Review

Algal proteins: Production strategies and nutritional and functional properties

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HIGHLIGHTS

- Potential use of algal proteins as sustainable alternative to animal-based proteins.
- Algae-based foods placed on global market.
- Strategies to enhance protein content of macro- and microalgae.
- Nutritional and functional properties of algal biomass and its protein extracts.
- Integrated production of algal proteins towards "greener" process approaches.

GRAPHICAL ABSTRACT

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ABSTRACT

Animal-based proteins are the most consumed worldwide given their well-balanced nutritional composition. However, the growing demand for animal proteins will not be sustainable due to their low conversion efficiency and high environmental footprint. Specific consumers' dietary restrictions and modern trends emphasize the importance of finding alternative sustainable non-animal sources to meet future food (and, in particular, protein) global needs. Algal biomass is considered a relevant alternative, presenting advantages over terrestrial biomass such as higher growth rate, low water consumption, no competition for arable land, carbon–neutral emissions, and production of numerous bioactive compounds. This review provides an overview of recent research advances on algae as source of proteins, including production strategies from relevant protein-producing species. Particular emphasis will be given to algae protein current applications and forthcoming challenges of their use. Nutritional and functional aspects of algae biomass or its protein-enriched fractions will be overviewed.

1. Introduction

Estimates suggest that world population will reach 10 billion within the next 30 years, increasing food demand by 70%. This fact is in agreement with Food and Agriculture Organization (FAO) statements, which claim that global meat production is expected to double by 2050, making the search for new food sources and alternative food systems an objective of the utmost importance (Dopelt et al., 2019). Also, 30% of the Earth's land resources are involved in livestock production, contributing to several environmental issues (e.g., land degradation, water pollution, overgrazing, and desertification). Animal-based proteins, such as those from dairy and meat, are still the most consumed and nutritionally well-balanced. However, their growing demand will not be sustainable given the low conversion efficiency and high environmental footprint during their production process. In spite of the seek for new protein sources is becoming increasingly inevitable, the establishment of

food needs cannot be based solely on nutritional and safety factors. Sustainability is also a pillar to take into consideration. Consequently, alternatives to meat protein have been studied and evaluated, including insect, microbial, vegetable and algae proteins (Fasolin et al., 2019; Kazir et al., 2019). Algal biomass arises as a potential source of non-animal protein, being envisaged as a relevant alternative due to several advantages over terrestrial biomass such as higher growth rate, low water consumption (or even growth in seawater), no competition for arable land, carbon–neutral emissions, bioremediation (waste treatment), and the possibility of producing a wide range of bioactive compounds. The term algae, although very wide, can be defined as a functional group of autotrophic photosynthetic, aquatic, and non-embryophytes organisms – including both bacterial (cyanobacteria) and eukaryotic organisms with simple reproductive structures. They can be unicellular, colonial, present filaments or be composed of simple tissues (Guiry, 2012). Microalgae are ubiquitous unicellular organisms. Due to their ability to adapt to different environmental conditions, microalgae can be found in all Earth's ecosystems, from the desert to polar seas (Geada et al., 2017). Since most of the microalgal production is based on batch cultivation systems, typically entailing low productivity rates, high harvesting costs, and irregular product quality, this is hindering their entry in the food market. For human consumption, *Arthrospira*, *Chlorella*, *Aphanizomenon*, *Dunaliella*, and *Haematococcus* are the most widely used, even though not all of them are approved by the European Food and Safety Authority (EFSA), limiting its consumption as food in Europe (Niccolai et al., 2019). Microbial proteins from unicellular and multicellular algae, also known in particular cases as single-cell protein (SCP), present interesting benefits from the production and nutritional point of view. SCPs are dried cells (usually from bacteria, fungi or microalgae) used as protein supplements to food and feed. In the particular case of microalgae, they also constitute an important source of bioactive compounds and, therefore, might be a promising alternative to integrate future human food choices (Sathasivam et al., 2019). Macroalgae or seaweed are macroscopic, eukaryotic, photosynthetic, and marine algae. Structurally, they present a thallus, reproductive "sori" and gas bladders, and/or stipes (Littler and Littler, 2011). They can be classified into three higher taxa according to their pigmentation: red (*Rhodophyta*, around 6000 species), brown (*Ochrophyta*, about 1750 species), and green (*Chlorophyta*, approximately 1200 species) (Guiry, 2012).

This review provides an overview of recent research on algae (both macro- and microalgae) as an emergent source of protein, including the most relevant protein-producing species and strategies to enhance protein content in algae feedstocks. Particular emphasis will be given to the production of commercially implemented and emergent algae species. Nutritional and functional aspects of algae biomass or protein-enriched fractions extracted from it will be overviewed. This review will also address an update about current applications and forthcoming challenges of algae-based protein use.

2. Protein sources

It is common to think of animal proteins as the only protein source available, leading to huge consumption of, for example, turkey and beef – approximately 63% and 50% protein content, respectively (Adeyeye and Ayejuyo, 2007; Wu et al., 2016).

At a first glance, vegetable proteins seem to be the obvious alternative to meat, not only because of their nutrients-rich composition (i.e., vitamins, minerals, fibres, and antioxidants), but also due to lower environmental impact and higher sustainability compared to animal-based proteins (Lynch et al., 2018). Different vegetable sources – e.g., soy (35% protein content) and chickpea (18% protein content) – have been used for a long time. However, despite the attractive protein content, they do not generally contain all the essential amino acids (EAAs) (Carrera et al., 2011; Yan et al., 2006). Therefore, the combination of more than one protein source is a common and recommended practice.

In addition, factors such as protein digestibility and bioavailability must be analysed (Lynch et al., 2018). Another potential protein source emerging as an alternative to animal-based proteins are insects. Insect farming leads to a lower ecological footprint due to the lower impact on deforestation and soil fertility and also owing to the lower amount of water required for their production when compared to traditional farming (Fasolin et al., 2019). Although their nutritional characteristics are directly dependent on the species, it seems consensual that insects are rich in protein (content varying between 7 and 91%, with an average of 60%), containing all the EAAs (de Castro et al., 2018; Fasolin et al., 2019).

Marine resources are raising big expectations in the context of the EU bioeconomy. The development of commercial algae food and feed products reflects the consumers' preferences for ecological, vegan, natural, and healthy products (European Comission, 2018a; Vigani et al., 2015). From a nutritional point of view, algae biomass is considered an important and sustainable protein source. Microalgae's protein content is typically high and can go up to 70%; seaweeds' content is usually lower (9–22%) – though this is compensated by their high availability – reaching up to 47% in specific red seaweed species (Bleakley and Hayes, 2017). Similarly to vegetable proteins, algae present a wide range of nutrients and bioactive compounds in their composition, making them nutritionally complete food products or ingredients. However, comparing to vegetable/plant-like protein sources, algae present: i) higher growth and production rates; ii) higher photosynthetic efficiency; iii) low water consumption (in line with insects production); iv) no competition for arable land; v) absence or low lignin content (facilitating extraction processes); and vi) carbon storage ability and carbon–neutral emissions. Furthermore, oceans and seas occupy>70% of the planet's surface, which enables the sustainable cultivation and harvesting of a huge quantity of this potential feedstock. Regarding environmental aspects, the cultivation of algae can be performed in smaller spaces when compared to traditional agriculture and their production can be derived from agro-industrial by-products, thus creating not only an environmental friendly system, but also boosting circular economy, as discussed in Section 8. Moreover, they are capable to remove 10–50 times more CO_2 than land plants, making them the perfect candidate to supply a very significant fraction of food demand with the least environmental impact (de Mendonça et al., 2021). However, algae have some challenges with respect to their use as food product or ingredient, namely the intense undesirable colour and strong "sea" taste and flavour associated with pigments and sulphur-containing compounds and lipid-derived volatiles, respectively, negatively influencing the sensorial attributes of the end product (Batista et al., 2013; Lafarga, 2019; Roohinejad et al., 2017). Likewise insects, and despite displaying an interesting and balanced nutritional value, algae often present a lower protein digestibility in their unprocessed form (i.e., as SCP or whole cell) due to their cell wall composition and structure, generally containing a high content in fibers and eventually polyphenols (Harrysson et al., 2018). However, the lack of information on proteins' digestibility and bioavailability and the need for more studies in this field of knowledge is a common aspect to all the alternatives to animal-based proteins mentioned previously (except for some vegetable sources).

2.1. Microalgae

Generally, microalgae are considered a feasible protein source (Bleakley and Hayes, 2017), containing an interesting profile of amino acids (AA), rich in EAAs, and even similar to some animal protein sources, such as egg (Koyande et al., 2019; Wells et al., 2017). However, despite the wide range of opportunities that microalgal biomass offers and the known health benefits of this protein source (see section 6), only a limited number of food products containing microalgae or microalgae-based molecules reached the market. The European regulations on novel foods, food safety, and nutrition and food health claims, clearly affect

the marketing of microalgae products (Vigani et al., 2015). The safety of microalgae must be assessed before their commercialization, but restrictive regulatory requirements can delay their access to the market due to long and expensive authorization processes (Lafarga, 2019). Recently, the European Commission (EC) included *Odontella aurita*, *Haematococcus pluvialis*, *Tetraselmis chuii*, *Euglena gracilis*, *Schizochytrium* sp., and *Ulkenia* sp. in the Union's list of novel foods, specifying the conditions under which they can be used (Fig. 1). On the contrary, microalgae strains such as *Arthrospira platensis* and *Chlorella vulgaris*, which were significantly consumed before May 15, 1997, are not subject to the Novel Food Regulation (NFR). For this reason, most of the microalgae-derived foods that are currently commercialized contain *Arthrospira platensis* or *Chlorella vulgaris* (Lafarga, 2019). In summary, eleven microalgae species are authorized by EFSA and only two species are currently submitted for application under the NFR, *Phaeodactylum tricornutum* and *Galdieria sulphuraria*. Moreover, applications for extended use of *Schizochytrium* WZU477 (to include DHA-rich algal oil from this species) and to modify specifications for dried *Tetraselmis chuii* have also been submitted. Except *Haematococcus pluvialis*, *Schizochytrium* sp. and *Ulkenia* sp., that are sources of oil extracts, all the authorized microalgae species are exploited as whole cells. Until today, the evolution of microalgae production in the EU was remarkable but, in order to achieve a solid position in the microalgae-based food and feed sector, along with Asia and US (where the regulation for microalgae food applications is less restrictive), the EU would need: i) to maximize production volumes and protein content, ii) to decrease production costs, and iii) a wider range of EFSA-approved microalgae, suitable for a broader range of food applications (European Comission, 2018a; Lafarga, 2019; Vigani et al., 2015).

2.2. Macroalgae

As opposed to microalgae, macroalgae have been used in human diet for many years, meaning that most of them did not need new approval for human consumption (Banach et al., 2020). Thus, the number of EFSA-approved macroalgae species is significantly higher than microalgae (Fig. 1). In comparison to Asia, macroalgae production in Europe is still very immature. In Asian diets, macroalgae have been used as essential components, being directly consumed; in Europe, their production is mainly oriented towards the export market and to supply the processing industry of hydrocolloids extraction (alginate, agar-agar, and carrageenan), especially related to pharmaceuticals, cosmetics, textiles,
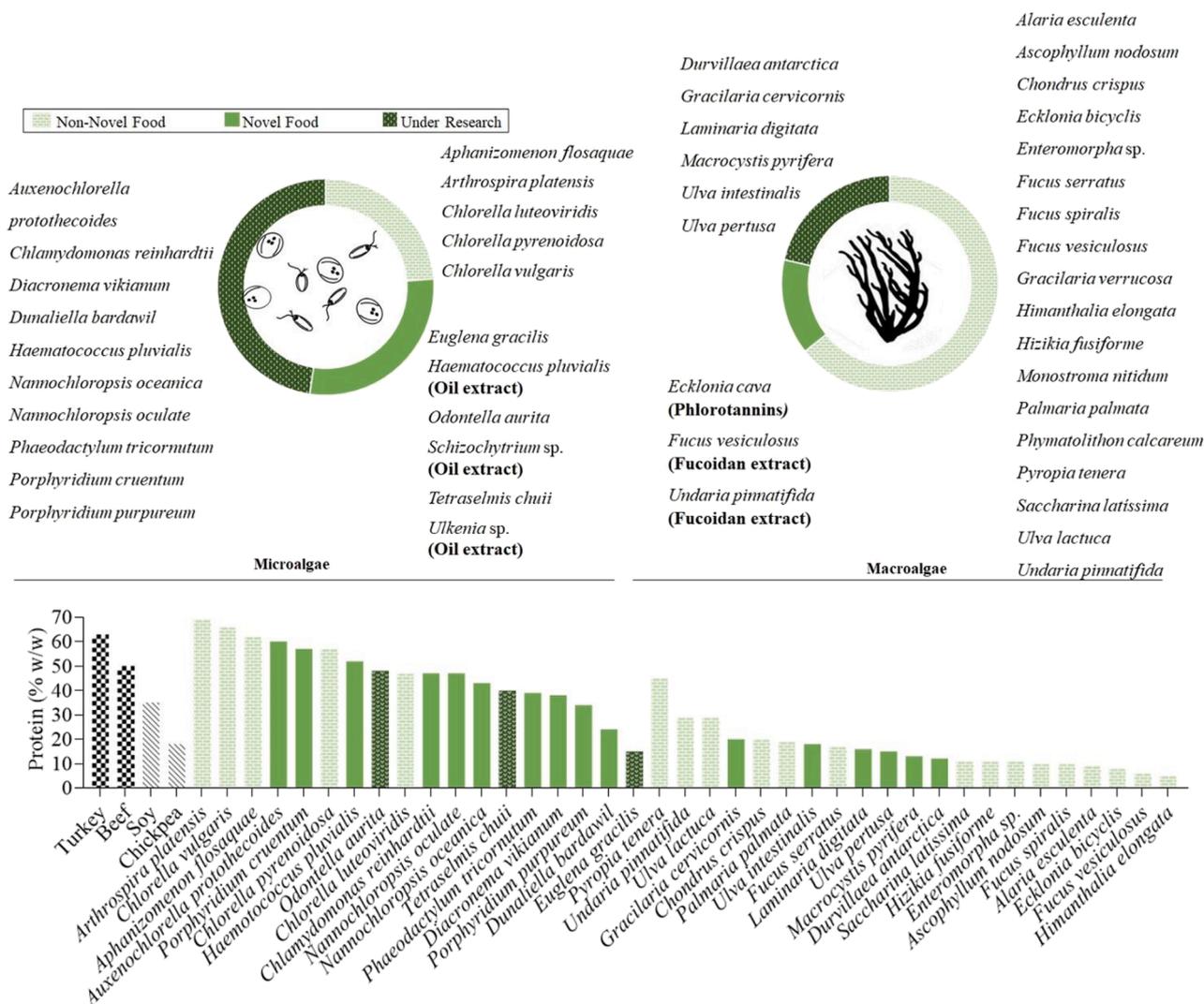

Fig. 1. EFSA approved micro- and macroalgae species for human consumption in EU as Novel (Regulation (EU) 2020/1820) and non-Novel Food (Regulation (EU) 2018/1023) and potential protein-rich microalgae under research (based on information from: Adeyeye and Ayejuyo, 2007; Batista et al., 2013; Benjama and Masniyom, 2011; Carrera et al., 2011; Figueroa-Torres et al., 2020; Kumari et al., 2014; Lynch et al., 2018; Mæhre et al., 2014; Marinho et al., 2015; Morais et al., 2020; Niccolai et al., 2019; Rolls and Phillips, 1990; Rupérez and Saura-Calixto, 2001; Wu et al., 2016). Note: Non-Novel Food products were on the market (as food or a food ingredients) and were consumed to a significant degree before May 15, 1997.

microbiology media and food-processing industries (European Comission, 2018a). In 2016, the EU exported 101,594 tons of macroalgae (only 4,607 tons were used for human consumption) and imported 178,467 tons (of which 15,184 tons were used for human consumption) (FAO, 2018). The level of discrepancy between the amount of macroalgae exported and imported for human consumption is associated with both the production technologies applied and species exploited. Whereas Asian production is mostly based on controlled macroalgae cultivation, which allows increasing their macronutrient profiles, the European macroalgae industry is mainly based on the harvesting of *Laminaria hyperborea*, *Laminaria digitata*, and *Ascophyllum nodosum* (European Comission, 2018a; FAO, 2018). On the other hand, despite their nutritional value and the fact that several other protein-rich macroalgae are already approved by EFSA, such as *Chondrus crispus* (20% of proteins), *Porphyra tenera* (44%), *Palmaria palmata* (19%), *Ulva lactuca* (29%), *Undaria pinnatifida* (29%), and *Fucus serratus* (17%), the exploitation of macroalgae for human consumption remains marginal at the moment (European Comission, 2018b). The growing interest in algae-containing products has, however, triggered the EU's effort to increase cost-effective production of cultivated macroalgae, instead of harvested ones, to boost their incorporation in food and feed markets and to improve their food safety (Banach et al., 2020; European Comission, 2018a).

3. Strategies to enhance protein content

Light exposure, mixing, and CO_2/O_2 concentration are some of the parameters that might cause serious limitations on algae growth and production yields, especially when cultivated inside a photobioreactor (PBR). Particularly for microalgae, the level of mixing in a PBR strongly affects their growth, being sometimes challenging to find the most suitable conditions. Inadequate mixing causes thermal stratification, low nutrients diffusion, and inefficient photosynthetically generated O_2 removal. Regarding the accumulation of dissolved O_2, inhibiting photosynthesis, it might present high oxidative tensions which, combined with laminar flow typically found especially in tubular bioreactors, results in cell precipitation and wall growth, being one of the main reasons for industrial failure of microalgae cultivation (Geada et al., 2017). The appearance of light gradients when mixing is not appropriate also determines a negative influence over algae's productivity by exposing cells to irregular flow patterns and average light irradiances, as well as inconvenient light–dark cycles. On the other hand, in the case of highly dense cultures, the regions close to PBR's surface are subject to light intensities that are often greater than the saturation value of algae species, causing photoinibition (Fernandes et al., 2015), while more inner regions remain in the dark due to optical absorption and self-shading of the cells, causing photolimitation. The supply of CO_2 to algal culture systems is one of the main difficulties that need to be solved. The principal point relating to the CO_2 budget is that it must not reach an upper concentration that induces inhibition but, at the same time, should never fall below the minimum concentration that limits growth, and this balance is not easily achievable.

Downstream processing represents another economic limitation to the production of algal low-cost commodities (e.g., fuels, feeds, and foods) and also to the extraction of higher value compounds, considering the specificity of the processes that need to be applied. In the particular case of microalgae, given the relatively low biomass concentration obtained in cultivation systems (typically in the range of 1–5 g.L^{-1}) and the small size of cells (typically in the range of 2–20 μm in diameter), costs and energy consumption for biomass harvesting are major bottlenecks towards industrial-scale processing, representing up to 20 – 30% of the total cost of producing the biomass (Fernandes et al., 2015). Increasing the volume of production facilities has been done by increasing the number of units in a production plant. Though, this method can become expensive considering that each unit requires the installation of a series of devices that control a wide range of growth factors (e.g., pH,

temperature, aeration, CO_2 and nutrients supply). Alternatively, it is possible to increase the working volume by increasing length or/and diameter; however, this strategy is limited by the existence of changes in the performance of the PBR at different scales. Based on the aforementioned limitations, the economic feasibility of algae cultivation is therefore dependent not only on the high biomass productivity, but also on high productivities in terms of the product of interest. To make algal proteins competitive as a food product or ingredient, algae should be capable of attaining high biomass and protein productivities at low cost.

For cultured or bioreactor-grown algae, changing the medium composition is a straightforward way to improve protein content. For instance, the increase of nitrogen, phosphate, and CO_2 seems enhance protein accumulation in algae (Kumari et al., 2014; Tossavainen et al., 2019; Toth et al., 2020). As an example, Tossavainen et al. (2019) concluded that, in high-nitrate medium (0.5 g/L of ammonium sulfate), protein content of *Euglena gracilis* varied between 17.51 and 18.56% of dry weight, while under lower concentrations or absence of nitrogen (0.2 g and 0.0 g/L of ammonium sulfate, respectively), algal proteins in biomass ranged between 10.99 and 12.09% of dry weight. However, the impact of increasing nitrogen concentration in the medium aiming at enhancing protein content, is also limited. Using *Isochrysis galbana* cultures, Zarrinmehr et al. (2020) reached maximum (326.1 mg/L) and minimum (56.9 mg/L) protein yields when applying nitrogen concentrations of 72 and 0 mg/L, respectively. Nevertheless, nitrogen concentrations above 72 mg/L were associated with lower protein contents (Zarrinmehr et al., 2020). Different sources of energy and carbon were found to influence the protein content as well. As an example, *Nannochloropsis gaditana* mixotrophic culture has resulted in a higher protein content (an average of 30.1%) in comparison with autotrophic (23.4%) and heterotrophic (23.2%) cultures (Matos et al., 2017). Salt-tolerant freshwater species, on the other hand, seem capable to adapt to saltier environments (up to 3 ppt) by increasing protein content in these conditions, although the exact mechanism is not yet completely understood and additional studies are needed (Lawton et al., 2015). Other conditions, such as temperature, light, time, seasonality, latitude, and growth stage, also play a crucial role in protein content (Gaillard et al., 2018; Matos et al., 2017). For instance, the increase of temperature was reported to influence both positively and negatively the protein content and more studies need to be made in order to assess the true effect of this parameter. Regarding the seasonality, macroalgae samples collected during spring time presented a higher protein content when compared to samples collected during the autumn. These results may be due to more sunlight, which favours photosynthesis (Gaillard et al., 2018).

In *Nannochloropsis gaditana* cultivation, the light/dark cycle seems to be of extreme importance for the final protein content of the microalga. In mixotrophic cultures, protein content reached 44.8% of dry weight applying a cycle of 12 h light (L):12 h darkness (D) per day; when the number of light hours was lower (8L:16D), the protein content was around 17.9% of dry weight. On the other hand, increasing the number of light hours above 12 h did not enhance protein content since cycles of 24L:0D and 16L:8D resulted in 20.5% and 37.3%, respectively (Matos et al., 2017). Similarly, different wavelengths provided by light emitting diodes (LED) can have a real effect over protein productivity (Prates et al., 2020). As an example, the use of red LEDs in integral photoperiod (12L:12D) resulted in a 2-fold increase for *Spirulina*'s cultures protein productivity. After-harvesting strategies can also influence the macronutrient profile and can then be used to increase the protein content, especially in the case of macroalgae. These can be particularly important for wild-harvested species as some pre-harvest strategies may be difficult to control. One possible approach is to storage the algae under an environment with light and nutrients that favour the accumulation of protein for a certain time period. Literature has also reported that during seaweed soaking, the wash-out can partially remove minerals and other non-protein compounds through diffusion, thus improving the protein content (Harrysson, 2019).

4. Protein recovery

A good alternative to avoid the poor digestibility of algae's protein would be to isolate its extracts and apply them instead of the whole biomass (Grossmann et al., 2018; Schwenzfeier et al., 2012). Protein extracts can then improve the digestibility of algal protein, due to the absence of cell wall, and increase the selling price when compared with the whole biomass (Bleakley and Hayes, 2017). Furthermore, in the case of seaweeds, the presence of high viscosity neutral or anionic cell-wall polysaccharides (e.g., agar, alginates or carrageenans), can hinder the extraction process and hamper fractioning and recovery procedures. Depending on the algae wall characteristics and nature, the recovery procedure should be sustainably designed to address these issues. Disruption techniques are generally required as a first step to promote membrane breaking and allow full access to the internal constituents, thus facilitating the extraction in a certain solvent medium. The extraction conditions need to be selected according to the desired objective since the procedure will directly influence protein bioactivity, as well as bioavailability, technological functionality, and taste (Bleakley and Hayes, 2017). Conventional protein extraction methods applied so far are based on physical processes, including mechanical disintegration and non-mechanical extraction. Also, novel methods such as ultrasonication, ohmic heating (OH), pulsed electric fields (PEF), and microwaves application are already being used in order to prevent some problems of the traditional methods (i.e., time consumption and protein integrity loss) (Bleakley and Hayes, 2017). In combination with disruption techniques, protein fractioning and concentration may be performed when highly concentrated protein fractions are envisaged.

4.1. Physical methods

The main principle of the physical methods is that the cells are subject to high stress via pressure, abrasion, presence of electric fields, cavitation or shearing, allowing a high and efficient product recovery with technological readiness, making these methods suitable to be used in large scale cell disruption (Geada et al., 2018). Moreover, the physical disruption of cells prevents chemical contamination of the algal preparation, while preserving a good percentage of the internal cell components (Show et al., 2015). When physical methods are used, the protein yield has a range between 41 and 90 % for microalgae and 15–80% for macroalgae (Cermeño et al., 2020; Safi et al., 2014). The maximum protein yield found for microalgae, using only physical methods, was on *Porphyridium cruentum* (90%) under high-pressure conditions, where a 2.2 kW disruptor was applied in two passes at 2700 bar to a freeze-dried sample at a concentration of 2% of dry weight (Safi et al., 2014). In the case of macroalgae, the highest value was observed for *Gracilaria* sp. (80%) by means of ultrasounds, where the seaweed was dried and ground thalli were suspended in 10% (v/v) NaOH, followed by sonication for 2 h, filtration, and dialysis using a cut-off membrane against distilled water (Kazir et al., 2019).

4.1.1. Bead milling (BM)

BM is widely used at industrial scale for fine grinding of mineral, ceramic, and paint pigments and was then adapted for cell disruption in both small- and large-scale production. The method consists in a vertical/horizontal cylindrical compartment with a motor-driven central shaft supporting an agitating element that, at high-speed spinning, causes a cell breaking action through direct physical damage. Before its use, some operating parameters (i.e., bead diameter and bead density) need to be set according to the cell type, in order to enhance disruption efficiency. An increase in the number of beads increases the disruption degree, while also affects the heating and power consumption. Besides being established as one of the most efficient methods for cell disruption, BM is often used combined with chemicals in order to increase product and energy efficiency (Show et al., 2015).

4.1.2. High-pressure homogenization (HPH)

HPH can be an effective technology due to the possibility of using aqueous environments, avoiding the need of a previous drying step (Barba et al., 2014). This method uses pumps that accelerate the liquid flow and induce stresses (i.e., shear forces and cavitation) during the passage through the homogenization valve, which result in cell wall disruption (Saranya et al., 2015). However, it is difficult to achieve high rates of cell destruction using high shear homogenization systems and a big portion of the energy that is absorbed is converted into heat, which is undesirable for heat-sensitive extracts (i.e., proteins). Moreover, its use is mostly indicated for high-value products recovery since the method requires high levels of energy input (Saranya et al., 2015).

4.1.3. Ultrasounds

The ultrasound-assisted extraction (or ultrasonication when extraction is performed in aqueous medium) occurs by intense shock waves that create microbubbles in the liquid medium. These structures expand and collapse violently (cavitation), generating shock waves with high energy that cause cell wall disruption and even break covalent bonds. At the same time, the temperature and pressure inside the cavitation bubbles increase. All these factors working together result in a vigorous and effective non-specific cell disruption and consequent release of intracellular compounds (Bleakley and Hayes, 2017). However, this method is not applicable to large-scale since it involves the application of high energy inputs and, alone, it is not sufficient for complete extraction of proteins (Soto-Sierra et al., 2018). Additionally, during the process, some reactive hydroxyl radicals (e.g., H^+; OH^-) can be generated and react with biomolecules. Thus, to prevent damage by oxidative free radicals, some substances should be added (e.g., nitrogen) to the medium (Barba et al., 2014; Show et al., 2015).

4.1.4. Microwaves

Microwaves extraction is a green thermal method that consists on the application of electromagnetic waves (frequency from 300 MHz to 300 GHz) that enhance the vibration of water and other polar molecules within the wet biomass, resulting in a quick increase in temperature (by dipole rotation and ionic conduction mechanisms) and pressure. The significant pressure increase inside the cell causes an increase in porosity, resulting in higher solvent penetration into the cell matrix thus facilitating the extraction of target compounds (Barba et al., 2014; Chew et al., 2019).

4.1.5. Electric fields

PEF and OH are electricity-based physical methods that may cause cell disruption. Under optimal conditions, the electric current is applied (as pulses at high voltages – in PEF – or continuously at low voltages – in OH) through a semi-conductive material. In the case of OH, due to the moderate electric fields applied and no energetic restrictions, the resistance to the passage of electric current through the material leads to internal dissipation of heat (Joule effect). This causes thermal permeabilization of the cells/tissues that can be combined with electric disturbances promoted by presence of electric fields, acting in synergy. In PEF, due to application of electric fields of high intensity (>1 kV/cm) the electro-permeabilization of the cell membrane (i.e., phenomenon known as electroporation) can allow the diffusion of intracellular components (Geada et al., 2018). Moreover, and depending on the matrix, the adequate operation conditions (e.g., pulse shape, number of pulses) must be defined in order to obtain the maximum protein extraction yield using both PEF or OH. Generally, an increase in PEF/OH electric field strength increases the permeabilization effects, causing reversible electroporation (when the cell's physiological state is not affected) or permanent permeabilization and consequent cell lysis (Geada et al., 2018).

4.2. Non-physical methods

Biological and/or chemical agents are also used to promote cell wall disintegration and consequent release of intracellular components. There are various actuation mechanisms involved but, generally, they operate by destroying the cell wall of algae through the use of enzymes, solvents, osmotic pressure or by precipitating cell wall proteins. These methods can result in a protein yield ranging between 15.8 and 73.5% for microalgae and 25 to 42% for macroalgae (Cermeño et al., 2020; Hardouin et al., 2016; Safi et al., 2014). Similarly to physical methods, the maximum protein yield found for microalgae using only non-physical methods was attained with *Porphyridium cuentrum* (57.3 ± 3.84% dw) by means of a chemical treatment with pH changes and successive centrifugation steps. For macroalgae, the yield range is more homogeneous when compared with physical techniques; as an example, the protein yield in an *Ulva armorican* using 6% endoprotease resulted in a protein extraction of 41.4% (Hardouin et al., 2016).

4.2.1. Osmotic shock

The osmotic shock is based on a rapid alteration of the water concentration across the algal cell membrane that has proper functionality only in a strictly defined chemical environment (e.g., pH or salt concentration). In optimal conditions, cells have the capacity to control their internal conditions but a sudden change in cell's surrounding environment leads to an extreme shock, which results in cell death or disruption. In all cells, water moves from the place with the lowest solute concentration to the place with the highest solute concentration. Thus, the addition of a high concentration of a certain compound (e.g., salt, substrates and neutral polymers) causes an instant change in water movement, inducing a stressful environment that leads cells to rupture, releasing the intracellular components (Show et al., 2015).

4.2.1.1. Enzymatic hydrolysis.
Enzyme-assisted extraction is the most used technique for macroalgal cell wall disruption. Macroalgae's cell walls are constituted by several types of polysaccharides (e.g., cellulose, hemicellulose, galactans, and floridean starch), which are associated with proteins and ions (e.g., calcium and potassium) that have a negative influence on algal proteins' availability and decrease protein extraction efficiency (Bleakley and Hayes, 2017). Thus, some enzymes (e.g., cellulase, xylanase, viscozyme, and lysozyme) can be used to break these complex molecules and increase cell disruption efficacy, allowing higher protein yields. However, this technique requires knowledge about the composition and complexity of each algal cell wall in order to select the most reliable enzyme to break specific macromolecules, which could affect the efficiency of the process (Cermeño et al., 2020). On the other hand, enzymatic hydrolysis can be used as an alternative to chemical extraction because it is performed in an ecologically friendly and non-hazardous manner and is a low-energy procedure (Soto-Sierra et al., 2018).

4.2.1.2. Chemical extraction.
Among the chemical extraction methods (e.g., acids, bases, and surfactants), alkaline extraction (e.g., using NaOH) is the most common and it has been established as an effective method for solubilization of hydrophobic proteins by the degradation of chemical bonds (Cermeño et al., 2020). The disruption efficiency is strongly dependent on the choice of solvent because it will define the type and selectivity of interactions with the cell wall. However, it was observed that it is difficult to anticipate the mechanism involved in a chemical interaction due to the limited understanding of the affinity of each solvent to different microalgae.

4.3. Fractionation techniques

Membrane technologies, such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO), are non-thermal and environmentally-friendly techniques that can promote algal protein enrichment when conjugated with cell disruption methods by isolating protein (Bleakley and Hayes, 2017; Kumar et al., 2013; Wenten et al., 2017). MF can be used to remove algae's cell wall components with molecular weight>200 kDa, while UF is used to isolate proteins and other macromolecules with molecular weight between 1 and 200 kDa. NF is often used to remove monovalent salts in order to minimize osmotic pressure and RO is used to reduce fluid volume (Bleakley and Hayes, 2017; Kumar et al., 2013; Wenten et al., 2017). Depending on the desired effect, a combination of membranes can also be applied rather than a single-membrane system. Regarding microalgae, membrane technologies are used for harvesting biomass since these methodologies are cheaper than centrifugation or other harvesting methods. Also, these processes allow recycling nutrients, as well as the removal of contaminant microorganisms from the biomass. UF is the most used membrane mechanism in microalgae harvesting, presenting several advantages: i) less damage to cell integrity; ii) low or no chemical consumption; iii) easy to scale up; iv) flexible operation; v) low energy consumption (Wenten et al., 2017). Safi et al. (2014) studied a two-stage ultrafiltration process to separate multiple components of *Tetraselmis suecica* after cell disruption. In this experiment, the authors applied high-pressure homogenization to break the cell wall and, after centrifugation, the aqueous phase was submitted to UF with two consecutive membranes of different molecular weight cut-offs. At the first step, starch and pigments were retained with a 100 kDa membrane, allowing proteins and sugars to pass into the permeate. Then, using a 10 kDa, the protein content was retained in the membrane, allowing sugars to be concentrated in the permeate. Since the process is easy to scale up, the methodology can successfully be extrapolated to other microalgal species (with minor modification in the cut-off of the membrane) and used in combination with the extraction methods, aiming at an efficient separation of intracellular components.

4.3.0.1. Dry fractionation
Traditional wet fractionation, applied to obtain pure protein isolates (>90% protein), leads to a partial loss of native proteins' functionality due to pH changes and drying processes. Moreover, this type of fractionation uses high quantity of water, chemicals (e.g., alkalis or acids, hexanes and/or ether) and energy, generating acidic effluents containing proteinaceous material. Likewise, the insoluble proteins are excluded and some of their functionality may be lost in the process (Pelgrom et al., 2015). Thus, the implementation of dry fractionation techniques (i.e., milling with air classification) is an alternative method that does not require the addition of water nor energy-intensive dehydration and involves the physical separation between large starch granules/fibre-rich fragments and small protein-rich particles based on size, shape and density (air classification) after a mechanical detachment between protein bodies and other cellular components (Pelgrom et al., 2015; Ruiz et al., 2016). Although the protein content that is achieved with this method is not incredibly high (~30–80% protein) and the absence of heating causing the presence of other components (e.g., oil, fibres, anti-nutritional components), it is often sufficient to allow the application of the extracted compounds in food products (Ruiz et al., 2016). In addition to air classification, solid fractionation can also be done using the differences in particles' dielectric properties, instead of size and density (electrostatic separation). This method is based on a higher charge extension in proteins (due to the presence of ionizable groups in their AA residues) when compared to carbohydrates (that lack ionizable groups), allowing them to be separated according to their type and magnitude of charge. When used together with milling, smaller particles are formed, increasing the surface area and thus achieving a better charge density. However, an optimal milling speed needs to be set in order to prevent agglomeration through exposure of lipids (Assatory et al., 2019). As a means to increase the protein content, multi-steps of electrostatic separation can also be applied.

5. Nutritional aspects

Generally, algae are known for having an interesting nutritional label. Macroalgae are rich in vitamins, minerals, and dietary fiber. On the other hand, microalgae are rich in protein and bioactive compounds and their derived foods are marketed as "healthy foods" (Koyande et al., 2019). However, the exact protein content of algae is still controversial. The most common method used to quantify protein content is the Kjeldahl method, through which the total organic nitrogen content in the samples is obtained. This value is then multiplied by a conversion factor to determine the organic nitrogen-protein value. However, since macroalgae contain significant amounts of non-protein nitrogen, the most suitable conversion factors to be applied for each type of macroalgae are still under discussion. Usually, the protein content of brown

macroalgae is low (3%–15% (w/w) of dry biomass), while green (9%–26%) and red macroalgae (up to 47%) have higher values. For example, *Porphyra* sp. has protein levels comparable to those of a soybean meal, with authors reporting a protein content up to 44% (Garcia-Vaquero and Hayes, 2016).

In microalgae, the nitrogen-to-protein conversion factor also remains a hot topic since the use of the standard value (6.25) can result in either underestimated or overestimated results. An average factor of 4.78 is often used and even recommended, but current literature still frequently uses 6.25 as the nitrogen-to-protein conversion factor in microalgae experiments, leading to higher and inaccurate percentages.

Similarly to vegetable sources, algae protein content and its quality also depends on the EAA composition and the ability to be digested, absorbed, and retained by the body. There are nine EAA (histidine (His),

|		Thr	Val	Met	Ile	Leu	Phe	Lys	His	∑EAA
Microalgae	*Chlorella* sp.	4.7	6.1	2.2	4.4	9.2	5.5	8.9	2.0	**31.1**
	Dunaliella sp.	4.9	6.0	2.4	4.5	9.4	5.5	6.8	2.6	**36.1**
	Spirulina sp.	5.1	6.4	2.9	5.8	9	4.8	5.1	2	**41.1**
Macroalgae	Brown	4.65-5.24	5-6.5	1.49-2.18	3.71-5.4	5.81-8.1	3.74-4.8	6.26-8.3	1.9-2.31	**32,56-42.83**
	Green	4.26-5.35	5.06-6.71	1.02-1.78	3.75-4.64	5.46-7.25	3.74-4.84	4.84-9.8	1.75-2.55	**29.88-42.92**
	Red	3.61-6.09	4.21-7.53	1.09-1.91	3.97-5.73	5.52-8.60	3.41-5.89	5.87-9.71	1.26-2.29	**28.94-47.75**
Vegetable	Soy	2.3	2.2	0.3	1.9	5	3.2	3.4	1.5	**19.8**
	Chickpea	0.662	0.753	0.122	0.642	1.26	0.892	1.28	0.454	**6.065**
Animal	Turkey	2.92	4.15	2.63	3.47	6.46	4.3	6.48	2.54	**32.95**
	Beef	4.55	5.85	3.17	5.18	8.38	4.34	9.23	3.18	**43.88**
WHO*		2.3	3.9	1.6	3.0	5.9	3.8	4.5	1.5	**26.5**
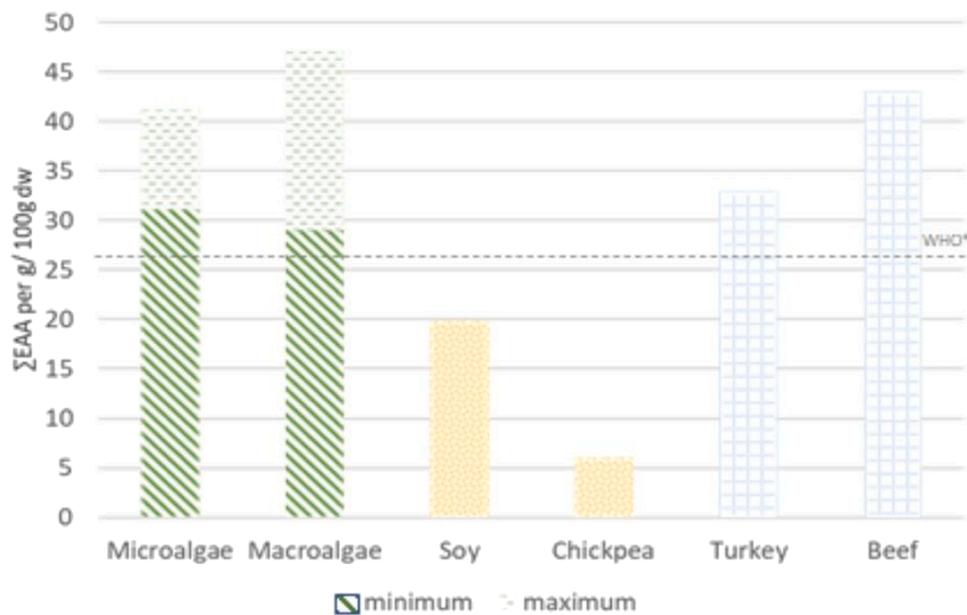
Fig. 2. EAA profiles in micro- and macroalgae species (brown (*Adenocystis, Lessonia,* and *Macrocystis*), green (*Ballia, Cladophora, Codium, Enteromorpha, Monostroma,* and *Ulva*), and red (*Ceramium, Heterosiphonia, Iridaea, Gigartina, Mazzaella, Nothogenia, Polysiphonia, Porphyra,* and *Sarcothalia*)), vegetable (soy and chickpea), and animal-based sources (turkey, beef) is expressed in g/100 g dw (based on information from: Adeyeye and Ayejuyo, 2007; Carrera et al., 2011; Holt and Snyderman, 2007; Koyande et al., 2019; Martínez-González et al., 2012; Rolls and Phillips, 1990; Torres-Tiji et al., 2020; Yan et al., 2006). *WHO recommendations of EAA requirements are presented in g/100 g protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

leucine (Leu), isoleucine (Ile), lysine (Lys), valine (Val), methionine (Met), phenylalanine (Phe), tryptophane (Trp), and threonine (Thr)), being the nutritional quality directly related to their bioavailability. The Protein Digestibility-Corrected Amino Acid Score (PDCAAS) is a method used to evaluate the quality of the protein source and can be calculated through the product of the Amino Acid Score (AAS) and the percentage of true fecal protein digestibility of the foodstuff.

Animal protein sources are generally a rich source of EAAs, whereas vegetable proteins are often considered an incomplete protein source due to their lack in some EAA. Among marine macroalgae, protein content and AA balance can have seasonal changes. Therefore, harvesting should occur when the protein content is more favorable. Although brown seaweeds have the lower protein content, it appears that they are rich sources of Thr, Val, Leu, and Lys. Met, on the other hand, seems to be the limiting EAA both in micro- and macroalgae, while Trp is not possible to quantify in some cases due to protein degradation during the extraction methods applied. It is also interesting to note that both in micro- and macroalgae, the EAA profile showed to be better than in vegetable sources (e.g., soy beans and chickpea) and similar to animal sources (e.g., turkey and beef) (Fig. 2). Although microalgae present

higher potential as protein supplier (as far as protein content is concerned), they appear to have an identical or even more incomplete EAA profile than macroalgae. However, in general, both algae showed to be a potential source to fulfill WHO's EAA requirements for healthy adults. Nevertheless, more studies need to be performed to support this statement.

In general, algae present a lower digestibility (around 80%) compared to animal sources due to the presence of high amounts of cell wall anionic polysaccharides; however, digestibility may be increased with the application of a proper extraction technique. Cell's morphology and composition determine the technique to be applied and, sometimes, a pretreatment is required (e.g., heat treatment) in order to disrupt cellulosic cell walls and make intracellular components more accessible (Bleakley and Hayes, 2017).

Considering the bioavailability, it is important to analyze both concepts entailed by this parameter: bioaccessibility and bioactivity. Due to its ethical and practical issues, studies on algae's bioactivity are based on short-term *in vitro* tests, which can lead to lack of information on the behavior of the algae (and/or their components) in the human body. Consequently, current knowledge on functional and nutritional algal
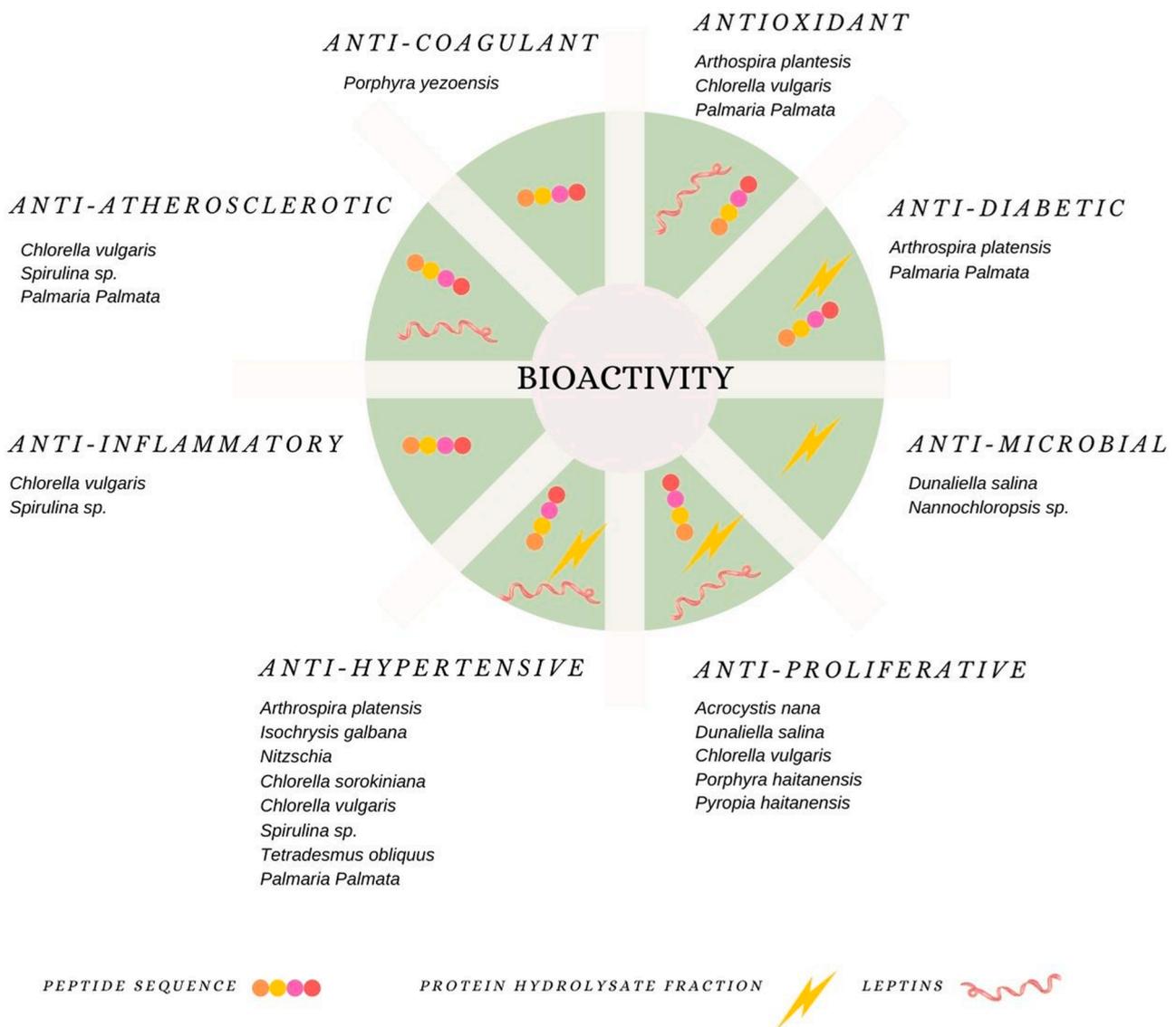

Fig. 3. Micro- and macroalgae bioactive compounds and their bioactivity in humans (based on information from: Anam et al., 2017; Anekthanakul et al., 2019; Barkia et al., 2019; Barton et al., 2016; Darvish et al., 2018; Fan et al., 2017; Fitzgerald et al., 2013; Furuta et al., 2016; Hannan et al., 2020; Indumathi and Mehta, 2016; Li et al., 2019; Mao et al., 2017; Verdugo-González et al., 2019; Wu et al., 2014).

value is limited. Nonetheless, Amorim et al. (2020) concluded that *Arthrospira*'s PDCAAS is 48%. It is extremely important to develop further research on micro- and macroalgae protein bioavailability, incorporating PDCAAS estimations (Wells et al., 2017). As an example, the use of analytical methods, such as simulated gastrointestinal digestion and genetic techniques, could play an important role on gathering more information about algae's bioavailability(Amorim et al., 2020).

6. Functional aspects

6.1. Bioactivity

Micro- and macroalgae are made of polysaccharides, proteins, lipids, pigments, fibers, and polyphenols, among others. Besides the potential as an alternative protein source, algae proteins can also provide bioactive peptides (BAPs) and other proteinaceous compounds with biological value and beneficial impact on health by exhibiting antioxidant, anti-proliferative, anti-inflammatory, anti-hypertensive, anti-diabetic, anti-atherosclerotic, anti-coagulant, and anti-microbial properties (Pimentel et al., 2019; Fan et al., 2017) (Fig. 3). BAPs are sequences of 2 to 20 AA produced by digestive proteases and, in appropriate amounts, they can be absorbed in the intestine and enter directly into the bloodstream, inducing interesting health effects (Pimentel et al., 2019). Due to their particularities, BAPs have gained especial interest in the food field, because they exhibit higher bioactivity and biospecificity for target cells when compared to synthetic molecules (Bleakley and Hayes, 2017) and are very rarely associated with adverse effects (Bleakley and Hayes, 2017; Li et al., 2019). Still, their activity depends essentially on their chemical structure, length, and on the hydrophobic/hydrophilic characteristics of the AA chain (Pimentel et al., 2019).

6.2. Technological functionality

Besides the interesting effects in human health, proteins have several functions in foods that go beyond their nutritional value, such as the implementation of desirable characteristics and their physical behavior during preparation, transformation, and storage. Thus, micro- and macroalgae can play an important role through their structural biopolymers, having both techno- and bio-functional applications (e.g., emulsification and foaming properties) (Bernaerts et al., 2019).

6.2.1. Solubility

Protein solubility, which is related to the hydrophobic and hydrophilic interactions in water, can vary from zero to hundreds of milligrams per milliliter and is an essential requirement when intended to be applied in the food industry (Grossmann et al., 2020). In addition of being a good indicator of the potential protein extracts applications, its solubility influences other functional properties (e.g., emulsifying/foaming capacity and aggregation state), being determined by the AA composition, native/denatured state, and environmental factors (e.g., temperature and pH) (Zheng et al., 2020). Moreover, protein solubility is also important for low-viscous foods to prevent gravitational separation and turbidity. Regarding microalgae, protein solubility depends on the pH and can be compared to other food proteins, since they have low solubility for acidic pH (2.0 – 6.0) – or close to their isoelectric point – and high solubility in neutral or basic environments. Teuling et al. (2017) studied protein extracts from different microalgae sources (i.e., *Arthrospira maxima* and *Nannochloropsis gaditana*) and demonstrated that, despite the differences in protein isolates' composition, using the same isolation procedure and at low ionic strength, proteins were completely soluble at pH > 6.5, presenting lower solubilization yields at lower pH values (4.0–4.5), which was explained by the proximity to their computed isoelectric point.

6.2.2. Emulsification

Proteins are already used as emulsifiers as they can stabilize the interface between aqueous and organic/oily phases. This property is extremely important since many foods are made of lipidic and aqueous phases. The emulsifying ability of proteins, also called emulsifying capacity (EC), is defined as the maximum amount of oil that can be dispersed in an emulsifier solution, without creating destabilization in its structure by coalescence, creaming, flocculation or sedimentation over a defined time period (Kumari et al., 2014). The formation and stability of protein–polysaccharides is influenced by changes in pH and an increase in ionic strength, due to the existence of mostly electrostatic interactions (Schwenzfeier et al., 2012). Typically, the EC is higher for higher pH values (7–10) and minimal for low pH values (≈3), being related to the extraction/purification process applied. Also, the addition of divalent cations (e.g., Ca^{2+}) can negatively influence the emulsion due to a decrease in electrostatic repulsion and ion bridging (Grossmann et al., 2020). However, since polysaccharides (e.g., uronic acid) can also be applied as emulsifiers, they can be combined with proteins yielding in beneficial and more stable emulsifying complexes, which can be used in food products (e.g., gum Arabic) (Grossmann et al., 2018; Schwenzfeier et al., 2012).

6.2.3. Gelation

The establishment of the gelation mechanism and gel nature of protein gels are directly related to several factors: i) protein type and concentration, ii) pH, iii) ionic strength, iv) reducing agents, v) denaturants, and vi) miscible solvents due to possible changes in protein native form, net charge, and electrostatic interactions. Bashir et al. (2016) studied the functional properties of protein isolates from *Spirulina platensis* and observed that the isolates exhibited good gelling properties when compared to *Spirulina* cell suspension.

6.2.4. Foaming

Protein foams are found in bread, cakes, cookies, meringues, ice creams, and several bakery products and consist in dispersions of gases in a liquid or solid phase, being related to its amphiphilic behavior. In foams, proteins play the specific role of forming an elastic and dense interfacial film between the two phases, as they have the ability to retain air, improving desirable textural attributes. This property is affected by surface hydrophobicity, ligand binding, molecular flexibility, and structure stability of proteins, which is directly influenced by the extraction procedure, as well as the drying method (Grossmann et al., 2020). Also, in a recent study developed by Benelhadj et al. (2016), it was verified that proteins foaming properties were also affected by pH changes and treatment time. Using a protein isolate extracted from *Arthrospira platensis*, the foaming properties showed to be minimum at pH 3 and maximum at pH 10. Although the hydrophobic interaction was weaker at pH 10, protein flexibility was greater, which resulted in higher foaming properties (Zheng et al., 2020). Moreover, after 30 min of treatment, an improvement in foaming properties was observed at pH 10, possibly due to an increased solubility and surface activity of the soluble proteins.

7. Current applications

Food products containing algae can be divided into two main groups: those that contain the whole algal biomass and those that contain algae-derived compounds. Macroalgae are mainly used to produce hydrocolloids (e.g., alginate, agar-agar, and carrageenan). These macroalgae-derived compounds are used for meat and poultry processing, dairy, canned fish, desserts, and jelly, because of their thickening, gelling, and stabilizing properties (European Comission, 2018b). The addition of other macroalgae extracts and whole biomass might improve food properties as well due to their bioactive compounds, as summarized by Roohinejad et al. (2017). Table 1 reports some commercialized food products containing macroalgae (Lafarga, 2019; Nova et al., 2020). Apart from the examples shown in Table 1, Europeans also consume macroalgae as sea vegetables in the following cuisine recipes: cannelloni

Table 1
Currently available products containing macro- and microalgae on food market (adapted from Lafarga (2019) and Nova et al. (2020)).

Food product	Brand	Product description	Macroalgae content	Origin
Microalgae				
Snacks	Simply Raw Protein RAW BA	Fruit bar rich in proteins	*Arthrospira platensis* (5%)	Germany
	Mavericks	Vegan breadsticks rich in fibre and free from added sugar	*Arthrospira platensis* (2%)	UK
Drinks	Frecious Slow Juice	Vegetable juice	*Chlorella vulgaris* (2.4%)	Netherlands
Pasta	Ametller Origen	Spelt noodles	*Arthrospira platensis* (20%)	Spain
	Nutrecentis di Ab	Spirulina pasta	*Arthrospira platensis* (10%)	Italy
Biscuits,Crackers,Cookies orCandies	Gullón Vitalday	Oat and Rice Cakes	*Arthrospira platensis* (1%)	Spain
	Helga	Sea Salt Algae Cracker	*Chlorella vulgaris* (5%)	Germany
	Casino Bio	Spirulina and cranberry biscuits	*Arthrospira platensis* (2.6%)	France
	Próvida	Bio matcha and Spirulina biscuits	*Arthrospira platensis* (1%)	Portugal
	Earth of Eco	Organic fudge	*Chlorella vulgaris* (1.2%)	Poland
Chocolate	Algenheld	Vegan Algae Chocolate	*Arthrospira platensis* (5 g)	Germany
	Zitronen zauber	Lemon chocolate truffles	*Arthrospira platensis* (1.2%)	Germany
Baking ingredients and mixes	Better & Different	Peanut spread	*Arthrospira platensis* (1.2%)	Israel
Meat substitutes	Bottega Vegetale Alga Gurme	Vegetable burgers	*Arthrospira platensis* (1.5%)	Italy
Macroalgae				
Snack	Tomy'z/Tomizawa/ Tomiz	Nori and wasabi coated peanuts	*Porphyra* sp. (0.07%)	China
Drinks	Alter Eco	Rice and Sorghum Drink with Calcium	*Lithothamnium calcareum* (0.4%)	France
Pasta sauces	Bio-verde	Fresh Vegan Algae Pest	Unspecified algae (37%)	Germany
Pasta	Sottolestelle	Bio Vegando Lentils, Red Seaweed and Thyme Wholegrain Fusilli Super Pasta	*Lithothamnion calcareum* (1% w/w)	Italy
Rice	Miss Algae	Seaweed Rice	*Palmaria palmata* (38%)*Ulva* sp. (38%) *Porphyra* sp. (38%)	France
Prepared meals	Carlota Organic	Chickpeas Stew	*Laminaria japonica* (0.6%)	Spain

bean salad (*Alaria esculenta*), chocolate molasses meringues (*Pyropia yezoensis*), Welsh laver-bread cakes, and dulse-cheese scones (*Palmaria palmata*) (Wells et al., 2017). On the other hand, some microalgae are currently being commercialized as dietary supplements and are sold as capsules, tablets, or dried powder (Lafarga, 2019). Whole cell protein, from *Arthrospira* and *Chlorella* species, is the most popular microalgae product used for human consumption, without any kind of processing except drying. Besides of the whole biomass, products containing specific microalgae-derived compounds are also being delivered nowadays. Most of them are infant formulae containing *Schizochytrium*-derived docosahexaenoic acid (DHA) or astaxanthin-rich oleoresin from *Haematococcus pluvialis*, a carotenoid available as dietary supplement, food additive, or pigment (Enzing et al., 2014; Lafarga, 2019). The incorporation of microalgal biomass into conventional food products, because of their nutritional properties, is a global trend that fostered the launch of several products worldwide. Once again, the majority of these products contain either *Arthrospira* or *Chlorella*, mainly because of their long history of use and protein content (Lafarga, 2019; Nova et al., 2020). The very low concentrations used in some products suggest that microalgal biomass is mostly applied as a colouring agent or for marketing purposes focused on vegan consumers as well as on consumers who decide to purchase organic or ecologic products, rather than for the nutritional or technological advantages of microalgae as a food ingredient (Lafarga, 2019). In fact, several authors evaluated the effect of macroalgae and microalgae biomass incorporation into foods. In general, authors reported that higher algae concentrations (depending on algae species and end product) resulted in negative effects on colour and flavour of the final product, which decrease consumers' acceptance (Arufe et al., 2018; Batista et al., 2013; Jiménez-Colmenero et al., 2010). Protein extracts would allow improving consumers' acceptance, at least from a sensorial point of view. For this reason, the effect of different protein processing methods on yield, digestibility, bioactivity, colour, and flavour of the resulting protein extract needs to be evaluated, in view of the final application (Grossmann et al., 2018; Schwenzfeier et al., 2012).

8. Environmental impact and economic prospects of algae proteins

Given the worldwide guidelines pointing towards more sustainable and "greener" processes based on circular economy and zero-waste concepts, it is possible, for instance, to improve biomass and metabolites' productivity while treating industrial wastewater streams or taking advantage of the great organic load of agro-industrial by-products to supress the nutritional needs of algae (Kazir et al., 2019). In the particular case of algal proteins, it is known that the growth using nitrogen- or phosphorus-rich substrates might trigger their overproduction, enabling the potential application of the aforementioned underrated streams as means of increasing algae's protein content (Kumari et al., 2014; Tossavainen et al., 2019; Toth et al., 2020).

Algae are frequently rich in other highly valuable fractions: macroalgae are usually rich in carbohydrates (hydrocolloids) and minerals and many microalgae have significant amounts of proteins and lipids with application potential; other minor fractions such as pigments, besides being potentially used as natural colorants, have well documented antioxidant and anti-inflammatory activities. Considering the predicted nutrient shortage and the algae overall nutritional value, recovering only the protein fraction represents, therefore, a waste of resources we cannot afford. Additionally, delivering the algae as a whole to be directly consumed can hinder the bioavailability and digestibility of algae proteins, being also important to understand how all algae nutrients can be efficiently used. The major limitation for turning all macronutrients bioaccessible in microalgae is the presence of a cell wall; in macroalgae, this is combined with the "overall" tissues' arquitecture. These can resist to the digestion process (Nethravathy et al., 2019) due to their structure and composition; they can be thick, bi-layered, composed of cellulose and hemicellulose, pectic compounds, glycoproteins or other polysaccharides. To overcome these issues, holistic strategies supporting a circular economy, enabling the sustainable and efficient use of available feedstocks while targeting minimal environmental impact and zero wastes, are in order. In this context, several pretreatment strategies and cascade biorefinery approaches have been established as an attempt to disrupt or weaken algae's structure and cell

wall, and extract algae-based products of interest, aiming at recovering the maximum fractions as possible while contributing to environmental sustainability and economic feasibility. A general approach may include preliminary recovery of the water-soluble fractions (phycobiliproteins and minerals) and low polar fractions, such as lipids and pigments, with solvents, followed by hot-water soluble phycocolloids (or vice-versa). Other protein fractions may then be recovered, for instance, by an alkali or enzymatic treatment. Furthermore, the lipidic fraction may also be considered for esterification and biodiesel production. However, this fraction is often rich in polyunsaturated fatty acids (PUFAs) and may find higher added value applications. Lower valued fractions, such as the residual cellulosic fraction, may be considered for hydrolysis and saccharification, producing fermentable sugars to be used in biofuels production or other fermentation processes (Del Río et al., 2020). This fraction may also be used as feed in the production of SCP from microalgae, further reinforcing the concept of circular economy, or in anaerobic digestion processes for the production of biogas and fertilizers. Residual non-hydrolysable fractions can be fed to thermochemical conversion processes to deliver syngas, bio-oil, biochar or simply used for direct combustion in the production of electricity. Finally, the ash fraction, consisting of minerals (e.g., potassium, calcium, iron and magnesium) and trace elements, often represents a relevant fraction also with valorization potential in food, feed, and agriculture, as fertilizer (together with the fiber fraction that can act in moisture retention). The residual algal cells have also been described as efficient biosorbents to be used in bioremediation of industrial effluents or wastewaters.

Currently, the greatest challenge regarding the application of algal biotechnology for food and feed purposes is related with their true economic and environmental benefits – are algae a profitable and green source of beneficial nutrients? This question is still controversial. Techno-economic analysis and life cycle assessment (LCA) are crucial tools to take reliable conclusions about sustainability and viability of algae-based processes. Several studies related with LCA and economic viability are available but mainly for the use of microalgae in biodiesel production. There is still scarce or limited information about an adequate analysis towards protein production for food/feed applications (Smetana et al., 2017). In the context of biodiesel production, LCA and economic analysis are somehow limited to their specificities, depending on factors such as the type of cultivation system (open systems or photobioreactors), species and respective strains, production scale, type of equipment and experimental apparatus, location (which influences the prices of electricity, for example), and lack of a target product or a defined objective. Smetana et al (2017), using a LCA tool, unveiled that: i) there are high impacts associated to microalgae, more relevant than those of the most conventional/feed protein sources, which are related to heat, energy, and nutrients input; ii) the combination of heterotrophic production of microalgae using food waste as a source of carbon and renewable energy (such as photovoltaic) can result in one of the most sustainable sources of protein concentrate. Using this strategy, *Chlorella pyrenoidosa* cultivation resulted in 2.25 kg CO_2eq (CO_2 equivalents), 28.4 MJ (energy), and 0.31 m^2 (land occupation in area year) per kg, which are quite below the ones correspoding to egg protein, for example, corresponding to 23.4 kg CO_2eq, 183.1 MJ, and 40.1 m^2 (Kim et al., 2013; Smetana et al., 2017).

The integration of a biorefinery concept within the cultivation of microalgae seems to decrease energy inputs and to be the key of success for a sustainable and viable process towards production of proteins. However, the establishment of a harmonized protocol able to guarantee that techno-economic and LCA studies are made under identical experimental conditions or assumptions could be valuable in bringing more reliable information about how strategic changes in the up- and downstream processes may impact the viability of these so-considered promising sources of food proteins.

9. Future challenges

Several strategies might play an important role on leveraging a broader commercialization of algal products. From a technical point of view, the development of innovative PBR designs envisaging the optimization of mass transfer properties and the retention of CO_2 in algal cultures, might improve its uptake leading to higher protein content, since CO_2-rich environments seem to favour protein production by algae (Toth et al., 2020). However, attention must be paid to the prevalence of CO_2 in the medium for large periods of time once it can be responsible for unwanted acidification of the culture and jeopardize the whole growth process. Alternative environmentally friendly approaches, such as the application of electric fields, have the potential to suport up- and downstream processing by maintaining control over the chemical environment and enhancing the extraction of biocompounds, if properly used and optimized (Geada et al., 2018). Besides the economic and environmental advantages, processing alternatives that allow simultaneous or sequential refining of more than one stream with added value may also address important processing issues, while delivering products with improved purity (by removing possible contaminants from the main protein stream) and functionality. For instance, in the case of seaweeds, recovering the gelling polysaccharides by thermal treatments will facilitate the subsequent protein extraction by decreasing viscosity and solubility problems. Another important issue to be considered is the strong flavour of some algae species that can pass to the algae-based protein products. In this context, it is extremely important to consider the removal of the contaminants responsible for those undesired flavours early in the process design. For instance, in the case of microalgae, designing the process to efficiently remove the lipidic fraction may decrease the strong characteristic "sea" flavour that hampers consumers' acceptance of the algae protein-based products. Furthermore, bitter off-flavours may also be liberated when solubilizing the protein fraction. Specific enzymes, such as Flavorzyme®, have been developed to break the proteins into less bitter AA and may be used in mild protein solubilisation protocols.

Bioactivity of microalgae can be inherently related with their composition but also result from downstream processing and gastrointestinal digestion upon their consumption. Likewise, safety aspects of microalgae fractions are intrinsic to the product, but can also result from production and processing strategies adopted. Information regarding the assessment of bioaccessibility and toxicity/safey aspects of microalgae and its relationship with production and processing is still scarce. This assessment is even more crucial in a time where biorefinery strategies are being established, allowing the use of microalgae as CO_2 mitigators or the use of growth media that can result partially from industrial by-products (e.g., wastewaters), and genetic engineering of microalgae is now being envisaged for the commercial production of high-value metabolites (Nethravathy et al., 2019). Regarding safety, it is then important to evaluate and control the different stages of upstream and downstream processing. Nethravathy et al. (2019) pointed out some "check-points" about safety of microalgae biomass, which are briefly summarized as follows: i) quality of water, that will be dependent on if microalgae biomass results from a controlled environment or natural habitats; ii) the need of periodic tests for undesired chemical or biological contaminants, such as toxins that are very frequent in cyanobacteria; iii) high probability of microalgae biomass presenting significant contents of nucleic acids, which can result in adverse health effects; iv) the need of establishing an analysis of potential physical, biological, and chemical hazards during all stages of production (upstream to downstream and packaging); and v) take into consideration the risk of allergies, which are very complex and depend not only on the individual consumer but also on processing and imposed physical and chemical changes. In this context, valorization of microalgae for development of healthy and functional food will be intrinsically related with the following sequential aspects that are aligned with observations made by Pina-Pérez et al. (2019): i) optimal upstream conditions aiming

production and accumulation of bioactive metabolites; ii) guarantee an efficient production of EAA and fatty acids in comparison with other emergent protein sources; iii) scientific evidence that microalgae biomass or enriched fractions can bring nutritional and health-enhancing properties and ensure safety upon their consumption.

The use of *in vitro* and *in vivo* digestion models is important to assess the bioaccessibility profiles of macromolecules, such as proteins, and verify their relationship with downstream processing. This would provide a better knowledge about the impact of the production process and gastrointestinal digestion on protein hydrolysis, development of bioactive peptides, and would allow determining whether these are absorbed in the intestinal mucosa or not. Algae biomass and its peptides' fractions envisage several health allegations– which include antioxidant, antihypertensive, immunomodulatory, anticancerogenic activities, among others (Pimentel et al., 2019) – but advanced characterization of bioactive molecules from microalgae fractions is still overlooked. As an example, protein-pigment complexes are often found, being difficult to understand if their biological value is controlled by the individual protein or not. It is, therefore, crucial to develop a fundamental understanding about the structure and function of the molecules of interest that can be retrieved from algae biomass. Although the application of macroalgae species in the food market is more varied than that of microalgae – dominated by *Arthrospira* sp. and *Chlorella* sp. –, both algae groups remain far from their full potential usage, especially in Europe (Lafarga, 2019; Nova et al., 2020). Given the previous and ongoing research on algae, it is expected that the number of algal species and products applied suffer a sharp increase within the next decade. However, to be successful on the complete harnessing of algal proteins, it would be of the utmost importance to have a simpler and quicker Novel Food process approval, as well as the reappreciation of the (sometimes highly) restrictive regulations established, namely in the case of EFSA, always bearing in mind consumers' safety and health (Lafarga, 2019). Among the most promising algae to become approved protein sources (>40%) in a near future, it is possible to highlight, for example, the microalgae *Haematococcus pluvialis, Nannochloropsis oceanica, Nannochloropsis oculata, Nannochloropsis gaditana, Porphyridium cruentum,* and *Scenedesmus obliquus.* Some of them, as the case of *Nannochloropsis* sp., are also known to present interesting contents of PUFAs (Enzing et al., 2014). Moreover, other species such as *Auxenochlorella prototothecoides* (60% of proteins), *Chlamydomonas reinhardtii* (47%), and *Dunaliella bardawil* (24%) are already reported as GRAS (Generally Recognized as Safe) under the FDA (U.S. Food and Drug Administration) regulations (FDA, GRN N°. 330, 773, 351), which might anticipate the same outcome from EFSA's evaluation when required (Fig. 1).

10. Conclusion

The use of algae as food protein supplements or ingredient, besides contributing to food sustainability, could be an important pathway to improve human health due to their rich composition in other macronutrients, but also because of the presence of bioactive molecules, such as carotenoids, PUFAs, and bioactive peptides, within their enriched fractions. However, detailed economic and life cycle assessment considering different algae sources, culture and harvesting systems, production pathways, scale up needs, fractions to exploit, and safety of final products, will be needed to further enlighten the real application potential of algae-based proteins and products.

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.

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