifuged at 4000 rpm for 5 min. The supernatant was removed and stored at 4 °C, while the residual compound was used to repeat the extraction process two more times. Finally, the extracts obtained (15 ml for each sample) were stored at −20 °C until further analyses (TPC, ABTS, and FRAP).

Ex vivo digestion

Gastric and intestinal fluids were collected at the slaughterhouse from pigs ($n = 20$) aged 50–110 days. After collection, the fluids were centrifuged at 4000 x g for 10 min to remove undigested components (particulate fraction) and used to form gastric and intestinal pools to reduce variability. Gastric and intestinal fluids were frozen at −20 °C for up to 48 h before digestion. Subsequently, pH and enzyme activity were checked to match the gastric and intestinal phase and corrected if necessary. For the digestive process, 5 mL of gastric fluids were added to the previously ground and weighed samples (0.500 ± 0.05 g) and incubated at 39 °C, for 2 h, under agitation. At the end of the first incubation, 5 mL of intestinal fluids were added to the samples and incubated under the same conditions. Different aliquots were taken at each digestive phase [beginning of gastric phase (0 h), end of gastric phase (2 h), end of intestinal phase (4 h)], frozen at −20 °C and used to monitor TPC and antioxidant activities (ABTS and FRAP) trends during *ex vivo* digestion. The digestive process was replicated three times by taking two technical replicates for each digestion phase.

Total phenolic content

For TPC, the protocol of Attard (2013) was used with minor adaptations as reported by Lanzoni et al. (2023). Values were expressed in terms of tannic acid equivalent (mg TAE/100 g of dried algal material).

ABTS assay

The antioxidant activity was assessed using the ABTS method according to the protocol of Re et al. (1999) with minor adaptation as reported by Lanzoni et al. (2023). Values were expressed in terms of Trolox equivalent (mg TE/100 g of dried algal material).

FRAP assay

The FRAP assay was performed following the protocol of Abdelaleem and Elbassiony (2021), with minor modifications (Lanzoni et al. 2023), with the only exception that ferrous sulphate ($FeSO_4$) was used as the standard for monitoring antioxidant activity. Values were expressed as mg $FeSO_4$/100 g of dried algal material.

Statistical analysis

The chemical analysis, growth yield, paramylon content, and TPC, ABTS, and FRAP data of the chemical extracts were analysed by one-way Anova followed by Tukey's multiple comparison test, while TPC, ABTS, and FRAP data of *ex vivo* digestion were analysed by two-way Anova (*Time x Treatment*) followed by Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1 (GraphPad Software Inc., San Diego, CA, USA).

All data are expressed as means ± standard error of the mean (SEM) of at least three independent experiments (biological replicates). Values are considered statistically significant for a 95% confidence interval (p-value = 0.05).

Results and discussion

Growth yield and paramylon content

In Figure 1, the growth yield and paramylon content values, measured after one week (7 days), are shown.

As observed in Figure 1, EgM showed a higher growth yield (6.94 ± 0.25 g/L), with statistically significant differences ($p < 0.05$) compared to ETX (5.75 ± 0.14 g/L), and DOE-ETX (4.72 ± 0.17 g/L). The same trend was observed for paramylon content, with statistically significant differences ($p < 0.05$) between EgM (4.35 ± 0.13 g/L), ETX (3.16 ± 0.08 g/L), and DOE-ETX (2.25 ± 0.05 g/L).

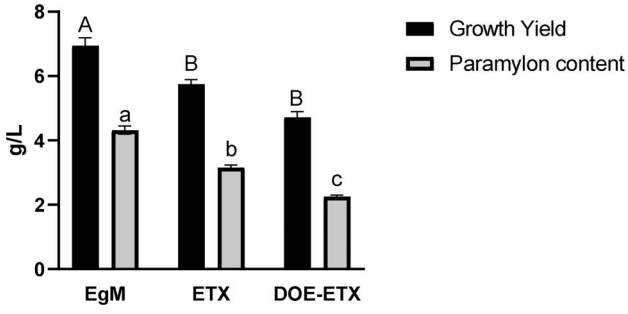

Figure 1. Growth yield and paramylon content in EgM, ETX, and DOET-ETX after 1 week (7 days). Values are expressed in g/L. Capital letters indicate statistically significant differences in mass growth, small letters in paramylon content ($p < 0.05$).

As reported in the literature, the growth rates of *E. gracilis* are strongly influenced by the components in the culture medium (Schwarzhans et al. 2015). Optimisation of medium composition is highly important in any bioprocess. It must be aimed at reducing time and cost, thereby increasing the recovery of the desired product. In particular, the media to be optimised must support the growth of targeted cells or the production of specific metabolites, while maintaining cell viability (Ivušić and Šantek 2015). It is known that to proliferate, *E. gracilis* requires the supply of vitamins, such as B1 and B12, a source of carbon and nitrogen (Oda et al. 1982; Ivušić and Šantek 2015). Vitamins B1 and B12, present in all the media conditions studied, play a key role in growth yield. The former, also known as thiamine, although not universally required among *Euglena* species, is essential in *E. gracilis* for its growth. More specifically, as reported by Cook (1968), the addition of at least 30 nM of vitamin B1 is required to support maximum growth. In parallel, *E. gracilis* shows a high requirement for vitamin B12. As demonstrated by Watanabe et al. (2017), *E. gracilis* requires at least 22.000 molecules of vitamin B12 per cell to ensure normal growth. In particular, *Euglena* has the ability to take up and accumulate this vitamin in an exogenous form due to the presence of numerous non-enzymatic proteins in the cytosolic, mitochondrial, chloroplast, and microsomal fractions that can bind and internalise it (Watanabe et al. 2017). The carbon source in the present study is provided by the addition of glucose in all media (20 g/L). As reported by Ivušić and Šantek (2015), the addition of 20 g/L maximised growth and paramylon content in *E. gracilis*. As demonstrated by the authors, glucose as well as fructose are the most efficient carbon sources under heterotrophic culture conditions, as they are easily metabolisable by the cell.

The differences found in the growth yield of the samples under study are due, most probably, to the type of nitrogen source, which differed in the microalgae tested. In EgM, the nitrogen source is yeast extract (3 g/L), which is widely used in the literature (Bhattad et al. 2021). More specifically, Bhattad et al. (2021) demonstrated how the addition of yeast extract (5 g/L) stimulated the growth yield, reaching biomass values of 6.4 g/L (Bhattad et al. 2021), which are highly comparable with those obtained in the present study (6.9 g/L). The microalgae ETX and DOE-ETX, on the other hand, were grown in media enriched with an AAs extract (containing all AAs) (5 g/L). As reported in the literature by Oda et al. (1982), not all AAs are required for growth stimulation. More precisely, as

claimed by the authors, AAs, such as glutamine, asparagine, alanine, and serine are only good sources of nitrogen when glucose is present in the medium as a carbon source. Branched-chain AAs, glycine, threonine, and proline are only utilised by cells in the presence of light, while basic, aromatic, and sulphur-containing AAs are unable to stimulate the growth of *E. gracilis* (Oda et al. 1982). Specifically, in the study conducted by Oda et al. (1982), AAs, such as methionine, cysteine, threonine, leucine, and phenylalanine proved to be growth inhibitors, most likely due to their competition for cellular transport systems. So it is possible that all the AAs in the extract did not allow differences in cell growth and proliferation to be observed.

Finally, although microelements, such as iron, copper, manganese, zinc as well as KH_2PO_4 are known to promote growth in *E. gracilis* (Hilt et al. 1987; Marčenko 1972), no statistically significant difference in growth yield was observed between ETX and DOE-ETX, as shown in Figure 1. As reported in the literature, it is complex to identify the exact concentration of these compounds in the culture medium, due to their toxicity at a high inclusion level that would arrest their growth and affect cell morphology (Chen et al. 2019).

As previously reported *E. gracilis* is known to produce high amounts of paramylon, β-1,3-glucan, which is characterised by high functional and immunomodulatory properties (Bhattad et al. 2021). This ability is also typical of yeasts, such as *Saccharomyces cerevisiae*. But whereas the latter is capable of producing paramylon up to 15% of its dry weight, *E. gracilis* reaches the values of 20–75% (Bhattad et al. 2021). This was confirmed in our study, where EgM, ETX, and DOE-ETX produced 62.7, 54.9, and 47.7% of their final weight, respectively. These differences are in line with those found with the growth yield, again underlining the importance of the culture medium also in the final production of paramylon.

Chemical analysis

The nutritional profile of EgM, ETX, and DOE-ETX is shown in Table 1.

As reported, *E. gracilis* is characterised by a high nutritional profile. DM recorded is in line with studies reported in the literature (Aemiro et al. 2019). In fact, as demonstrated by the authors, DM in *E. gracilis* is around 96%, a value that is a function of multiple factors, including the characteristics of the culture medium. More precisely, EgM (98.24 ± 0.04%) shows

Table 1. Chemical composition of EgM, ETX, and DOE-ETX (% w/w on DM basis).

Sample	DM	ASHES	CP	EE	CF
EgM	98.24 ± 0.04^{a}	1.71 ± 0.01^{a}	15.60 ± 0.30^{a}	30.37 ± 0.27^{a}	6.17 ± 1.33^{a}
ETX	$95.71 \pm 0.17^{a,b}$	2.14 ± 0.19^{a}	18.61 ± 0.30^{b}	15.16 ± 0.07^{b}	6.95 ± 0.58^{a}
DOE-ETX	94.52 ± 0.05^{b}	3.63 ± 0.07^{b}	25.11 ± 0.35^{c}	11.26 ± 0.06^{c}	6.18 ± 0.97^{a}

DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fibre.
Data are presented as mean ± standard error of mean (SEM), ($n = 3$). Different superscript letters in columns indicate statistically significant differences ($p < 0.05$).

statistically significant differences ($p < 0.05$) compared to ETX (95.71 ± 0.17%) and DOE-ETX (94.52 ± 0.05%), the same trend in the growth yield recorded and shown in Figure 1. The opposite trend was observed for the ashes content. As reported by Aemiro et al. (2019), their value is variable and can reach 3.5%, confirming what we observed for DOE-ETX (3.63 ± 0.07%). The latter shows statistically significant differences to ETX (1.4 ± 0.19%) and EgM (1.71 ± 0.01%). This difference is most likely due to the addition of microelements in the DOE-ETX medium, which led to the uptake by the *E. gracilis*, appearing in the total ashes content. However, the nutritional profile of a food/feed matrix is primarily a function of protein, fat, and fibre content. The protein content of *E. gracilis*, as reported in the literature, can reach up to 30.9% (Aemiro et al. 2019). In fact, microalgae can accumulate large amounts of protein at the intracellular level, characterised both by the presence of all 20 proteinogenic AAs and a high digestibility as shown by *in vitro* and *in vivo* studies, thus representing an interesting alternative to more traditional sources of dietary protein, such as meat and fish (Ritala et al. 2017; Gissibl et al. 2019). However, as previously reported, cultivation methods can affect the total protein content, especially when comparing photoautotrophic, heterotrophic, and mixotrophic growth conditions, with maximum levels for microalgae growing under heterotrophic conditions (Gissibl et al. 2019). These differences are also observed depending on the type of nitrogen that is added to the growth medium. As can be observed in Table 1, DOE-ETX (25.11 ± 0.35%) showed statistically significant differences compared to ETX (18.61 ± 0.30%) and EgM (15.60 ± 0.30%). These differences are most likely due to both the source of nitrogen (increasing trends were also observed for ETX compared to EgM, although not statistically significant) and the addition of microelements in the culture medium. In the first case, as shown by Oda et al. (1982), AAs, added to the culture medium, are easily metabolised by cells and consequently used for protein synthesis. In the second case, as reported in the literature, microelements play an important role in the

protein synthesis process of plants (Kozera et al. 2013). More specifically, the application of zinc, copper, molybdenum, manganese, and boron (all microelements present in the DOE-ETX culture medium) resulted in an increase in the total protein content in the bean. These microelements are essential parts of living tissue and perform many specific essential functions as components of many enzymes that control the metabolic processes of organisms. Among these, manganese is not only responsible for increasing the intensity of photosynthesis, but also stimulates nitrogen assimilation and protein biosynthesis; a function similar to that of boron (Cakmak 2000; Kozera et al. 2013). However, regardless of the cultivation method, *E. gracilis* would be a viable alternative for the feed industry due to the high protein content outlined above. At the same time, *E. gracilis* is characterised by a high lipid content. Lipids play a key role in microalgae cells as they ensure their survival during night periods and provide energy for vital biological processes, such as cell division, in particular DNA replication, and nuclear division (Kottuparambil et al. 2019). The lipid content of *E. gracilis* is characterised by the presence of 13 different types of fatty acids (50% unsaturated fatty acids), the production of which increases under photosynthetic, heterotrophic, and anaerobic conditions (Kottuparambil et al. 2019). As reported in the literature, the lipid content can exceed 20% of dry weight (Kottuparambil et al. 2019), confirming our data. More precisely, EgM (30.37 ± 0.27%) showed a higher lipid content, with statistically significant differences, than ETX (15.16 ± 0.07%) and DOE-ETX (11.26 ± 0.06%).

The modulation of lipids under heterotrophic culture conditions has led to conflicting results in the literature. While Kottuparambil et al. (2019) reported that lipid production is only a function of the type and concentration of the carbon source and independent of the nitrogen source, Jung et al. (2021) observed how the latter influenced the total lipid content in *E. gracilis*. The data obtained in this work would corroborate the findings of Jung et al. (2021). More specifically, the authors observed that lipid production was highest in the groups treated with the highest nitrogen concentrations compared to the microalgae grown under starvation conditions.

For this reason, as the samples in this study were grown under the same carbon source, it is very likely that the lipid content of EgM, ETX, and DOE-ETX was also modulated in this case by the nitrogen source, and that the addition of the amino acid extract, although it increased the total protein value, was in this case a limiting factor, a result previously confirmed in the growth yield and total paramylon production. These results would confirm the yeast extract as an important source of nitrogen in *E. gracilis*, previously reported.

Finally, the addition of microelements in DOE-ETX resulted in a further reduction in lipid content. As noted by Abou-Shanab et al. (2012), the addition of zinc, cobalt, copper, and manganese did not allow dose-response effects in lipid production to be observed in *Micractinium pusillum*, a freshwater alga. More precisely, the enrichment of culture medium observed a decrease in their total production, underlining the need for further investigation.

With regard to crude fibre content, no differences were observed between the EgM (6.17 ± 1.33%), ETX (6.95 ± 0.58%), and DOE-ETX (6.18 ± 0.97%) groups, suggesting that differences in culture media, in this case, did not affect the final total content. In the literature, few studies focus on the determination of fibre in *E. gracilis*, being a negligible component of its nutritional profile. In fact, as reported in the literature, the maximum value observable in crude fibre content can reach a maximum of 10–11% (Metsoviti et al. 2019), confirming the data we obtained.

In light of the above, *E. gracilis* represents a food/feed matrix characterised by a high nutritional profile, especially in terms of protein and lipid, also confirmed by *in vivo* trials conducted on broilers (Choi et al. 2004; Pieniazek et al. 2016). However, the variability in their yields, which can be observed following modulation of cultivation methods, requires further study and investigation to delineate the best characteristics of culture media that guarantee high nutritional profiles.

Total phenolic content of chemical extractions

Figure 2 shows the TPC of EgM, ETX, and DOE-ETX following chemical extractions (H2O:EtOH).

As can be seen in Figure 2, the highest TPC in each of the microalgae tested was obtained following chemical extractions with 50% EtOH and 75% EtOH. More specifically, although ETX algae showed a higher TPC following 50% EtOH (307.11 ± 5.40 mg TAE/100 g) with statistically significant differences compared to the other concentrations tested ($p < 0.05$), EgM and DOE-ETX showed a better trend with 75% EtOH (507.54 ± 19.68 mg TAE/100 g; 1104.40 ± 68.18 mg TAE/100 g, respectively), not statistically significant compared to extraction with equal concentrations of H_2O and EtOH (377.02 ± 6.77 mg TAE/100 g; 1030.33 ± 66.43 mg TAE/100 g, respectively). These

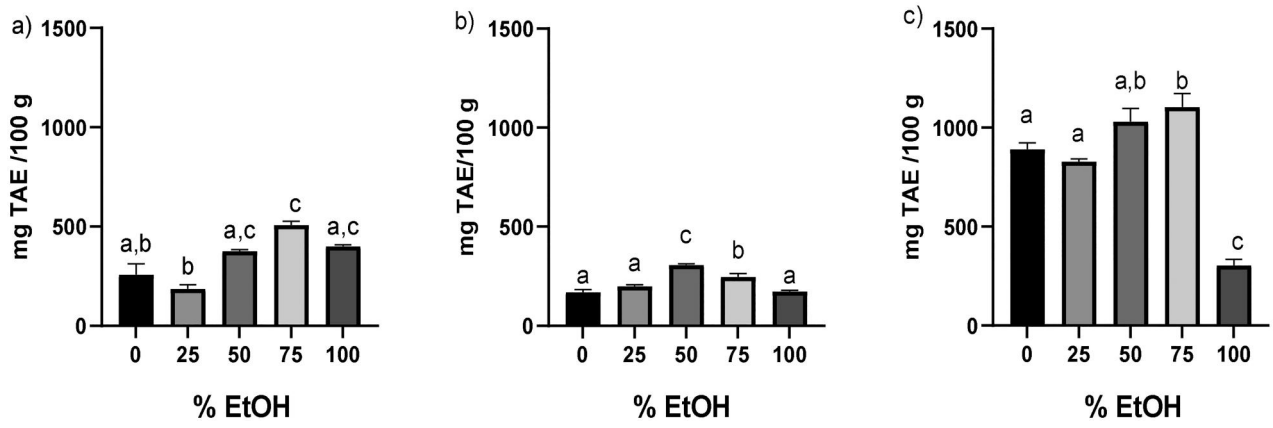

Figure 2. Total phenolic content of chemical extractions. (a) EgM. (b) ETX. (c) DOE-ETX. TAE: tannic acid equivalent. Data are presented as mean ± standard error of mean (SEM), ($n = 3$). Different superscript letters in columns indicate significant different data ($p < 0.05$).

results are confirmed in the study conducted by Čagalj et al. (2021). More specifically, the authors demonstrated how the 50% EtOH extraction tested on *Padina pavonica* (brown alga) resulted in a higher TPC. This occurs due to the *'like dissolves like'* principle where water dissolves polar compounds while ethanol, an organic solvent, dissolves less polar ones (Lim et al. 2019). This result is also confirmed by Tierney et al. (2013). As reported by the authors, the best phenolic extraction in different macroalgae was found in aqueous organic mixtures. However, although the results obtained following extraction with 0% EtOH were in line with those observed by Čagalj et al. (2021), higher concentrations of 50% EtOH reported different results. More specifically, the authors reported how extraction with 70% EtOH resulted in a decrease in TPC, a trend different from what was observed in this study.

Phenols are secondary metabolites produced by plants as defensive weapons against abiotic and biotic stresses. They are therefore recognised for their intrinsic antioxidant effect that protects cells against oxidative stress (Sorrentino 2021). For this reason, most studies in the literature characterise phenols following heavy metal contamination to see modulation in TPC. However, few studies characterise phenolic compounds under normal conditions (Bernard and Guéguen 2023). As reported by Bernard and Gueguen, phenols production is stimulated by the carbon source. In particular, glucose is not only used by cells to produce energy but also for the production of biological compounds including phenolic compounds *via* the phosphoenolpyruvate pathway (Bernard and Guéguen 2023). However, in our study, glucose represents the common carbon source for all treated groups. For this reason, it is conceivable how the microelements added in the medium of DOE-ETX resulted in high TPC values compared to EgM and ETX. No TPC values are reported in the literature following the addition of these microelements in the culture medium of *E. gracilis*. However, Klimek-Szczykutowicz et al. (2019) demonstrated how the addition of copper, iron, and zinc stimulated the production of phenolic compounds in *Nasturtium officinale* R. Br., an aquatic or semi-aquatic plant. As reported, besides not observing a dose-dependent effect, the concentrations tested were higher than those added in DOE-ETX. For this reason, although these data suggest an involvement of these microelements in the stimulation of phenolic compounds, they require further investigation to better characterise this phenomenon.

At the same time, the involvement of microelements in the production of phenolic compounds is reported by Sampaio et al. (2011). The authors demonstrated how copper, iron, manganese, and zinc stimulate phenol production in the leaves of *Lafoensia pacari*, a plant recognised for its antipyretic and anti-inflammatory properties (Sampaio et al. 2011).

The relationship between these microelements and phenol production can be explained by several factors. Firstly, these microelements, particularly iron, represent nutrients associated with the process of photosynthesis, with the synthesis of phenolic compounds, such as simple phenolic acids (Sampaio et al. 2011). Furthermore, as reported by Kováčik and Klejdus (2008), copper is directly involved in phenol production by acting as an activator of the phenylalanine ammonia-lyase (PAL) pathway, an important enzyme involved in the biogenesis of phenolic compounds. A further explanation for the accumulation of phenols may be associated with the copper tolerance mechanism, as copper is a catalyst for redox reactions that

can lead to the production of damaging free radicals. Consequently, the increase in TPC could have two meanings: decreasing the concentration of free copper through its reaction with phenols and reducing the damaging effects of the free radicals formed, thanks to the antioxidant action of phenolic compounds (Sampaio et al. 2011). Finally, manganese and zinc are involved in phenols synthesis and conversion processes as enzyme cofactors (metalloproteins) (Sampaio et al. 2011).

Antioxidant activity of chemical extractions

The next step was to assess the antioxidant activity of each microalgae by ABTS and FRAP assay. In Figure 3, only the values for DOE-ETX are shown, having obtained for EgM and ETX non-detectable values in both assays, in almost all the concentrations tested.

More specifically, EgM and ETX showed no detectable values for FRAP in all concentrations tested. For the ABTS assay, the only positive value for EgM was recorded following 75% EtOH extraction (21.50 ± 2.32 mg TE/100 g), whereas for ETX following 50% EtOH (17.92 ± 0.42 mg TE/100 g) and 75% EtOH (35.94 ± 5.04 mg TE/100 g) extraction, with statistically significant differences. This trend was also confirmed for DOE-ETX, although showing no significance between the two concentrations, confirming the results obtained in TPC content.

In general, it was observed that the values recorded by the ABTS assay are higher than those of the FRAP assay. This disparity in antioxidant activities at different H_2O and EtOH concentrations indicates that the type and amount of recoverable antioxidant compounds in *E. gracilis* are influenced by EtOH concentrations, as confirmed by Lim et al. (2019). In fact, as can be seen in Figure 3, varying the polarity of the solvent from very polar (0% EtOH) to less polar (100% EtOH), also varies the solvent's ability to dissolve selected groups of antioxidant molecules by modifying their activity (Turkmen et al. 2006; Abbasi et al. 2015). This observation is consistent with the results observed in this study, in which the antioxidant capacities of the extracts were sensitive to the polarity of the solvent, which may also be related to TPC.

As previously reported, the values recorded using the ABTS assay are much higher than those observed using the FRAP method. As reported in the literature, *E. gracilis* is characterised by the presence of compounds characterised more by the antioxidant action of free radical scavenging rather than the metal chelation action, the principle of action of the ABTS and FRAP assays, respectively (Gissibl et al. 2019). Of these, vitamins E and A are the most important. Vitamin E includes several isoforms of tocopherol (α, β, γ, δ). These isoforms in addition to being powerful antioxidants, are a very important source of *E. gracilis*. More specifically, although the α-tocopherol is considered to be the most important for animal physiology, γ-isoform is the main consumed in countries where the vegetable oils consumed are predominantly derived from soybean and maize, leading to an insufficient intake of α-tocopherol. However, although *E. gracilis* possesses a plant-like α-tocopherol pathway, it almost exclusively produces the α-tocopherol isoform, thanks to the enzyme γ-tocopherol methyltransferase that converts γ-tocopherol to α-tocopherol using S-adenosyl methionine (Gissibl et al. 2019).

In parallel, vitamin A plays a key role in the antioxidant action of *E. gracilis*. Vitamin A comprises a group of compounds, such as retinol, retinoic acid, and various retinoic esters (Gissibl et al. 2019). It is only found in products of animal origin and human supply is currently supplemented with provitamin A of plant origin

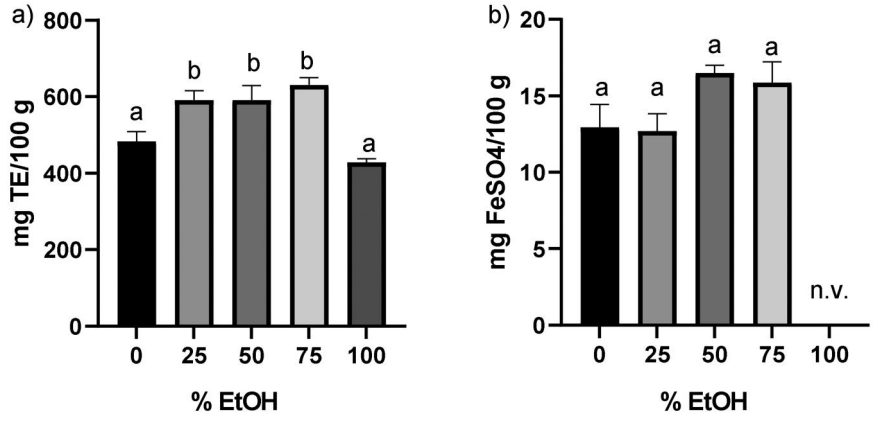

Figure 3. Antioxidant activity of DOE-ETX. (a) ABTS assay. (b) FRAP assay. Data are presented as mean ± standard error of mean (SEM), ($n = 3$). Different superscript letters in columns indicate significant different data ($p < 0.05$). n.v.: not valuable.

(β-carotene). Like other photosynthetic microorganisms, *E. gracilis* produces β-carotene as a protective pigment to avoid photo-oxidative damage to chloroplasts, which is why beta-carotene titres may be higher under phototrophic conditions. However, as shown in the literature (Gissibl et al. 2019), β-carotene levels in *E. gracilis* can be easily increased by changing cultivation conditions.

However, although the values reported for FRAP are lower than those observed for ABTS, they are, especially following 50 and 75% EtOH extractions, comparable to other matrices widely used in the feed industry due to their high nutritional and functional profile, such as flaxseed and soya protein extract, as reported by Lanzoni et al. (2023). Most likely, microelements also seem to be involved in this case. Indeed, as explained by Jin et al. (2007), phenolic compounds can complex with Fe ($Fe3^{+}$) and be transported to other tissues, facilitating the mobilisation of this mineral between different tissue types and participating in the reduction of Fe^{3+} to Fe^{2+}, the principle of action of the FRAP assay.

Total phenolic content and antioxidant activity of DOE-ETX *ex vivo* digested

In light of the interesting results obtained with DOE-ETX, it was decided to test the TPC and antioxidant activity following the *ex vivo* digestion process, as shown in Figure 4.

The TPC trend shows a peak at the end of the gastric phase (584.98 ± 6.77 mg TAE/100 g) with statistically significant differences to the other digestion phases (beginning of the gastric phase (0 h) (501.01 ± 12.05 mg TAE/100 g) and end of the intestinal phase (4 h) (465.24 ± 37.36 mg TAE/100 g). This trend is confirmed in the literature on different matrices, such as hemp-based products, bamboo leaves, Butia and Carob fruits, digested with *in vitro* digestion protocols (Goulas and Hadjisolomou 2019; Ma et al. 2020; Lanzoni et al. 2023). The peak observed at the end of the gastric phase, as argued in the literature, is due to the low pH value (2 ± 0.05), which allows the release of phenols following the breaking of bonds within the matrices, including proteins and polysaccharides (Goulas and Hadjisolomou 2019). Subsequently, as anticipated, a decrease with statistically significant differences is observed at the end of the intestinal phase. This result, as shown by Friedman and Jürgens (2000) and Lanzoni et al. (2023), could be a direct consequence of the instability of phenolic compounds at high pH values (6.8 ± 0.05).

The antioxidant activity, assayed by the ABTS and FRAP methods, shows the same trend as that observed for TPC. As reported in the literature, the peak in the gastric phase (586.86 ± 32.10 mg TE/100 g for the ABTS and 50.30 ± 1.02 mg $FeSO_4$/100 g for the FRAP assay) is probably due to the release of phenolic compounds. Subsequently, a significant decrease is observed following intestinal digestion (208.78 ± 4.67 mg TE/100 g for ABTS and 25.38 ± 2.17 mg $FeSO_4$/100 g for the FRAP assay). As discussed by Ma et al. (2020), this result is due to the degradation of almost all phenolic compounds in the sample as a consequence of the basic pH.

As demonstrated in Figure 4, the values recorded by the ABTS assay are higher than those of the FRAP assay. As previously reported, the explanation lies in the multiple compounds characterised by scavenging action as opposed to metal chelation. Finally, the *ex vivo* digestion process proved to be a reliable model for characterising the TPC and antioxidant activity of *E. gracilis*.

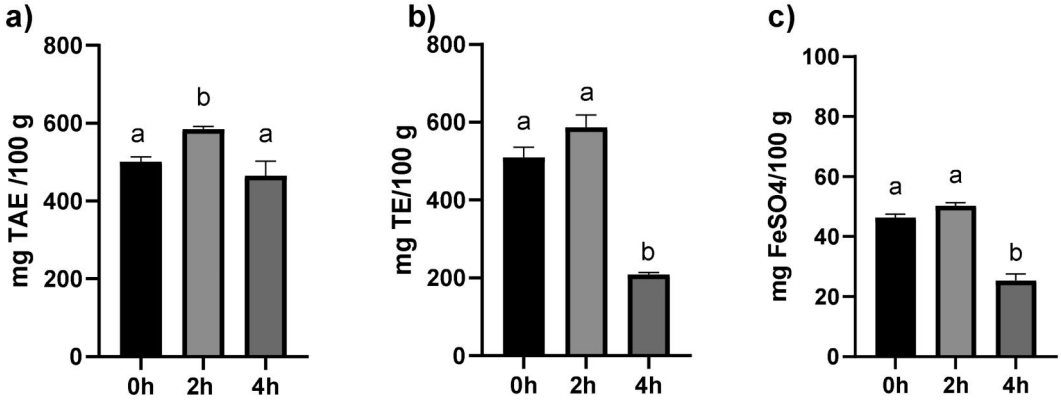

Figure 4. Total phenolic content and antioxidant activity (ABTS and FRAP) of *ex vivo* digestion of DOE-ETX. TAE: tannic acid equivalent; TE: trolox equivalent. Data are presented as mean ± standard error of mean (SEM), ($n = 3$). Different superscript letters in columns indicate significant different data ($p < 0.05$). (a) Total phenolic content; (b) ABTS assay; (c) FRAP assay. 0 h: start of gastric phase, 2 h: end of gastric phase, 4 h: end of intestinal phase.

In light of the above, *E. gracilis* has great potential for the food/feed industry due to its high growth rate and ability to store high quality lipids, proteins, carbohydrates, and functional compounds (Klinthong et al. 2015). In parallel, as reported in the literature by Klinthong et al. (2015), *E. gracilis* plays a key role in environmental protection due to its ability to store CO_2 through the process of chlorophyll photosynthesis to directly fix carbon in cells. This role is also crucial in the energy sector. Indeed, the pollution-free conversion of CO_2 into chemicals and fuels through this approach is a promising way to reduce environmental CO_2 (Klinthong et al. 2015).

Conclusions

In conclusion, the modulation of the culture conditions of *E. gracilis* plays a key role in the growth yield, in paramylon content, and in determining the nutritional and functional profile of this microalgae. In particular, although DOE-ETX showed lower growth yield and paramylon content than EgM and ETX, it demonstrated a greater functional profile. More precisely, DOE-ETX presented a higher TPC and antioxidant activity following chemical extraction, for each condition tested (H_2O:EtOH), deepening the role of nitrogen sources and microelements in the modulation of the functional aspect. However, to be able to confirm the large-scale use of *E. gracilis* in the feed industry, further studies and in-depth investigations are required to characterise the best cultivation methods for this microalgae, to obtain a homogeneous, constant, and non-variable matrix; fundamental requirements to ensure animal welfare and guarantee high standards of production performance in farm animals. Furthermore, given the high nutritional, functional profile and the important role in the environmental protection of *E. gracilis*, a possible application in the food sector should be considered.

Ethical approval

Not applicable.

Disclosure statement

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Data availability statement

The dataset generated during and/or analysed during the current study is available from the corresponding author on reasonable request.

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