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Fermentation of *Chlorella vulgaris* and *Aphanizomenon flos-aquae* biomass improves the antioxidant profile

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ABSTRACT

Microalgae, known for their rich nutritional profile and bioactive molecules, have promising potential in nutraceutical applications. However, their rigid cell wall often limits the bioaccessibility of bioactive compounds. Fermentation offers a viable approach to improve the nutritional and functional properties of microalgae by breaking down complex macromolecules enhancing antioxidant capacity. This study investigated the fermentation of *Chlorella vulgaris* (*Cv*) and *Aphanizomenon flos-aquae* (*Afa*) with *Saccharomyces cerevisiae* and *Lactobacillus plantarum* over 24 and 48 h. The results showed a significant time-dependent increase in total polyphenol content: from 2.41 ± 0.29 to 6.06 ± 1.54 mg GAE/g in *Cv* and from 10.7 ± 0.57 to 15.95 ± 2.38 mg GAE/g in *Afa* after 48 h, although the total flavonoid content decreased in both microalgae. Antioxidant activity, evaluated by DPPH and ORAC assays, exhibited different trends. *Cv* showed a 1.6-fold increase in DPPH and a 2-fold increase in ORAC at both times, while *Afa* showed improved DPPH and ORAC values only after 24 h. Particularly, the cellular antioxidant activity in human erythrocytes revealed a significant effect of *Afa* fermentation at both time points. These findings suggest that fermentation can improve the polyphenol content and antioxidant properties of microalgae, especially in *Aphanizomenon flos-aquae*, indicating its potential for functional and nutraceutical food formulations.

1. Introduction

Microalgae, including photoautotrophic eukaryotic protists and prokaryotic cyanobacteria (blue-green algae), are gaining great interest for their ecological roles and adaptability to diverse environments, such as brackish and saltwater ecosystems (Sultana et al., 2023). As highly sustainable sources of high-value compounds, microalgae offer a promising solution to addressing the growing global demand for food and health resources. Their dense nutritional profile, rich in proteins and essential amino acids, renders them one of the most balanced sources of nutrients for human consumption (Koyande et al., 2019). Moreover, microalgae contain essential metabolites, including polysaccharides and polyunsaturated fatty acids (PUFAs) such as omega-3s (eicosapentaenoic acid and docosahexaenoic acid), as well as essential minerals and vitamins. This versatile composition supports their application in diverse industries, from food and nutraceuticals to pharmaceuticals and biofuels (Liang et al., 2004; Yaakob et al., 2014; Singh and Saxena, 2015). Additionally, recent studies have evidenced microalgae as natural source of antioxidants, including ascorbic acid, tocopherols, carotenoids, and polyphenols, which broaden their potential uses in animal feed, aquaculture, and human health (Cai et al., 2021; Coulombier et al., 2021; Mavrommatis et al., 2023; Zhou et al., 2022). Notably, *Chlorella vulgaris (Cv)* and *Aphanizomenon flos-aquae (Afa)* are among the species with significant nutritional and nutraceutical potential (Nuzzo et al., 2019).

Chlorella spp. consists of 53 % proteins, 23 % carbohydrates, 9 % lipids, and 5 % minerals and oligoelements. Additionally, *Cv* contains significant amounts of B complex vitamins, especially vitamin B12 and β-1,3-glucan, a bioactive compound, which acts as an immune stimulant reducing free radicals and cholesterol in the blood (de Morais et al., 2015). *Afa*, known as *Klamath* algae, is a fresh water unicellular blue-green alga, with a significant source of blue photosynthetic

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pigment known as phycocyanin (PC), which has been shown to possess strong antioxidant properties (Benedetti et al., 2004). Furthermore, extract from *Afa* might have an anti-inflammatory and protective role against the mechanisms that lead to neurodegeneration (Nuzzo et al., 2018; Terzo et al., 2023).

However, the rigid cell wall, characteristic of many microalgae species pose a barrier to the bioaccessibiliy of these nutrients, which limits the effectiveness of these compounds for human health (Li et al., 2023; Machado et al., 2022). Fermentation has emerged as a promising technology to overcome this barrier by breaking down complex macromolecules, thus enhancing the antioxidant capacity of microalgae (de Marco Castro et al., 2019; Garofalo et al., 2022; Verni et al., 2019). Notably, lactic acid bacteria have been widely explored for their capacity to improve both the nutritional and sensory properties of fermented food. *Lactobacillus plantarum* is frequently used in fermentation for its ability to metabolize carbohydrates into organic acids and breakdown proteins into small peptides and free amino acids, enhancing flavors and the bioavailability of nutrients (Gao et al., 2022). Moreover, probiotic cultures typically require extended fermentation times to achieve low pH values. However, the food industries always prefer shorter fermentation periods to increase production efficiency and minimize the risk of microbial contamination. A promising solution to this challenge is the use of mixed or co-culture fermentations, which can accelerate acidification while enhancing the sensory and functional qualities of the final product. The co-culture fermentation, which involves the use of two or more microorganisms, can produce unique growth dynamics that enhance the organoleptic and functional properties of the food (do Amaral Santos, da Silva Libeck, & Schwan, 2014; du Toit et al., 2020).

Building on previous findings that indicate synergistic interactions between yeast and lactobacilli strains in various naturally fermented foods and beverages (du Toit et al., 2020; Ponomarova et al., 2017), this study investigated whether a co-culture fermentation of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* can enhance the nutraceutical properties of *Cv* and *Afa*. We hypothesized that this approach will increase the bioavailability of polyphenols and flavonoids, thus amplifying the antioxidant properties of these microalgae. Therefore, to elucidate the impact of fermentation on microalgae, *Cv* and *Afa* biomass were fermented and screening for the total polyphenols and flavonoids content, as well as the antioxidant activities, using both *in vitro*, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) antiradical activity and the oxygen radical absorbance capacity (ORAC), and *ex vivo,* cellular antioxidant activity (CAA), tests. The CAA test, conducted on human erythrocytes, provides a more comprehensive and physiologically relevant assessment of how antioxidants may perform within human systems (Blasa et al., 2011). This study contributes to the development of fermented microalgae as a valuable resource for enhancing human health and nutritional well-being, with implications for food and health industries.

2. Materials and methods

2.1. Lactobacillus and yeast strains preparation

Stock freeze cultures (20 % glycerol) of *Lactobacillus plantarum*, supplied by Institute of sciences of food production (ISPA) of the National Research Council (CNR) of Milan, were grown about 48 h in MRS Broth (Oxoid, Basingstone, UK) at 37 °C. The concentration of *L. plantarum* used for the fermentation process was 10^8 cells/mL based on the work of Bottani et al. (2018). To reach the target concentration, an optical density of 0.8 was measured at 600 nm (OD_{600}) using the FLUOstar Omega (BMG LABTECH) spectrophotometer (Trabelsi et al., 2013).

Saccharomyces cerevisiae was obtained from commercial yeast (Lievital, Lesaffre Italia) and was cultivated in liquid complete medium containing 25 g of glucose, 10 g of bactopeptone and 5 g yeast extract (provided by Oxoid (Basingstone, UK) dissolved in 500 mL of double distilled water, at 30 °C until reach the stationary/growth phase. Then 10^6 cells/mL was used for the fermentation process.

2.2. Microalgae fermentation conditions and extract preparation

Chlorella vulgaris was supplied by the Institute of Biology and Biotechnology (IBBA)-CNR, and *Aphanizomenon flos-aquae* was supplied by Institute for Biomedical Research and Innovation (IRIB)-CNR, Palermo.

For fermentation process, 1 g of algae powder was suspended in 20 mL of double distilled autoclaved water, then added the *L. plantarum* and *S. cerevisiae* inoculum, and left to stir at 120 rpm, for 0 h (T0), 24 h (T1) and 48 h (T2) at 37 °C in a ventilated flask. After each time 5 mL of the samples were collected and store at −80 °C. The frozen samples were lyophilized for three days using a freeze-dryer Lyovac GT 2 (SRK Systemtechnik, Riedstadt, Germany).

For the extraction, the lyophilized samples were resuspended in 80 % methanol at a concentration of 25 mg/mL and left stir in the dark overnight. The samples were then centrifuged at 4000 rpm for 25 min at 4 °C using the Jouan CR 3i centrifuge and the supernatant was stored at −20 °C, until used. The extraction was carried out in triplicate.

2.3. Total polyphenols and flavonoids content

Total polyphenols content of algae extracts was determined by the Folin-Ciocalteu colorimetric method (Singleton et al., 1999). Briefly, 100 μL of the sample extracts and 500 μL diluted (1:10 with distilled water) Folin-Ciocalteau reagent, were incubated in the dark at room temperature. After 5 min 400 μL of sodium carbonate (Na_2CO_3) 0.7 M were added. After 2 h incubation in the dark at room temperature, the absorbance was measured at a wavelength of 760 nm in a microplate reader (FLUOstar Omega). The total polyphenol content was expressed as milligrams of gallic acid equivalent (GAE)/grams of dry weight (dw), determined through a standard calibration curve obtained from appropriate dilution of a gallic acid, ranging from 0 to 200 μg/mL.

Total flavonoids content of algae extracts was measured by the aluminium chloride colorimetric method described in the work of Chiellini et al. (2022). Briefly, 100 μL of the samples extract were mixed with 400 μL of distilled water and 30 μL of sodium nitrite ($NaNO_2$) 5 %. After 5 min, 30 μL of aluminium chloride ($AlCl_3$) 10 % were added and incubated for 6 min. Finally, the solution was neutralized with 200 μL sodium hydroxide (NaOH) 1 M and added 240 μL of distilled water to the volume of one mL. Absorbance was measured after 30 min at 430 nm in FLUOstar Omega microplate reader. The total flavonoid content was quantified as milligrams of quercetin equivalent (QUE)/grams of dry weight (dw), using a standard curve obtained from serial dilutions of quercetin, ranging from 0 to 500 μg/mL.

2.4. In vitro antioxidant activities

2.4.1. DPPH radical scavenging assay

The antiradical activity of the algae extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by Colosimo et al., (2020) slightly modified. In detail, 25 μL of appropriate dilution of algae extract were added to 975 μL of 60 μM methanolic DPPH solution. After 30 min in the dark, the reduction of DPPH radicals was measured at 517 nm and the anti-radical activity (ARA) was quantified using the following equation: $ARA = [1- (A_S/A_{DPPH})] \times 100$, where A_S and A_{DPPH} represent absorbance of the sample and DPPH solution respectively. The exact concentration corresponding to 50 % of DPPH inhibition (EC_{50}) was measured by interpolation of ARA results and the concentration of samples. Lower EC_{50} values indicate higher antioxidant activities.

2.4.2. Oxygen radical absorbance capacity (ORAC) assay

The Oxygen Radical Absorbance Capacity was performed follow the

protocol of Ninfali et al., (2005) with some modification. An aliquot of 100 μL of diluted sample or 50 μM Trolox, were mixed with 800 μL of 40 nM fluorescein sodium salt in 75 mM phosphate buffer, pH 7.4, as fluorescent probe and with 100 μL of 2,2′-azobis 2-amidinopropane dihydrochloride (AAPH) 400 mM, as peroxyl radicals' generator. Fluorescein fluorescence decay was read at 485 nm excitation and 520 nm emission using a FLUOstar Omega (BMG LABTECH) microplate reader. The ORAC value was expressed as micromoles of Trolox equivalents (TE)/grams dried weight (dw).

2.5. Cellular antioxidant activity test in red blood cells (CAA-RBC)

The *ex vivo* antioxidant capacity of algae extract was performed using the CAA-RBC assay as described by Blasa et al., (2011). Blood samples were collected in tubes treated with ethylene diamine tetraacetic acid (EDTA) and centrifuged for 10 min at 2300×*g* at 4 °C to remove plasma, platelets, and buffy coat. Then 250 μL of diluted algae extracts and 250 μL of 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), 15 μM final concentration, were added to 2 mL of red blood cells (RBC) (diluted 1:100 with PBS pH 7.4). Trolox 500 μM was used as standard. The RBCs were incubated for 1 h in dark at 37 °C under gentle stirring and then washed twice with PBS and resuspended in 1 mL of cold PBS. An aliquot was transferred to a microplate and AAPH solution (1.2 mM final concentration) was added to the cell suspension. The fluorescence was read using a FLUOstar Omega microplate reader at 485 nm excitation and 535 nm emission. Values were expressed as CAA units using the following formula: CAA = [1 - (∫SA/∫CA)] × 100, where ∫SA and ∫CA are respectively the integrated area of the sample and control curve.

Blood samples were obtained from healthy donors, upon informed consent for the use of residual blood for research purposes according to the Italian regulations and in particular, the regulations of "Fondazione G. Monasterio CNR-Regione Toscana".

2.6. Statistical analysis

The results are expressed as mean ± standard deviation (SD), of three different experiments, for each test. One-way analysis of variance (ANOVA) with the Tukey's post hoc test was used for multiple comparisons; *p*-values <0.05 were considered statistically significant. All analyses were carried out using the SPSS version 27 software package (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Fermentation enhances polyphenols content

The co-fermentation process of microalgae powder with *L. plantarum* and the *S. cerevisiae* was conducted in a 100:1 ratio, according to Bottani and colleagues (2018), who reported that lactic acid bacteria (LAB) are typically abundant, reaching concentration of 10^8 CFU/g, while yeast populations generally remain below 10^7 CFU/g. This ratio was found in sourdough where LAB establish some durable microbial associations whit yeasts (Corsetti & Settanni, 2007).

The temperature of 37 °C used during fermentation is beneficial for *L. plantarum* activity, as higher temperatures reduce yeast competition, promoting fermentation activity and overall lactic acid production in various substrates (du Toit et al., 2020; Khan et al., 2020). After 24 and 48 h of fermentation, the pH decreased and stabilized around 4.7, supporting LAB activity in acidification.

Total polyphenols (TPC) and flavonoids content (TFC) of *Cv* and *Afa* lyophilized extracts were assessed at 0 (T0), 24 (T1), and 48 h (T2) of fermentation. As showed in Table 1, in *Cv*, TPC was lower at the beginning of fermentation process and increased 2 and 2.5-fold at T1 and T2 respectively, whereas TFC remained stable at T1 and greatly decreased at T2 time point of fermentation compared to T0 and T1. Similarly, *Afa*, exhibited notable increase in TPC over time, while TFC showed a declining trend, peaking at T0. The increase in TPC after fermentation could be related to the release of glycosylated polyphenols by microbial enzymes, such as decarboxylase, reductase, and tannase. This hydrolytic process, wherein tannic acid is converted into gallic acid and glucose, has been well-documented (Curiel et al., 2015; Septembre-Malaterre et al., 2018; Wang et al., 2021). These findings are in agreement with those reported by Taniguchi et al. (2019) who observed an increase in TPC in fermented *Afa* with L. *plantarum and Lactococcus lactis* and their mixture. In addition, our results are consistent with those reported previously in some cyanobacteria and microalgae species (de Marco Castro et al., 2019; Kaga et al., 2021; Niccolai et al., 2019). de Marco Castro et al., (2019) observed an increase in TPC in spirulina (*Arthrospira platensis*) after 24 and 48 h of fermentation with *L. plantarum* compared to non-fermented one. Likewise, the authors did not report a significant difference in TPC between the two fermentation time points. Otherwise, exposure of microalgae to fermentation temperature for long time may influence the stability and the bioactivity of flavonoids (Gao, Xia, et al., 2022). Yu et al. (2020) observed a significant reduction of TFC in spirulina following 72 h of fermentation with mixture of LAB but, to date, data in literature are controversial. This difference in TPC and TFC content may reflect the complex interplay of fermentation conditions, such as species type, microbial strain, and fermentation time and temperature which determines the changes in compounds during fermentation (Krishna, 2005).

Table 1
Effect of fermentation at different times of 0 (T0), 24 (T1) and 48 (T2) hours on total polyphenol and flavonoid content of *Chlorella vulgaris* (*Cv*) and *Aphanizomenon flos-aquae (Afa*) *Cv* and *Afa* biomass using a combination of *S. cerevisiae* and *L. plantarum*. Total polyphenols and flavonoids content are expressed as milligrams of gallic acid equivalents per grams of dry weight (mg GAE/g dw) and milligrams of quercetin equivalent per grams of dry weight (mg QUE/g dw) respectively. Different letters within each column indicate significant differences according to One-way ANOVA with the Tukey's multiple-range test. Results are expressed as mean ± SD (n = 3).

Time	Cv		Afa	
	Polyphenols (mg GAE/g dw)	Flavonoids (mg QUE/g dw)	Polyphenols (mg GAE/g dw)	Flavonoids (mg QUE/g dw)
T0	2.41 ± 0.29b	23.59 ± 1.64a	10.70 ± 0.57b	13.67 ± 1.68a
T1	4.87 ± 0.23a	27.74 ± 5.22a	14.68 ± 2.61a	4.56 ± 0.95b
T2	6.06 ± 1.54a	8.05 ± 1.08b	15.95 ± 2.38a	4.86 ± 1.62b

3.2. In vitro antioxidant capacity

Phenolic compounds are characterized by an aromatic ring and constitute significant contributors to antioxidant and anti-inflammatory property (Barsanti et al., 2022). To better understand the impact of fermentation on antioxidant properties, *in vitro* bioactivity through DPPH, a method widely used to predict the ability of flavonoids to transfer H atoms to radicals (Tsimogiannis and Oreopoulou, 2006) and ORAC tests were performed on fermented extracts from *Cv* and *Afa*.

In *Cv*, radical-scavenging activities significantly increased after 24 h of fermentation (T1), with DPPH (Fig. 1A) reaching 1.47 ± 0.09 mg/mL EC_{50} value and ORAC (Fig. 1B) at 124.36 ± 12.66 μmoliTE/g. Both T1 and T2 showed comparable EC_{50} and ORAC values. *Afa* also exhibited a notable rise in antioxidant activity at T1, as indicated by decreased EC_{50} in the DPPH assay (Fig. 2A) and a 1.4-fold ORAC (Fig. 2B) increase relative to T0, followed by a slight decline at T2. A strong antioxidant activity after 24 h of fermentation may be due to a high TPC observed at the same time, suggesting that fermentation facilitates the release of phenolic components, significantly contributing to antioxidant capacity (Mousavi et al., 2013). These findings are in line with the results obtained by de Marco Castro et al. (2019) in fermented *Arthrospira platensis* where the greatest DPPH radical scavenging capacity and ORAC was observed after 24 and 48 h of fermentation respectively. Similar results

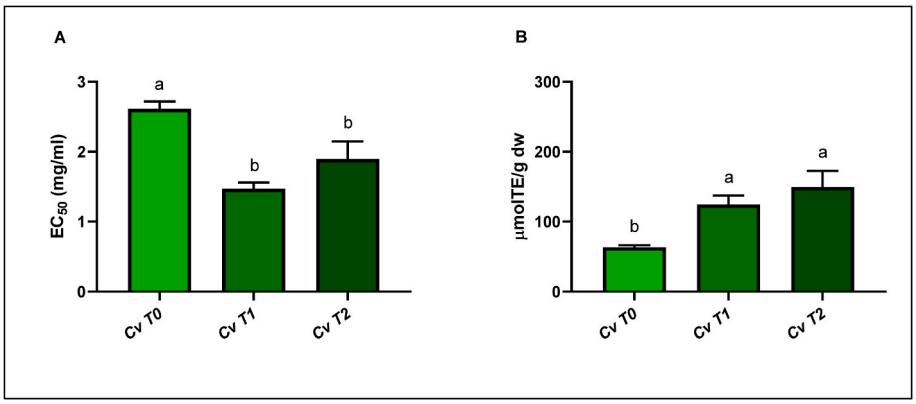

Fig. 1. Antioxidant capacity of *Chlorella vulgaris (Cv)* extracts, at different times of 0 (T0), 24 (T1) and 48 (T2) hours of fermentation. A) DPPH assay expressed as mg/ml of extract required to inhibit 50% of DPPH radicals (EC_{50}); B) ORAC assay expressed as µmol Trolox equivalent (TE)/grams of dry weight (dw). Different letters indicate significant differences according to One-way ANOVA with the Tukey's multiple-range test. Results were expressed as mean ± SD (n = 3).

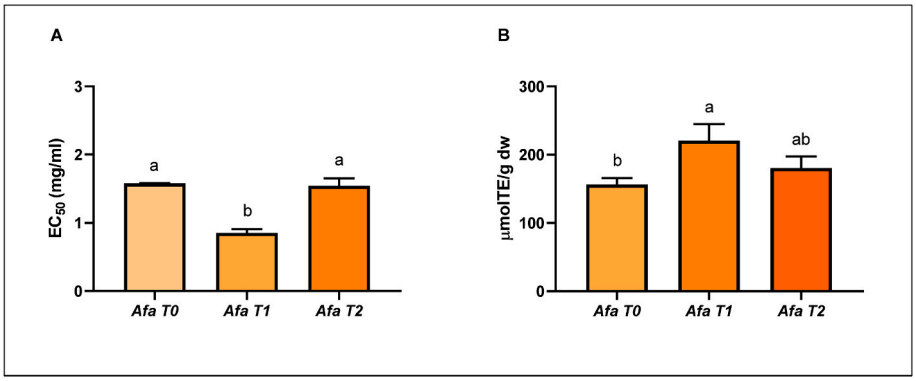

Fig. 2. Antioxidant capacity of *Aphanizomenon flos-aquae (Afa)* extracts at different times of fermentation: 0 (T0), 24 (T1) and 48 (T2) hours. A) DPPH assay expressed as mg/ml of extract required to inhibit 50% of DPPH radicals (EC_{50}); B) ORAC assay expressed as µmol Trolox equivalent (TE)/grams of dry weight (dw); Different letters indicate significant differences according to One-way ANOVA with the Tukey's multiple-range test. Results were expressed as mean ± SD (n = 3).

have also been reported by Jamnik et al. (2022) who observed a higher antioxidant capacity of 24 h fermented *Arthrospira platensis* with *L. plantarum* compared to the unfermented ones. However, some studies showed that pigments present in algae, especially chlorophyll in *Cv* (Andriopoulos et al., 2022) and phycobilin in *Afa* (Benedetti et al., 2010), could further explain the antioxidant activity, as pigments directly quench radicals.

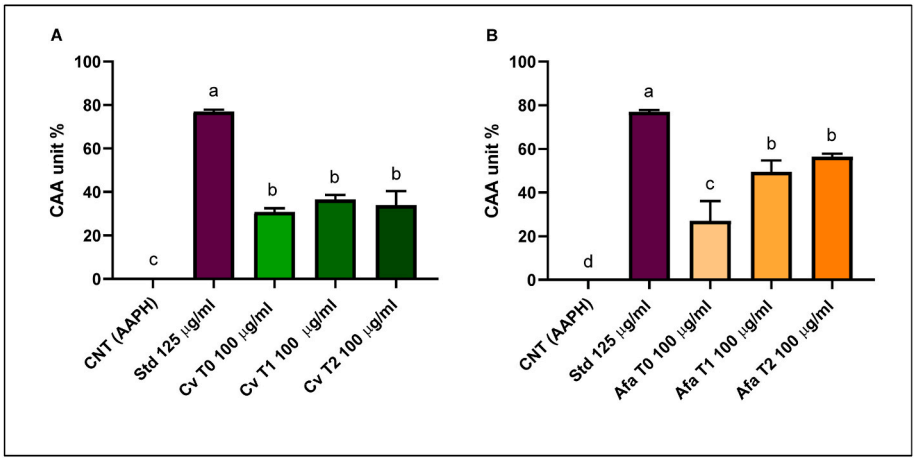

Fig. 3. Effect of 100 µg/mL of A) *Chlorella vulgaris (Cv)* and B) *Aphanizomenon flos-aquae* (*Afa*) extracts on the cellular antioxidant activity of human red blood cells under oxidative conditions (AAPH) at different fermentation timepoints (T0, T1 and T2 are 0, 24 and 48 h respectively) Trolox was used as the standard. Different letters indicate significant differences according to One-way ANOVA with the Tukey's multiple-range test. Data were expressed as mean ± SD (n = 3) and obtained from blood samples of healthy and distinct volunteers.

3.3. Antioxidant effect on human red blood cells

Chemical tests for antioxidant activity may not always correlate with *in vivo* effectiveness. Therefore, we applied the Cellular Antioxidant Activity (CAA) assay on human red blood cells (RBC) to evaluate the antioxidant capacity of microalgae in a biological context (López-Alarcón & Denicola, 2013).

All treatments as well as Trolox, used as a standard, significantly enhanced the cellular antioxidant activity on human RBCs respect to the control cells, exposed only to oxidative stress (CAA unit = 0). Unfermented *Cv* (Fig. 3A) demonstrated some radical-scavenging ability against AAPH-induced oxidative stress, though no differences were observed across extracts from different fermentation timepoints. Benedetti and colleagues (2004) showed a protective role of increasing concentrations of *Afa* against haemolysis induced by oxidative stress; however, our results (Fig. 3B) have shown better protection from AAPH-induced oxidative damage in fermented *Afa* at both time (T1 and T2), respect the unfermented (T0). The difference between *in vitro* and cellular antioxidant activities stems from the complexities of cellular environments. In cells, antioxidants must cross the membrane, face metabolic transformations, and interact with cellular components, all of which can alter their efficacy compared to the straightforward chemical reactions measured *in vitro*. Factors like bioavailability, stability and cellular uptake lead to variations in observed activity between the two tests, highlighting the need for cellular assays to assess physiological relevance (Blasa et al., 2011; López-Alarcón & Denicola, 2013). Moreover, the fermentation process can also lead to the creation of new phenolic derivatives with unique bioactive properties. For instance, *L. plantarum* degrades tannic acid into gallic acid, glucose and pyrogallol, further enriching bioactivity (Rodríguez, de las Rivas, Gómez-Cordovés, & Muñoz, 2008).

4. Conclusion

Microalgae represent an invaluable resource in addressing the global demand for sustainable food and nutraceuticals, particularly due to their potential for high biomass yield, nutritional richness, and environmental adaptability. In this study, we demonstrated that the fermentation of *Chlorella vulgaris* and *Aphanizomenon flos-aquae* with a co-culture of *L. plantarum* and *S. cerevisiae* enhances the bioavailability of polyphenolic compounds, thereby boosting their antioxidant properties. Notably, the fermentation process increased TPC, while decreasing TFC, suggesting that fermentation conditions can be optimized to improve the bioactive profile of microalgal products.

The results indicate that a 24-h fermentation period achieves similar outcomes to a 48-h period, suggesting that shorter fermentation could be both time- and energy-efficient. This approach holds promise for industrial applications, where efficiency and productivity are critical. The improvements in antioxidant capacity observed through both chemical (DPPH, ORAC) and cellular antioxidant activity assays, reinforce the potential for fermented microalgae to serve as functional food ingredients and dietary supplements with health-promoting benefits.

While this study has provided valuable insights, further research could focus on the metabolic pathways activated during fermentation to identify and quantify new bioactive compounds formed in the process.

Additionally, variations in fermentation parameters (e.g., temperature, pH, fermentation time and microbial strains) could allow for more tailored nutrient profiles that maximize health benefits enhancing not only antioxidant capacity but other functional properties, such as anti-inflammatory and immunomodulatory effects.

Ultimately, optimizing fermentation processes for microalgae could expand their applications in food, pharmaceutical, and cosmetic industries, advancing the role of microalgae as a sustainable and functional resource for human health.

CRediT authorship contribution statement

Elena Tomassi: Writing – original draft, Methodology, Investigation, Data curation. **Nafiou Arouna:** Writing – original draft, Data curation. **Maria Grazia Caruso:** Investigation. **Antonella Girgenti:** Investigation, Funding acquisition. **Pasquale Picone:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Domenico Nuzzo:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Laura Pucci:** Writing – review & editing, Supervision, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Domenico Nuzzo, Salvatore Picone, Antonella Girgenti reports financial support was provided by Sicilian Region Department of Regional Agriculture Rural Development and Mediterranean Fisheries. If there are other authors, they 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

No data was used for the research described in the article.

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