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Nutritional protein quality and digestibility changes during food processing

Cambios en la calidad nutricional y digestibilidad de la proteína durante el procesamiento de alimentos

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Abstract

Proteins can be broadly classified by their origin (plant or animal based), amino acid composition (essential versus non-essential; complete versus incomplete), and their digestibility. The composition or quality of various proteins may be so unique that their influence on physiological function in the human body can be quite different. The ability to accurately and objectively define protein quality plays an important role in addressing human nutrition requirements, nutrition policy, trade, and product development. Such quality is influenced by the availability of amino acids, which depends on various factors like protein origin, previous processing treatments, and interactions with other food components. We review the advances in dietary protein evaluation, focusing on the bioavailability of proteins and the changes suffered during food processing. The awareness of all the multidimensional factors involved, have allowed food scientists and technologists to tailor better processing conditions for improving protein overall quality, functional properties, digestibility, and bioavailability, and for consumers to form criteria for selecting appropriate food products.

Keywords: Protein, amino acid, nutritional changes, digestibility, food processing.

Resumen

En general, las proteínas se pueden clasificar por su origen (vegetales o animales), composición de aminoácidos (esenciales vs no-esenciales; completos vs incompletos), y por su digestibilidad. La composición o calidad de varias proteínas puede ser tan única que pueden influir en la función fisiológica del cuerpo humano de forma disímil. La habilidad para definir la calidad de la proteína con precisión y objetivamente juega un papel importante en el establecimiento de los requerimientos nutricionales del ser humano, políticas nutricionales, comercio y desarrollo de productos. Su calidad está influenciada por la disponibilidad de aminoácidos, que depende de varios factores como lo son el origen de la proteína, tratamientos previos, e interacciones con otros compuestos de los alimentos. Aquí se revisarán los esfuerzos históricos para evaluar la proteína dietética, enfocándonos en la biodisponibilidad de las proteínas y los cambios que sufren durante el procesamiento de alimentos. El conocimiento de todos los factores multidimensionales involucrados, ha permitido a los profesionales en ciencia y tecnología de los alimentos diseñar condiciones de procesamiento que para lograr una mejor calidad global, propiedades funcionales, digestibilidad y biodisponibilidad de la proteína y a que los consumidores formen criterios para seleccionar sus alimentos apropiadamente.

Palabras clave: Proteína, aminoácido, cambios nutricionales, digestibilidad, procesamiento de alimentos.

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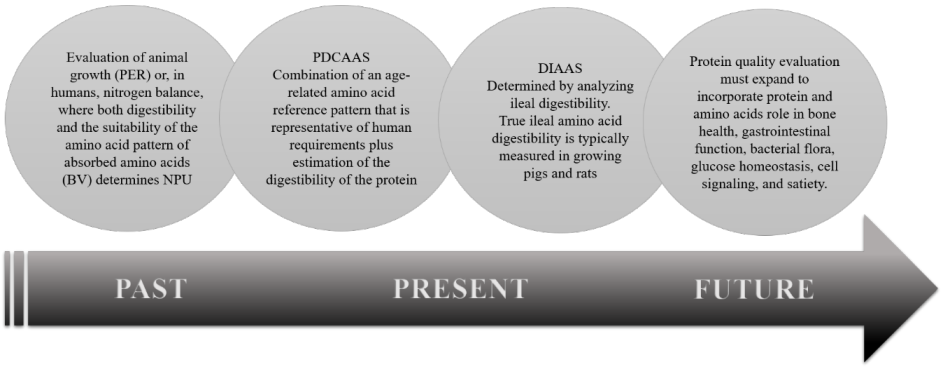

Figure 1. Protein quality evaluation through time. Protein efficiency ratio, PER; net protein utilization, NPU; biological value, BV; protein digestibility-corrected amino acid score, PDCAAS;

1 Introduction

A food matrix is formed by water, polysaccharides, lipids, proteins, and other minor components. All these components are required mainly as building blocks for tissues and as energy source. In this way, the nature and efficiency of absorption and utilization of food components is a major issue from economical, health and social standpoints. Food science and engineering research is concerned with the fabrication of food products with tailored nutritional features. This problem is of importance as different population segments require particular nutritional needs, for instance, ageing populations which require a higher intake of high-quality protein for maintaining muscle mass and strength (Lonnie *et al*., 2018). Proteins are important components of food products, with extensive and detailed studies from a nutritional viewpoint. Major concerns are related to the composition and factors linked to digestibility and utilization of amino acids. Hsu *et al*. (1977) proposed an *in vitro* multienzyme method that allowed to predict the apparent protein digestibility within 1 h which was highly correlated with the in vivo apparent digestibility of rats. However, there does not exist a clear classification of proteins in terms of digestibility properties. This has led to a continuous search of how food design and processing affect protein overall quality and digestibility (Zahir *et al*., 2018; Bhat *et al*., 2021a).

Thus, the aims of this review are to: (1) provide a brief description of methods, concerns, and processing effects regarding the digestibility of proteins, and (2) to motivate the food science and engineering community to increase research of protein digestibility for improving the design and processing of food matrices with prescribed digestibility characteristics.

2 Dietary protein evaluation

Proteins are of great nutritional value and are directly involved in the chemical processes essential for life. There are many animal and vegetable sources of protein available to humans. However, their ability to achieve defined metabolic actions or quality may differ substantially depending on their (essential) amino acid composition and digestibility within a food matrix (Millward *et al*., 2008). Since protein quality is an important aspect of any consideration of human protein needs, there have been extensive efforts to measure quality and standardize those measurements (Figure 1). For many years, bioassays, mainly with rats, were the methods of choice to assess the nutritional value of proteins. This value was expressed in parameters such as protein efficiency ratio (PER), net protein utilization (NPU) and biological value (BV) (Schaafsma, 2000). The PER compares the growth response of young rats, fed a marginal amount of a test protein, with that of control rats, fed a similar amount of casein, and the NPU is, in fact, the product of digestibility (digestion and absorption) and biological value (the amount of utilized nitrogen divided by the amount of absorbed nitrogen) (Schaafsma, 2012). However, the rat may be able to digest more poorly digestible proteins than humans (Deglaire and Moughan, 2012). Furthermore, the rat amino acid requirement pattern differs to that of humans, so that the only correct measure

of protein quality was nitrogen balance evaluation in humans, which is too expensive for routine use (Schaafsma, 2012). Practical difficulties encountered with the nitrogen balance method led to the adoption of the protein digestibility-corrected amino acid score (PDCAAS). The PDCAAS, adjusts the level of protein in a food by using the indispensable amino acid content of the food (mg/g protein), indispensable amino acid requirements of a reference population (mg/g protein), and the weighted average true fecal nitrogen digestibility of the sources of protein in the test food (Marinangeli and House, 2017). This method has proved to be of considerable value in practice. Nevertheless, the use of fecal nitrogen to estimate digestibility has been recognized as a notable limitation of PDCAAS method. This estimation of the crude protein digestibility over the total digestive tract leads to an overestimation of the number of essential amino acids absorbed (Callaghan *et al*., 2017). Therefore, some food products may claim high protein content, although they are not providing readily digestible essential amino acids in quantities that correspond to human requirements (Leser, 2013). Moreover, a low ileal protein digestibility increases the flow of nitrogen into the colon. In humans, an association exists between high protein intake and fermentation metabolite concentrations in patients with inflammatory bowel disease. Fermentation of undigested protein in the hindgut may result in the formation of toxic compounds such as ammonia, phenolic and indolic compounds, biogenic amines, hydrogen sulfide and nitric oxide that may cause severe intestinal disease and the risk of colon cancer (Gilbert *et al*., 2018). For these reasons, the FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition (FAO, 2013) recommended replacing the PDCAAS method with the digestible indispensable amino acid score (DIAAS). The DIAAS determines amino acid digestibility, at the end of the small intestine, providing a more accurate measure of the amounts of amino acids absorbed by the body and the protein contribution to human amino acid and nitrogen requirements (Leser, 2013). The use of the DIAAS method was a step forward on the evaluation and perfection of techniques to directly measure the bioavailability of protein-bound dietary amino acids in humans. However, as research continued to evolve in assessing protein's role in optimal health at higher intakes, there was also a need to continue to explore implications for protein quality assessment (Millward *et al*., 2008). Methods using metabolomics approaches and relating complex metabolite profiles from plasma and urine samples to protein and amino acid true ileal digestibility and availability offer a promising perspective for the evaluation of dietary protein quality in humans (FAO, 2013).

3 Protein digestibility

A second important issue in quality evaluation relates to the bioavailability or digestibility of a protein or the capacity to provide metabolically available nitrogen and amino acids to tissues and organs. A protein can be predicted as being of good quality based on its amino acid score, but in practice may be of poor quality because it is poorly digested and/or absorbed. Thus, when making recommendations for protein requirements, factors which might affect digestibility or absorption should also be considered (Millward *et al*., 2008).

There are many factors that affect protein digestibility as can be observed in Table 1. Some proteins in raw products are by nature poorly digestible because of structural peculiarities. Others become difficult to digest due to protein processing treatments, such as extruding, boiling, fermentation, homogenization or heat treatment that could modify the conformation of the protein and consequently its susceptibility to enzymes (Becker and Yu, 2013). Fourier transform infrared spectroscopy (FT-IR) and Fourier transform Raman (FT-Raman) have been used to determine modifications on the secondary structure of proteins by effect of different processing treatments such as proteolysis, thermal, high electric field, cold-pressure and isoelectric precipitation (González-Cruz *et al*., 2020). The modifications on the conformational structure of proteins occur mainly about the secondary structure as the transitional effect of α-helix structure to the β-sheet structure during protein gelation, unfolding of proteins, interactions between CO_2 and specific amino acids, and generation of disordered structures and random coil have been mainly about those occurring on the secondary structure (González-Cruz *et al*., 2020). Changes in α-helix and β-sheet structures start to occur at heating temperatures above 55 °C, and an almost complete loss of secondary and tertiary structure, as well as cleavage of disulfide bonds, occurs at temperatures above 70-80 °C (Hellwig and Henle, 2014). At the same time, because of protein denaturation, irreversible intermolecular interactions may result in protein aggregation and cross-linking reactions between amino acids, e.g.,

Table 1. Factors which impair enzymatic protein digestion.

Naturally occurring limiting structures	Animals	Scleroproteins such as collagen, elastin, keratin, and silk fibroin that form supporting structures in the body and are resistant to digestion due to their unusual structures.
	Plants	Plant proteins have lower digestibility due to their relative insolubility, intracellular organization in discrete protein bodies, and protective covering of the seed by the seed coat. They usually require some processing to improve the protein digestibility.
Processing treatments	Heat-treatment	Enhances polymerization and changes in secondary structure which decreases enzymatic digestibility of sorghum proteins.
	Maillard reaction	Causes a decrease of protein nutritional quality due to a condensation reaction between the carbonyl group of a reducing carbohydrate and the free amino groups of a protein, which originates Maillardized peptides that cannot be absorbed by the gut.
	Irradiation	Reduces protein digestibility due to cross-linking and to the formation of Maillard products, which inhibit enzymatic protein digestion.
Anti-nutritional factors	Tannins	They have been linked to weight gain reduction due to their inhibition of digestion of dietary proteins. Apparently, they reduce feed digestibility by the formation of tannin-nutrient complexes.
	Protease inhibitors	Inhibit the activity of trypsin and chymotrypsin, thus preventing protein digestion.
Changes in chemical structures	Disulfide bonds	They stabilize the protein structure making it more resistant to proteolytic degradation.
	Cross-linking	Lowers the digestibility of food because the cross-linked, aggregated protein is less accessible to digestive enzymes.
	Oxidation	Impairs protein function, leading to an increase of protein hydrophobicity, which results in the formation of toxic aggregates. Diminishes the sensory and nutritional protein quality due lysin and sulfur amino acids loss.

Adapted from Becker and Yu (2013)

through the formation of lysinoalanine (LAL). These heat-induced conformational changes of food proteins, may affect digestion and absorption of proteins/peptides by the intestinal epithelium, as well as their recognition by immune cells (de Oliveira *et al*., 2016). Furthermore, heat-induced treatment combined with other treatments, i.e., microfluidization, may affect the particle size, stability and surface roughness of the protein aggregates, and affect their digestion/absorption (Monroy-Rodríguez *et al*., 2021). Food and feed products may also contain anti-nutritional factors or protease inhibitors that may adversely affect protein digestibility and amino acid availability. Some anti-nutritional factors may occur naturally, such as trypsin inhibitors and hemagglutinins in legumes, tannins in legumes and cereals, phytatesin cereals and oilseeds; and some, like Maillardized peptides and oxidized amino acids, are formed during heat/alkaline processing of protein products (Gilani *et al*., 2012). Moreover, the food matrix in which a protein is consumed or processed also impacts on the bioavailability of amino acids (Millward *et al*, 2008). In this sense, the microstructural arrangement of the food matrix and molecular interactions (protein-protein, protein-polysaccharide, or protein-lipid) could modify the enzymatic susceptibility of a protein and its behavior in the acidic pH of the stomach (Morell *et al*., 2017). Thus, it can be concluded that food processing, an issue that will be addressed further on, has a significant effect on protein digestibility, since it influences the matrix structure and protein conformation, and therefore the digestion and absorption kinetics of amino acids.

The digestion rate of a protein is another factor that should be taken into consideration when talking about protein digestibility. The concept of 'slow' and 'fast' proteins, according to the speed at which proteins are digested and amino acids are absorbed from the gut, was introduced by Boirie *et al*. (1997). They studied the effect of two milk proteins, casein and whey protein, in postprandial whole-body protein metabolism, and found that although whole milk (casein) and whey (lactoglobulins) contain all essential amino acids, they are digested and absorbed differently. Casein coagulates in the acidic environment in the stomach reducing gastric emptying and inducing a slow postprandial increase in plasma amino acids (Bendtsen *et al*., 2013).

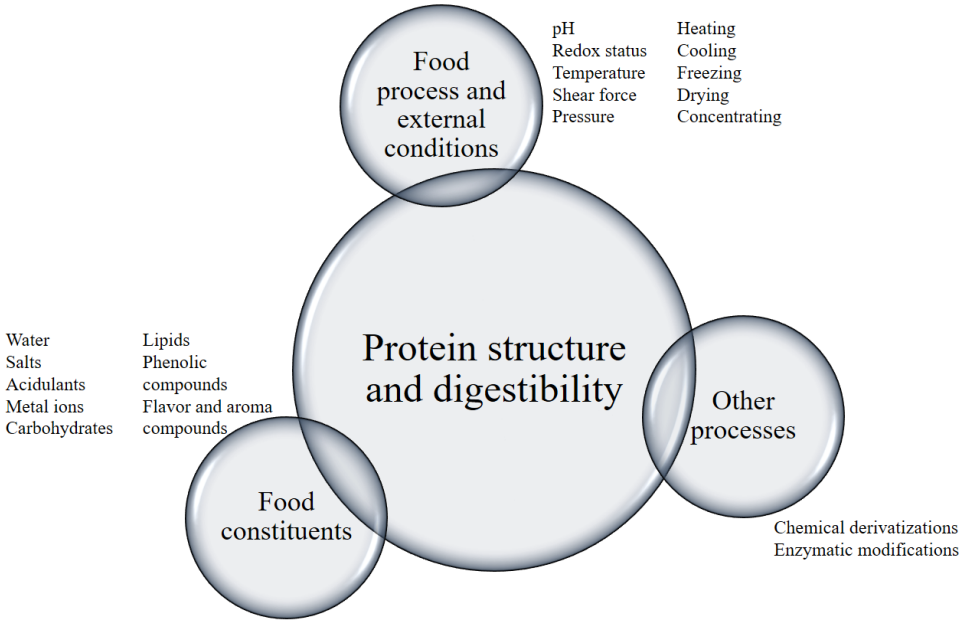

Figure 2. Factors which determine the structure and digestibility of food proteins. Adapted from Yada (2018).

Meanwhile, whey proteins pass rapidly through the stomach leading to a faster delivery of amino acids. Such findings led to the conclusion that slow and fast proteins differentially modulate whole-body protein deposition after a meal. The results suggest that a slowly digested protein induces a better postprandial utilization than a fast one (Dagnin *et al*., 2001). Still, more studies evaluating protein digestion rate effect on nitrogen retention were needed, since factors as age may be an important determinant of the mechanisms of nitrogen retention. Either way, the concept of slow and fast proteins offers several therapeutic possibilities for patients with wasting disorders (He and Giuseppin, 2014), a topic requiring further investigation.

4 Food processing and protein digestibility

The benefits of food processing are diverse and range from food safety to convenience, making it a routine procedure that is somewhat under studied. Processing conditions encompass a wide range of chemical and physical environments, a variety of components with different properties coexisting in foodstuffs, so that interactions between them are inevitable. Non-covalent forces, such as hydrogen bond, hydrophobic interaction, electrostatic interaction, and van der Waals forces, are mainly responsible for these interactions. The interplay between proteins/enzymes and other food components affects food quality and protein structure (Lv *et al*., 2017). Moreover, during food processing, protein sources are treated with heat, oxidizing agents, organic solvents, alkalis, and acids for a variety of reasons such as to sterilize/pasteurize, improve flavor and texture, deactivate antinutritional factors, and prepare concentrated protein products (Vagadia *et al*., 2017). Such processing treatments may cause the formation of Maillard compounds, protein cross-linking, and racemization of amino acids, which, as was previously mentioned, tend to make the protein less digestible (Table 1). However, there is not a full understanding of how the different processing (especially those named non-thermal treatment) impact on digestibility or bioavailability, therefore this has become an increasing research area.

Commonly, processing results in protein denaturation, which modifies protein functionality and exposes reactive groups that can react with each other as well as with other food components. Many of these chemical reactions, such as the formation of color and flavor compounds, are the reason why the food processing is performed (Fayle *et al*., 2002). However, many of those changes also impact on protein quality and digestibility. In Table 2, it was mentioned that some of the last decade studies

Table 2. Selected food processing methods effect on selected proteins digestibility.

Food product	Effect on protein digestibility	Reference
Vegetable feed ingredients	During thermal processing, proteins reacted with reducing sugars to produce Maillard products that decreased the digestibility of the protein	Salazar-Villanea *et al*. (2016)
Lentil and faba bean concentrates	High pressure processing produced greater gastric digestibility	Hall and Moraru (2021)
Faba bean isolate	Ultrasonication treatment decreased protein digestibility	Martínez-Velasco *et al*. (2018)
Soybean milk	Microwave treatment increased soy proteins digestibility	Vanga *et al*. (2020b)
Shrimp	Microwave treatment (125 °C, 15 min) decreased the allergenicity of tropomyosin and *in vitro* digestibility	Dong *et al*. (2021)
Beef	Freezing-then-aged treatment (FA) was applied and compared to an only aged control. Post *in vitro* digestion (14 days aged) showed that FA had enhanced protein digestibility	Lee *et al*. (2021)
Muscle foods	Ultrasound can induce denaturation and affect de unfolding-refolding of proteins, affecting the diffusion of proteases into the protein matrix and their accessibility to cleavage sites, increasing digestibility	Bhat *et al*. (2021b)
Milk protein	Milk proteins exposed to various heat treatments (temperature, time) induced changes on the digestibility of the protein, which could be used for tuning the gastric coagulation behavior of milk proteins.	Li *et al*. (2021)
Buffalo and cow milk	Microfluidization improved lactose and protein stability and in vivo Wistar rat digestibility.	Kumar *et al*. (2019)
Liquid whole eggs	Showed no *in vitro* digestibility differences when thermally treated at 60°C for 10 min with untreated control, but digestibility was improved when treated at 80 °C for 10 min.	Bhat *et al*. (2021a)
Egg white proteins	Thermally treated at 65°C for 30 min, exhibited higher digestibility than when treated at 56°c for 32 min or !00 °C for 5 min. Applying HPP in the range of 400?700 MPa led to the formation of aggregates stabilized mainly by SS bonds. Increasing pressures increased the formation of protein aggregates, which were more prone to enzymatic hydrolysis.	Farjami *et al*. (2021)

focused on the effect of food processing methods on the digestibility of different protein-rich products. Almost every heat treatment alters digestibility, and only the treatments aiming to reduce antinutrients showed an increase on protein digestibility due to partial removal of antinutrients that probably created a large space within the matrix, which increased the susceptibility to enzymatic attack and consequently improved the digestibility of the protein (Rehman and Shah, 2005). Changes in digestibility are apparently time and heat dependent, suggesting that different processing treatments affect physicochemical properties of food proteins in different ways which influence accessibility and digestibility of the protein. Moreover, the inherent characteristics of the protein, the treatment used, the intensity of the treatment, the environment condition, the food matrix, and the combination of these determine the impact of processing on the quality and digestibility in a food protein (see Figure 2). Therefore, every time a new production process is introduced vast array of protein modifications occur. Such modifications affect the structure, functionality, and digestibility of the proteins, but also can lead to the production of detrimental (toxic) or bioactive compounds, that often remain uncharacterized or overlooked (Meade *et al*., 2005). Additionally, rare attention has been paid in the literature to understand if the reheating treatment at homes, i.e., in ovens or microwave, has any additional influence on the digestibility of the proteins ingested (Laguna *et al*., 2016). Hence, despite years of research, much remains to be learned about the effect of the chemical modification of proteins undergo during food processing due to the complex nature of the changes involved and to the difficulties imposed when analyzing proteins within a food matrix. Moreover, the consequences of such changes on stomach emptying rate, gut motility, and gut hormones secretion require future research.

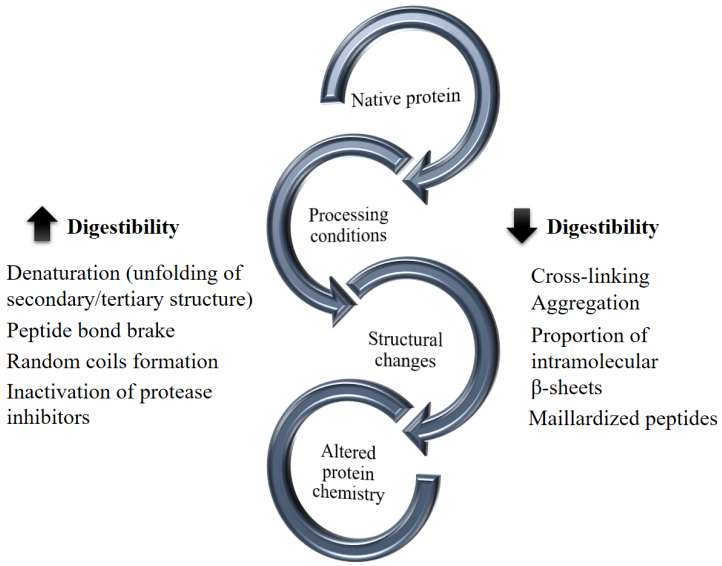

Figure 3. Structural changes that modify protein digestibility.

5 Protein structural changes during processing

So far, it is known that protein structure influences the accessibility of enzymes for digestion, and that the impact of the physical and chemical processing on the structure of food proteins is diverse. Now, the effects of processing on the physicochemical changes of proteins and the consequences of these changes on protein digestion will be reviewed (Figure 3). During food processing, the exposure of proteins to harsh conditions, like extreme pH values, heat, or pressure, causes protein denaturation which facilitates the accessibility of proteases to the peptide bonds and modifies the protein structure. Such changes can result either in the formation of random coils, which exposes groups that are not usually accessible, thus making it more susceptible to enzymatic hydrolysis; or in protein aggregation and cross-linking that decreases protein digestibility (Carbonaro *et al*., 2012). However, there is not a consensus on how a protein will respond since the extent of the structural changes in proteins depends on the conditions employed during processing, the amino acid composition, and the net charge of the protein (Tang *et al*, 2009).

Traditional food processing technologies (i.e., thermal processing, chilling, freezing, acidification) are being displaced by emerging new food process and preservation technologies (i.e., high pressure, ultrasound, microwave, microfluidization) (Bhat *et al*., 2021b). Following, we present some recent applications of these technologies to diverse protein food sources, and relevant reported findings.

5.1 Preservation methods

5.1.1 Thermal processing

The use of high temperature processing is known to induce important changes in the structure of proteins. It is widely used for improving the digestibility of proteins or to eliminate antinutritional factors. However, during thermal processing, simultaneously to the inactivation of antinutritional factors and the formation of random coils (due to denaturation), proteins react with reducing sugars to produce Maillard products that reduce the digestibility of the protein (Salazar-Villanea *et al*., 2016). Moreover, thermal processing originates changes in the proportion of intramolecular β-sheets in the secondary structure of proteins, which has been related to a decrease in protein digestibility (Carbonaro *et al*., 2012). Thus, the overall effect of thermal treatments seems to be a combination of the positive and negative effects on each individual protein fraction, and therefore, the effect of thermal treatments on protein digestibility could be linked to the nature of the protein (Salazar-Villanea *et al*., 2016). Recently, Li *et al*. (2021) made an excellent review of the fate of milk proteins, which are exposed to various heat

treatments during the processing and storage of dairy products. The milk proteins undergo modifications at the molecular, microstructural, and macrostructural levels. The type and extent of the heat-induced changes on the digestibility of the protein depended on the product matrix and the heating conditions. They considered that heat treatment may be used as a tool for tuning the gastric coagulation behavior of milk proteins to attain different digestive outcomes. Tarhan and Kaya (2021) investigated the structural changes of cow milk proteins when processed to cheese. The effect of heating, of culture/enzyme activity, and other treatments on protein composition and molecular structure were evaluated. Curdling after pasteurization produced newborn peptides not found in milk. Similarities in peptide profiles in cheeses to that of raw milk indicated that high-temperature pasteurization induced protein unfolding followed by extensive proteolysis.

The effect of diverse thermal processes during steaming, microwave, baking and extrusion on the *in vitro* protein digestibility of highland germinated barley were analyzed. All the thermally processed samples displayed an increased protein digestibility, especially the extrusion processing (Huang *et al*., 2021). Also, the boiling of soybeans produced a significant increase in protein digestibility (48.71 %), that was comparable to that produced by fermentation (50.21 %) and superior to that achieved by soaking (20.58 %) (Ketnawa and Ogama, 2021). Fermented soybean exhibited the maximum antioxidant activity at the end of the simulated digestion, followed by boiling. The improvement overall digestibility and antioxidant properties after the digestion, could be probably attributed to a synergistic combination of concentration of peptides/free amino acids, low molecular weight peptides, and high percentages of high antioxidative amino acid residues (bioactive peptides). Bhat *et al*. (2021a) made a review of the effect of processing conditions on the digestibility of egg proteins. Eggs are an important vehicle for *Salmonella* infection so that they are increasingly pasteurized, and conditions used are very broad. For liquid whole eggs, temperatures fluctuate between 60-68 °C, for 2-5 min. In general, no significant effect was observed in the protein susceptibility to *in vitro* gastrointestinal digestion simulation when heated at 60 °C for 10 min compared to an untreated control. When eggs were heated at 80 °C for 10 min, a significant increase in protein susceptibility occurred, and the protein ovoalbumin and protein aggregates were completely hydrolyzed during the first 30 min of peptic digestion. Farjami *et al*. (2021) heated egg white proteins (EWPs) at different conditions (65 °C for 30 min; 56 °C for 32 min; and 100 °C for 5 min). EWPs (65 °C, 30 min) showed the higher peptic hydrolysis than those treated at 56 °C for 32 min and 100 °C for 5 min, attributed to proper denaturation of EWPs, which exposed hidden hydrolysis sites. EWPs (100 °C, 5 min) underwent extensive aggregation, that could hide some cleavage sites. At supramolecular scale, the formation of aggregated structures with different sizes and morphologies can influence the digestion of EWPs. The extent of proteolysis was the greatest for linear aggregates that were almost entirely digested after only 10 min of *in vitro* simulated gastric digestion.

5.1.2 High pressure processing (HPP)

HPP is an environmentally friendly emerging mild processing technology, reported to induce several effects in food proteins such as modification of the secondary structure, unfolding or improved digestibility of proteins (Bhat *et al*., 2021a). Hall and Moraru (2021) studied the effects of HPP (600 MPa/5 °C/4 min) on the *in vitro* digestibility and tripsin inhibitor activity of protein concentrates (15 g protein/100 g for digestibility; 5 g protein/100 g for tripsin inhibitor activity) from lentil and faba bean. HPP produced different hydrolyzed peptide patterns after gastric digestion. HPP resulted in comparable or greater gastric digestibility than untreated controls, but superior to heat treatment (95 °C/15 min). Neither treatment impacted overall *in vitro* protein digestibility. Heat treatment was superior for reducing trypsin inhibitor activity than HPP, that only achieved a slight reduction compared to both lentil and faba bean controls. It was concluded that neither HPP o heat treatment impacted the overall lentil or faba bean protein quality. Mune *et al*. (2020) subjected Bambara bean protein isolate to 200-600 MPa range at different pH (4.5, 7 and 9). Detailed information at atomic level on the structural changes of the main proteins was obtained by molecular modeling. In short, HPP resulted in decrease of the total number of hydrogen bonds stabilizing the structure of proteins, which altered exposure of both Tyr and Trp residues. Wang and Moraru (2021) studied the effect of pH (6.6-5.1) and calcium (24 to 36 mg of Ca/g of protein) on the structural properties of gels created by HPP (600 MPa, 5°C, 3 min) of milk protein concentrate (MPC, 12.5% protein) were evaluated. A pressurization time of 3 min was sufficient to induce gel formation.

Increasing pH from 5.3 to 6.6 increased gel strength. Adjusting the pH or calcium affected the structure of the HPP-milk gels by influencing electrostatic interactions and shifting the Ca-phosphate balance. Reducing the pH or increasing the calcium content produced more porous structures. Farjami *et al*. (2021) reported that applying HPP in the range of 400-700 MPa led to the formation of aggregates stabilized mainly by SS bonds in egg white proteins (EWPs) solution (9.6 mg/mL). By increasing pressure (from 400 to 700 MPa), protein solubility decreased, and turbidity of solution increased, indicative that protein aggregates were formed. The susceptibility of these aggregates to enzymatic hydrolysis was improved. Thus, simultaneous HPP and proteolytic enzymatic treatment are recommended for obtaining more extensive proteolysis of ovalbumin.

High pressure thermal processing (HPT; 100-140°C and 0.1-600 MPa for up to 60 min) was used to study the proteolysis, lactosylation, and non-enzymatic browning kinetics of skim milk (Devi *et al*., 2013). The rate of lactosylation and browning of milk increased with temperature at atmospheric pressure. The rate of browning at 110 °C increased three-fold at 400 MPa. Lactosylation of milk proteins steadily slowed with increasing pressure. At 130 °C, a remarkable reduction (from 60 to 20%) of the lactosylation degree was obtained when the pressure was increased from 0.1 to 600 MPa. Proteolysis of milk was accelerated with increasing temperatures at all tested pressures. However, hydrophobic and hydrophilic peptides increased up to two and three times faster at 600 MPa, respectively, compared to the same temperature at atmospheric pressure which possibly explains the observed accelerated browning of milk under HPT conditions.

5.1.3 Ultrasonic processing

The application of high-intensity ultrasound (HIUS) for modifying protein properties has become a frequently employed emerging technology processing technique due to its simplicity and eco-friendly attributes. Protein functionality such as solubility, gelation, emulsification, foamability and digestibility have been improved by HIUS, possibly due to cavitation, heating, agitation, shear stress and turbulence, which induce chemical and physical changes in protein structure (Martínez-Velasco *et al*., 2018). HIUS (72.67% amplitude, 17.29 min) was applied to faba bean protein isolate. Ultrasonication produced an increase in β conformations (6.61% β-sheet, 19.6% β-turn, 0.8% anti-parallel β-sheet) and decreases in inter-molecular aggregates (43.54%) compared to the native faba bean protein. As a result, the surface and foaming properties of the ultrasonicated faba bean protein improved but induced a slight unsignificant decrease on the *in vitro* digestibility. Khatkar *et al*. (2020) sought to restructure the closed-packed globular structure of soy protein employing HIUS (30% amplitude, 10 min) for improving the *in vitro* digestibility and structural attributes. New FT-IR peaks after ultrasonication confirmed that the protein secondary structure was altered, improving water and oil holding capacity, higher gel strength at lower protein concentration, and an improved *in vitro* digestibility. Vanga *et al*. (2020a) applied pulsed ultrasound to almond protein. FT-IR analysis showed a slight relocation of the ordered and unordered structures in the sonicated almond protein and Circular Dichroism spectroscopy revealed a restructuring of the α-helices into β-sheets. The *in vitro* digestibility of ultrasonicated almond protein increased slightly with respect to that of the native protein, but the increase was non-significant. Bhat *et al*. (2021b) claimed that protein digestibility of muscle foods can be improved by emerging technologies by affecting the structural (quaternary, tertiary or even secondary) and muscle microstructure, making the muscle proteins more susceptible to gastrointestinal proteases. Ultrasound can induce denaturation and affect de unfolding-refolding of proteins, affecting the diffusion of proteases into the protein matrix and their accessibility to cleavage sites. This is a nascent area of research where the need to expand our understanding of processing conditions, effects on muscle protein, and their behavior under gastrointestinal environments.

5.1.4 Microwave

In microwave processing, energy is supplied by electromagnetic waves typically at frequencies of 915 or 2450 MHz directly to the food (Bhat *et al*., 2021a). This results in a rapid heating throughout the food thickness with a low-temperature gradient within the food. Microwave processing induces minimum effects on food compounds and on its nutrition and sensory quality. Nevertheless, it has been reported to affect the structural and functional properties of food proteins (Bhat *et al*., 2001a). Vanga *et al*. (2020b) applied microwave (2450 MHz, 70-100 °C, 2-10 min) treatment to soymilk, finding that the digestibility of soymilk significantly increased to 93% after 10

min microwave processing at 85 °C compared to the untreated control. The improvement in digestibility was attributed to changes in the secondary structure of the microwaved soybean proteins. Dong *et al*. (2021) applied microwave (2.45 GHz, 1000 W, 75-125 °C, and 5-15 min) treatment to shrimp. SDS-PAGE that tropomyosin band intensity was lowered by increasing temperature-time treatment. At 125 °C treatment for 15 min, the allergenicity of tropomyosin dropped by 75%. However, microwave treatment caused a reduction of between 30-75% in total soluble protein and peptide contents, and on *in vitro* digestibility. These changes were associated with the increase in β-sheets, and the loss in α-turns in the shrimp protein secondary structure. High temperature mainly affected the helical and β-sheet regions, and the electric field induced changes in the hydrophobicity of the protein surface.

5.1.5 Miscellaneous

Other processing includes static external electric field that was applied to β-lactoglobulin with a strength 3.0 V/nm and temperatures of 300, 400 and 500 K to evaluate the dependence of the electric field on temperature (Baruah and Borgohain, 2020). The combined effect of high temperature and static external electric field induced significant changes in the structural conformation of β-lactoglobulin. Xie *et al*. (2021) applied low voltage electrostatic field (LVEF) to the freezing process of prepared beef steak. LVEF-assisted freezing (LVEFF) minimized the gaps in the cross-section between muscle fibers, improving fiber compactness. A decreased carbonyl content and increased total sulfhydryl content pinpointed that protein oxidation by freezing was hindered. Changes in secondary and tertiary protein structures were minimized during the freezing process. Lee *et al*. (2021) investigated the *in vitro* protein digestibility of freezing-then-aged (FA) beef in an infant digestion model and compared with only aged control (AO). Carbonyl and free sulfhydryl were the same for FA and OA after 14 days aging. Freezing did not affect the beef myofibrillar tertiary protein structure. Higher caspase-3 activity and a higher 10% trichloroacetic acid-soluble amino acid content was shown by FA than by OA. Post *in vitro* digestion of the 14 days aged showed that FA had a higher content of α-amino groups and proteins under 3 kDa digested. Thus, FA enhanced the protein digestibility of beef. Trigo *et al*. (2021) obtained a novel protein source from seaweed (Ulva fenestrata) by the use of pH-shifting for the extraction. Protein degree of hydrolysis ($\approx$ 28 to 36%) and amino acid accessibility ($\approx$ 57 to 73%) were improved. *Ulva* and concentrated protein extracts were as bioavailable as casein. Kumar *et al*. (2019) microfluidized (2500, 15000, 22000 and 30000 psi) cow and buffalo milk. They found that at 22000 psi the cholesterol level was reduced by 42% in cow and 46 % in buffalo milk. Microfluidization improved melting properties of fat, protein and lactose stability. Microfluidization significantly improved *in vitro* trypsin digestibility and in vivo Wistar rat digestibility.

6 *in vitro* techniques for estimating protein quality and digestibility

Human nutritional studies are still being considered the “gold standard" for addressing diet-related questions; however, they are expensive and laborious. *in vitro* methods have the advantage of being more rapid, less expensive, less laborious, and do not have ethical restrictions. Moreover, *in vitro* models are very suitable for mechanistic studies and hypothesis building due to reproducibility, choice of controlled conditions and easy sampling at the site of interest (Minekus *et al*., 2014). Therefore, *in vitro* protein quality and digestibility techniques are often a first step in measuring protein quality due to their rapidity and sensitivity.

For many years, food scientists and nutritionists have discussed the need for measuring protein nutritional quality. The rat-based protein efficiency ratio (PER) bioassay did not serve the fast-paced needs of the food industry. The researchers sought to develop fast rapid assays that could broadly be in three types: (i) Proteolytic enzymes assays, used to predict one aspect of protein quality, protein digestibility; (ii) Assays with microorganisms, used to measure the availability of certain selected essential amino acids (EAA) in proteins, and thereby describe protein quality based upon EAA availability; and (iii) Assays utilizing amino acid profile, where protein quality is compared with a revised reference pattern for EAAs. While a valuable tool for screening protein quality, it has a major flaw, it assumes that all amino acids are totally available (Satterlee, 1984). A collaborative expert panel developed the *in vitro* C-PER (Calculated Protein Efficiency Ratio) assay and its DC-PER

subassay for predicting protein efficiency ratio as measured by rat bioassay (Sattelee *et al*., 1982). Seven laboratories tested 6 foods each (non-fat dried milk, cooked chicken muscle, protein-fortified dry breakfast cereal, textured soy protein, oat-based dry breakfast cereal, and durum wheat flour), using as control casein. Foods were assayed for *in vitro* apparent protein digestibility, amino acid composition and PER via rat bioassay. C-PER was able to rapidly predict rat PER, with an accuracy of 0.26 (Satterlee, 1984). Up to date, C-PER is routinely used for evaluating protein quality where other methods are not cannot discriminate statistically protein differences. Barrón-Hoyos *et al*. (2013) evaluated the protein quality of lean muscle of terrestrial (beef, pork, chicken, turkey) and aquatic (tilapia, shrimp, sole, shark) species. They used various *in vitro* methods: digestibility%, total amino acid analyses (HPLC), PDCAAS, computerized PER calculations (C-PER and DC-PER) and total collagen contents. Both, the C-PER and DC-PER methods were more exact in their results and were able to detect significant differences among samples of different origin. With regards to *in vitro* methods for assessing protein digestibility, despite the several *in vitro* techniques with varying protease types and concentration, incubation conditions and end-product analysis techniques that have been used to measure protein digestibility of various foods (Table 3), there has not been a consensus. Some authors have used a single enzymatic system (Gulati *et al*., 2017; Mertz *et al*., 1984; Price *et al*., 1979; Rehman *et al*., 2005). while others have used a two-step enzymatic system to reproduce in vivo digestion sequence (Gauthier *et al*., 1982). Then, the information of the earlier reported methods led to the use of enzymes mixtures to simulate gastric digestion (Ketnawa and Ogawa, 2021; Martín-Cabrejas *et al*., 2009; McDonough *et al*., 1990). In recent years, efforts have focused on validating and/or correlating *in vitro* data with in vivo findings (Nosworthy *et al*., 2017, 2018), and in developing methods that accurately simulate in vivo conditions (Bryan *et al*., 2018; Egger *et al*., 2016; Laguna *et al*., 2016; Minenkus *et al*., 2014). Such investigations have significantly advanced our understanding of protein digestion; however, the variation in the digestion parameters has reduced the possibility to compare results across research-groups and to deduce general findings (Minenkus *et al*., 2014). Given the fact that protein structures are related to their breakdown properties under gastric conditions, understanding their *in vitro* proteolysis is vital to understand how they are metabolized in vivo and their effect on stomach emptying rate, gut motility, and gut hormones secretion. Therefore, despite the many assays established, there is an urgent need to develop a standardized, independently validated *in vitro* protein and amino acid digestibility assay, based on the current state of knowledge on in vivo digestion conditions.

Table 3. *In vitro* methods for assessing protein digestibility.

Food product evaluated	Enzymes used	End-product analysis technique	Relevant information	Reference
Pinto beans & buckwheat flours, red and green lentils	Trypsin, chymotrypsin, protease	Calculated from the pH change in ten minutes	A good correlation between *in vitro* PDCAAS and PDCAAS was found.	Nosworthy *et al.* (2017, 2018)
Skim milk powder, pea protein	Salivary amylase, Amylase, porcine pepsin, porcine trypsin, bovine chymotrypsin, porcine pancreatic lipase, bile salt.	Protein hydrolysis was analyzed with Coomassie-stained SDS PAGE. The degree of free amino acid release was analyzed by HPLC.	Static *in vitro* digestion model based on physiologically relevant conditions gathered from humans. Validated in a wide inter-laboratory trial and on-going efforts to correlate findings of protein digestibility with in vivo trial in pigs and biochemical assays with humans.	(Egger *et al*., 2016; Laguna *et al*., 2016; Minekus *et al*., 2014)
Tamales, bread	α-amylase, pepsin, pancreatin, bile extract		Simulates gastrointestinal digestion	Elles *et al*., 2000; Gawlik-Dziki *et al*., 2013; Rodríguez-Huezo *et al*., 2018; Tan *et al*., 2000)

Chickpea, lentil, and bean	Trypsin, chymotrypsin, peptidase	The uptake of titrant during enzymatic digestion was used to calculate estimates of digestibility.	Digestibility of different protein sources was estimated by six laboratories with a mean relative standard deviation of 1% for repeatability and 2.5 for reproducibility.	Martín-Cabrejas *et al.*, 2009; McDonough *et al.*, 1990
Proso Millet, sorghum, wheat, maize, rice	Pepsin	Nitrogen determination	The method simulates the digestion values found in children	Gulati *et al.*, 2017; Mertz *et al*, 1984
Casein, soy protein isolate, rapeseed protein concentrate	Pepsin, pancreatin	Nitrogen determination	A two-step proteolysis method that performs the enzyme digestion inside a dialysis bag to allow the separation of proteolysis products	Gauthier *et al.*, 1982
Red kidney beans, black grams, chickpea	Pepsin		Single enzymatic system	Price *et al.*, 1979; Rehman and Shaw, 2005

Conclusion and future research directions

Food processing technologies have evolved over the years, and while traditionally their main objective preserve and stabilize foods, their focus today has shifted to enhance health and nutritional aspects, improve textural and sensory attributes, achieve sustainable production, food security, and food diversity. The growing array of new technologies have the potential to replace and improve conventional processing technologies, capable to deliver better quality products. However, the tracking down of the changes suffered by the food components is a painstaking and slow process. With regards to protein quality and digestibility, it is known that any food processing modifies the conformation of proteins and consequently their susceptibility to digestive enzymes. However, the effect will depend on the nature of the protein and on the specific processing treatment and conditions used. In general terms, novel and innovative methods, particularly non-thermal processes, seem to indicate that an increase overall protein quality and digestibility compared to conventional (thermal) processing methods is achievable. Nevertheless, more studies have to be conducted to establish this, as the results published in the literature are often contradictory for a given protein and process. Moreover, food researchers should focus on developing a suitable standardized *in vitro* digestibility method that mimic in vivo physiological conditions, capable of predicting proteins effect on newly emerging actions such as emptying rate, gut motility, gut hormones secretion and therefore, satiety. The combination of enzymatic methods and metabolomics seems to be a promising perspective in this area. Also, the effect of the food matrix deserves more investigation due to that some proteins are not available to proteolytic enzymes present in the digestive system.

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