Emergent food proteins – towards sustainability, health and innovation

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ABSTRACT

There is an increasing demand for alternative and sustainable protein sources, such as vegetables, insects and microorganisms, that can meet the nutritional and sensory pleasantness needs of consumers. This emergent interest for novel protein sources, allied with “green” and cost-effective processing technologies, such as high hydrostatic pressure, ohmic heating and pulsed electric fields, can be used as strategies to improve the consumption of proteins’ from sustainable sources without compromising food security. In addition to their nutritional value, these novel proteins present several technological-functional properties that can be used to create various protein systems in different scales (i.e., macro, micro and nano scale), which can be tailored for a specific application in innovative food products.

However, in order for these novel protein sources to be broadly used in future food products, their fate in the human gastrointestinal tract (e.g., digestion and bioavailability) must be assessed, as well as their safety for consumers must be clearly demonstrated. In particular, these proteins may become novel allergens triggering adverse reactions and, therefore, a comprehensive allergenicity risk assessment is needed.

This review presents an overview of the most promising alternative protein sources, their application in the production of innovative food systems, as well as their potential effects on human health. In addition, new insights on sustainable processing strategies are given.

Keywords: novel proteins; green process; structure; digestibility; allergenicity

1. Introduction

Currently, a significant number of trends at planetary scale are compromising the sustainability of food and agricultural systems, and the main reason for this can be explained by global population increase. The world's population is now more than 7.7 billion persons, and this number is presently growing at a rate of around 1.07 % per year, which is expected to reach the 10 billion mark by 2050 (more than 30 % of current population) (Worldometers, 2019). This will inevitably mean agricultural expansion and productivity growth, which in turn will overpressure natural resources by increasing deforestation, greenhouse gas emissions and water consumption, thus contributing to world's ecological insufficiency and climate changes (FAO, 2017). For example, from 2010 to 2050 it is projected that meat and dairy products world consumption will increase about 173 % and 158 %, respectively (FAO, 2011). This continued expansion of food production and increasing demand for animal protein is causing serious concerns. The resources needed to convert vegetable matter into animal-derived proteins like meat or milk proteins are inefficient by 7:1 – i.e. 7 kg of vegetable food is required to produce 1 kg of milk or meat for human consumption (Nadathur, Wanasundara, & Scanlin, 2017). Since April 2016, the United Nations Decade of Action on Nutrition is ongoing with main objective "to eliminate malnutrition in all its forms", but also to develop "sustainable, resilient food systems for healthy diets" following the framework agreed at Second International Conference on Nutrition in 2014 (FAO, 2014). For this reason, much has been discussed not only about food alternatives, but also about all the dimensions that integrate the concept of food safety and sustainability.

It is then necessary to adopt a more sustainable production of the conventionally used proteins and to start rebalance the contributions between animal and plant proteins (or other alternatives), thus contributing to the sustainability of food systems, biodiversity and eventually, to a more efficient distribution of high quality proteins for the entire world population (Chardigny & Walrand, 2016; Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). The global context, the pressure of government and non-governmental policies, along with the current intention of consumers to include more plant-based proteins in their daily diets (Niva, Vainio, & Jallinoja, 2017), are bringing to discussion the importance of a greater knowledge about the use of alternative protein sources and their impact on human health. Some examples of considered emergent and sustainable protein sources include grains (e.g., wheat and zein), seeds (e.g., chia), leaves (e.g., moringa), pulses (e.g., beans, lentils, peas), microalgae, fungi (e.g., mycoproteins), milk (e.g., whey proteins) and insects.

In a context where not only socioeconomic, environmental, but also, health dimensions are increasingly intertwined with food production systems and food consumer perception, the impact that these emergent protein sources bring to health and wellbeing upon consumption should be thoroughly discussed. The use of encapsulated biomolecules is now considered as an important innovative trend (De Vries et al., 2018). Vegetable and animal protein-rich fractions due to their functional and technological properties – i.e., ability to interact with biomolecules to form gels, hydrogels or emulsions – can be designed as delivery systems (at nano or micro-scale) to protect and deliver bioactive compounds with intended functionalities at specific sites in the human body. An outstanding example of this versatility is the case of whey serum from milk, recently considered as dairy waste, it is now used as rich-protein fraction with interesting properties regarding nutritional value (e.g., balanced amino acid profile), functionality (e.g., enhanced digestibility, gelation, foaming and emulsifying capacities) and bioactivity (e.g., antimicrobial, antiviral and anti-carcinogenic) (Ramos et al., 2017). Among the various unit operations in the food industry, processing such as heating and enzymatic treatments are probably the ones that most affect proteins, thus influencing the outcomes of the gastrointestinal digestion process and consequent sensitization of the immune system. Fundamental research is still needed to achieve a comprehensive understanding of the biochemical function of emergent food proteins and adequacy of sustainable processing strategies for a better maintenance of nutritional profile and reduced risk of allergenicity.

Today, food production needs to evolve to sustainable exploitation of natural resources and at the same time, meet the growing demand for a balanced diet focused on healthier products (van der Goot et al., 2016). Recently, De Vries et al. (2018) highlighted the importance of three main innovation directions for the future food systems: 1) better efficiency – "mass production at the lowest possible price"; 2) innovation opportunity driven by consumer trends and the need to balance the use animal-origin proteins - development of new perceptions/sensations and niche products with high value at small scale; and 3) development of functional foods targeting health and wellbeing upon consumption. The use of alternative protein sources allied to the use of eco-innovative and cost-effective technologies is then aligned with this paradigm shift.

This review intends to address the most promising alternative protein sources, while providing new insights on sustainable processing strategies that can bring innovation and added value for underrated protein rich fractions. The potential health-related implications upon consumption will be also critically discussed.

2. Sustainable aspects of alternative proteins sources

Nutrition is the main function of foods, and good nutrition is a dimension of food security and sustainability. However, as summarized in Figure 1, assessing social and environmental aspects and economic costs of emergent foods is important for evaluating their long-term sustainability. According to FAO definition (FAO, 2010), sustainable food systems are those that deliver food security and nutrition for all in a way that economic, social and environmental sustainability is not compromised for future generations. In this way, this section will address sustainability aspects of some emerging proteins.

2.1. Vegetable proteins

When thinking about replacing conventional proteins (e.g., meat and egg proteins), vegetable sources seem to be a natural substitute, since they are naturally present in people's diets, bring health and environmental benefits and have lower production associated costs. Plant-based foods have lower greenhouse gas emissions and tend to be less resource-intensive and environmentally destructive than animal husbandry. In addition, vegetable proteins reduce the risk of spreading diseases such as bovine spongiform encephalitis (Elzoghby, Samy, & Elgindy, 2012; Tarhini, Greige-Gerges, & Elaissari, 2017). For such reason, encouragement of partial replacement of proteins from animal husbandry by vegetable-based ones could have a positive impact on decreasing climate changes and biodiversity loss (Joyce, Dixon, Comfort, & Hallett, 2012; Stoll-Kleemann & Schmidt, 2017). On the other hand, agriculture still has a negative impact as a result of decreasing soil fertility levels, polluting water resources with agrochemicals and contributing to deforestation and desertification due to the high demand of cropland areas (Gahukar, 2016). However, in a general way, plant protein utilization can reduce the demand for animal protein sources and consequently their environmental impact (Tian, Bryksa, & Yada, 2016).

Concerning nutritional aspects, different foods such as seeds, legumes, nuts, fruits and vegetables can be not only alternative protein sources but also, provide numerous health promoting nutrients such as vitamins, minerals, fibers, antioxidants and anti-inflammatory agents that are important in healthy diets (Kojima et al., 2018; Msambichaka et al., 2018). Despite of the high protein content of some vegetable sources (Table 1), it is known that conventional animal proteins have a high quality, while vegetable proteins are generally deficient in essential amino acids (Lonnie et al., 2018). However, the essential amino acids content is not the only factor to classify the nutritional quality of proteins. Digestibility and bioavailability also affect their utilization and must be considered (Lynch, Johnston, & Wharton, 2018). Combining plant sources in the right balance is a good solution to achieve adequate essential amino acid profiles, and such diet is supported and promoted by the Academy of Nutrition and Dietetics (Melina, Craig, & Levin, 2016).

Moreover, it has been well documented that a partial exchange of animal for vegetable protein sources is related to beneficial effects on gut microbiota, and reduced risk of type 2 diabetes, cardiovascular diseases and of other mortality causes (Busnelli, Manzini, Sirtori, Chiesa, & Parolini, 2018; Malik, Li, Tobias, Pan, & Hu, 2016; Song et al., 2016; Tharrey et al., 2018). For these reasons, in the last decade, the number of works unraveling the nutritional and technological functionality of vegetable protein increased exponentially.

Vegetable proteins can be divided into albumins, globulins, prolamins and glutelins that possess different technological properties (Day, 2013). Rapeseed shows great foam and emulsion-stabilizing properties due to the presence of cruciferin and napin. Proteins from legumes (e.g., lupin, chickpea, lentil and pea) and nuts (e.g., cashew nut) possess strong potential for stabilization of emulsions and foams, and/or gel formation (Berghout, Boom, & van der Goot, 2015; Burgos-Díaz et al., 2018; Djoullah, Husson, & Saurel, 2018; Ladjal Ettoumi, Chibane, & Romero, 2016; Liu et al., 2018; Tabilo-Munizaga et al., 2019). Soybean protein presents gelling properties but also phenolic compounds that may reduce the nutritional and functional quality. However, soy protein gels' elasticity increases when phenolic compounds are removed (Alu'datt, Rababah, & Alli, 2014). Furthermore, food industrial vegetable-based by-products have been used for protein isolation, such as apple pomace, oat bran, sugar beet leaves and orange pulp (Huc-Mathis, Journet, Fayolle, & Bosc, 2019; Tamayo Tenorio, Gieteling, Nikiforidis, Boom, & van der Goot, 2017; Wallecan, McCrae, Debon, Dong, & Mazoyer, 2015).

2.2. Insect proteins

Insects are probably one of the most controversial alternative to animal protein source because it conflicts with cultural habits in some populations Although their consumption is widespread in Eastern, African and some Latin American countries, with over 2,000 species classified as edible (Jongema, 2017), their introduction on Western eating habits is not well accepted and there are some issues that must be overcome (Hartmann & Siegrist, 2016; Piha, Pohjanheimo, Lähteenmäki-Uutela, Křečková, & Otterbring, 2018). Most consumers still not associate insects to food, instead they relate potential consumption as a primitive and disgusting behavior (Lensvelt & Steenbekkers, 2014; Woolf, Zhu, Emory, Zhao, & Liu, 2019). On the other hand, the use of processed insects as powder ingredient has been an alternative that could enhance consumers' acceptance (Hartmann & Siegrist, 2016; Piha et al., 2018). Their use in pastas, tortilla chips and breads has been assessed in terms of nutritional value and structural and sensory features (Duda, Adamczak, Chełmińska, Juszkiewicz, & Kowalczewski, 2019; Hartmann & Siegrist, 2016; Osimani et al., 2018; Roncolini et al., 2019). Nevertheless, insects are considered a sustainable food system, once besides their nutritional value, insect breeding has also positive ecological, environmental and economic impacts (de Castro, Ohara, Aguilar, & Domingues, 2018; Sun-Waterhouse et al., 2016). Among the groups generally consumed there are Coleoptera (31 %), Lepidoptera (18 %), Hymenoptera (14 %), Orthoptera (13 %) and Hemiptera (10 %) (Sun-Waterhouse et al., 2016).

There are several advantages when comparing insect farming with traditional agriculture and animal husbandry. For example, it has less impact on deforestation and soil fertility reduction since they have a smaller land-use footprint with low environmental contamination (Oonincx, 2017). Cultivating them also requires less water consumption, using up to 50 % less in some cases (Miglietta, De Leo, Ruberti, & Massari, 2015). Also, they are responsible for relatively low emissions of greenhouse gases and ammonia compared to traditionally farmed cattle, poultry, fish and seafood (Poma et al., 2017). In fact, one of the main reasons to be considered as potentially sustainable alternative protein source is their high feed conversion efficiency (van Huis, 2013), short life-cycles and high reproduction rates (Sun-Waterhouse et al., 2016). Moreover, insects can grow with a wide range of foods, including by-products from food processing and high-impacting waste streams (Smetana, Palanisamy, Mathys, & Heinz, 2016). These aspects make insects as one of the most environmentally beneficial and economical viable crops.

Regarding their nutritional aspects, great differences can be found mainly because there is a large quantity of species. However, insects are rich in protein and fat, and can provide a certain amount of minerals and vitamins (de Castro et al., 2018). Iron, zinc, potassium, sodium, calcium, phosphorus, magnesium, manganese, copper, riboflavin, pantothenic acid, biotin and folic acid can be found in insects (de Castro et al., 2018; van Huis, 2013). Indeed, in some species, zinc and iron concentrations can be similar to beef and higher than chicken and pork (Mwangi et al., 2018). Fat represents the second largest fraction of the nutrient composition, typically between 5 % and 40 % of dry matter. However, its content is dependent on life stage and can reach over 70 % (Roos, 2018). In addition, (Stull et al., 2018) showed evidences that cricket supplementation improved gut health and reduced systemic inflammation.

Protein content of most insects is around 60 % and this value can vary between 7 % and 91 % (dry weight) (van Huis, 2016) and some examples are shown in Table 1. Besides the differences among the species, other factors as development stage (Mishyna, Martinez, Chen, & Benjamin, 2019; Roos, 2018) and sex (Kulma et al., 2019) can exert effect on protein content. In general, protein values make some insect protein content comparable to meat and also plant sources (Yi et al., 2013). However, it is worth mentioning that information regarding protein may be dubious. Most of papers use Kjeldahl standard protocol to quantify protein content, considering that all the nitrogen present is in the form of protein, using the conversion factor of 6.25 (well accepted for foods). But arthropods have an exoskeleton built primarily of chitin fibers and polysaccharides containing nitrogen atoms (Jonas-Levi & Martinez, 2017). This exoskeleton and some fraction of insect proteins are not digestible by humans. Thus, the use of Kjeldahl method with conversion factor of 6.25 overestimates the protein content (Jonas-Levi & Martinez, 2017; Mishyna, Martinez, Chen, & Benjamin, 2019). Indeed, Janssen et al. (2017) estimated lower values for *Tenebrio molitor*, *Alphitobius* *diaperinus*, and *Hermetia illucens*. In addition, Mishyna et al. (2019) showed that the development stages also exert influence, estimating a nitrogen-to-protein conversion factors of 4.5 for adult grasshopper, and 4.9 and 5.6 for pupae and larvae of honey bee, respectively. Nevertheless, insect protein is better in terms of nutritional properties than other sources, since they contain all the essential amino acids (Zielińska, Baraniak, Karaś, Rybczyńska, & Jakubczyk, 2015). Recent works have exploited the solubility, foamability, gelling ability and emulsifying properties of insect proteins, unrevealing their technological potential (Gould & Wolf, 2018; Hall, Jones, O'Haire, & Liceaga, 2017; Mishyna, Martinez, Chen, & Benjamin, 2019; Mishyna, Martinez, Chen, Davidovich-Pinhas, & Benjamin, 2019; Zielińska, Karaś, & Baraniak, 2018). Regarding food applications, insect proteins are being used mainly as dry powder or meals, but more knowledge about technological feasibility and functionality of these proteins are still needed (Lamsal, Wang, Pinsirodom, & Dossey, 2019; Sosa & Fogliano, 2017).

2.3. Microbial protein

Microbial protein, or "single" cell protein (SCP), is the designation of protein derived from unicellular or even multicellular microorganisms, mainly fungi (yeasts and filamentous fungi), microalgae (cyanobacteria and unicellular eukaryotes) and bacteria. The use of microbial protein for protein supplementation in human diets and animal feeding is not a novel concept, once yeasts have been reported to be employed to supply protein requirements since World War I. However, some drawbacks, such as costs limitation, product quality, protein recovery, high level of nucleic acid and other technical problems, have delayed microbial protein large-scale successful production as we know currently (Goldberg, 1985; Otero, Wagner, Vasallo, García, & Añón, 2000; Reihani & Khosravi-Darani, 2019).

Microorganism cultivation does not require a large amount of lands as in crops and animal husbandry since microorganisms are usually grown in tanks or reactors, despite the existence of some open ponds systems that are used for microalgae production (Laurens et al., 2017). Another great advantage of microbial protein production is the eco-friendly approach when it is associated with waste treatment. Utilization of agro-industrial by-products, which are low-cost and abundant substrate sources, is a way to produce microbial protein while these wastes are treated by reducing the chemical oxygen demand (Kurcz, Błażejak, Kot, Bzducha-Wróbel, & Kieliszek, 2018; Reihani & Khosravi-Darani, 2019; Ukaegbu-obi, 2016). A high content of protein (up to 92 %) was reported to photosynthetic bacteria *Rhodopseudomonas* sp. during biogas slurry treatment under high salinity and high ammonia conditions (Yang et al., 2017). Microbial protein may also be a co-product to be recovered after industrial processes, such spent yeast (US 9,963,671 B2, 2018) or algae (Chandra, Iqbal, Vishal, Lee, & Nagra, 2019; Laurens et al., 2017) from biorefineries and brewery industries (Pietrzak & Kawa-Rygielska, 2013; Vieira, Cunha, & Ferreira, 2018). Additionally, molecular biology techniques have been employed to improve or add value to the processes, such as improvement of metabolic routes for the use of agro-industrial wastes in simultaneous production of SCP and lipase by *Yarrowia lipolytica* (Yan et al., 2018). Although, the use of genetically modified organisms (GMO) lacks public acceptance and stills an issue of discussion (Ritala, Häkkinen, Toivari, & Wiebe, 2017).

Microbial protein content (Table 1) and quality, in terms of essential amino acids, are variable and depend not only on microorganism species and type of substrate, but also on cell growth stage, nutrient sources and environmental growth conditions (Laurens et al., 2017; Reihani & Khosravi-Darani, 2019). In general, microbes are considered sources of high-quality protein since they are able to produce essential amino acids in amounts close to FAO/WHO reference value of 40 % (FAO/WHO, 2007; Matassa, Boon, Pikaar, & Verstraete, 2016). Up to 38 % of *Chlorella vulgaris* protein is composed of essential amino acids, mainly by leucine (8.2 %) and valine (6.7%) (Ursu et al., 2014). Similar amounts of essential amino acids were observed in protein from *Saccharomyces pastorianus* brewery spent yeast (Vieira et al., 2018). Slight lower levels (33 %) of essential amino acids were described for *Fusarium venenatum* ATCC 20334 which were mainly lysine, valine and phenylalanine (Hosseini & Khosravi-Darani, 2011).

Protein extracted from microalgae showed emulsifying, foaming and gelling properties (Ba, Ursu, Laroche, & Djelveh, 2016; Benelhadj, Gharsallaoui, Degraeve, Attia, & Ghorbel, 2016; Schwenzfeier, Helbig, Wierenga, & Gruppen, 2013) and may be further explored as a functional agent in foods. In addition to protein content and quality, microbial proteins are sources of nucleic acids, lipids and fats, carbohydrates, vitamins, minerals and, in some cases, pigments (Becker, 2007; Kurcz et al., 2018; Ukaegbu-obi, 2016). Then, microbial protein may be classified as food/feed (regarding nutrition) or additives (as preservatives, colorants, texture modifiers, among others) when aiming at improving or adding functionalities to food/feed preparations (Ritala et al., 2017). In a recent review, Ritala et al. (2017) approached the commercial exploitation of microbial protein and the advances in microbial protein patents for the last two decades. According to these authors, bacteria have been used mainly in animal feed, whereas microalgae and fungi have been used for human consumption as food ingredients or supplements, being *Arthrospira* (*Spirulina*), *Chlorella*, *Dunaliella*, *Aphanizomenon*, *Saccharomyces*, *Torula*, *Fusarium* and *Torulopsis*, the main commercially available genera.

3. Green Processing

Measures to a more efficient and sustainable agri-food industry should not be limited to primary food production and reduced use of products from animal origin, but also be applied to all stages of food supply chain, such as processing set-up, distribution and food by-products management (van der Goot et al., 2016). The disruption of food chain due to spoilage is a key driver for the increase of food waste and an inefficient food distribution, particularly for sub-developed countries, thus contributing to one of the main food paradoxes in the world: obesity *versus* malnutrition. The development of more efficient and sustainable food processes is then of utmost importance and should guarantee enhanced quality and safety, but also increased energy efficiency, reduced water consumption and mitigation of gas and effluent emissions. Over the last decade, the so-called "green" processing technologies – such as the case of High Hydrostatic Pressure (HHP), Ohmic Heating (OH), Pulsed electric Fields (PEF) – bring new opportunities to re-design food processing, while reducing the environmental footprint and improving nutritional quality of food products without compromising preservation (Pereira & Vicente, 2010). Figure 2 shows an example about how OH, PEF and HHP technologies can introduce new variables on food processing (e.g. pressure and electric fields) and contribute to a greener processing by reducing the number of processing operations thus avoiding the excessive use of water, gas emissions and energy. In this section, these technologies will be briefly overviewed, addressing potential contributions and implications on functionality of proteins.

3.1. HHP

Technologies such as HHP and PEF are in the frontline of non-thermal processing and they have recently been the subject of major interest for the preservation of foods with minimal degradation of its sensorial and nutritional quality. In case of

HHP, food materials are subjected to isostatic pressures roughly ranging from 100 to 1000 MPa. Application of these high pressures typically can occur at room temperature but can also be combined with adiabatic heating depending on the intended level of microbial inactivation. In accordance with Le Chatelier–Braun principle, the applied pressure level induces molecular counter reactions, despite being irrelevant for small molecules (e.g., vitamins, amino acids and pigmented flavor compounds), that foster non-thermal denaturation of microorganisms and enzymes affecting also the structure and functionality of bio-macromolecules such as proteins (De Maria, Ferrari, & Maresca, 2016; Pereira & Vicente, 2010; Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). Depending on treatment conditions – i.e., pressure, temperature and treatment time – HHP processing can affect from quaternary to secondary structure of proteins, thus influence their unfolding process and protein-protein interactions in a similarly way to that of thermal processing (De Maria et al., 2016). Recent research suggests that HHP can promote conformational changes in globular proteins such as β-lactoglobulin contributing to its altered allergenicity (Meng, Bai, Gao, Li, & Chen, 2017).

3.2. PEF

Regarding PEF, high voltage electrical pulses (usually between 20 kV/cm and 80 kV/cm) are delivered through a food material in microseconds treatment time scale with the objective of inducing electro-permeabilization of biological membranes – a phenomenon known as electroporation (Raso et al., 2016; Rocha et al., 2018). Depending on the electrical protocol applied (number of pulses, pulse width and intensity, and treatment time) it can be used as an alternative to thermal microbial inactivation of cell suspensions or to permeabilize plant tissues enhancing mass transfer operations (e.g., extraction of biocomponents and nutrients, juice pressing, drying and osmotic dehydration). Combination of PEF treatments with mild heating can be a necessary condition to the inactivation of resistant forms of microorganisms (such as spores and enzymes), but can also be an interesting strategy to increase higher efficiency in the extraction of valuable proteins from vegetables sources (Barba et al., 2015; Puértolas, Luengo, Álvarez, & Raso, 2012).

3.3. OH and moderate electric fields

OH is in the origin of electric food processing and supported the appearance of electric field-based technologies such as PEF (Sastry, 2014). Similarly to PEF, its working principle is based on the application of an external electric field through a semi-conductive food material. In the case of OH, the electric fields are of moderate to low intensity (< 1000 V/cm) with an assigned frequency (i.e. typical from 50 Hz up to 20 kHz) (Pataro et al., 2014), being continuously applied in time, allowing internal heat dissipation in a very fast and volumetric way. This technology is bringing a new paradigm to thermal food processing by both reducing excessive thermal load (not dependent on conduction and convection heat transport mechanisms) while benefiting from non-thermal effects of electric fields. Much in part because of these electric effects, another common designation for OH is moderate electric fields (MEF) processing. Over the last decade it has been highlighted the importance of these MEF on enhancing inactivation of certain type of microorganism and foods enzymes (Cappato et al., 2017; Jaeger et al., 2016; Knirsch, Alves dos Santos, Vicente, & Penna, 2010; Machado, Pereira, Martins, Teixeira, & Vicente, 2010). More recently, research studies using β-lactoglobulin rich fractions as a model system have been pointing out the potential implications that these electrical effects exert on the dynamic behavior and conformational state of protein structure during denaturation and aggregation processes (Pereira et al., 2016; Pereira, Teixeira, & Vicente, 2011). It was shown that the fast internal heating combined with electrical effects has the ability to reduce size of protein aggregates, as well as to change its morphology and physicochemical aspects during thermal denaturation. These biophysical changes are suggested to be linked with molecular motion imposed by the oscillating electric field particularly when protein structure is more susceptible to thermal structural changes (Rodrigues, Vicente, Petersen, & Pereira, 2019). These outcomes bring novel perspectives on how OH can be used to design protein structured systems - such as complexes, emulsions, acidified gels, hydrogels or nanohydrogels - seeking intended functionalities (e.g., development of carrier systems of bioactive compounds, food textural enhancers and improved intestinal absorption). However, because of the effects outlined before, OH can also change immunoreactivity of produced protein aggregates and the pathways of gastrointestinal digestion which can bring relevant consequence on protein allergenicity (Pereira et al., 2018).

3.4. Processing and protein functionality

Overall, the use of the processing technologies aforementioned can enhance preservation, extraction and transformation of important (bio)-macromolecules. Depending on the applied pressure or electric fields intensity, it is possible to induce changes within a given protein structure, thus altering its functional and technological aspects. Novel processing approaches can also be established by a combination of methods such as the case of pulsed ohmic heating (combination of PEF and OH), or even fermentation and enzymatic hydrolysis under the influence of MEF. Recently it has been shown that electric fields associated to an electrical frequency and treatment temperature can be used to modulate enzymatic activity of important food enzymes (Samaranayake & Sastry, 2016a, 2016b, 2018). These combined approaches may bring synergistic effects and contribute to a larger extent to change biophysical properties of proteins such aggregation, allergenic potential and also digestibility. It is also important to highlight that the effects on protein function of other emergent non-thermal processing methods such as ultrasounds, gamma irradiation, ultraviolet pulsed light and high voltage electrical discharge, as well as thermal ones (such as the case of microwave heating and radiofrequency) are also being discussed but available information on protein function is still limited. The majority of conducted research about the impact of novel food processing have been using preferentially whey and milk proteins (e.g., β-lactoglobulin and bovine serum albumin) as model systems due to their well-known physicochemical and structural properties. Knowing that these green technologies are increasingly taking a leadership position towards innovation, quality and sustainability in the actual context of food processing, to our understanding is then important to establish more fundamental knowledge about their impact on the safety, quality and functionality aspects of emergent protein sources previously highlighted (Section 2).

4. Innovative protein systems

In addition to nutritional factors, several technological-functional properties of proteins in food have been reviewed. Also, the development of protein structures as protective and delivery systems for bioactives have been extensively reported mostly for conventional protein sources. In the past few years, there have been advances in assessing alternative protein sources but there is still much to explore regarding the technological functional properties of emerging proteins. Moreover, with the exception of vegetable proteins, decreasing the working scale to micro and nanosystems using alternative proteins (e.g., insect and microbial protein) still represents a challenge and is a relatively unexplored research field. In this section, the properties of some innovative protein systems in different structure scales are addressed according to the recent findings.

4.1. Macrosystems and bulky behavior

During the last decade, many exploratory studies are bringing novel insights about the rheological behavior of these polymers, as well as their behavior in the presence of salts, ions, pH, among others (van Huis, 2013; Yi et al., 2013; Zhao, Vázquez-Gutiérrez, Johansson, Landberg, & Langton, 2016). Also, solubility/hydrophobicity, thermal behavior, water and oil holding capacity, emulsifying and foamability properties have been characterized for proteins isolated from some vegetables, insects and microbes as shown in Table 2.

The functionalities of vegetable proteins have been explored for longer, thus there are more studies evaluating and comparing their aggregation ability on edible coatings, films and gel formation. Wheat and soybean proteins are probably the most studied vegetable sources. For instance, active films based on glycerol-plasticized wheat gluten protein with thyme essential oil addition were prepared by a thermoplastic process. At the same time, the increase in essential oil content allowed to prepare biodegradable edible films with antioxidant and antimicrobial properties; it also led to more deformable films with lower storage modulus (Ansorena, Zubeldía, & Marcovich, 2016). In turn, gelling properties of soybean protein from different raw materials (whole and laminate soybean seeds, soy meal obtained of oil extraction residue and dried residue) were recently evaluated (Monteiro & Lopes-da-Silva, 2019). The authors showed that some extent of pre-denaturation decreased the gelation temperature and produced elastic and stiff gels. However, extensive protein denaturation originated water insoluble macro-aggregates that were less available to form a stable three-dimensional network. Due to its known properties and wide applicability in semi-solid foods, gels from soy protein also have been compared to other vegetable proteins. Berghout et al. (2015) showed that lupin protein isolate behaved differently from soy protein isolate, being unable to form gels with similar consistency and deformability, which has been attributed to the different concentration of free sulfhydryl groups. Lam, Can Karaca, Tyler, & Nickerson (2018) published a review about pea protein isolates, where they included results about cold-set gelation and effect of pH, salts, structural changes on its heating-set gelling behavior. On the other hand, López et al. (2018) evaluated the effects of extraction pH on the functional properties of chia protein isolates. They showed that the heat-induced gelation of proteins extracted at pH 10 and 12 resulted in weak gels. Besides wheat and soybean proteins, lupin, chickpea, zein, moringa, amaranth, quinoa and rapeseed are examples of the numerous vegetables that had their technological-functional properties unraveled recently. There are interesting recent works and reviews about these proteins, addressing their functionality besides their structure and extraction procedures. Jones, (2016) published recent advances about the functional properties of some oilseed and pulse proteins and prolamins. The author concluded that soybean, rapeseed, pea and chickpea proteins have best functional properties.

Insect proteins as gelling agents are still poorly investigated, while to the best of our knowledge there are no works using these proteins to produce edible films and coatings. For this reason, the aggregation and gelation mechanisms of proteins from insects are still not well understood. For example, yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), superworm (*Zophobas morio*), and Dubia cockroach (*Blaptica dubia*) proteins gelled only at neutral and alkaline pH at a concentration of 30 % (w/v) (Yi et al., 2013). They evaluated the gelling behavior at pH 7 through changes in rheology and found that gelation occurred from about 51 ºC to 63 °C for all these species. On the other hand, Mishyna et al. (2019) evaluated the heat-induced aggregation of proteins from honey bee brood (*Apis mellifera*) and grasshopper (*Schistocerca gregaria*). Honey bee brood showed significantly higher coagulation than grasshopper (Mishyna, Martinez, Chen, Davidovich-Pinhas, et al., 2019). Moreover, proteins from honey bee brood showed maximum aggregation at 85 °C (for pH 5 and 7). The authors attributed the mechanism of aggregates formation to covalent and non-covalent intermolecular interaction. Moreover, hydrophobic domains were more exposed under heating at pH 5 and 7, contributing to higher protein aggregation (Mishyna, Martinez, Chen, Davidovich-Pinhas, et al., 2019).

Most studies regarding microbial protein have focused on characterization of protein extracts according to nutritional quality in terms of essential amino acids composition (Gerde et al., 2013; Vieira, da Silva, Carmo, & Ferreira, 2017), while protein functionalities were investigated in a lower extent (Table 2). Minimum critical gelling concentration for protein isolate from *Arthrospira platensis* (*Spirulina platensis*) was 12 % (w/w) after boiling and cooling (Benelhadj et al., 2016). Lower values for other strains of the same species were reported by Chronakis (2001). In this case, the protein isolate was described as a protein-pigment complex and its least gelling concentration was 1.5 % (w/w) in pH 7 buffer solution, or 2.5 % (w/w) with salt addition (0.02 M $CaCl_2$). Authors suggested that hydrophobic interactions showed a significant role in protein gelation, contributing to molecular association, initial aggregation, and stability of the gels (Chronakis, 2001). The yeast *Saccharomyces cerevisiae* also showed complete gelation at low concentration (3.5 % w/v) (Bacha, Nasir, Khalique, Anjum, & Jabbar, 2011). However, in this case, proteins were not previously extracted, and gelation properties may be substantially enhanced by interaction with other cellular components. Indeed, it is shown that proteins properties and functionalities are dependent on extraction conditions (Ursu et al., 2014), purity degree of extracts and microbial source (Otero et al., 2000). Moreover, according to (Otero et al., 2011; Sceni et al., 2009), membrane and cell wall lipoproteins and mannoproteins show best surface properties while hydration and gelling properties are driven mainly by cytoplasm and nuclear proteins. These properties may correlate with decreasing water solubility of edible films after incorporation of yeast cell wall from *Saccharomyces pastorianus* (Peltzer, Salvay, Delgado, de la Osa, & Wagner, 2018).

4.2. Microsystems

Proteins obtained from vegetable sources can be designed as effective wall materials for microencapsulation of different bioactive compounds. Pea protein has been successfully used to microencapsulate conjugated linoleic acid (Costa et al., 2015), α-tocopherol (Pierucci, Andrade, Farina, Pedrosa, & Rocha-Leão, 2007), ascorbic acid (Pierucci, Andrade, Baptista, Volpato, & Rocha-Leão, 2006) and Propolis extract (Jansen-Alves et al., 2019). Also, chick pea protein has been used to encapsulate folate, conferring greater stability to folate relative to un-encapsulated folate (Ariyarathna & Nedra Karunaratne, 2015). In this case, values of 62.19 ± 2.05 % and 10.18 ± 0.89 % have been obtained for encapsulation efficiency and loading capacity, respectively and a gradual release of folate was observed in the pH range of 2-8.

Vegetable proteins such as lentil (Can Karaca, Nickerson, & Low, 2011), chickpea (Can Karaca et al., 2011), lupin (Burgos-Díaz et al., 2018) and soy (Liu & Tang, 2016) have been also used as emulsifiers to facilitate the formation, improve the stability and provide specific physicochemical properties to emulsions (Burgos-Díaz, Wandersleben, Marqués, & Rubilar, 2016). However, the pH sensitivity of vegetable-based proteins could not be neglected, being frequently necessary to find strategies to further increase emulsions' stability. Combination of vegetable proteins with polysaccharides may improve their emulsifying properties and stability against extreme conditions. In fact, it has been shown that multilayer emulsions (with droplet diameter less than 80 µm) can be prepared by the layer-by-layer technique using a protein isolate from the novel high yielding protein lupin crop (AluProt-CGNA) combined with chitosan and xanthan gum (Burgos-Díaz, Gallardo, et al., 2016). Although, lupin protein-stabilized emulsions showed to be highly unstable to aggregation at pH values around their isoelectric point (pH ∼4.6) and temperatures of 30–90 °C, their stability to aggregation over a wide range of pH values, temperature, and salt concentrations have been improved by the addition of chitosan and xanthan gum layers. In the same way, other authors used unmodified protein isolates from lupin, pea and broad beans as emulsifiers and showed that all emulsions precipitated at their isoelectric point, whereas emulsion stability increased by the presence of a polysaccharide (Makri, Papalamprou, & Doxastakis, 2005). Also, it has been shown that structural changes in pea proteins, as a result of alkaline pH treatment, improved the physical and oxidative stability of emulsions (Jiang, Zhu, Liu, & Xiong, 2014).

Although much less explored, proteins extracted from insects and microalgae can be used as emulsifiers in oil-in-water emulsions. For example, protein extracted from mealworm (*Tenebrio molitor*) have shown interfacial activity and fast adsorption kinetics at the oil/water interface. Also, the mealworm protein stabilized oil-in-water emulsions showed to be stable to changes in pH, salt and temperature, except for flocculation after heating at 90 ºC and pH close to proteins' isoelectric point (Gould & Wolf, 2018). Similarly, a soluble protein fraction isolated from the green microalgae *Tetraselmis* sp allowed the formation of stable emulsions in the pH range of 5-7 at low protein concentrations (Schwenzfeier et al., 2013).

4.3. Nanosystems

Nanotechnology is an emerging field in the food industry due to its great potential to improve food productivity by enhancing food processing conditions, as well as to allow obtaining high quality, safer and healthier food products (Cerqueira et al., 2017; Simões et al., 2017). Hence, the use of materials at nano scale (10^{-9} m) may display distinct physical-chemical and biological properties that can lead to novel material functionalities, in comparison to those in the bulk form, due to the higher surface area-volume ratio obtained at this scale (Madalena, Pereira, Vicente, & Ramos, 2019). In food industry, the use of nanomaterials can be particularly useful for the encapsulation and delivery of bioactive compounds (e.g., vitamins, nutrients, minerals, antioxidants, antimicrobials, prebiotics and probiotics) intended for the development of novel and more efficient functional food products. Due to the reduced size of nanosystems, they can enhance the solubility and sensorial features (e.g., mask unpleasant flavors), preserve the activity, prevent oxidative reactions, or even improve the bioaccessibility and bioavailability of bioactive compounds (Acosta, 2009; Durán & Marcato, 2013).

The development of functional foods enriched with nanosystems has been an emerging focus of food industry as a novel approach either to 1) fight the rising world malnutrition, particularly relevant in underdeveloped countries; 2) address the micronutrient deficiency that frequently result in severe health-related problem in developing countries due to modern eating habits and inadequate diets; or 3) face the growing consumer demands for healthy foods with additional properties (e.g., antioxidant, anti-cancer and anti-inflammatory) in addition to their nutritional value (Đorđević et al., 2015; Guiné, Ramalhosa, & Valente, 2016; Ramos et al., 2017)

However, the successful use of nanosystems in food applications dependents on the full replacement of non-food-grade materials by food-grade and generally recognized as safe (GRAS) alternatives (Cerqueira et al., 2014), and on the consumers' acceptance of nanotechnology-based products (Cerqueira et al., 2017). For food industry applications, nanosystems can be produced from a wide range of food-grade materials such as proteins, polysaccharides and lipids, or their combination, to form complex delivery systems (e.g. capsules, hydrogels and emulsions) (Cerqueira et al., 2014; Simões et al., 2017; Madalena et al., 2019). Among the distinct food-grade materials, protein-based nanosystems have particular interest because they are biodegradable, metabolizable, easily manipulated and functionalized (e.g. surface alteration and/or modification) for covalent binding with bioactive compounds (Madalena et al., 2016; Monteiro et al., 2016; Ramos et al., 2017; Tarhini et al., 2017).

Vegetable proteins such as zein and gliadin are GRAS materials that have shown a great potential to be used in the design of nano delivery systems. These proteins may not need the use of chemical agents or physical treatment for the development of nanosystems due to their high hydrophobicity. Moreover, they are less expensive than animal proteins and also have important functional groups able to adsorb or to covalently bind agents capable of altering the targeting properties of nanoparticles. Motivated by this, Hao Li et al., (2019) developed nanoparticles from zein as base material and soybean as stabilizer for quercetin encapsulation. These systems exhibited a particle size of ca. 200 nm and encapsulation efficiency of 82.5 %, showing to be relatively stable at high ionic strength and temperature (Hao Li et al., 2019). Patel et al. (2010) synthesized polymeric colloidal nanoparticles from zein for curcumin encapsulation. These nanoparticles displayed particle sizes between 100-150 nm, and curcumin loading and encapsulation efficiency from 1.6 to 4.1 % and 71.1 to 86.8 %, respectively, and exhibited a good colloidal stability at an extensive range of physiologically relevant pH (1.2, 4.5, 6.7 and 7.4) and in simulated gastrointestinal conditions (Patel et al., 2010). Wu, Luo, & Wang (2012) designed nano delivery systems from zein using the liquid–liquid dispersion method for encapsulation of thymol and carvacrol essential oils to improve their solubility without affecting their intrinsic antimicrobial and antioxidant properties. These nanosystems showing particle sizes below 320 nm and an encapsulation efficiency higher than 50 % for both essential oils, were able to improve the solubility of thymol and carvacrol up to 14-fold without hindering their ability to scavenge free radicals or to control *Escherichia coli* growth, for example (Wu et al., 2012).

Hu & McClements (2015) developed promising nano-delivery systems from zein (as core matrix) and alginate (as shell) exhibiting a high stability (at pH ranging from 3 to 8 and at ambient and refrigeration temperatures) for encapsulation and controlled release of bioactive molecules. In another work, Wu, Kong, Zhang, Hua, & Chen (2018) produced nanoparticle with wheat gliadin proteins (as base material) and gum arabic (as stabilizing agent), exhibiting good stability (at pH comprised from 4.0 to 7.0 and at 80 °C), which are important properties for successful delivering bioactive compounds. Wang et al. (2015) developed innovative nanofibrous membranes from poly (vinyl alcohol) and wheat gluten as base matrix that showed improved release rates of nisin, and thus better antimicrobial activity against *Staphylococcus aureus*. These are very promising characteristics that can be highly explored in drug delivery, wound dressing and active food packaging. In another study, Verdugo, Lim & Rubilar (2014) used protein concentrate from microalgae *Botryococcus braunii* residual biomass as base material to develop nano and microfibers by electrospinning. The work presented by these authors revealed a great potential of these fibers for many end-use applications, including in the food and biomedical industries.

The use of nanosystems for food applications, although promising and prepared from renewable and sustainable sources (mainly from vegetable proteins), may present risks for human health that should not be overlooked. Therefore, potential risks should be clearly identified regarding the unknown effects of such nanosystems in the human body and within the ecosystem (Cerqueira et al., 2014; Simões et al., 2017).

5. Health considerations

When introducing proteins from novel sources into the human diet, it is essential to take in consideration their behavior and bioavailability throughout the gastrointestinal tract and also assess their possible cytotoxic effects or other negative impacts on human health (e.g. allergic reactions).

5.1. Digestibility

Protein or protein fractions, able to resist human digestion and to be directly absorbed by the intestinal epithelium, have the potential to affect consumers' health by either exerting positive (e.g., nutraceutical) or negative (e.g., antinutritional and allergenic) effects (Ribeiro et al., 2017). In fact, stability to gastrointestinal digestion is one of the major characteristics shared by allergenic proteins (Astwood, Leach, & Fuchs, 1996) being, therefore, *in vitro* digestion experiments (i.e., testing proteins for their resistance to gastric fluids) frequently used to assess the allergenic potential of novel food proteins. The digestion susceptibility of lupin seed globulins has been evaluated and it was shown that globulins are completely hydrolyzed by pepsin, pepsin followed pancreatin or chymotrypsin, whereas, pancreatin and trypsin did not hydrolyze all globulins. The protein fraction resistant to the action of these enzymes was γ-conglutin, which retained its antigenic properties after digestion. Its insensitivity to hydrolysis by pancreatin and trypsin was attributed to the formation of complexes with the flavonoids released from other protein connections during digestion, as well as to the low number of cleavage sites for trypsin (Czubiński, Siger, & Lampart-Szczapa, 2016). Other authors identified and characterized chickpea seed proteins as being able to resist to *in vitro* simulated human digestion. It was found that the majority of these proteins were members of the 7S vicilin and 11S legumin seed storage protein classes, which are reported to exhibit bioactive functions (Ribeiro et al., 2017). However, the results of proteins' digestibility should be analyzed with care once, contrary to what would be expected, it was found that some potent allergens are not stable in gastric fluids, being rapidly digested (Fu, Abbott, & Hatzos, 2002). This indicates that using digestion stability as criteria provides important, but not sufficient, information to predict the allergenic potential of proteins (Untersmayr & Jensen-Jarolim, 2008).

It is known that proteins' digestion kinetics may be influenced by different factors including processing conditions, pH during processing and interactions with other components present in the food matrix. Recently, the influence of pH and processing conditions on the digestibility of pea protein isolate have been investigated (Laguna, Picouet, Guàrdia, Renard, & Sarkar, 2017). These authors found that HHP processing enhanced the degree and rate of proteolysis, which can be attributed to globular pea protein subunits unfolding. Also, the initial pH showed a strong effect on extent and degree of digestibility, being pea protein at pH 6.2 more digestible owing to their higher solubility at this pH. Other authors showed the influence of different processing treatments and the use of enzymes (isolated or in combination) on lentil protein digestion (Aryee & Boye, 2016). It was evidenced that mild processing methods (e.g., pre-hydrolysis using enzymes) could be used to render peptide bonds more accessible to digestive proteases.

Protein quality and postprandial protein gain are not only influenced by the amino acid content but also by the bioavailability of the protein, which in part depends on their digestibility. Different species of edible insects have been analyzed in terms of their protein digestibility (Ramos-Elorduy et al., 1997) and values from 76 % to 96 % have been obtained, which are comparable to the ones found for egg proteins (95 %) or beef (98 %) and higher than the ones found for many vegetable proteins (Kouřimská & Adámková, 2016). Also, digestibility of globulin proteins isolated from fava bean and lupin (using a rat small intestine) has been demonstrated to be well over 90 % (Rubio, Grant, Caballé, Martinez-Aragón, & Pusztai, 1994). In turn, protein from microalgae appear to have similar digestibility to that of seaweed, with *Scenedesmus obliquus, Spirulina sp.* and *Chlorella sp.* having digestibility coefficient values of 88.0 %, 83.9 % and 89.0 %, respectively (Becker, 2007). However, there is a major obstacle for microalgae utilization as protein source that is the presence of a cellulosic cell wall, representing about 10 % of their dry weight, which is not digested by humans. Therefore, in order to make microalgae protein accessible for digestive enzymes, effective processing treatments are typically necessary to disrupt the cell wall.

The effect of microbial protein produced by *Corynebacterium ammoniagenes* on ileal amino acid digestibility in pigs has been also evaluated and compared with soybean proteins (Wang, Kim, Kim, & Kim, 2013). A lower digestibility of microbial protein by pigs has been observed, which has been attributed to its higher non-protein nitrogen content (e.g., nucleotide) as compared with soybean.

Also, it is important to mention that the resistance of specific protein domains to digestion is not the only condition for exerting an immunological response. Protein allergenicity during/after gastrointestinal digestion could increase due to the creation of neoepitopes resulting from native state protein digestion, or due to the increase in epitope concentration and their affinity to immune system cells (e.g., B cells) (Groell, Jordan, & Borchard, 2018). Moreover, digestion induced conformational changes (e.g. unfolding or aggregation) of the linear (sequential) or conformational (discontinuous) epitopes which could influence the allergenicity (Matsuo, Yokooji, & Taogoshi, 2015; Picariello et al., 2010). For instance, Ara h 1 (a major peanut allergen) can form a stable trimer complex that offer protection from protease digestion and denaturation, which could allow Ara h 1 (with intact IgE-binding epitopes) to pass through the small intestine, leading to allergenicity (Sen et al., 2002).

5.2. Cytotoxicity

Many novel proteins and peptides present a great prospective as functional food ingredients or supplements, but not only their resistance to human digestion but also their quality and availability must be evaluated. Complementary safety information may be mandatory to investigate whether proteins or peptides from different sources such as seaweed, microalgae and insects, could be considered safe for consumption (van der Spiegel, Noordam, & van der Fels-Klerx, 2013).

Proteins and peptides toxicity take a central part in the regulation of body functions. Protein chemical or non-chemical modifications could result in nutritional value changes, possible toxic peptides or amino acid derivatives formation, and contamination by toxic chemicals harmful for health (Hurley et al., 2016; Zimmermann et al., 2018). Analyses intending to establish the proteins' cytotoxicity properties have to consider a range of factors (e.g., proteins' effect on the gastrointestinal tract and susceptibility to digestion – see section 5.1). Therefore, the application of *in vitro* assays as screening tools can assist in estimating the potential proteins toxicity prior to their test in animal models and clinical studies. The cytotoxic effects of proteins usually are evaluated using *in vitro* assays such as 3-(4,5 dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) test based on mitochondrial dehydrogenase activity detection in living cells and lactate dehydrogenase (LDH) released from dead cells (Hurley et al., 2016). Additionally, necrosis, apoptosis and/or cell cycle disturbances activities are assessed in many research works with the purpose to clarify about cell death mechanism stimulated by protein or peptides (Chalamaiah, Yu, & Wu, 2018). Recently, it has been shown that protein derived from the fungus *Cordyceps militaris* increased cell cytotoxicity (measured using MTT and LDH assays) in murine primary cell line possibly through mitochondrion-dependent apoptosis (Bai & Sheu, 2018). Zimmermann et al. (2018) evaluated the cell viability by LDH release, MTT conversion and barrier integrity of human intestinal epithelial cell monolayers (Caco-2 and T84 IEC) exposed to hazardous (e.g., wheat germ agglutinin) and non-hazardous (e.g., bovine serum albumin (BSA)) proteins for 24 h, 48 h and 72 h. The authors demonstrated that non-hazardous proteins did not have influence on Caco-2 and T84 IEC cells after 24 h of exposure. On the other hand, barrier integrity or cell viability decreased after exposure to hazardous proteins for 24 h, being this result more evident after 48 h and 72 h for both intestinal epithelial cells. Also, the safety of insect-derived protein hydrolysates has been evaluated using *in vitro* cytotoxicity assays. Zielińska et al. (2015) studied the cytotoxicity effect of raw, cooked and baked protein hydrolysates from *Tenebrio molitor*, *Schistocerca gregaria* and *Gryllodes sigillatus* to human skin fibroblasts CRL-2522. Authors indicated that hydrolysates from *T. molitor* and *G. sigillatus* had no cytotoxic effects (before and after heat treatment). On the other hand, *S. gregaria* hydrolysate (particularly, raw samples) showed cytotoxic effects (up to 40 % cell death) towards fibroblast CRL-2522.

Moreover, cytotoxicity studies have provided evidence that proteins derived from non-animal and animal sources could exhibit anticancer and antioxidant activities (Beltrán-Barrientos et al., 2017). For example, Rayaprolu et al. (2017) characterized protein hydrolysate fractions from a high oleic acid soybean. The factions obtained (10-50 kDa fraction peptides) were tested on blood (CCRF-CEM), colon (Caco-2 and HCT-116), and liver (HepG-2) cancer cell lines for their inhibitory effects on cancer cell proliferation. Peptides were found to exhibit anti-proliferation activity on the three types of cancer cells. In another study, Chalamaiah et al. (2015) assessed the anticancer properties of protein hydrolysates derived from rohu (*Labeo rohita*) roes (eggs) by enzymatic hydrolysis (using pepsin or trypsin) against human colon cancer cell line

(Caco-2). The results showed that pepsin protein hydrolysate exhibited 65 % antiproliferative activity on Caco-2 cells. Furthermore, antioxidant activity and cytotoxicity properties of peptides derived from microalgae *Navicula incerta* (by enzymatic hydrolysis) in HepG2/CYP2E1 cells have been studied (Kang, Qian, Ryu, Kim, & Kim, 2012). Results showed that *N. incerta* peptides attenuated the ethanol-induced cytotoxicity of HepG2 cells. Other study showed that *Chlorella pyrenoidosa*-derived peptide provides protective effects against UVC-induced cytotoxicity in human skin fibroblasts (Shih & Cherng, 2012).

Also, experimental animal models for human risk assessment were used to determine protein cytotoxicity. For instance, Canistro et al. (2017) studied rapeseed and sunflower protein hydrolysates toxicity and metabolic effects in a murine animal model. This study showed no toxicity effect of the protein hydrolysates diet on mice, since no changes were detected on growth, organ weight, blood biochemical and food intake parameters.

5.3. Allergenicity

Food allergies are growing considerably among the world population, particularly in infants. Very severe allergic reactions against ingested foods, mainly to dairy proteins, lead to anaphylaxis. Food allergic reactions are described as adverse reactions to health (from local and transient effects to systemic anaphylaxis) that induce specific immune response in susceptible subjects following dietary exposure to relevant allergens in food (Verhoeckx et al., 2015). In a broad sense, allergic food reactions can include immunoglobulins (IgE) and non-IgE-mediated primary immunological sensitivities, non-immunological food intolerances, and secondary sensitivities (Taylor & Hefle, 2001). The main form of immune-mediated allergic reactions to foods is linked to IgE formation against food allergens (Type I reactions). Some frequent food inducers of IgE-associated allergy are egg, milk, fish, wheat, nuts, vegetables and fruits (Valenta, Hochwallner, Linhart, & Pahr, 2015). Normally, an allergic reaction occurs when IgE antibodies are produced and bind to the ingested proteins. Consequently, IgE is bound to the surface of effectors' cells (basophils or mast cells), leading them to release mediators such as histamine, leukotrienes and cytokines, resulting in allergic response directly at the sites of allergen contact (e.g., mouth and intestine) or in other organs, when allergens are able to pass through the mucosa barrier into the blood circulation (Valenta et al., 2015).

Peptides derived from diverse sources (e.g., milk, egg, soybean, fish and nuts) have been shown to strongly influence the immune system. Vegetable proteins have attracted significant interest compared to animal proteins, due to their potential non-allergenicity. Thus, in addition to cytotoxicity, the incidence of allergic reactions following novel protein consumption (e.g., from insect origin) has been assessed (Parenti, Santoro, Del Rio, & Franceschi, 2019). The majority of studies explored the proteins' immunomodulatory properties and their influence on inflammatory cytokine production, antibodies production, and lymphocyte activity and proliferation (Parenti et al., 2019). One study reported that oral administration of carp egg protein hydrolysates (CEPHs) (pepsin hydrolyzed) to female BALB/c mice significantly enhanced humoral immune response because immunoglobulin A (IgA) antibody production increase in serum. Moreover, the splenic natural killer (NK) cells cytotoxicity increased in the gut, which indicates a host capacity improvement to fight against tumor cells and virus-infected cells (Chalamaiah, Hemalatha, et al., 2015).

In this framework, many technological novel and emergent food process applications (e.g., OH, PEF, HHP and enzymatic treatment) could contribute to avoid or limit proteins' allergenicity, at least for some classes of allergic groups, by changing the allergen appearance or cleaving the allergenic protein epitopes. Some reviews (Chizoba Ekezie, Cheng, & Sun, 2018; Rahaman, Vasiljevic, & Ramchandran, 2016; Verhoeckx et al., 2015) have gathered information about the potential effect of food processing technologies on the reduction of various proteins' allergenicity. For instance, ovalbumin (one of the most important sensitizing ingredients in allergens of egg albumin) was submitted to a pre-heating treatment integrated with glycation, and allergenicity was estimated by indirect competitive ELISA. The results showed that allergenicity decreased because IgG/IgE-binding capability of ovalbumin was dramatically reduced due to IgG and IgE epitopes cover and ovalbumin structural changes (Liao et al., 2018). It should be also highlighted that protein allergenicity could increase, as well the formation of neoallergenic species, due to food processing. For example, allergenicity of soy protein isolate (SPI) treated by enzymatic hydrolysis with alcalase, trypsin, chymotrypsin, bromelain, or papain was evaluated by *in vitro* IgE binding. Results showed that SPI enzymatic hydrolysis did not reduce the allergenicity, and chymotrypsin or bromelain hydrolysis could increase SPI allergenicity (Panda, Tetteh, Pramod, & Goodman, 2015). Moreover, research works showed that non-thermal treatments, like ultrasonication, microwave and HHP could possibly change food allergenicity (Pojić, Mišan, & Tiwari, 2018). For example, SPI allergenicity for infant formulae decreased 24.7 % due to a microwave treatment that changed proteins' secondary structure (Huijing Li, Zhu, Zhou, Peng, & Guo, 2016). The same authors also decreased SPI allergenicity by 18.9 %, 29.8 % and 46.6 % using high-intensity ultrasound, high-pressure homogenization and HHP, respectively (Li et al., 2016). In another study, Li, Zhu, Zhou, & Peng (2012) demonstrated the reduction of SPI allergenicity (48.6 %) as compared to non-treated SPI due to HHP at 300 MPa for 15 min.

Another way to reduce the potential protein allergenicity is to use capsules (e.g., nanocapsules) as protein carriers to avoid the activation of immune cells through antibodies identification (Pohlit et al., 2015). Gamazo et al. (2014) reviewed the use of nanoparticles as delivery systems to carry protein allergens.

Furthermore, a complete allergenicity risk assessment is needed (according to the European Food Safety Authority (EFSA) legislation) to approve novel protein sources as potential food ingredients taking into consideration various aspects such as route (e.g., oral) and dose of protein exposure, protein properties (e.g., physicochemical properties) and how protein is perceived by each person's immune system (Parenti et al., 2019). Different strategies can be used to assess allergenicity risks (Crevel et al., 2014). One of these strategies is a "weight-of-evidence approach" used for food derived from genetically modified plants (K. Verhoeckx, Broekman, Knulst, & Houben, 2016). The allergenicity risk assessment conclusion is the probability of a protein being an allergen. This strategy focuses on cross-reactivity; however, the novel (or modified) proteins can also cause a risk of *de novo* sensitization, which could lead to novel food allergies (Pali-Schöll et al., 2019). Some relevant methods for protein allergenic risk assessment were described by other authors (Mazzucchelli et al., 2018). The assessment of protein transport across the intestinal barrier, as well as its implication on epithelial permeability are important factors in allergenicity risk assessment (Reitsma, Westerhout, Wichers, Wortelboer, & Verhoeckx, 2014). The application of diverse epithelial cell models to study these characteristics has been reviewed (Cubells-Baeza et al., 2015). For instance, Hurley et al. (2016) used an *in vitro* human intestinal model system with epithelial cell lines (T84, Caco-2, and HCT-8) to evaluate potential protein hazards. Indicators of cytotoxicity (LDH release, MTT conversion), monolayer barrier integrity ([3H]-inulin flux, horseradish peroxidase flux and transepithelial electrical resistance (TEER), and inflammation (IL-8, IL-6 release) were monitored. These authors reported that this model system effectively distinguished between hazardous and non-hazardous proteins through combined analysis of multiple cell lines and assays.

6. Conclusions

Alternative protein sources such as vegetables, insects and microbial proteins, can address different pillars and the goals of food security and sustainability. Their cultivation and use can contribute to preserve natural resources and biodiversity, reducing environmental damage and climate changes. Moreover, they are a source of macro and micronutrients, including high quality proteins that can be related to healthy diets and also to the prevention of diverse diseases.

As previously mentioned, HHP and electrotechnologies are recognized as environmentally friendly processes due to their high energy efficiencies and possibility to reduce the use of non-renewable resources (e.g., water and fossil energy). From the processing point of view, they bring electrical and pressure effects that can be combined with heat (if needed), offering a great versatility of processing strategies towards preservation, extraction and functional aspects of food proteins. However, the implications of these effects on the biological value of emergent food protein fractions, such as the ones related with nutritional composition, digestibility and allergenicity is far from being understood. Fundamental research is still necessary to unravel many of the potential benefits of these technologies and to develop innovative products based on alternative protein-rich fractions.

Regarding emergent proteins used as ingredients, their functional-technological properties are still at an exploratory level in most of the cases. While vegetable proteins were well described in the last decade, insects and microbial proteins were less explored. The effect of extraction procedures on proteins' final properties and on their aggregation and gelling mechanisms have been reported. Therefore, studies are unraveling such proteins as promising ingredients to replace conventional proteins in the food industry.

Furthermore, proteins from novel sources can be used to prepare a wide range of structures at different scales (e.g., micro and nanostructures) for bioactive compounds' delivery, which can be tailored for a specific application in innovative products, following the current "green" trend in the food industry.

Future evaluation regarding emergent proteins' fate in human gastrointestinal tract (e.g., digestion and bioavailability) is of utmost importance since reduced protein digestibility may play a role in food allergenicity. Moreover, numerous proteins have been reported to have cytotoxic or allergenic effects against human cells. Thus, more studies are needed to determine if the use of proteins from novel sources will result in more benefits than risks. Particularly, the information on nanotechnology safety exploring the use of alternative proteins remains limited or less explored and requires further risk assessment, particularly for long-term toxicity and allergenicity.

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8. Authors contribution

The authors Luiz H. Fasolin, Ricardo N. Pereira, Joana T. Martins, Ana C. Pinheiro, Cristiane C. P. Andrade and Oscar L. Ramos contributed to conception, design, writing and revision of the manuscript. The author António A. Vicente contributed to revision of the manuscript. All authors read and approved the submitted version.

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