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Improvement of the probiotic growth-stimulating capacity of microalgae extracts by pulsed electric fields treatment

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ABSTRACT

This study aimed to investigate the nutritional character (carbohydrates, proteins, pigments, and phycocyanin), total antioxidant capacity (TAC) and the capability of simulating the growth of *Lactobacillus rhamnosus* of different extracts from *C. vulgaris* and *A. platensis* by means of the application of conventional aqueous extraction procedure and pulsed electric field (PEF) extraction technology. It was confirmed a significantly improved nutritional profile of *Chlorella* and *Spirulina* extracts obtained by PEF technology pre-treatment (3 kV/cm, 100 kJ/kg), with specifically higher values in total carbohydrate, Chlorophyll *a*, Chlorophyll b, and carotenoids content, and TAC. Additionally, *Spirulina* PEF extract showed a probiotic's growth-stimulating capability of 1 log10 cycle when fermented by *Lactobacillus rhamnosus*, with a metabolomic profile specifically rich in bioactive short chain fatty acids (SCFAs) and organic acids (3-phenyl lactic acid). The present study points out the applicability of PEF extraction technology under optimized conditions to improve the nutritional and functional character of microalgae and cyanobacterial-derived ingredients.

Industrial relevance: Nowadays, microalgae and cyanobacterial different species are exploited as sustainable and valuable sources of natural bioactive compounds. Several innovative commercial food products are including *Chlorella vulgaris* and *Arthrospira platensis* (*Spirulina*) compounds in their formulation, having an additional healthy-nutritious value due to their enrichment into proteins, complex polymers with prebiotic character, or antioxidant properties. This study demonstrates the applicability of Pulsed Electric Fields for obtaining extracts enhanced in biomolecules and functional properties.

1. Introduction

The constant increase in world population and life expectancy makes it necessary to explore sustainable nutritional alternatives that meet the demand for food and at the same time have a positive impact on consumers' health. The high biological diversity that inhabits the seas represents a great reservoir of fauna, flora and microbiota, and an important source of functional bioactive compounds. Among all this great repertoire, algae present unique physiological adaptations, which allow them to produce a wide variety of compounds that have been studied as a source of high-value metabolites for human and animal health (sterols, proteins, polysaccharides, antioxidants, pigments and polyunsaturated fatty acids) (Esquivel-Hernández et al., 2017; Wang et al., 2022). In recent years, the use of matrices of marine origin as a source of protein and other bioactive compounds has positioned itself, as a very interesting alternative, given the sustainable nature of their extraction and the low environmental impact of their cultivation (Alagawany, Taha, Noreldin, El-Tarabily, & Abd El-Hack, 2021). For all these reasons, it is well established that compounds of marine origin have great potential for use in the pharmaceutical, food, nutraceutical and cosmetic industries (Lordan, Ross, & Stanton, 2011).

Although the use of microalgae in the diet provides great benefits, only a few species have achieved the status of Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) (Torres-Tiji, Fields, & Mayfield, 2020). *Arthrospira platensis*, commonly known as *Spirulina*, is one of the best-valued nutraceuticals today, due to its high

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content of nutrients such as proteins of high biological value and complex polysaccharides. Its nutritional richness is characterized by the presence of amino acids, vitamins, beta carotene, minerals, essential fatty acids, polysaccharides and proteins up to 70% of its dry weight (Ali & Saleh, 2012; Wang et al., 2022). In addition, it has chlorophylls, carotenoids and phycobiliproteins, which are also useful in the food industry as natural colourants (Lafarga, Fernández-Sevilla, González-López, & Acién-Fernández, 2020).

The *Chlorella* genus includes various species of unicellular green microalgae, such as *Chlorella vulgaris*. They have a high protein content (up to 61.6% of their dry weight), lipids, carbohydrates, minerals and vitamins (Ahmad, Shariff, Yusoff, Goh, & Banerjee, 2020), in addition to chlorophylls *a* and *b* as main pigments and antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid and α-tocopherol (Bhuvana, Sangeetha, Anuradha, & Ali, 2019). It is a very interesting source of polyunsaturated fatty acids (PUFAs) (Tokuşoglu & Uunal, 2003).

Both Chlorella and Spirulina have been also studied due to their valuable prebiotic character, which is related to their high content of oligosaccharides, polysaccharides and phenolic compounds (Barros de Medeiros et al., 2021). According to the ISAPP (Gibson et al., 2017), the prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit". Polysaccharides from algae can be digested and fermented by the gastrointestinal microbiota, giving rise, among other compounds, to short-chain fatty acids (SCFAs), tryptophan and organic acids (Barros de Medeiros et al., 2021). These SCFAs are involved in reducing inflammation of the intestinal mucosa and improving cardiovascular health, among other benefits related to human health (Han et al., 2019; Sanders, Merenstein, Reid, Gibson, & Rastall, 2019).

Despite all the advantages associated with microalgae exploitation as food, the economic and environmental cost corresponding to the conventional extraction and purification processes of algae metabolites continues to be an obstacle. These techniques generate many hazardous residues, use high temperatures (which in most cases can contribute to the degradation of metabolites), and are highly demanding time and energy (Esquivel-Hernández et al., 2017).

The bioactive compounds present in *Spirulina* and *Chlorella* have been extracted using different technologies (mechanical, thermal, physical, chemical and/or enzymatic) reaching different degrees of efficiency and purity depending on the applications for which they were intended (medical use, cosmetic industry, nutraceutical use, food industry) (Martí-Quijal et al., 2021). These conventional extraction methods, although effective, are very slow, in most cases showing little efficiency and selectivity, and can promote the degradation of some thermolabile bioactive (Martí-Quijal et al., 2021).

Over the last years, some alternative methods such as Pulsed Electric Fields (PEF) have been proposed as alternative extraction methods, as it affects in a less extent the quality of the extracted compounds and can be applied to obtain greater extraction efficiencies, minimize the use of solvents and, therefore, representing a more efficient and sustainable extraction alternative (Barba, Grimi, & Vorobiev, 2015; Carullo et al., 2018). This non-thermal technique is performed in a treatment chamber in which high-voltage electrical pulses are applied between two electrodes. Short pulses (1–100 μs) at different intensities (between 0.5 and 40 kV/cm) are used depending on different factors such as the type of cell, its origin, and the type of permeabilization desired. In this way, the formation of pores in the cell membrane is achieved (Martí-Quijal et al., 2021; Raso et al., 2016). Thanks to the formation of micropores in the plasma membrane of cells, the migration of components of great biological interest into the cytoplasm are promoted, with high selectivity and purity, without thermal degradation and in a short time (Poojary et al., 2016). The efficiency of this process depends on the different parameters applied in the process (strength of the electric field, treatment time, specific energy, pulse shape, pulse width, frequency and temperature), the medium (pH and conductivity) and the target cells (size, shape, type of membrane and type of envelope structure) (Arshad et al., 2020).

Therefore, the main aim of the present study is to compare conventional aqueous extraction procedures and PEF as a pre-treatment extraction technology of bioactive compounds from different types of microalgae, *A. platensis* and *C. vulgaris*. The effectiveness of both methodologies in the extraction of bioactive compounds from aqueous microalgae suspensions will be tested (concentration of carbohydrates, proteins, polyphenols, pigments) and compared in terms of functionality (total antioxidant capacity, and probiotic's growth-stimulating capability).

2. Material and methods

2.1. Conventional extraction procedure

The aqueous extraction process was applied as reference technology for the extraction of bioactive compounds from both algae samples under study, *A. platensis* and *C. vulgaris*. Powdered samples were mixed with deionized sterile water at a final concentration of 2% (*w*/w) (4 g of microalgae powder in 196 mL of deionized water). The extraction solutions were kept in glass flasks and homogenized using agitation at room temperature for 3 h, using a thermostatic orbital shaker, with a rotational speed of 500 rpm. Subsequently, the aqueous suspensions were filtered (through filter paper (Whatman No. 4)) and divided into aliquots that were maintained under −20 °C up to the moment to be analyzed. Each suspension was prepared in triplicate.

2.2. Pulsed electric Fields (PEF) assisted extraction procedure

Algae liquid suspensions were treated by PEF, using a laboratory-scale batch system PEF Cellcrack III (ELEA, Germany) located at the Faculty of Pharmacy of the University of Valencia (València, Spain). Briefly, the system consisting of a treatment chamber of 900 mL capacity, with a distance between the two electrodes fitted to 10 cm, was loaded in batches with samples of 200 g (196 g of water +4 g of microalgae sample). The electric field intensity (E, in kV/cm) was applied at 3 kV/cm, equivalent to a total specific energy input (WT, in kJ/kg) of 100 kJ/kg. The number of impaired pulses per treatment (Np) was 45, at a constant pulse duration τp (100 μs) and pulse repetition frequency (5 Hz), conditions enough to produce electroporation (Martínez, Luengo, Saldaña, Álvarez, & Raso, 2017). Temperature and conductivity were measured before and after each treatment, using a portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analytics, Weilheim in Oberbayern, Germany). PEF-treated suspensions were afterwards maintained under agitation for 3 h, at room temperature. Obtained extracts were filtered and stored at −20 °C up to the moment of analysis.

2.3. Chemical analysis of algae bioactive compounds

2.3.1. Total phenolic compounds (TPC)

Total phenolic compounds were determined using the Folin-Ciocalteu colourimetric method (Singleton, Orthofer, & Lamuela-Raventós, 1999) with some modifications (Martí-Quijal et al., 2021). Gallic acid was used as standard. In brief, 0.2 mL of microalgae extract samples, 1 mL of Folin-Ciocalteu (diluted with water at a ratio of 1:10, *v*/v) (Sigma–Aldrich, France) and 0.8 mL of a Na_2CO_3 solution (75 g/L) (VWR, France) were mixed and incubated in a water bath at 30 °C for 60 min, in the darkness. Then, the absorbances were measured at 765 nm using a spectrophotometer Perkin-Elmer UV/Vis Lambda 2. A standard calibration curve was prepared for gallic acid, to quantify the total polyphenols in the microalgae extracts. Each sample was analyzed in triplicate. Results of TPC were expressed as mg gallic acid equivalent (GAE)/g d.w.

2.3.2. Total carbohydrate content

In the present study, the phenol-sulfuric acid (PSA) spectrophotometric method was selected for the microalgae extract carbohydrate determination (Jain, Karibasappa, Dodamani, & Mali, 2017; Masuko et al., 2005). It is based on the dehydration of glucose to hydroxymethylfurfural in a hot acid medium. Hydroxymethylfurfural in the presence of phenol forms a yellow-brown complex that has an absorption maximum at 490 nm. In brief, 2.5 mL of 98% sulfuric acid and 0.5 mL of 5% phenol are added to 1 mL of the sample. After 30 min of incubation in darkness, spectrophotometric readings are made at 490 nm. All samples were analyzed in triplicate. Results were expressed as mg glucose/g d.w.

2.3.3. Total proteins

The concentration of proteins (Cp) (mg of bovine serum albumin equivalent/g of dry matter; mg BSA/g), was determined by bicinchoninic acid (BCA) microtiter plate assay (Bainor, Chang, McQuade, Webb, & Gestwicki, 2011). In this assay, protein levels are measured via the creation of a purple, $Cu^{+1}(BCA)_2$ chromophore (k_{max} = 562 nm). Initially, 25 μL of the algae extract was mixed with 200 μL of solution of BCA protein Assay Kit (Pierce Biotechnology, Inc.). The sample was incubated for 30 min at 37 °C. The absorbance (A) was measured at the wavelength of 562 nm. Bovine serum albumin (BSA) (Thermo Scientific, USA) was used for calibration. Results were expressed as mg BSA/g d.w.

2.3.4. Phycocyanin

Phycocyanin (PC) belonging to phycobiliproteins in microalgae (phycocyanin (PC) and allophycocyanin (APC)were estimated by a spectrophotometric method (Bennett & Bogobad, 1973). The samples were diluted 1/10 with distilled water and measured at 615 and 652. Phycobiliprotein concentrations (mg/mL) were calculated using the equations:

(PC mg/mL) = A_{615}–0.474(A_{652})/5.34.

(APC mg/mL) = A_{652}–0.208(A_{615})/5.09.

The total phycocyanin content was calculated by adding the APC and PC content.

2.3.5. Chlorophyll a, chlorophyll b and carotenoids

The contents of pigments in algae extracts, chlorophyll *a*, chlorophyll *b* and carotenoids, were estimated spectrophotometrically (Parniakov et al., 2015). Samples of algae extract were centrifuged for 5 min at 14000 rpm in a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France). The absorbance was estimated spectrophotometrically by analysis of filtrates at the wavelengths within the 350–800 nm range. The maximum absorbances of chlorophyll *a* (Ca), chlorophyll *b* (Cb) and total carotenoids (Cx + c) were measured at 664, 648 and 470 nm, respectively. The samples were diluted with ethanol at 1/10 to carry out the measurements. All samples were analyzed in triplicate. The concentration of pigments in the extracts was estimated using the following equations (Lichtenthaler & Wellburn, 1983):

Ca (μg/mL) = 13.36 A_{664}–5.19 A_{648}.

Cb (μg/mL) = 27.43 A_{648}–8.12 A_{664}.

Cx + c (μg/mL) = (1000 A_{470}–2.13 Ca − 97.64 Cb)/209.

where Ca, Cb and Cx + c are the concentrations (mg of pigment/g of dry matter, mg/g) of chlorophyll *a*, chlorophyll band total carotenoids, respectively.

2.4. Antioxidant capacity by means of TEAC and ORAC methodologies

To determine the total antioxidant capacity (TAC), the Trolox equivalent antioxidant capacity (TEAC) assay was used. The TEAC value (millimolar Trolox equivalents, mMTE) measures the antioxidant capacity of a given substance, as compared to the standard, Trolox (Sigma-Aldrich, Steinheim, Germany). TEAC was measured using the method proposed by Safafar, van Wagenen, Møller, and Jacobsen (2015) based on the application of the ABTS Decolorization Assay (Sigma-Aldrich, Steinheim, Germany). The ABTS radicals (ABTS•+) were generated using 440 μL of potassium persulfate (140 mM). The solution was diluted in ethanol (96% *v*/v) until the absorbance of 0.70 was reached at 734 nm. Once the radicals were formed, 2 mL of ABTS• + was mixed with 100 μL of extract and the sample was incubated for 20 min at 20 °C. The absorbance was measured at 734 nm. The results were expressed as μmol Trolox equivalents /g d.w.

The oxygen radical antioxidant capacity (ORAC) method was evaluated, following the methodology proposed by Al Khawli, Martí-Quijal, Pallarés, Barba, and Ferrer (2021). Sodium fluorescein and an AAPH working solution were prepared at a concentration of 0.015 mg/mL and 120 mg/mL, using a 75 mM phosphate buffer (pH 7). In a 96-well microplate, 50 μL of the sample extract was mixed with 50 μL of fluorescein, and the mixture was pre-incubated at 37 °C for 10 min. Then, 25 μL of the AAPH solution was added, and the plates were immediately placed in the VICTOR^3 1420 multilabel plate counter reader (PerkinElmer, Turku, Finland), and the fluorescence was recorded every minute for 60 min under an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The phosphate buffer was used as a blank, and Trolox (100 μM) was used as the antioxidant standard. Each extract was analyzed in five replicates, and the differences in areas under the curve (AUCs) of the fluorescein decay between the blank and the samples were used to calculate the antioxidant activity. The results were expressed as μmol Trolox equivalents/g d.w.

2.5. Probiotic's growth-stimulating capability of algae extracts

For the present study, the lactic bacteria *Lactobacillus rhamnosus* GG (CECT 288) was used as a probiotic, which was acquired from the Colección Española de Cultivos Tipo (CECT). The lyophilized culture was reactivated following the instructions of the CECT, all under sterile conditions. Briefly, the reactivation of the lyophilized culture consisted of the inoculation of the L. *rhamnosus* GG in 20 mL of sterile Man Rogose Sharpe Broth (MRSB) liquid medium. The rehydrated culture was kept shaking for 3 h at 30 °C. After that time, 20 mL of rehydrated culture was transferred to a flask containing 480 mL of sterile MRSB, which was kept under agitation in anaerobic conditions, at 30 °C for 48 h. Later the culture was centrifuged (8000 x*g*, 10 min, 25 °C). After the removal of the supernatant, the pellet was suspended in a sterile MRSB medium and subjected to a new cycle of centrifugation. The pellet from the second centrifugation was resuspended in 50 mL of a solution of MRSB and glycerol [80:20] (*v*/v). From the glycerinated suspension, 2 mL cryovials were filled, which were kept frozen (−80 °C) constituting the stock until the moment of use. Stock concentration was determined by thawing 3 vials and counting using the serial decimal dilution procedure (in 1% (*w*/*v*) buffered peptone water) (Sigma-Aldrich, Barcelona, Spain), and plating on sterile MRS Agar. The quantitative results obtained from the 3 vials were used to estimate a mean concentration: $5 \pm 0.4 \times 10^{10}$ CFU/mL. To carry out the growth-stimulating capability study, a preculture of L. *rhamnosus* was first prepared from the stock vials. To do this, 9.9 mL of sterile MRSB were inoculated with 100 μL of glycerinated from L. *rhamnosus* and incubated at 37 °C under anaerobic conditions for 24 h. In order to evaluate the prebiotic potential associated with *Chlorella* and *Spirulina* extracts, 5 different media were prepared, including control samples exclusively MRSB reference media; [1:1] (*v*/v) suspension including [MRSB: *Chlorella* aqueous extract]; [1:1] suspension including [MRSB: PEF *Chlorella* extract]; [1:1] (v/v) suspension including [MRSB: *Spirulina* aqueous extract]; [1:1] (v/v) suspension including [MRSB: *Spirulina* PEF extract]. All media was inoculated with L. *rhamnosus* precultured to an initial concentration of 10^4 CFU/mL. Growth dynamics for *L. rhamnosus* ($\log_{10}$ CFU/mL) were registered at constant time intervals, from 0 to 72 h.

2.6. Metabolomic profile analyzed by nuclear magnetic resonance (NMR)

Nuclear Magnetic Resonance (NMR) was used in the present study to

register the metabolomic profile of L. *rhamnosus* during the fermentation of processed *Chlorella* and *Spirulina* matrices (Tomita, Saito, Nakamura, Sekiyama, & Kikuchi, 2017). Samples were analyzed in the General Service of Metabolomics, University of Valencia. The algae-processed extracts were taken as aliquots of 2 mL and stored at −80 °C up to the moment of the analysis. Samples were centrifuged at 12000 rpm, for 10 min before being analyzed. In brief, 140 μL of each one of the supernatants was diluted in 560 μL of potassium buffer (KPi). After the centrifugation step, the clarified supernatant was transferred to the NMR device (probe QXI 5.0 mm O.D. × 103.5 mm; Norell, Landisville, NJ) to be analyzed. The NMR spectrum was analyzed by means of an Avance-500 spectrometer (Bruker BioSpin, Karlsruhe, Germany) with a CPDUL CryoProbe (Bruker BioSpin), with proton/carbon frequencies of 600 MHz. The SpinAssign - Platform for RIKEN Metabolomics (http://prime.psc.riken.jp/) program was used for the identification of the different metabolites present in the sample, in comparison with standard compounds. The base data used in the identification and quantification of present metabolites were "The Human Metabolome Database" (http://www.hmdb.ca/) and the "Biological Magnetic Resonance Data Bank" (http://www.bmrb.wisc.edu/).

2.7. Statistical analysis

The results were expressed as the measurement averages ± standard deviation. To study the differences between the samples and treatments analysis of variance (ANOVA) was used. A significance level of 95% ($p < 0.05$) was considered. Finally, the LSD (Least Significant Differences) test was performed to determine the differences between the means of the obtained values. The statistical analysis was performed with the software SPSS (SPSS Inc., Chicago, Ill., USA).

3. Results and discussion

3.1. Total carbohydrate and protein extraction

It is well established that microalgae from different genera, such as *Chlorella, Dunaliella, Chlamydomonas, Scenedesmus* and the cyanobacterium *Spirulina,* are specifically rich in complex carbohydrates, representing >50% of their dry mass. To prone, the extraction of this high carbohydrate content, promoting the conversion of carbohydrates into glucose by gastrointestinal (GI) microbiota, algae raw materials were subjected to different pre-treatment methods. In the present study, and as can be seen in Fig. 1, no significant differences were detected between the extracted carbohydrate fraction by conventional method (aqueous extraction), in both, *Chlorella* and *Spirulina*. Contrarily, significantly improved extraction results ($p < 0.05$) were achieved due to PEF treatment in both matrices (~ 40 mg/g).

According to the obtained results, PEF technology applied at 100 kJ/kg (electric field strength, E = 3 kV/cm) has been able to increase the carbohydrate extraction by close to a 100% extra fraction, compared to the conventional extraction procedure (conventional extraction = 20 mg/g; PEF extraction = 40 mg/g). In this regard, several previous studies have dealt with the comparison between different aqueous and innovative extraction methods using algae as a raw material. Certainly, it has been defined that conventional aqueous extraction methods, specifically long extraction time and high temperatures are factors positively influencing the extraction yield of carbohydrates from microalgae (Pez Jaeschke, Rocha Teixeira, Damasceno Ferreira Marczak, & Domeneghini Mercali, 2021). Despite this, a detrimental nutritional value can be impaired due to these intensive treatment conditions. Among the studied alternative extraction methods, relevant advances have been observed over the last decades, mainly concerning PEF technology, applied at ambient temperature on liquid algae suspensions, increasing the recovering potential of bioactive from microalgae (Carullo et al., 2018; Pez Jaeschke et al., 2021; Zhou et al., 2022). Pez Jaeschke et al. (2021) studied the PEF potential for the extraction of bioactive compounds from *Spirulin*a, considering that the highest PEF applied energy levels (100–122 kJ/kg) were the most effective increasing algae cell wall damage and consequently, favouring the diffusion and extraction of the bioactive components. A previous study carried out by Carullo et al. (2018), also agrees with the application of PEF treatments at energy values in the range of 60–100 kJ/kg to maximize the total carbohydrate extraction from *C. vulgaris* liquid suspension.

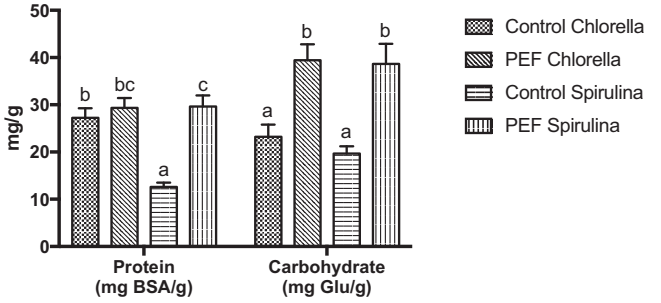

Fig. 1. Total protein (mg BSA/g; BSA: Bovine Serum Albumin) and carbohydrate content (mg Glu/g; Glu: Glucose) of extracts obtained by conventional aqueous and PEF technologies. Different superscript letters for each parameter, are indicating significant differences (p-value <0.05) in between obtained values.

Regarding the obtained results for protein extraction yield, the efficacy in protein recovery was significantly ($p < 0.05$) conditioned by both, the matrix under study and the extraction treatment applied. Regarding the matrix, when *Chlorella vulgaris* is processed, no significant differences in protein recovery were detected by conventional and PEF extraction methods (~30 mg/g) applied. However, the protein recovered fraction in *Spirulina* processed material was significantly higher (300% increase) when PEF treatment was applied. PEF resulted in similar values of protein extraction in both matrices (30 mg/g), meanwhile, conventional aqueous extraction was more effective to recover proteins from *Chlorella* than from *Spirulina.*

This fact is related to the different compositions of the cell wall in the microalgae *Chlorella vulgaris* and the cyanobacteria *Arthrospira platensis*. *C. vulgaris* cell walls are characterized by high levels of polysaccharides in the cell wall structure, such as glucose and mannose, and complex sugars such as arabinose, galactose, rhamnose, mannose and xylose. Also, algaenan is remarkable as an extremely resistant biopolymer, composed of long ω-hydroxy fatty acids chains linked by several types of chemical bonds. On the other hand, the cyanobacterium *Spirulina* contains peptidoglycans in its cell wall. This differential cell wall composition is important in the design and optimization of extraction treatments, mainly considering that PEF just generates slight-moderate electroporation of the cell, which means that high molecular weight proteins, or strongly linked proteins to the cellular structure, are not completely delivered to the media. Selective extraction of carbohydrates, and proteins by PEF, due to the permeabilization of the microalgae envelope, could be optimized by fitting PEF intensity applied and favouring the rapid diffusion and delivery of intracellular ionic compounds to the aqueous phase. The intensity of PEF treatment should be modulated to optimize protein extraction yield from these two different matrices. In this sense, Carullo et al., (2018) applied an electric field strength of 20 kV/cm on a *C. vulgaris* aqueous suspension to achieve a close to 30 mg/g protein extraction ratio. Even more, recently, Zhou et al. (2022) concluded that a combined multistep process including PEF and pressurized liquid extraction (PLE) multistep application, is the most effective option for maximizing *Spirulina* proteins extraction yield, polyphenols and pigments. According to Zhou et al. (2022), this combined multistep process PEF + PLE increased the protein and polyphenol values of *Spirulina* extracts by 1328% and 979%, respectively.

3.2. Pigments extraction: chlorophyll a, chlorophyll b and carotenoids

Fig. 2 shows the results obtained regarding the pigment extraction from *C. vulgaris* and *Spirulina,* by the application of the control process (conventional aqueous extraction) and PEF. As can be seen in the figure, PEF treatment enhanced the efficiency in pigment extraction from both, *Chlorella* and *Spirulina*, independently of the pigment under consideration.

Although *Chlorella* is recognized as the "*emerald food*" due to its high content in chlorophylls, in the present study, a maximized efficiency has been obtained regarding the extraction of chlorophyll *a* and b, and carotenoids from *Spirulina,* using both, conventional and PEF extraction methods. Previous studies have obtained larger concentrations of extracted chlorophylls and carotenoids from *Chlorella*, using conventional solvent extraction methods (Sumanta, Haque, Nishika, & Suprakash, 2014). To increase the efficiency of the pigment extraction, temperatures in the range of 50–65 °C have been conventionally applied, since at these temperatures cell walls become less resistant, increasing pigments delivery from structures, and also increasing the solubility of pigments into solvents (in this case water). However, above 70 °C chlorophylls and carotenoids can be affected, so optimization of parameters such as biomass-to-solvent ratio, type of solvent and temperature, should be fitted to maximize the pigments extraction from microalgae and cyanobacteria.

For the PEF extraction technique, it can be observed that enhanced rates of PEF pigment extraction from both matrices were observed in the present study (Fig. 2), achieving maximum incremented values of 66% in chlorophyll *a* extraction from *Chlorella,* and 400% increment in extraction yield for carotenoids from *Chlorella* and *Spirulina,* in relation to control aqueous extraction procedure. Luengo, Condón-Abanto, Álvarez, and Raso (2014) applied PEF intensities of 15 kV/cm to significantly increase the extraction yield of bioactive compounds from *Chlorella*. Even more, by increasing PEF treatment to 20 kV/cm and treatment time to 75–150 μs, the extraction yield for carotenoids, and chlorophylls *a* and b increased 1.2, 1.6, and 2.1 times, respectively, in relation to the control extraction procedure with ethanol (Luengo et al., 2014). According to previous studies, PEF as a selective physical extraction method could be optimized regarding the magnitude of electroporation achieve to maximize pigments extraction from functional matrices, not affecting thermally sensitive bioactive compounds as the main advantage.

3.3. Total phenolic compounds (TPC) and phycocyanin

Fig. 3 shows the results obtained for TPC and phycocyanin from both matrices, using the conventional aqueous extraction method and PEF technology. As can be seen in the figure, the extraction yield differed ($p < 0.05$) according to the matrix used and the treatment applied. The

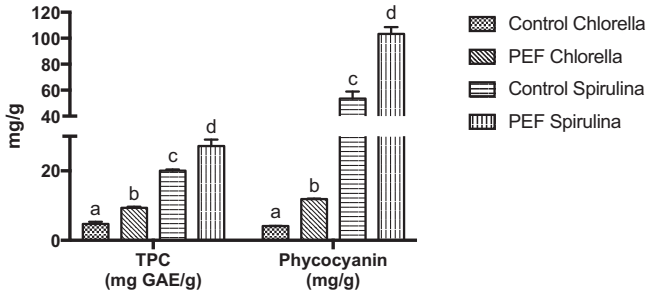

Fig. 3. Total phenolic compounds (TPC) expressed as mg GAE/g (Gallic Acid Equivalent) and total phycocyanin values expressed as mg/g. Superscript letters are indicating significant differences (p-value <0.05) between values inside each analytic group.

extraction of TPC and phycocyanin was significantly higher when extracted from *Spirulina* than from the *Chlorella* matrix. The extraction of TPC and phycocyanin from *Chlorella* was limited to values <5 mg/g using a conventional aqueous extraction procedure while PEF technology slightly increased these bioactive compounds extraction from *Chlorella* aqueous suspension (< 10 mg/g concentration of TPC and phycocyanin). Meanwhile, the extraction of TPC and phycocyanin from *Spirulina* achieved in all cases values in the range [20–40] mg/g and [60–100] mg/g, respectively. Regarding the comparison between technologies, it can be concluded that independently of the processed matrix, PEF treatment increases the TPC and phycocyanin extraction rates, achieving enhanced values of 50% in the case of TPC, and close to 70% in the case of phycocyanin extraction from *Spirulina*, in relation to the conventional aqueous extraction procedure.

TPC extracted from different microalgae species such as *Nannochloropsis gaditana, Phaeodactylum tricornutum*, or *Tetraselmis suecica*, achieved values in the range of 22–40 mg/g GAE by means of the application of solvent extraction procedures. Zhou et al. (2022) achieved values of TPC in *Spirulina* aqueous extracts processed by PEF close to 20 mg/g using similar operating conditions to the applied in the present study (3 kV/cm, 100 kJ/kg). Regarding phycocyanin, specifically relevant light-blue-water soluble natural pigment found in *C. vulgaris* and *A. platensis,* which exhibits healthy-functional properties, remains to date under the focus of different research technologies application to improve and increase the efficiency extraction (extraction yield and purity) (Gorgich et al., 2020). Previous studies by (Martínez et al., 2017) achieved an optimized method by PEF to maximize the phycocyanin extraction from *Spirulina,* applying an electric field strength close to 10 kV/cm and temperatures in the range of 10–40 °C (140 mg/g d.w.). In the present study, lower electric field strength (3 kV/cm) applied at room temperature was enough to achieve close to 120 mg/g (d.w.) of

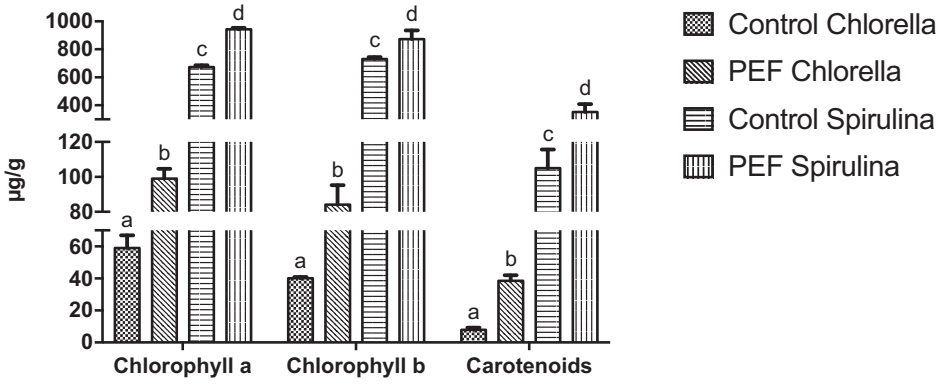

Fig. 2. Pigment extraction results (μg/g) under aqueous conventional extraction method and pulsed electric field (PEF) treatment. Different superscript letters are indicating significant differences (p-value <0.05) inside each considered group, chlorophyll *a*, chlorophyll *b*, and carotenoids.

extracted phycocyanin. These results point out the selectiveness and suitability of PEF technology as an efficient method in phycocyanin extraction yield increment in *Spirulina, Chlorella* and *Nostoc* genus, being a respectful method with its functional properties (not affected by temperature). Despite this, PEF technology would be not suitable as extraction pre-treatment to process other matrices such as *Oscillatoria okeni*, due to the cell wall's higher resistance (Chittapun, Jonjaroen, Khumrangsee, & Charoenrat, 2020).

3.4. Total antioxidant capacity (TAC)

One of the main valuable functional properties of some bioactive compounds extracted from microalgae is related to their antioxidant character. Fig. 4 shows the results obtained regarding the TAC measured by means of ORAC and TEAC in vitro methods in extracts from *C. vulgaris* and *A. platensis* fractions, after conventional aqueous and PEF treatments. Significant higher values of TAC expressed as μmol Trolox/g were found in all processed matrices by means of the ORAC methodology, indicating interesting ORAC values for both, control and PEF-treated matrices. By using ORAC measurements it can be established the scale regarding the antioxidant capacity of treated matrices in the following order: PEF Chlorella > PEF Spirulina > Control Spirulina > Control Chlorella. The PEF pretreated *Chlorella* suspension showed the highest TAC (close to 700 μmol Trolox/g), being also *Spirulina* conventionally treated and PEF treated in values in the range [500–700] μmol Trolox/g. Regarding the TEAC methodology, the *Spirulina* PEF-treated matrix showed the highest antioxidant capacity against ABTS reactive species. These maximum values of antioxidant capacity detected by ORAC showed a specifically good correlation with the extraction yields obtained for pigments, proteins, and polyphenols in PEF-treated matrices, *Spirulina* and *Chlorella* (*sections 3.1 and 3.3.*)

In a previous study, Zhou et al. (2022) also obtained higher TAC values for PEF-treated *Spirulina* samples by means of ORAC methodology (1500 μmol Trolox/g), meanwhile, the detection of TAC by TEAC was close to 600 μmol Trolox/g in PEF *Spirulina* aqueous extracts. The optimization of extraction methods to promote the fragmentation of *Spirulina* and *Chlorella* cells is crucial to maximize the diffusion of bioactive compounds (proteins, pigments, polyphenols) to the aqueous phase and ensure the antioxidant maximized capacity of the recovered ingredient.

3.5. Probiotic's growth-stimulating capability and metabolomic profile in fermentation

In the present paper, it is not our objective to demonstrate the prebiotic effect of microalgae. This prebiotic effect has been extensively attributed to *C. vulgaris* (Hyrslova et al., 2021). Our objective was to test

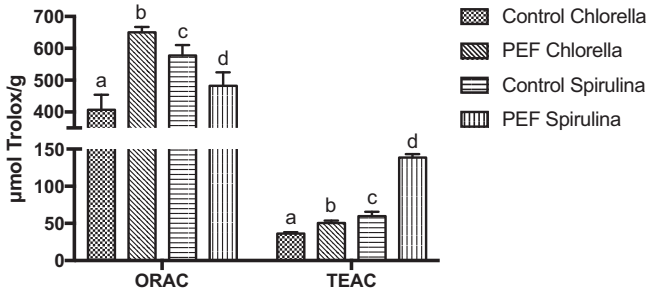

Fig. 4. Total antioxidant capacity (TAC) results obtained by means two different in vitro analytic techniques: Trolox equivalent antioxidant capacity (TEAC) method (μmol Trolox/g) and oxygen radical antioxidant capacity (ORAC) (μmol Trolox/g). Superscript letter for each column is corresponding with significant differences (p-value <0.05) between the values obtained for each analysis.

the maintenance of the capability of *Chlorella* and *Spirulina* extracts to enhance the *Lactobacillus rhamnosus* after processing. Fig. 5 shows the probiotic's growth-stimulating capability of *Chlorella* and *Spirulina* extracts after aqueous and PEF extraction. The L. *rhamnosus* growth ($\log_{10}$ CFU/mL) pattern in reference media (MRSB) was compared with growth boost in MRSB supplemented with algae-processed matrices [1:1] (*v*/v). Additionally, the metabolomic profile obtained from microbial fermentation of different substrates was also evaluated by NMR.

Regarding the probiotic's growth-stimulating capability observed under the studied conditions, it can be concluded that in that media supplemented with *A. platensis* subjected to the conventional aqueous extraction method, the stimulation of L. *rhamnosus* growth was equivalent to 1 $\log_{10}$ cycles, just after 24 h incubation under optimum conditions (anaerobiosis, 30 °C) (achieving 10.04 ± 0.37 $\log_{10}$ CFU/mL). When PEF technology was applied as a pre-treatment extraction process, the growth-stimulating capability associated with *A. platensis* promoting L. *rhamnosus* growth was affected. A probiotic's growth-stimulating capability, also close to 1 $\log_{10}$ cycle (achieving 9.77 ± 0.32 $\log_{10}$ CFU/mL), was observed after 72 h incubation in media supplemented with *A. platensis* extract processed by PEF.

L. rhamnosus growth was also promoted in MRSB-supplemented media with Spirulina pre-treated by PEF, but in this case, a longer incubation time (72 h versus 24 h) is required to start showing this increase. Beheshtipour, Mortazavian, Haratian, and Darani (2012), showed that the positive effects of microalgae on the viability of probiotics can be attributed to the reason that microalgae provide higher nutritious and stimulatory media for lactic acid bacteria and probiotic bacteria and stimulate their growth and activity. From these substances, exopolysaccharide, adenine, hypoxanthine, free amino acids, and essential vitamins and minerals can be mentioned. On the other hand, no stimulating capability effect on L. *rhamnosus* growth was detected in the MRSB media supplemented with *C. vulgaris* (8.63 ± 0.08 $\log_{10}$ CFU/mL), independently of the technology used to obtain the extract. This fact can be attributed to the *A. platensis* higher buffering capability (compared with *C. vulgaris*) thus leading to a more efficient probiotic's growth-stimulating capability until reaching the final pH of fermentation (pH ~ 4) as it was similarly reported previously (Beheshtipour et al., 2012).

In *Section 3.1.,* carbohydrates content extracted from *Chlorella* and *Spirulina* resulted specifically higher when these algae matrices were processed by PEF. The selective extraction of bioactive compounds from partial electroporation of microalgae and cyanobacterium cell walls promoted carbohydrate extraction (Zhou et al., 2022). Among these extracted carbohydrates, the high content of polysaccharides (PS) and their derivatives (non-digestible oligosaccharides, NDOs) extracted from *Spirulina,* are responsible for the observed growth-stimulating capability on L. *rhamnosus*. This probiotic's growth-stimulating capability was detected just after 24 h incubation when *Spirulina* was subjected to an aqueous extraction, meanwhile, the PEF *Spirulina* extract required 72 h to start reaching the same growth-stimulating capability on *L. rhamnosus* (1 $\log_{10}$ cycle). The different structures of the extracted carbohydrates can be the main factor explaining the probiotic's growth-stimulating capability between aqueous and PEF *Spirulina* extracts (Ścieszka & Klewicka, 2020). Ścieszka & Klewicka, (2020) also pointed out the increase in the specific growth rate dynamics of *Lactobacillus* spp. biomass when cultivated under the presence of microalgae. By comparison of technologies, aqueous extraction can favour the migration and solubilization of complex carbohydrates into the aqueous phase, meanwhile, the PEF technology promotes not only the electroporation and easy extraction of complex carbohydrates but also the partial hydrolyzation of these polymeric compounds into oligomeric or monomeric sugars. It seems that under PEF cyanobacterial cell wall electroporation, also partial hydrolysis of a polymeric form of intracellular or cytoplasmic reserve α-glucans, such as starch, amylopectin and/or glycogen, occurs. This fact is promoted during fermentation, being the utilization of sugars as follows: (i) first utilization of complex carbohydrates (aqueous *Spirulina* extract, just 24 h required to show prebiotic effect), and (ii)

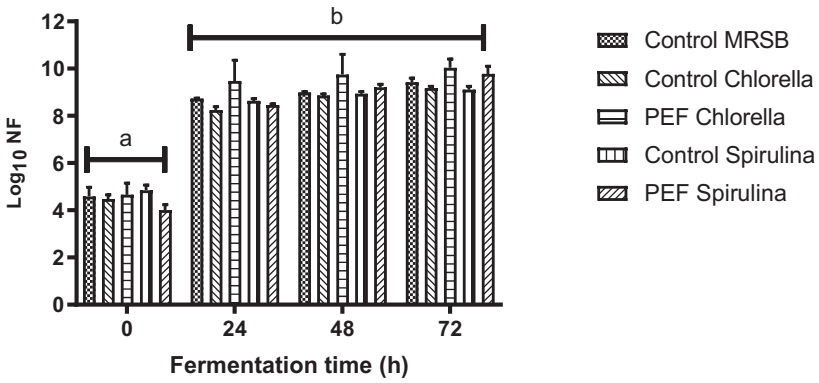

Fig. 5. Results of the dynamic bacterial growth for *Lactobacillus rhamnosus* corresponding with the prebiotic effect detected in each different media: MRSB, Man Rogose Sharpe Broth (reference media); MRSB supplemented with Chlorella extract obtained by conventional aqueous extraction; MRSB supplemented with Chlorella extract obtained by PEF extraction; MRSB supplemented with Spirulina extract obtained by conventional aqueous extraction; MRSB supplemented with Spirulina extract obtained by conventional PEF extraction treatment.

secondly, utilization of oligomers and monomers (PEF Spirulina extract, just 72 h required to show prebiotic effect).

Table 1 shows the metabolomic results obtained due to the fermentation of algae substrates by L. *rhamnosus* in the different formulated media. Via fermentation, significant differences ($p < 0.05$) were observed in generated metabolites by bacteria via enzymatic hydrolysis. In this task, both, the extractability, and bioavailability of nutrients in the aqueous and PEF extracts (proteins, carbohydrates, polyphenols, pigments, vitamins) and the biotransformation that bacteria make from it, are conditioning the final nutraceutical characteristic properties of the processed and fermented *Chlorella* and *Spirulina* extracts as ingredients. Short-chain fatty acids (SCFAs), amino acids, organic acids and carbohydrates were identified and quantified by NMR spectroscopy after fermentation via a heterofermentative pathway. SCFAs possess antimicrobial activity and promote homeostasis that affects the integrity of the colonic epithelium and metabolic, immunological, and central nervous system activity (Tan et al., 2014).

Table 1
Main metabolites (μg/mL) registered by Nuclear Magnetic Resonance (NMR) spectroscopy in *Lactobacillus rhamnosus* fermentation of *Chlorella vulgaris* and *Arthrospira platensis* (Spirulina) extracts supplementing the Man Rogose Sharpe Broth (MRSB) media (after 72 h incubation under optimum conditions).

Metabolite	MRSB Control	MRSB + Control *Chlorella*	MRSB + PEF *Chlorella*	MRSB + Control *Spirulina*	MRSB + PEF *Spirulina*
Carbohydrates					
Glucose	4.20 ± 0.15^{a}	1.92 ± 0.05^{b}	2.89 ± 0.07^{ab}	7.82 ± 0.18^{c}	6.65 ± 0.03^{c}
Fructose	1.04 ± 0.08^{a}	1.16 ± 0.03^{a}	1.11 ± 0.03^{a}	1.90 ± 0.22^{a}	1.49 ± 0.25^{a}
Arabinose	0.13 ± 0.01^{a}	0.15 ± 0.01^{a}	0.16 ± 0.01^{a}	0.11 ± 0.05^{a}	0.32 ± 0.08^{a}
Short Chain Fatty acids (SCFAs)					
Butirate	0.11 ± 0.01^{a}	0.14 ± 0.03^{a}	0.14 ± 0.02^{a}	0.24 ± 0.03^{a}	0.25 ± 0.01^{a}
Propionate	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}	0.04 ± 0.01^{a}	0.04 ± 0.005^{a}	0.14 ± 0.01^{a}
Acetate	18.44 ± 2.23^{a}	18.65 ± 1.27^{a}	19.31 ± 1.12^{a}	24.97 ± 3.22^{b}	33.35 ± 3.62^{c}
Isobutirate	0.34 ± 0.04^{a}	0.28 ± 0.05^{a}	0.16 ± 0.01^{a}	0.48 ± 0.09^{a}	0.63 ± 0.09^{a}
Aminoacids					
Arginine	1.08 ± 0.02^{a}	1.01 ± 0.04^{a}	0.70 ± 0.08^{a}	0.62 ± 0.02^{a}	1.75 ± 0.02^{a}
Phenylalanine	0.05 ± 0.001^{a}	0.04 ± 0.09^{a}	0.02 ± 0.001^{a}	0.07 ± 0.001^{a}	0.02 ± 0.001^{a}
Organic acids					
Lactate	0.77 ± 0.05^{a}	0.62 ± 0.01^{a}	0.92 ± 0.07^{a}	1.20 ± 0.36^{a}	1.49 ± 0.12^{a}
Succinate	0.08 ± 0.007^{a}	0.10 ± 0.01^{a}	0.10 ± 0.03^{a}	0.14 ± 0.04^{a}	0.15 ± 0.03^{a}
3-Phenyllactic acid (PLA)	0.01 ± 0.003^{a}	0.02 ± 0.01^{a}	0.10 ± 0.02^{a}	0.05 ± 0.002^{a}	0.61 ± 0.02^{a}

Statistical differences related to the metabolite (by row) are represented by different lower-case letters in the table ($p < 0.05$).

According to *Section 3.1.* the highest carbohydrate contents were obtained in *Spirulina* and *Chlorella* PEF extracts. Furthermore, and according to the NMR spectrum, glucose content was significantly higher in *Spirulina* extracts fermented by L. *rhamnosus* than in *Chlorella* extracts. By means of the NMR spectrum, it can be concluded the different utilization of carbohydrates by L. *rhamnosus* depends on the composition of the fermented media, which is also related to the marked probiotic's growth-stimulating capability detected for *Spirulina-supplemented* media. Meanwhile in MRSB media supplemented with *Chlorella* extract, the glucose content is reduced (range 2–3 μg/mL), in MRSB media supplemented with *Spirulina,* glucose content is increased (range 7–8 μg/mL) in relation to MRSB reference media (glucose 4.20 ± 0.15 μg/mL).

Complex extracted carbohydrates from microalgae and cyanobacterium are depolymerized and used as a carbon source during bacterial growth. According to Sauer Leal et al. (2017) carbohydrates in *Chlorella* extracts are specifically rich in glucose, rhamnose, xylose, and galactose; meanwhile, *Spirulina* resulted in a higher yield of glucose compared with the other hexoses, pentoses, and deoxysugars. Additionally, and specifically in the case of MRSB media supplemented with *Spirulina* extracts, exopolysaccharides (EPS) can be synthesized by the probiotic bacteria. The EPS are biopolymers consisting of a single monosaccharide such as glucose or fructose (homoEPS), or at least two different monosaccharides connected at different positions (heteroEPS) (Lee, Jeon, Yoo, & Kim, 2021; Tomita et al., 2017).

The major content of glucose and arabinose in the media after L. *rhamnosus* fermentation (see Table 1) is indicating both: (i) a higher extraction rate of complex carbohydrates from the matrix via the PEF electroporation of cell walls, and (ii) the promotion of EPS synthesis by L. *rhamnosus* in *Spirulina* supplemented media. This conclusion represents a positive impact of PEF technology on *Spirulina* extracts, since these extracts showed not only a significant probiotic growth stimulating capability, but also accumulate beneficial secondary metabolites from fermentation that have been described to be anti-cancerogenic, anti-inflammatory, antioxidant, anti-viral, anti-diabetic, and with exerted immunomodulatory activities (Tomita et al., 2017).

Also, regarding the SCFAs profile, it can be appreciated a significant ($p < 0.05$) higher content of butyrate, propionate, acetate, and isobutyrate in fermented MRSB media supplemented with *Spirulina* PEF extracts. Acetate was the predominant compound in all the studied media after 72 h of fermentation, being the acetate production increasing up to 83% ratio in MRSB media supplemented with PEF *Spirulina* extract in relation to MRSB not supplemented media. Tan et al., (2014) showed that acetate, propionate, and butyrate, have highlighted their effects on various systems both at the cellular and molecular levels.

Indeed SCFAs or their deficiency may affect the pathogenesis of a diverse range of diseases, from allergies and asthma to cancers, autoimmune diseases, metabolic diseases, and neurological diseases. This reinforces the idea that an increase in these SCFAs, thanks to the effect on the viability of probiotics such as L. *rhamnosus*, produced by Spirulina and Chlorella, can have great benefits for the organism. Furthermore, products of primary fermentation could be further converted through diverse pathways, called secondary fermentation, such as different forms of organic acids. Lactate can be oxidized and reduced to other forms of organic acids with valuable healthy properties. According to the obtained results (see Table 1) the lactate content was significantly higher in the MRSB media supplemented with Spirulina extracts, after 72 h fermentation. In fact, lactate production was specifically higher in media supplemented with PEF extracts, in both, *Chlorella* and *Spirulina* fermented substrates. Meanwhile, in *Chlorella* PEF extracts, the lactate content during fermentation increases up to 20% in relation to fermentation of MRSB reference media, and the lactate values in PEF *Spirulina* extract fermented by L. *rhamnosus* increased up to 93% compared with values in MRSB reference media.

Special mention can be done regarding the 3-phenyl lactic acid (PLA) content present in the different studied fermented media, depending on the microalgae and the extraction technology applied. PLA has valuable antibacterial and antifungal properties, and it is obtained from phenylalanine via enzymatic reduction (Lee et al., 2021). Regarding the fermented extracts, PLA content was higher in PEF pre-treated fermented samples, with values 10-fold higher in MRSB media supplemented with PEF *Chlorella* extract and 60-fold higher in MRSB media supplemented with *Spirulina* PEF extract (compared to fermented MRSB reference media).

4. Conclusions

The assessment of PEF technology as extraction pre-treatment of bioactive compounds from both, microalgae *Chlorella vulgaris* and cyanobacterium *A. platensis* led to promising conclusions regarding the added value of obtained extracts to be used as functional ingredients. The applied PEF conditions (3 kV/cm, 75 pulses) on aqueous suspensions of studied microalgae, showed to have a significant positive effect on the extraction of proteins, carbohydrates, chlorophylls, carotenoids, polyphenols and phycocyanin, with increased values specifically when *Spirulina* matrix was treated. These extractions with higher yields are due to the processes of electroporation and permeabilization of the cell membrane of the microalgae, including the disruption and breakage of cells produced by electrical pulses.

Improved and valuable antioxidant (500–700 μmol Trolox/g) properties and probiotic's growth-stimulating capability (1 $\log_{10}$ cycle increased L. *rhamnosus* growth) were also found in microalgae extracts by means of PEF processing as extraction technology. These results are evidencing the applicability of PEF technology under optimized conditions as an alternative extraction method that is more efficient, faster, and more sustainable, than other types of extraction methods (i.e., conventional thermal and chemical extraction procedures).

Credit author statement

All the authors contributed in the same way to the development of the research presented in this article.

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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