ANEXO II

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Update on natural food pigments - A mini-review on carotenoids, anthocyanins, and betalains

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ABSTRACT

Extensive structure elucidation has revealed a remarkable diversity of structures for carotenoids, anthocyanins, and betalains, the major natural pigments in plant-derived foods. Composition, stability, influencing factors, processing effects have been widely investigated. Carotenoids isomerize and oxidize while anthocyanins undergo hydrolysis, nucleophilic attack of water, ring fission, and polymerization during thermal processing. Betacyanins suffer deglycosylation, isomerization, dehydrogenation, hydrolysis, and decarboxylation. Biotechnological production dominates research on carotenoids as food colorants while the search for plant sources continues with anthocyanins and betalains. Stabilization studies presently focus on microencapsulation and nanoencapsulation. For anthocyanins, co-pigmentation has also been intensely researched. Carotenoids have been the most studied in terms of health effects, involving epidemiological, cell, animal, and human intervention studies, yet some inconsistencies in the results persist. A wide range of biological activities have been attributed to anthocyanins and betalains, based mainly on cell and animal studies; human clinical studies are lacking.

1. Introduction

Renewed interest on natural pigments has been stimulated by their potential health benefits and concern about the safety of artificial food dyes. The principal research topics are: structure elucidation, analytical methods, composition in foods along with the influencing factors, processing effects including the chemical reactions involved and the influencing factors, bioavailability, stability and stabilization methods, production of food colorants, and health effects.

2. Carotenoids

Carotenoids, the most studied of the natural food pigments, are lipophilic pigments responsible for the yellow, orange, and red colors of plant foods (e.g., corn, carrot, papaya, tomato, watermelon) and the red color of some fish (e.g., salmon) and crustaceans (e.g., cooked lobster, crab, and shrimp). Known for structural diversity, their most distinctive feature is a centrally located conjugated double bond system, which serves as the chromophore and is essential to multiple functions and actions. However, it also renders the carotenoids prone to isomerization and oxidation.

The intense work done to develop reliable quantitative methods motivated worldwide investigation of the carotenoid composition of foods and the influencing factors (e.g., cultivar/variety, maturity, climate or season, cultivation site, agronomic practices, harvesting and post-harvest handling, processing, and storage) (Maiani et al., 2009; Rodriguez-Amaya, 2015; Rodriguez-Amaya, Kimura, Godoy, & Amaya-Farfan, 2008).

As with other natural pigments, instability is a problem with carotenoids, influenced by the nature of the carotenoid (carotene or xanthophyll, *E*- or *Z*-configuration, esterified or unesterified) and the food matrix (fruit, root, leaf, juice). Degradation increases with destruction of the food cellular structure, greater surface area or porosity, duration and severity of processing conditions, duration and inadequate conditions of storage, permeability of packaging material to O_2, and exposure to light (Rodriguez-Amaya, 2015).

Thermal processing provokes variable often substantial losses of carotenoids, but moderate heat treatment increases bioavailability. Non-thermal processing (e.g., high pressure processing, high-intensity pulsed electric field processing) results in no or insignificant losses, but has mixed results for bioaccessibility (Barba et al., 2017).

Loss of carotenoids during processing and storage is due to physical removal (e. g., peeling) and enzymatic or non-enzymatic oxidative degradation. Nonenzymatic oxidation (also called autoxidation) is accompanied by isomerization, and both the *Z*- and *E*-isomers are oxidized (Fig. 1) (Rodriguez & Rodriguez-Amaya, 2007, 2009). Oxidation

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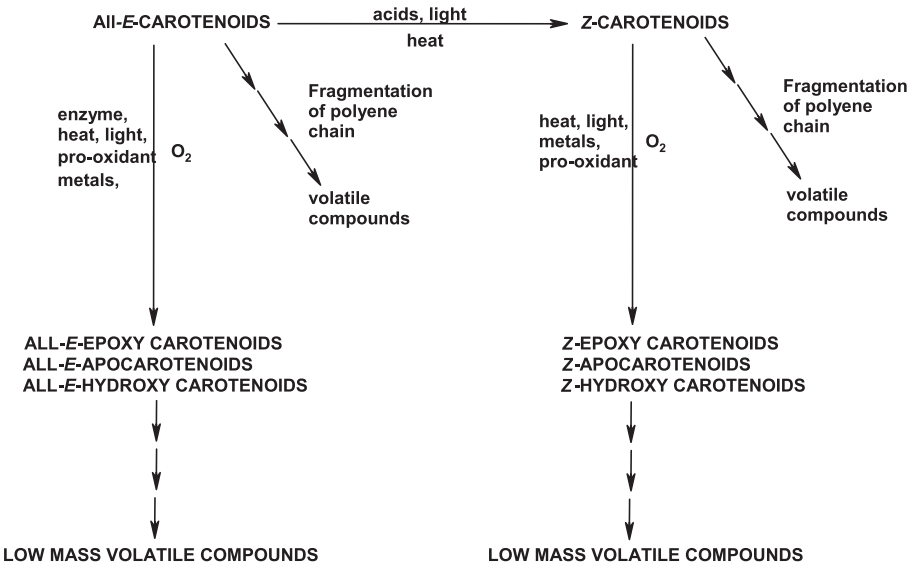

Fig. 1. Overall scheme for the oxidative degradation of carotenoids. Taken from Rodriguez-Amaya (2015).

initially involves epoxidation, cleavage to apocarotenals, and hydroxylation (Rodriguez & Rodriguez-Amaya, 2007, 2009; Zepka & Mercadante, 2009). Subsequent fragmentations result in compounds of low masses (volatiles). Direct cleavage of the polyene chain and modifications of the cleavage products also occur (Kobori, Wagner, Padula, & Rodriguez-Amaya, 2014), increasing the range of volatiles formed. Colorless and devoid of the biological activities of carotenoids, these compounds contribute to the desirable flavor or off-flavor of foods and beverages.

Carotenoids commercialized as food color additives and supplements continue to be mostly products of chemical synthesis. Colorants are also produced by extraction from rich natural sources (annatto, paprika, saffron, tomato lycopene, marigold lutein) and by microbial fermentation (β-carotene by *Dunaliella* sp., astaxanthin by *Haematococcus pluvialis*, and β-carotene by the fungus *Blakeslea trispora*). Biotechnological production has dominated research in this area. Two strategies have been followed: (1) fermentative production in a microorganism, followed by isolation, and (2) genetic engineering to biosynthesize the desired carotenoid in plants foods. Current stabilization efforts has centered on microencapsulation and nanoencapsulation (Rodriguez-Amaya, 2015).

The long established function of carotenoids in terms of human health is the provitamin A activity. In more recent years, the focus has been on reduction of the risk of developing chronic degenerative diseases. Cancer, cardiovascular diseases, cataract, and macular degeneration have been the most investigated disorders (Krinsky & Johnson, 2005; Rao & Rao, 2007). Other health effects have been attributed to carotenoids, including: protection of cognitive functions (Devore, Kang, Stampfer, & Grodstein, 2013; Johnson, 2012), protection of the skin from sunlight (Stahl & Sies, 2012), reduced risk of rheumatoid arthritis (Pattison et al., 2005), low prevalence of depressive symptoms (Beydoun, Beydoun, Boueiz, Shroff, & Zonderman, 2013; Niu et al., 2013), and protective role for mineral density in older men and women (Sahni et al., 2009).

Carotenoids' action against diseases has been attributed to their antioxidant activity. Oxidative stress is widely postulated to be a causative factor in the development of degenerative diseases, and human studies have demonstrated that carotenoid supplementation decreased oxidative stress markers (Basu & Imrhan, 2007; Wang et al., 2013). However, non-antioxidant mechanisms are increasingly cited, such as modulation of carcinogen metabolism, regulation of cell growth, inhibition of cell proliferation, enhancement of cell differentiation, stimulation of intercellular gap junction communication, modulation of DNA repair mechanisms, induction of detoxifying enzymes and filtering of blue light (Krinsky & Johnson, 2005; Rao & Rao, 2007).

Carotenoids vary in their bioefficacy. Examining research findings on β-carotene, a group of carotenoid experts concluded that its primary role is its provitamin A activity (Grune et al., 2010). Lycopene has been associated with reduced risk of prostate cancer (Giovannucci, 2002; Wertz, Siler, & Goralczyk, 2004), and lutein and zeaxanthin with reduced risk of age-related macular degeneration (Carpentier, Knaus, & Suh, 2009; Sabour-Pickett, Nolan, Loughman, & Beatty, 2012) and cataract (Ma et al., 2014), and the maintenance of cognitive function (Johnson, 2012).

Carotenoid intake has been reported to have significant or no association/effect on disease incidence. This inconsistency has been mainly attributed to differences in the experimental protocols of the different studies (Costa-Rodrigues, Pinho, & Monteiro, 2018). There are, however, at least three findings that make studies on this topic especially challenging (Rodriguez-Amaya, 2015). First, the effect of bioactive compounds on the human body may be very small over relatively short period, but can contribute significantly throughout life as part of the daily diet. The health effect may not be observed during the study period. Second, it is now known that reduction of the risk of chronic diseases is not due to a single class of compounds but to the combined effects of bioactives in foods. Thus, the evidence for the effects of foods is generally more compelling than that of individual compounds. Third, health effects are further masked by considerable variation in inter-individual responses to the administered bioactive compound, which has been attributed to genetic factors.

The consensus is that there is sufficient evidence to support increased consumption of carotenoid-rich foods, but caution and more investigations are recommended to evaluate the benefits and risks of supplements. β-Carotene supplement at doses much higher than physiological levels, was shown to increase rather than lower lung cancer incidence in smokers and asbestos-exposed workers (ATBC, 1994; Omenn et al., 1996).

3. Anthocyanins

Anthocyanins are water-soluble pigments that confer red, blue, and purple colors to fruits, vegetables, and beverages (e.g. strawberry, cherry, cranberry, blueberry, red cabbage, eggplant, red wine). They are glycosides or acylglycosides of anthocyanidins, which are flavylium

structures consisting of two aromatic rings linked by a three carbon heterocyclic ring that contains oxygen. The conjugated double bonds of the anthocyanidin moiety constitute the chromophore.

Six anthocyanidins are commonly found in food (pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin), varying in the number and degree of methylation of hydroxyl groups in ring B. In general, hydroxylation increases blueness and reduces stability, whereas methylation increases redness and raise stability.

Glycosylation and acylation give rise to a diversity of anthocyanins, varying from simple (e.g., the grape cyanidin-3-glucoside) to complex structures (e.g., the purple sweet potato peonidin-3-caffeoyl-*p*-hydroxybenzoylsophoroside-5-glucoside) (Song et al., 2013), with consequent variation in stability and hues. The most common sugar is glucose, but other sugars are also conjugated to anthocyanidins. Glycosylation occurs typically at C-3 or at both C-3 and C-5, but it can also take place at C-7, C-3′, C-5′, and C-4′. The sugar moieties may be acylated with aromatic and/or aliphatic acids. Complex anthocyanins have shown remarkable stability.

The anthocyanin composition of food is affected by cultivar/variety, maturity, cultivation practices, growing area, season/climate, processing and storage. Amarowicz et al. (2009) reviewed extensively the influence of postharvest processing and storage on the contents of flavonoids (including anthocyanins), concluding that the effect of storage and processing on the polyphenol content is negligible in comparison with the differences between different varieties of plants.

Various factors affect anthocyanin color and stability, such as the chemical structure and concentration of the anthocyanin, temperature, pH, light, oxygen, enzymes, metallic ions, other flavonoids and phenolics, ascorbic acid, sugars, sulfites (Cavalcanti, Santos, & Meireles, 2011; Patras, Brunton, O'Donnell, & Tiwar, 2010; Schwartz, Cooperstone, Chichon, von Elbe, & Giusti, 2017).

Anthocyanins in nature are stabilized by copigmentation, self-association, and metal complexing. Comprehensively reviewed recently by Trouillas et al. (2016), copigmentation has drawn a lot of attention. It can be intramolecular, by stacking of the hydrophobic acyl moiety covalently bound to sugar and the flavylium nucleus or intermolecular, by van der Waals interactions between the planar polarizable nuclei of the anthocyanin with colorless copigment (e.g., phenolics) (Mazza & Brouillard, 1990). This protects C-2 of the flavylium chromophore from the nucleophilic attack of water, thereby preventing color loss (Boulton, 2001). Many factors influence the magnitude of copigmentation, including the structures and concentrations of the anthocyanin and copigment, their molar ratio, the pH, temperature, and ionic strength (Boulton, 2001; Gordillo, Rodríguez-Pulido, Escudero-Gilete, González-Miret, & Heredia, 2012).

In aqueous medium as in food, anthocyanins undergo reversible structural transformations with pH, manifested in color change. At pH below 2, the red flavylium cation predominates. At pH 3 to 6, rapid hydration of the flavylium cation occurs at C-2 to form the colorless carbinol pseudobase, which can undergo ring opening to a yellow chalcone. At slightly acidic to neutral conditions, deprotonation of the flavylium cation generates the blue quinoidal base.

During thermal processing, anthocyanins undergo deglucosylation, nucleophilic attack of water, cleavage, and polymerization (Fig. 2) (Schwartz et al., 2017; Stintzing & Carle, 2004). In alkaline or acid medium, or in the presence of the enzyme β-glucosidase, anthocyanin is hydrolyzed, releasing the less stable anthocyanidin The latter can be transformed first into the colorless carbinol base and subsequently into α-diketone, which can polymerize, forming brown products, or fragment into an aldehyde and a derivative of hydroxybenzoic acid. Anthocyanin can also be converted to colorless carbinol and later into chalcone and coumarin.

Utilization of anthocyanins as food colorants and functional ingredients has been limited by their low stability and interaction with other compounds in the food matrix. Grape skin extract has been used as colorant for a long time. Current research centers on the search for better sources, and the enhancement of extraction efficiency and stability. Since acylation improves color and pigment stability, suitable sources of acylated anthocyanins are sought, such as radishes, red potatoes, red cabbage, black carrots, and purple sweet potatoes (Giusti & Wrolstad, 2003).

Stabilization methods include addition of copigments, exclusion of O_2, and encapsulation (Cavalcanti et al., 2011; Cortez, Luna-Vital, Margulis, & de Mejia, 2017). Microencapsulation of anthocyanins has been investigated to develop natural colorants with improved stability, solubility, dispersibility, and bioavailability (Bakowska-Barczak & Kolodziejczyk, 2011; Mahdavi, Jafari, Ghorbani, & Assadpoor, 2014).

A wide range of biological activities has been reported for anthocyanins, such as antioxidant, anti-allergic, anti-inflammatory, antiviral, antiproliferative, anti-microbial, antimutagenic, anti-tumor activities; microcirculation improvement; peripheral capillary fragility prevention. Anthocyanins are associated with low prevalence or alleviation of some disorders such as cancer, cardiovascular diseases, diabetes, obesity, cognitive decline (He & Giusti, 2010; Pojer, Mattivi, Johnson, & Stockley, 2013). However, these assertions are mostly based on in vitro cell and animal studies. Long-term, properly designed intervention trials are deemed necessary to provide conclusive proof of efficacy.

4. Betalains

Betalains are water-soluble nitrogenous pigments consisting of the red-violet betacyanins (e.g., betanin of red beet) and yellow-orange betaxanthins (e.g., indicaxanthin of cactus pear). They are immonium conjugates of betalamic acid with cyclo-Dopa (cyclo-3,4-dihydroxyphenylalanine) and amino compounds (amino acids, amines, or derivatives), respectively. The conjugated double bond system of the betalamic acid moiety constitutes the chromophore. The aromatic ring's double bonds of cyclo-Dopa raises the absorption of betacyanins 50 to 70 nm higher compared to betaxanthins.

As with anthocyanins, glycosylation and acylation diversify the betacyanin structures. The betanidin aglycone is usually linked with glucose, occasionally with glucuronic acid, sophorose, rhamnose and apiose. Further modification occurs by aliphatic or aromatic acid esterification of the sugar moiety.

Betalains are found in numerous sources (flowers, fruits, roots, leaves, stalks, seeds, grains) in the plant kingdom but have limited occurrence in foods. For a long time, the red beet was practically the only known food source. Other edible sources have now been investigated, principally prickly (cactus) pear (Moβhammer, Stintzing, & Carle, 2006; Stintzing, Schieber, & Carle, 2003) and the red-purple pitaya (dragon fruit) (Herbach, Maier, Stintzing, & Carle, 2007; Stintzing, Schieber, & Carle, 2002). Betaxanthins have received much less attention, food sources of which are yellow varieties of Swiss chard, beet, and cactus fruits (Kugler, Stintzing, & Carle, 2004).

Stability of betalains is lowered by degrading enzymes (peroxidases, polyphenol oxidases, glucosidases), low degree of glycosylation and acylation, high a_w, metal cations, pH < 3 or > 7, high temperature, exposure to light, O_2, and H_2O_2 (Herbach et al., 2007; Herbach, Stintzing, & Carle, 2006).

Betacyanins undergo C_{15}-isomerization, deglycosylation, dehydrogenation, hydrolysis, and decarboxylation during processing, as shown for betanin in Fig. 3. The predominating reaction differs among betacyanins (Herbach, Stintzing, & Carle, 2005). Betanin was mainly hydrolyzed into betalamic acid and cyclo-Dopa 5-*O*-glucoside. Decarboxylation and combined decarboxylation/dehydrogenation predominated with hylocerenin (3-hydroxy-3-methyl-glutaryl-betanin) and phyllocactin (malonyl-betanin), Additional decarboxylation of the malonic acid moiety as well as generation and subsequent degradation of betanin after phyllocactin demalonylation make phyllocactin degradation more complex.

Betacyanins are generally accompanied by their respective

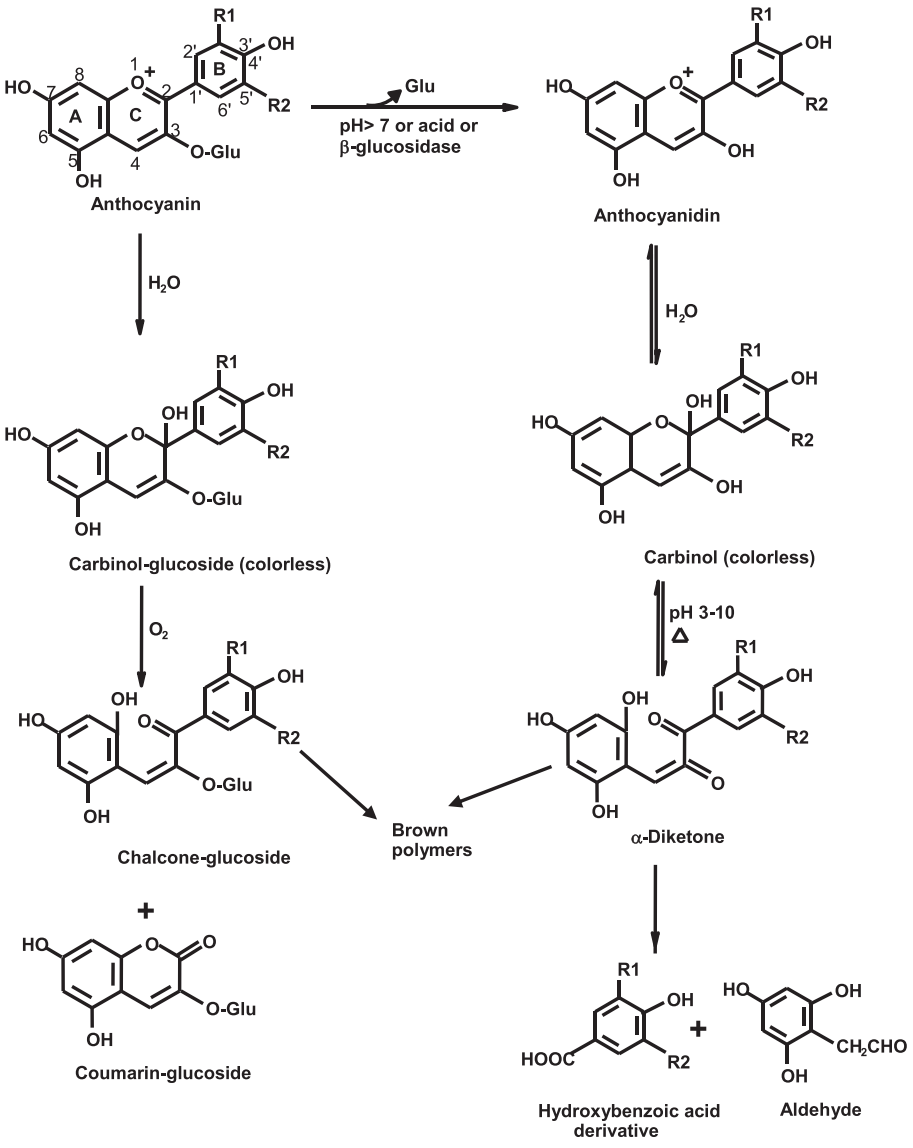

Fig. 2. Chemical changes of anthocyanins during processing and storage of foods. Based on Schwartz et al. (2017) and Stintzing and Carle (2004). R_1, R_2 = −OH, $-OCH_3$, or −H.

isobetacyanins, but isomerization can be induced by low pH or thermal treatment (Herbach et al., 2004; Herbach et al., 2006). Cleavage of the glucose moiety of betanin occurs in the presence of β-glucosidase, under highly acidic conditions, or at high temperature (Herbach et al., 2006). Deglucosylation results in increased susceptibility towards oxidation (Stintzing & Carle, 2004). Although an endogenous pigment, neobetanin can be generated by heat exposure (Herbach et al., 2004). At pH above 6 or during thermal processing, betanin is cleaved to the bright yellow betalamic acid and the colorless cyclo-Dopa-5-*O*-β-glucoside. Partial regeneration of betanin occurs after short-term heating (Herbach et al., 2006).

Betanin may be decarboxylated at the C-2, C-15, or C-17 position (Herbach et al., 2005). The monodecarboxylated betanins are subsequently transformed to di- and tridecarboxylated betanins (Wybraniec, 2005). Decarboxylation at either C-2 or C-15 do not alter the betanidin chromophore, thereby maintaining the color of the original betacyanin (Herbach et al., 2006). On the other hand, a carboxyl substituent in conjugation with the conjugated double bond system is eliminated in 17-decarboxybetanin, which thus exhibit an orange hue. Isobetanin and neobetanin are also decarboxylated in a manner similar to that of betanin (not shown in Fig. 2) (Wybraniec, 2005).

Mixtures of mono-, di-, and tridecarboxylated betacyanins, together with their corresponding neobetacyanins were identified in heated beet root juice and purified extracts of red beet and purple pitaya (Wybraniec, 2005; Wybraniec & Mizrahi, 2005). The main products were 17-decarboxy-betanin, 17-decarboxy-isobetanin, 2-decarboxy-betanin, 2,17-bidecarboxybetanin, and 2,17-decarboxyisobetanin, and 14,15-dehydrogenated-neobetanin. During heating of *Basella alba* L. fruit juice, the principal decarboxylation products of major gomphrenin pigments were 2-, 17-, and 2,17-decarboxy-gomphrenins (Kumorkiewicz & Wybraniec, 2017).

Betanin from beetroot has been commercialized as a natural food colorant. Its use is, however, restricted by its high nitrate content and its earthy flavor caused by geosmin and pyrazine derivatives (Lu, Edwards, Fellman, Mattinson, & Navazio, 2003; Stintzing & Carle, 2004). Current research on the production of betacyanin colorant involves the search for new and economically viable sources, appropriate pretreatment and extraction methods, advances in processing technology and stabilization, availability of wide spectrum of color, better quality of pigments devoid of off-flavor, possibility of developing betalain-fortified formulations containing certain other bioactive and nutritional components (Celli & Brooks, 2017; Khan & Giridhar, 2015;

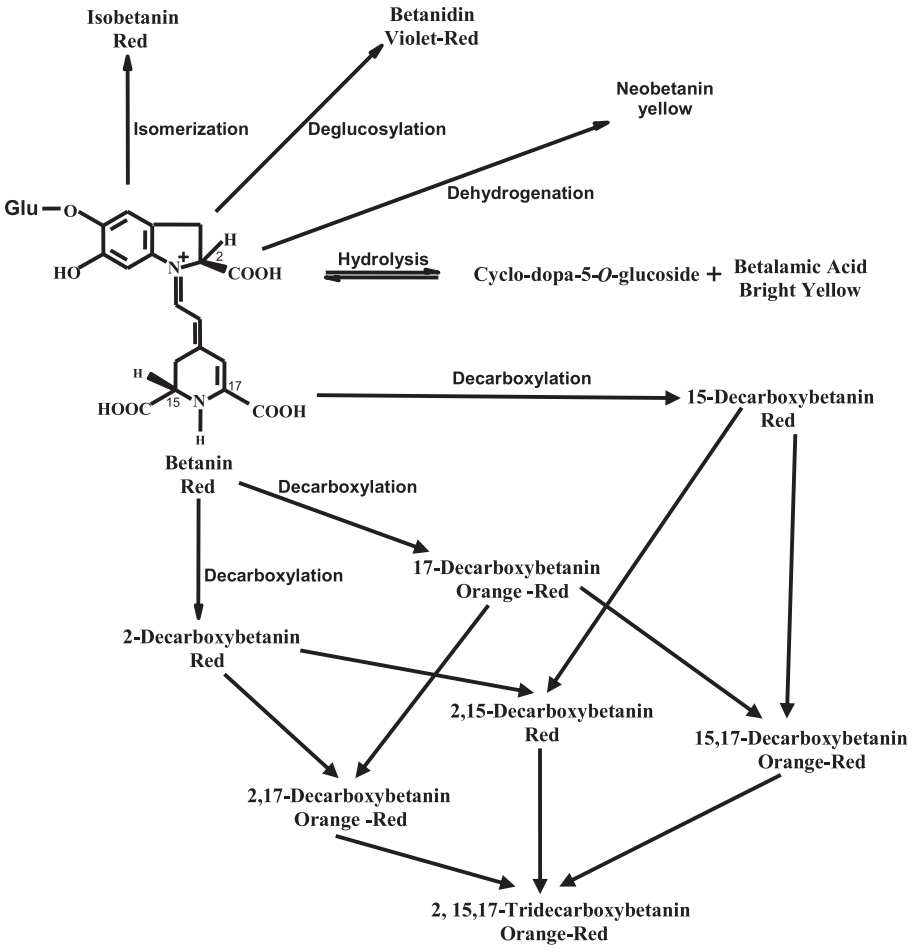

Fig. 3. Chemical changes of betanin during processing and storage of foods. Based on Herbach, Stintzing, and Carle (2004) and Herbach et al. (2006).

Ngamwonglumlert, Devahastin, & Chiewchan, 2017). Microencapsulation of betalains can be a successful strategy to improve stability, make handling easier during processing, ensure bioavailability (Otálora, Carriazo, Iturriaga, Nazareno, & Osorio, 2015) and biological activities (Rodriguez, Vidallon, Mendoza, & Reyes, 2016).

Various health-promoting biological activities have been attributed to betanin and betalain-rich foods, such as scavenging of free radicals/reactive oxygen species, inhibition of lipid peroxidation and LDL oxidation, prevention of DNA-damage, induction of antioxidant and phase II detoxifying enzymes, gene regulatory activity, anti-inflammatory, antiproliferative and antimicrobial activities (Esatbeyoglu, Wagner, Schini-Kerth, & Rimbach, 2015; Gandía-Herrero, Escribano, & García-Carmona, 2016; Gengatharan, Dykes, & Choo, 2015). Most of the studies, however, have been conducted with cell lines and animal models; human studies are lacking.

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A comprehensive review on heat treatments and related impact on the quality and microbial safety of milk and milk-based products

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ABSTRACT

This review presents a comprehensive understanding of the dynamics of the microorganisms in milk and their growth and the heat treatments used to minimize their number. Milk and milk-based products provide a suitable environment for the growth of microorganisms such as yeasts, molds, and a wide range of bacteria. The variation in the microbial count is attributable to the degree of contamination and the processing temperature at various stages of milk processing. It has been acknowledged that the heat treatment of milk, such as pasteurization, permits its safe devouring with regards to food-borne illness. At the same time, its negligence in sufficient amount ensued in both withdrawals of products and food disease breakouts. In this review, different aspects of heat treatments that have an impact on relevant microbes, that encompasses the illustration of D and Z values as a way to calculate heat resistance, factors affecting D- values, and the effect of heat treatments on microbes present in dairy foods. Also, mild methods for food preservation have been developed as a result of the increasing demand for fresh-like foods. These methods preserve the sensory, nutritional, and other functional properties of foods due to the inactivation of microorganisms at sub-lethal temperatures.

1. Introduction

Milk is a nutrient rich fluid that, by definition, is an excellent culture system for a wide range of organisms. It contains various nutrients, such as vitamins, proteins, lipids, and carbohydrates, so it nutritionally supports a vast spectrum of microorganisms in optimal growth conditions (Islam et al., 2018). Since dairy products contain all of the essential elements that are important for bone and muscle health, so considered food for people of all ages. In milk, magnesium, calcium, selenium, riboflavin, Vitamin B12, pantothenic acid, and other minerals are found. As a result of the rising demand for "healthy foods," milk and dairy products are fortified with calcium or vitamin D, and the preference for lactose-free milk has grown importance. Customers' expectations for milk and dairy products to be more stable and have a longer shelf life has risen rapidly, in addition to their revival of interest in products with health benefits (van den Oever and Mayer, 2021). Dairy products are a good source of dietary proteins because they are abundant in essential amino acids (EAAs) and have enhanced digestibility and bioavailability. Numerous milk products, including liquid milk, yogurt, and cheese, are good sources of milk proteins and are consumed due to their numerous capabilities (Li et al., 2021).

Despite the benefits of milk for human development, it also serves as an excellent breeding ground for microorganisms such as yeast, bacteria, and molds, which are frequent pollutants that cause milk deterioration (Fusco et al., 2020). The constituents of milk microbiota are rather dynamic in that it is associated with a number of host-related and environmental factors. Inside and outside of the udder, handling surfaces, & storage equipment are the three key origins of microbial contamination in milk. Other sources that cause heavy contamination include the nearby environment, such as manure and bedding that might contain microbes up to 10^5 CFU/ml. The number of microorganisms in raw milk when it leaves the udders of healthy cows is usually relatively low, and generally, it contains microorganisms such as Micrococci, Streptococci, and Corynebacterium bovis in concentrations ranging between 10^{-2} and 10^{-3} CFU/ml. The population of microorganisms was found to be increased more if the cow suffers from diseases such as mastitis, brucellosis, tuberculosis, and so on.

The most common type of microbe found in dairy products is psychrotrophic microorganisms. The microbes can get into milk through

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the cow, the air, the feedstuffs, and the milking equipment. These microorganisms cause health hazards such as food infection, poisoning, as well as spoilage of milk. Several investigations have been conducted to evaluate the constituents of cow milk microbiota. Endogenous and environmental factors have a significant impact on the microflora found in milk. Despite the fact that their presence can have an impact on the microbial population, the typical constituents of the cow milk microbiota revealed a heterogeneous composition characterized by the abundance of LAB such as Lactococcus spp. (10^1–10^4 CFU/ml), Lactobacillus spp. (10^2–10^4 CFU/ml), Enterococcus spp. (10^1–10^3 CFU/ml), *Leuconostoc* (10^1–10^3 CFU/ml) and Streptococcus spp. (10^1–10^4 CFU/ml) (Deeth, 2017; Quigley et al., 2013; Masoud et al., 2012). Understanding the processes involved in the inactivation of milk microbiota would help the growing number of readers who are venturing into the fascinating world of dairy and dairy products. Based on the above fact, this review aims to provide a full overview of the dynamics of the microorganisms found in milk, their growth, and the heat treatments used to reduce their population. There is also a discussion of the kinetic parameters of heat-induced changes.

2. Microorganisms in milk

Microorganisms are omnipresent in nature & can be found in various environments, like air, water, and soil. Bacteria, fungus, protozoa, and certain algae are examples of microorganisms. At the same time, viruses are the only non-living and cellular microorganisms that are capable of replicating inside the cells of a host organism, which can be a bacterium, plant, or mammal. Microorganisms are both single-cell and multicellular. Bacteria, some algae, and protozoa are included in the single-celled microorganisms, while algae and fungi are included in the multicellular microorganisms. Based on the structure of the cell, they are classified into prokaryotes and eukaryotes. Bacteria and archaea are included in the class of prokaryotes, but both unicellular and multicellular organisms, such as fungi, plants, and animals, are considered eukaryotes. Bacteria are one of the major spoilage microorganisms in milk because milk provides an excellent growth medium for microorganisms. It houses microbes like gram-negative bacteria, coliforms, aerobic psychrotrophic, spore-forming bacteria, yeast, and molds.

2.1. Psychrotrophs in milk and milk products

Psychrotrophs are the bacteria found in higher concentrations in raw milk, particularly *Pseudomonas.* They are gram-negative, and rod-shaped in nature. Pseudomonas species are the major psychrotrophs isolated from milk (Griffiths et al., 1987). Pseudomonas can grow at temperatures ranging from 3 to 70°C. They hydrolyze proteins and lipid molecules in order to grow. They can lower the diacetyl content of sour cream buttermilk and can produce a green or yogurt-like flavor (Wang & Frank, 1981). Cottage cheese and creamed curd have pH values of 4.5 - 4.7 and 5.0 - 5.3, respectively. As a result, gram-negative psychrotropic bacteria create an atmosphere that is normally favorable to their proliferation. Despite its presence, salt in the cheese is unable to prevent bacteria from growing, bringing about a reduction in the storage life of cottage cheese. Cottage cheese quality gets degraded if psychrotrophs are present in raw milk at a concentration of 10^6 CFU/ml or higher (Machado et al., 2017; Mohamed & Bassette, 1979; Fairbairn & Law, 1986).

2.2. Coliforms in milk and milk products

Coliforms belong to the *Enterobacteriaceae* family and are gram negative bacteria that are facultative in nature and have a rod-shaped structure. Coliforms are not a natural part of raw milk's microbiota, but they can get into it through the environment, milking machinery, or the udder (Metz et al., 2020). They also can break down the diacetyl content of sour cream and lactose, which helps in the production of a yogurt-like flavor (Wang & Frank, 1981). E.coli, E. aerogenes etc., are some of the species which are also commonly found in milk. Coliforms can produce gas in cheese when the starter culture produces lactic acid slowly due to their short generation time. When the pH of the soft, mold-ripened cheese rises, it creates an ideal environment for the coliform to grow. They do not indicate a serious illness, but their presence confirms the presence of pathogens of fecal origin.

2.3. Yeast in milk and milk products

Yeast is one of the primary causes of yogurt &fermented milk spoilage because they thrive in low pH environments. To have a shelf life of 3, 4 weeks at 5°C, a good quality yogurt ought to have not over than ten cells. Because they can grow at a lower pH than any cultured product, like, sour cream or buttermilk, they develop an off-flavor, which is then referred to as yeasty or fermented. They reduce the diacetyl, resulting in a yogurt-like flavor. Geotrichum candidum is the most commonly found yeast that causes cottage cheese spoilage. When the yeast count in yogurt reaches 10^5-10^6 CFU/g, it results in the production of off-flavors as well as gas production. Cheese has a high nutritional value as well as a low pH, surface moisture, lactic acid, amino acids, and peptides. All of this promotes yeast growth, which results in the production of alcohol and CO_2. If yeast is present, it causes the swelling of cheese packets packed in a vacuum or modified atmosphere. Candida spp., G candidum, Pichia spp. Kmarxianus and Debaryomyceshansenii are some of the most commonly found yeasts that cause contamination.

2.4. Spore forming bacteria in milk and milk products

In milk products, the commonly found spore formers are Bacillus licheniformis, B. mycoides, B.subtilis, B. megaterium, & B. cereus, where raw milk acts as a medium. They are also present if the packaging is not done properly even after heat treatment. Many studies have shown that many spores become activated after pasteurization and develop when they reach the appropriate temperature (Cromie et al., 1989). Some microorganisms can also withstand the ultra-high temperature of milk, such as B.stearothermophilus (Muir, 1990). They cause "flat sour" in canned milk products because they produce acid but not gas (Kalogridou-Vassiliadou, 1992).

2.5. Molds in milk and milk products

Molds are most commonly seen growing on the surface of cheese when there is an abundance of oxygen, but some can also grow in low oxygen tension. They are seen to be growing in cheeses that are vacuum packed, especially Penicillium spp. and Cladosporium spp. Degradation of sorbic acid & potassium sorbate occurs in sorbet-containing cheese. Therefore, it leads to the formation of trans-1, 3- pentadiene and develops an off odor, and that flavor is termed as "kerosene". At 92°C, the D value of various heat resistant variants of *B. nivea* ascospores in cheese and milk was found to be 1.3–2.4 s. As a result, the cream cheese would have a 1% spoilage rate (Engel & Teuber, 1991).

2.6. Lactic acid bacteria in milk and milk products

LAB (Lactic Acid Bacteria) is a gram-positive bacteria. They have the potential to ferment, which allows them to enrich nutrients, improve organoleptic characteristics, improve the safety of food, as well as provide therapeutic benefits (Steele et al., 2013). Perishability of milk, LAB is employed to extend its life span through fermentation of milk. This also aids in the preservation of milk's nutritious constituents. The use of LAB in milk fermentation results in high-quality dairy products with excellent organoleptic characteristics. Lactic Acid Bacteria (LAB) variants from the *Lactobacillus* genus are widely used for health-promoting bacteria (Griffith and Tellez, 2013). Several *Lactobacillus* variants are thought

to have immunological mediator, antihypertensive, anti-cancer properties and calcium-binding, LAB fermentation, as per some research, protects against diarrheal disorders by altering the structure of microbes in the gut. Bacteriocins, which are protein antibacterial compounds, are also produced by LAB (Rakhmanova et al., 2018).

3. Microbiological growth

The freshly drawn milk has a temperature of almost 38°C, which makes the growth of bacteria effortless, resulting in the spoilage of milk. This could be achieved by lowering the temperature of the milk to 16°C in order to avoid rapid growth and spoilage by microorganisms. At this temperature, the activity of enzymes is lowered, slowing the growth of bacteria and allowing milk to be preserved for longer periods of time. Before processing, fresh milk is often kept at low temperatures, such as 4°C, to inhibit the growth of most bacteria. On the other hand, psychotropic bacteria can grow and lead to the spoiling of ultra-high temperature (UHT) treated & sterilized milk and other products with a longer storage life owing to their ability to synthesize heat-resistant extracellular enzymes peptidases and lipases (Machado et al., 2017). According to Reguillo et al. (2018), saprophytic bacteria with glycolytic, lipolytic, and proteolytic activities are found in raw milk. The presence and multiplication of these organisms can be constrained by storing fresh milk below 7°C, which favors the selection of psychrotrophic species such as Pseudomonas spp., Micrococcus, B. cereus, Alcaligenes spp, and Lactobacillus, Streptococcus, as well as Enterobacteriaceae.

Psychotropic bacteria, like, *Pseudomonas spp*., can form lipolytic or proteolytic enzymes, which break down raw milk constituents, resulting in flavor and texture let-downs (Hahne et al., 2019; von Neubeck et al., 2015). High bacterial counts in raw milk are caused by inadequate cleanliness and biofilm formation in storage tanks and milking equipment (Hahne et al., 2019; Marchand et al., 2012). All of them contribute to the growth of microflora in milk.

The normal souring process of milk is advantageous as it leads to the formation of an acid that is beneficial during the processing of butter by helping in the extraction of fat during churning. Pathogens are also unable to grow in low pH environments, but molds can. This natural souring process also helps develop many products made from milk, such as cheese, sour cream, ripened buttermilk, and yogurt. The products are a form of preservation of milk. They are formed due to fermentation by bacteria on lactose, which makes them easily digestible compared to fresh milk. Milk containing 10^5 cells/ml of bacterial cells shows poor handling and hygiene conditions. In order to assure the safety and security of consumers, rapid tests are conducted to test the bacteriological quality of milk before processing.

4. Microorganisms occurring in milk and milk-based products

B. cereus, C. jejuni, Brucella spp., Coxiella burnetii, Shiga toxin-producing E.coli (particularly E. coli O157:H7), Mycobacterium paratuberculosis, L. monocytogenes, Yersinia enterocolitica, Salmonella spp., & S.aureus have been linked to milk-borne outbreaks (Dhanashekar et al., 2012). *Listeria, Salmonella, Campylobacter, & Vero toxigenic E. coli* seem to be the four leading bacteria causative for milk-borne illnesses. Moreover, *B. Cereus, S. Aureus* & *M. Paratuberculosi* are developing microorganisms that are important in dairy contamination (CDC, 2016a).

L. monocytogenes is a Gram-positive, non-spore-forming, facultatively anaerobic rod including a diameter of 0.5m & length of 1–1.5m. *L. monocytogenes* is one of the principal genera concerned with human infection, despite its encompassing around 17 genera (Orsi & Wiedmann, 2016). *L. monocytogenes*, unlike numerous other infectious infections, can survive and flourish in a broad range of temperatures (0.4°C to 50°C) & pH conditions (Yang et al., 2005). Raw milk and soft cheese are both major sources of *L. monocytogenes*. Numerous investigations showed that certain variants of L. monocytogenes may develop in dairy with variable fat content at different temperatures, and cell numbers have increased in the existence of cane sugar & cocoa powder, owing to its distinctive growth characteristics. In general, invasive & non-invasive diseases have been associated with outbreaks of dairy products, with a fatality rate of 20%–30%. (FDA, 2012). Listeriosis, a gastrointestinal condition characterized by diarrhea, mild fever, muscle pains, nausea, & vomiting, is generally associated with non-invasive *L. monocytogenes* diseases. Invasive diseases, which include meningitis &septicemia, have more severe symptoms. Listeriosis can last anywhere from a few hours to two to three days for persons with healthy immunity. However, in pregnant ladies, neonates, the elderly, or persons with a weakened immune system, the disease can continue for up to 3 months & cause nervous system damage (Lund, 2015).

Salmonella spp.: Salmonella is a Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacilli, motile pertaining to the Enterobacteriaceae family with a size in the range of 2–4m. *Salmonella* could transform nitrite to nitrate, generate gas from glucose, manufacture hydrogen sulphide on triple-sugar iron agar, use citrate as a sole source of carbon & energy, and develop unfavorable effects when oxidatively deaminate phenylalanine & tryptophan are oxidatively deaminate. It also has a lysine positive, ornithine decarboxylase positive, indole negative, & urease negative phenotype (Coburn et al., 2007). *S. Typhi* & *S. Paratyphi A* are 2 of the most well-known *Salmonella* species for inducing typhoid fever, which is more severe and also has a 10% death rate. Typhoid fever typically takes 1–3 weeks to develop, as well as the main symptoms include a high temperature, headache, gastrointestinal aches, and diarrhea. Septicemia or a persistent infection might also happen. Nontyphoidal *salmonellosis* is a disease that is caused by serotypes other than *S. Typhi* & *S. Paratyphi A.* (Kubota et al., 2011). Although salmonellosis can be fatal in certain situations, it is usually more self-limiting and far less dangerous than typhoid fever. Nontyphoidal salmonellosis manifests itself 6–72 h of exposure, with symptoms including nausea, stomach cramps, vomiting, fever, diarrhoea, and headache. *S. Dublin, S. Heidelberg, S. saint paul, S. typhimurium, S.Newport, S. Kottbus,* as well as *S. newbrunswick* are among the *Salmonella* serotypes linked to milk infections in the United States (Sulaiman and Hsieh, 2017). Raw milk and cheese have been the main sources of infection in these outbreaks, with the exception of two outbreaks induced by pasteurized milk. The milk outbreak resulted in a lot of *S.enteritidis* isolates. Nonetheless, since 2000, one of the most common species linked with the milk outbreak seems to have been *S. Typhi,* as well as the potentially contaminated carriers have largely been cheese or cheese-related goods (CDC-Food Net, 2016).

E. coli & *STEC* are Gram-negative bacteria that are typically found in the small intestines of warm-blooded animals, such as humans. In the human upper gastrointestinal tract, *E. coli* is usually innocuous; nevertheless, it can be hazardous to persons who have a weak immune system. Pathogenic *E. coli* infections can cause urinary tract infections, meningitis, sepsis, & enteric diarrheal illnesses when contrasted to non-pathogenic *E. coli* infections (FDA, 2012). There are four types of E. coli strains that cause diarrhea: (1) EIEC (Enteroinvasive E. coli), (2) STEC/EHEC (Shiga toxin-producing E. coli), (3) ETEC (Enterotoxigenic E. coli), and (4) EPEC (Enteropathogenic E. coli) (Hunt, 2010). STEC are primarily found in raw milk or other milk products and have also been linked to a number of food-borne outbreaks (Farrokh et al., 2013). Shiga-like toxins (stx) are produced by STEC, with stx1 and stx2 being the most important pathogenic factors for toxicity (Oliver, 2015). 37°C is the appropriate temperature for STEC development. However, research revealed that it might flourish at temperatures ranging from 7 to 50°C and in an acidic (pH 4.4) or low-water activity environment. STEC infection is most common in raw milk & cheese goods among all milk products. Amid the manufacturing of lactic cheese, cream cheese, & feta cheese at pH 4.5, E. coli O157:H7 was detected (Sung & Collins, 2000). Some O157:H7 bacteria have been identified as acid resilient, with these strains capable of flourishing at pH 3.0. Although the E. coli O157:H7 variant can be destroyed through pasteurization (72°C for 16 s), certain strains were identified to persist even at 80°C when heat-

Table 1
Details of inadequate heat treatment resulting food borne disease or dairy product recalls

Food product	Date	Product withdrawn/ food borne illness	Country
Milk	1986	Foodborne illness was due to *Camphylobacter*	USA
Semi soft cheese	2000	Foodborne illness was due to*L. Monocytogenes*	Italy, Germany, Austria
Milk	2001	Foodborne illness was due to*Listeriosis*	Sweden
Fresh cheese	2003	Food borne illness was due to *Zooepidemicus*	Spain
Morra bocconcini cheese	2004	Foodborne diseases was due to *L. monocytogenes*	Canada
Cream and milk	2013	Withdrawn	United Kingdom
Chocolate milk	2015	Withdrawn	USA
Yogurt	2016	Withdrawn	USA

ing mozzarella cheese (Trevisani et al., 2014). Amid the cheese-making process, STEC was also discovered in low-water activity & high-salt percentage conditions.

S. aureus: Staph aureus (Staph) is a Gram-positive *coccus bacterium* that can be found in many places, such as the air, dust, sewage, water, animals, & humans (inside their nasal & respiratory tract & skin). The majority of Staph strains are not harmful. Meanwhile, it is dangerous to young children, the elderly, and those that are immuno-compromised (Kadariya et al., 2014). It generates enterotoxins (SE), which are the most common source of food-borne gastroenteritis in humans (Argudn et al., 2010; Ortega et al., 2010*)*. Despite the fact that S. aureus might not be the main cause of milk outbreaks, it is nevertheless a new bacterium that has raised questions about the safety of milk products. Despite this, Staph outbreaks in dairy have increased over the years. *Staph* has infected 37.5% of raw milk, 32.8% of pasteurized milk, 10.8% of unpasteurized milk, & 18.8% of milk with such an uncertain treatment among the numerous dairy food products (Paulin et al., 2012). Infection usually causes milk contamination in a cow's mammary gland or poor hygiene practices during the milking operation (Hu & Nakane, 2014). *S. aureus* has also been recognized in the beginning stages of the cheese-making procedure, as well as in low-concentration milk. Several food strains from outbreaks tested positive for SEA, SEC, & SED (Kadariya et al., 2014). *S. aureus* has also been found at a variety of temperatures (7–48°C), pH (4–10), & water activity levels (between 0.83 and 0.99). Nonetheless, *S. aureus* grows best at 37°C with a pH of 6–7 as well as a water activity of 0.98 (Paulin et al., 2012), & this can persist in 15% NaCl. In particular, raw milk is rapidly infected with *S. aureus,* posing a high chance of infection in raw milk cheese (Fagundes et al., 2010).

5. Thermal processing of milk

Specific treatments such as pasteurization and sterilization were employed in order to make milk free from any pathogenic and spoilage microorganisms as well as preserve it for a long time. Standard milk processing methods include high temperatures for short periods of time (HTST) or low temperatures for long periods of time (LTLT) pasteurization, along with ultra-high temperature (UHT) processing. They have been used in inactivating enzymes and bacteria in foods to assure food safety for a long time. Thermal processing of milk products protects people from foodborne outbreaks (Costard et al., 2017), but inadequate thermal treatment has culminated in product recalls as well as outbreaks of foodborne disease (Table 1). Microorganisms, such as protozoans, viruses, yeasts, and molds, are largely thermal sensitive; hence, bacteria are the main focus of this review. Six major types of thermal treatments used in the manufacturing of dairy products are emphasized (Table 2) and summarised in the next section.

5.1. Pasteurization

Thermal pasteurization, which is common in the dairy industry, kills and inactivates microbial flora resulting in a safer, long-lasting food (Liu et al., 2020). Heat treatments have different impacts on milk components depending on how severe they are, but they are all linked to unpleasant changes in color, consistency, and nutritional qualities (Liu et al., 2020). Pasteurization is the short-term heating of liquids to an elevated temperature. High temperatures for short periods of time (HTST) or low temperatures for long periods of time (LTLT) are the commonly used pasteurization techniques. For the production of cheese, LTLT is mostly used. The milk is heated for 30 min at 62.8°C until being cooled to 40°C.HTST includes heating milk to 72°C for 15 s before cooling it at 40°C. The primary goal of both techniques is to eliminate *C. burnetii,* which is the bacteria that is regarded as being the most thermally stable and non-spore-forming in nature (Enright et al., 1957). Pasteurization can be run in two ways: batch and continuous. The cream also needs to be pasteurized prior to the production of butter. In batch processing, a fixed amount of milk is heated to 63°C for 30 min and then cooled to 40°C before being packaged. During pasteurization, the cream layer minimizes as the fat globule membrane gets denatured at such temperatures. While the association of fat globules is greatly reduced at this point, resulting in less creaming, it has no effect on milk fat content. In the case of pasteurized milk, no major changes in the nutrients are observed apart from a modest loss of Vitamin A and Vitamin C. This kind of milk can be stored for 2,3 days at maximum before it gets spoiled by putrefactive organisms rather than acid development.

5.2. Ultra-high treatment (UHT)

The ultra-high treated milk (UHT) process is typically carried out at 135–150°C for 1–10 s, depending on the product being treated. The process's target microorganisms are mostly *Geobacillus*, a thermophile &spore-forming bacteria, and the goal is to achieve a 9-log reduction in its initial microflora. The UHT process is divided into several stages: pre-heat, high-heating, cooling, homogenization, and sterile or aseptic packaging. In industries, UHT systems can be either direct or indirect. In the first one, the product & heating medium is in direct contact with each other. The product is promptly heated and cooled. In the latter system, the product &heating medium are not in direct contact; rather, heat is transmitted to the product via a heat exchanger. Direct heating is preferable for keeping the flavor profile over indirect heating, which is considered more severe (Lindsay et al., 2021). ESL milk has a storage life of 21–45 days when kept under refrigerated conditions (Deeth, 2017).

5.3. Batch pasteurization

There are several different batch pasteurization temperatures & time frames used in the dairy industry. In most cases, the materials are thermally treated to inhibit any vegetative microorganisms present before being homogenized in a batch container. As examples, consider the following: (i) milk is heated to 90-95°C for 3–5 min as a starter culture for yogurt production, with the goal of destroying vegetative bacteria, bacteriophages, and possibly some spores, eliminating antagonistic compounds, denaturing some protein, and liberating dissolved oxygen;(ii) To inactivate vegetative cells, processed cheese batch ingredients are normally heated to a temperature equal to or greater than pasteurization, and a variety of process parameters are employed to achieve this (Lindsay et al., 2021).

Table 2
Various heat treatment techniques to inactivate microbe in milk and milk products

Heat treatment		Time/ Temp (°C)	Inactivation of microbe
Batch Pasteurisation	Cheese milk	>95/ 4-15 min	Non-spore-forming bacteria, some psychrotrophic and mesophilic spores (depending on heat treatment)
Pasteurisation	LTLT	63/ 30min	Non-spore-forming pathogens, psychrotrophic spoilage bacteria, such as gram-negative *Pseudomonas Enterobacteriaceae* (not spores or thermoduric bacteria, such as *Enterococcus, Streptococcus*
	HTST	72-75/ 15 s	
In container sterilisation	110-120/ 10-20 min	Except for those microbes which are exceptionally heat-resistant, both spore-formingand non-spore-forming.	
UHT	135-150/ 1-10 s	Except for very heat-resistant spores of *B. sporothermodurans*and*G. sterothermophilus,* all non-spore-forming and spore-froming bacteria; produces 'commercially sterile product'	

5.4. In container sterilization

Cans of evaporated/condensed milk are packed and pasteurized, either in batches or continuously. During sterilization, the cans are continuously moving to disperse the heat more efficiently & evenly throughout the cans. Any protein that forms amid the thermal processing is dispersed uniformly across the milk (Lindsay et al., 2021). The milk is sterilized at 110–120°C for 15–20 min before being chilled to storage temperature. Due to Maillard'sreaction, thermal treatment is vigorous, resulting in a light brown coloration (Bylund, 2003). The inactivation of pathogenic spores, such as *Clostridium botulinum,* is a food safety concern for 'commercially sterile' goods (i.e., There may still be some heat-resistant spores present, which is known as 'commercially sterile' in the industry). As milk is a low-acid product pH(6.6), the food safety goal is to reduce *C. botulinum* by 12 decimal points (the same requirement applies to sterile foods).

The minimal *botulinum* cook (time/temperature combination) yields a product that is free from microbiological contamination (i.e., *C. botulinum* spores are inactivated), not sterile ('commercially sterile'). *G. stearothermophilus* and *B.sporothermodurans* are two of the more heat-resistant spores that can induce spoilage (Lewis, 2003). As a result, the thermal treatments used in milk processing that control these more thermal resistant spores would also suppress *C. botulinum* spores. It is worth noting that C. botulinum is infrequently seen in fresh or pasteurized milk products (Doyle et al., 2015).

5.5. Thermisation

Thermisation takes place around 57 and 68°C for 5 s to 30 min. This is a technique that is occasionally used during the production of cheese (E.g: thermisation and raw milk cheese) also targets vegetative psychrotrophs (those that produce thermal-resistant lipases and proteinases) and or to prolong the shelf life of milk during chilled storage before any kind of processing (Lindsay et al., 2021).

6. Mechanism of inactivation by heat

The mode of action of heat on microbes has been thoroughly researched, with several cellular changes observed. Before going into detail about the specific changes caused in microbial cells, it is vital to understand the terms "alive cell" & "inactivated cell". The living cell is defined as a "cell that has the capability to replicate indefinitely under suitable conditions" (Mackey & Mañas, 2008). The cell's ability to grow in liquid medium, solid media, or other experimental setup is used to demonstrate viability in this context. The basic goal of food preservation technologies is to inactivate microbial cells, which means they lose their ability to proliferate. This alteration entails changing one or more cellular structures or functions, which are collectively referred to as cellular targets. The nucleoid, the cell's RNA, the ribosomes, and many enzymes are the cellular structures or targets that have been commonly thought to be affected by cell inactivation by heat (Cebrián et al., 2017). Despite decades of investigation, the ultimate reason for cell inactivation by heat remains unknown. The majority of authors believe that heat-induced microbial inactivation is the multi-target phenomenon. It is unknown how important the damage is done to each individual cellular target is in the end result—inactivation or survival of cells from different microbial groups and under different treatment circumstances. This brings to a crucial concept: the critical component (Miles, 2006).

A critical component is a component whose loss can cause cell death. It must meet two criteria: it must be essential for the cell's self-maintenance and duplication, and it must be irreplaceable if it becomes inoperable. The primary criterion includes all cellular components that seem to be products of important genes, while the secondary criterion eliminates any compound that may be synthesized after treatment if environmental conditions allow it. RNA polymerase and ribosomes are two obvious examples of essential components. Moreover, each microbial cell contains a large number of ribosomes, and it is unclear how much ribosome loss a cell can tolerate (Niven et al., 1999). The cytoplasmic membrane is treated in a similar way: it is recognized as a critical component, but it must be damaged to a certain extent—where it cannot be repaired. Furthermore, linkages between distinct cellular compounds might be extremely high (Mackey and Mañas, 2008).

In last, the lethality of heat treatment will be determined by the changes of at least one critical compound beyond a critical threshold, either as a direct result of heat on the critical target or as a result of contemporaneous changes of other cellular targets (which might not be necessarily lethal). Furthermore, the intrinsic resistance of each cell target may differ depending on the kind of microbe and the surrounding conditions, i.e., the treatment media composition (Mackey and Mañas, 2008). As a result, the extent of changes in a single cell is numerous. Undoubtedly, various in between circumstances occur in practice, such as cells with damages in non-critical or in critical compounds, although at a low. It is directly attached to a crucial phenomenon: cellular sub-lethal injury and recovery. Damage to cellular structures and functions occurs in sub-lethally wounded cells, which the cellular machinery can only restore if environmental conditions are favorable (Mackey, 2000). Ultimately, it is necessary to keep in mind that most bacterial species are capable of developing resistance to the stressful effects of specific physical and chemical stimuli. The so-called "heat shock response" is a classic example of bacterial cells developing heat resistance after being exposed to sublethal temperatures (Yura et al., 2000). These reactions enhance the thermostability of specific cellular components as well as the cells'

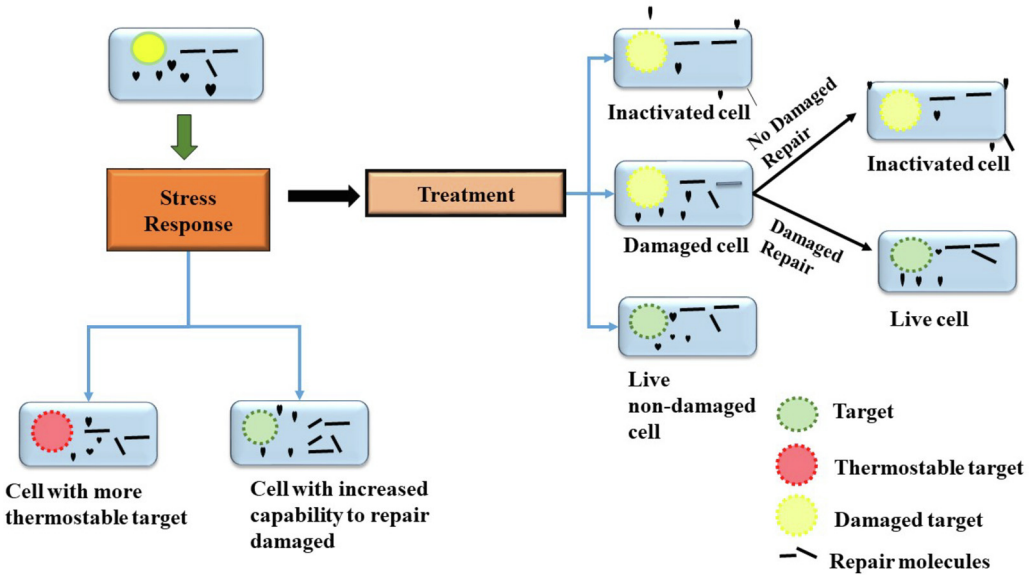

Fig. 1. Different scenarios for determination of cellular survival or inhibition upon thermal exposure

capacity to regenerate sublethal injuries (Cebrián et al., 2009). Fig. 1 depicts the different situations that could happen when vegetative bacterial cells are exposed to thermal treatment, assuming the most basic scenario, i.e., a single target molecule that is also a bacterial cell.

6.1. Effect of heat on cellular targets

One of the components influenced by heat is the outer membrane of Gram-negative cells. The degradation that occurs to that component has been demonstrated through the sensitization of cells to lysozyme, hydrophobic antibiotics, or bile salts (Mackey, 2000). It has also been reported that vesicles and lipopolysaccharide molecules are released into the treatment media (Tsuchido et al., 1985). Heat has an effect on the peptidoglycan wall as well. According to Mackey (2000), D-alanine is released from teichoic acids by *S. aureus* cells, causing binding of Mg ions in the cell wall, inhibiting their utilization in some critical metabolic activities. According to Teixeira et al. (1997), thermal treatment of *L. bulgaricus* at 64°C disrupted the cellular wall since heated cells were more sensitive to the presence of penicillin. Several authors have studied the involvement of the cytoplasmic membrane in heat inactivation. Several researchers have reported the loss of intra-cytoplasmic material, such as UV absorbing compounds and ions from heated bacteria of diverse species (Mackey & Mañas, 2008). The synthesis of membrane vesicles & the depletion of membrane material was described by Mackey (2000). This depletion of membrane integrity after thermal treatments has also been used to reveal via Propidium iodide staining, the most extensively used indicator for membrane integrity (Kramer & Thielmann, 2016; Marcén et al., 2017). Several authors have noted that the membrane is associated with cell inactivation based on its loss of internal homeostasis and accompanying the partial loss of functionality: modifications to the entry and exit points of a number of components (Kramer & Thielmann, 2016). Osmotic and pH homeostasis are lost as well as respiratory activity (Mackey & Mañas, 2008; Leguérinel, Spegagne, Couvert, Coroller, & Mafart, 2007).

For bacterial survival, nucleoid is a critical molecule and has been shown to directly inhibit inhibition by other technologies, such as ionizing radiation and UV light. It has been demonstrated that DNA is one of the most thermostable biological constituents, with denaturation occurring only at sterilization temperatures, particularly under hot and dry heat circumstances (Cebrián et al., 2017; Mackey et al., 1991).

Many studies have been done on the effect of heat on ribosomes and RNA. These compounds are even more susceptible to temperature than that of DNA (Earnshaw et al., 1995). Denaturation of RNA was first found as amongst the most obvious outcomes of heat exposure in bacteria (Iandolo & Ordal, 1966). Ribosome damage has also been found (Cebrián et al., 2017). Because ribosomes are mostly composed of protein and RNA, it was hypothesized that they could be a key target, and DSC tests were conducted to test this theory (Mackey et al., 1991; Mohácsi-Farkas et al., 1999; Nguyen et al., 2006; Lee & Kaletunç, 2002). According to published research, irreversible denaturation of ribosomes happens in the majority of cases at temperatures at which bacteria become non-functional. Meanwhile, other important proteins, such as subunits α & β of the RNA polymerase compounds, also disintegrate at similar temperatures (Nguyen et al., 2006). Another important topic in ribosome stability is the impact of Mg ions that are believed to be necessary for the persistence of subunit attachment. Tolker-Nielsen and Molin (1996) found that adding magnesium to the treatment medium preserves ribosomes from the thermal effect and improves *S. enterica* var *Typhimurium* heat resistance. As a result, it is plausible to expect that a reduction in magnesium in the cell, such as a result of membrane damage, would result in ribosome destabilization, contributing to cell death.

Proteins are present in almost every microbial cellular structure, in either structural proteins or as enzymes. Denaturation of proteins can lead toa significant decline in functionality in a variety of ways. Detoxifying enzymes like proteases and superoxide dismutase, chaperones, catalase (DnaK, DnaJ, GroEl/GroES, Clpx, and others), and DNA repair enzymes could all be targeted. Heat denaturation also affects transport pumps and channels (Cebrián et al., 2017). Furthermore, if a bacterial cell has been suffering from other sub-lethal damage, such as those in the membrane, significant protein aggregation may be hard to accomplish. Individual proteins were first dismissed as potential thermal inactivation targets because of the likelihood of resynthesis following treatment in most cases, but they have now been evaluated as key possible targets. Although this has not been clearly confirmed, it is presently thought that perhaps RNA polymerase could be a viable critical target (Nguyen et al., 2006).

In addition to the direct effects of heat on cellular components, some indirect events could be involved in cellular inhibition under specific climatic conditions. This is the case with ROS build-up, which has been observed in *Cronobacter sakazakii*, and *B. cereus, E. coli* when they are heated (Baatout et al., 2005; Arku et al., 2011; Mols et al., 2011). ROS can react with almost every biological component involving membrane lipids, DNA, and proteins. It is yet unknown where these ROS come from

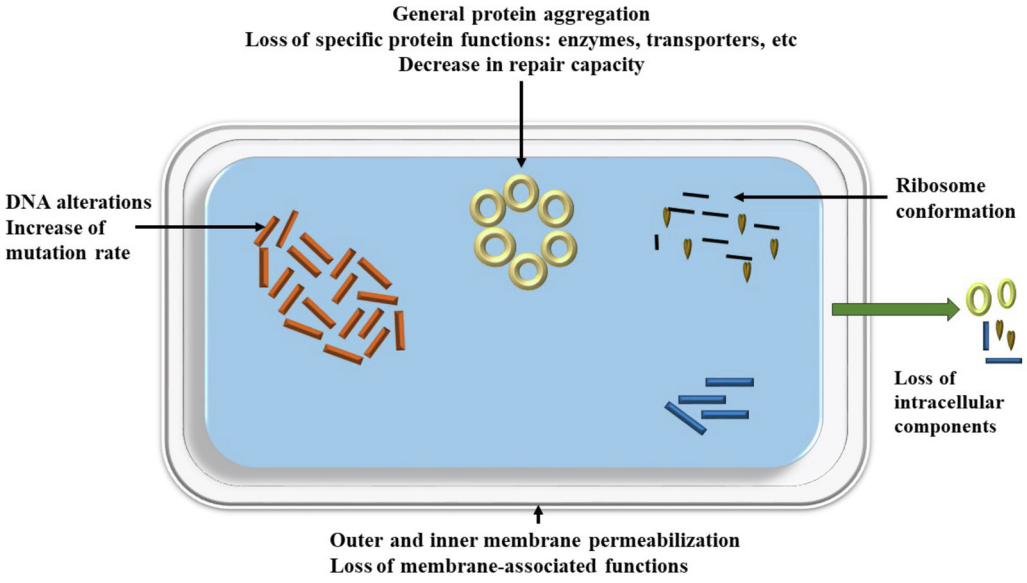

Fig. 2. Changes in vegetative cells due to exposure of heat

or their role in cell inactivation. Recent research suggests ROS production in *E. coli* is not the result of membrane damage and consequent electron chain disruption. There is no link between membrane permeabilization to propidium iodide and ROS production. According to the authors, other processes, such as destabilization of some of the other components or lack of activity of microbial defense systems, could thus be responsible for the increased amount of ROS.

Fig. 2 describes the main activities which are often seen in a vegetative bacterial cell subjected to heat. The reader should keep in mind that all these occurrences may be connected in some way. Under diverse experimental conditions, Heat has the potential to destabilize a wide variety of structures & cellular functions, resulting in cell inhibition. As a result, heat is a multitarget agent which does not function in an “all-or-nothing manner”. This causes sublethal damage to the cells in a varying number of the population, and its restoration is dependent on a variety of circumstances, including treatment intensity, treatment medium, microbe species, and recovery conditions (Cebrián et al., 2017).

7. Thermal kinetic parameters

The term "thermal death point" was originally used to characterize the thermal sensitivity of bacteria. It was stated as the time it takes for a suspension of an organism (including prokaryotic & eukaryotic cells) to be effectively inactivated at a single specific temperature (Tischer & Hurwicz, 1954). According to Lindsay et al. (2021), the thermal death point for tubercle bacilli in milk was 1 min at 68°C. As it is dependent on the type of organisms present and the size of the population, the thermal death point is no more widely used and gives the idea of an instantaneous kill at a specific temperature. The D-value calculation is much more informative because it gives details about bacteria’s heat resistance (Juffs and Deeth, 2007).

The thermal kinetic parameters are the time and temperature parameters involved in the heat processing that will ensure the destructive effect on the microorganisms. Understanding the dynamic variables in heat treatments would allow process strategies to accomplish desired antimicrobial properties at the end of the heat operation, preserving the product by maintaining bioactivity throughout the product’s shelf life (Sant’Anna, Utpott, Cladera-Olivera, & Brandelli, 2010, 2012). According to Peleg (2021), TDT is "how long it takes to kill a specific bacterium at a specific temperature." And the lethality of a heat process, called as F_0 or the F value, is "the level of microbial destruction obtained by thermal treatment expressed in minutes or, to be more precise, in equivalent minutes of exposure to 121.1°C". In the food sector, obtaining TDT and Fo values from experimental data and using them to determine microbiological safety is common practice. TDT is defined as "how long it takes to kill a certain bacterium at a specific temperature", according to Peleg (2021).

7.1. Decimal reduction time (D value)

D values are frequently employed in food processing to explain first-order reactions (TDT concepts). Espachs-Barroso et al. (2006), defines D-value as the time needed to minimize the initial activity by tenfold at a specific temperature. The general inactivation rate constant and D-value are expressed in terms of first-order reactions, as shown in Eq. (1).

$$D = \frac{Ln(10)}{k} = \frac{2.303}{k} \tag{1}$$

Where k is the rate constant.

BelowEquation (Eq. (2)) can also be used to estimate the D-value graphically and mathematically:

$$D = \frac{t}{\log N_0 - \log N_t} \tag{2}$$

N_0 is the microflora at time 0 or at starting, N_t is the microflora at time t.

At a particular temperature in a given medium, the D-value is the amount of time needed to eradicate 90% of vegetative cells or spores of a known microbe. The D-value of microorganisms can be graphically determined using Eq. (2). Eq. (3) can also be used to express the relationship between D-value and Z-value.

$$\log_{10}\left[\frac{D(T)}{D(T_{ref})}\right] = \frac{T_{ref} - T}{z} \tag{3}$$

Here, T_{ref} denotes the standard temperature, which is 121.1°C (traditional heat sterilization temperature). Z is the variance in temperature that causes D(T) to rise or fall by a factor of ten. Eq. (3) can be used to calculate the Z value of a specific microorganism in a food or beverage product, and the results can then be combined with the temperature profile of a proposed thermal process to calculate F_0 or process lethality.

Research being carried out has reported distinct D values for various microorganisms found in milk. A study reported in ICMSF (1996) showed the relationship between inactivation temperature and D value for *S. aureus*, a prevalent non-spore forming bacteria extensively known to cause several foodborne outbreaks across years.

Table 3
D-values and Z-values of spore and non-spore-forming bacteria in processing of milk and milk based products

Non-spore forming Temp of inactivation (°C)	D-value (min)	Z-value (temp)	Dairy Product	Bacteria	Refs.
64	0.385	7	Ice cream mix	*E. coli*- ATCC 9637	(Desmarchelier and Fegan, 2003)
68	0.116	6.71	Reconstituted skim or UF whole milk (27% TS)	*L. monocytogenes*	(Szlachta et al., 2010)
85	0.08	4.81	Galacto-oligosaccharide syrup (74% total solids)	*Cronobacter spp*	(Bang et al., 2017)
60	˜0.2	7.29	Milk	*Salmonella*	(Lindsay et al., 2021)
70	0.1	6.42	Milk	*S. aureus*	(Lindsay et al., 2021)
Spore forming					
95	2.6	6.7-13.8	Whole milk	*B. cereus*	(Le, 2019)
125	2-13	6.7-13.8	Raw milk	*B. sporothermodurans*	(Burgess et al., 2010; Le, 2019)
140	0.06-1	6.7-13.8	Milk powder	*Geobacillus*	(Eijlander et al., 2019; Hill & Smythe, 2004; Le, 2019)
105	0.3	6.7-13.8	Low fat processed cheese (requeij~ao cremoso)	*Paenibacillus*	(Oliveira et al., 2018; Le, 2019);;
100	4-8	6.7-13.8	General	*B. licheniformis*	(Lindsay et al., 2021; Le, 2019)

The study recorded a D-value of 0.1 and 0.02 min at a temperature of 70 and 75°C, respectively, in milk. A D-value of 6.3 min was recorded against a temperature of 95°C in infant formula for *B. cereus,* which is a spore-forming bacteria. The spores are hard to destroy, which is why we can see a higher D- value as compared to *S. aureus.* The D value of *Cronobacter* spp (*sakazaki* DPC 6529) was investigated at an inactivation temperature of 58°C by Huertas et al. (2015) and Osaili et al. (2009) for reconstituted infant formula (RIF), RIF (Whole milk), RIF (Low fat milk), RIF (Skim milk) that reported a D value of 0.55, 0.68, 0.62, 0.51 min, respectively. An increasing trend in D value was observed in the products as the fat content increased. A higher D value was associated with increased fat content & vice versa. Casadei et al. (1998), found a similar effect by investigating the D value of *L. monocytogenes* (strain 1151, non-spore forming) for three different milk products, seemingly butter, half cream, and double cream over the same temperature (68°C). The D- value of 0.19, 0.15, and 0.13 min was observed for the three, respectively. Another research by Szlachta et alSzlachta et al. (2010)observed a D-value of 0.116 min for *L. monocytogenes* under the same temperature condition in UF whole milk with 27% TS. A decreasing trend of D value was observed in this study which was the result of higher fat content in butter (0.19 min) as compared to half cream (0.15 min) and double cream (0.13 min). Since D values differ from one to another microbes, we can only observe a trend rather than an exact value for it.

Both spore-forming and non-spore-producing bacteria can be found in dairy and dairy products (Table 3). In comparison to non-spore formers, spore formers with the ability to generate spores in unfavorable conditions require a higher inactivation temperature. Different inactivation temperatures for non-spore producing bacteria like *Salmonella* typhi have been reported in various investigations, including 57, 60, 63, 73-74, and 85°C.This is lower as compared to the pasteurization temperature for the spore forming bacteria *Paenibacillus* found in milk products like full fat and low-fat processed cheese. The inactivation temperature was observed to be as high as 105°C for the bacteria (Oliveira et al., 2018). Research work by Stoeckel et al. (2014) investigated a D-value of 2.6 min required at a temperature of 95°C to destroy B. Cereus in whole milk. B.cereus is a spore producing bacteria seen in dairy products such as whole milk, cream, infant formulas, skim milk, etc.

Recent research has shown that minerals found in milk have also been proven to enhance or reduce the thermal resistance of several bacteria. At 63°C, magnesium and calcium were seen to improve the thermal resistance of *S. Seftenburg* 775 W, but higher quantities of phosphate were shown to reduce the thermal resistance of *Geobacillus* spores at 110°C (Mañas et al., 2001).

The influence of heat treatment on the antimicrobial peptide P34 present in dairy was described by Sant'Anna et al. (2012). According to the study, the thermal treatment affects not just microorganisms but also antimicrobial chemicals. D-value of 32.26 min was recorded against a Z-value of 37.7°C & a D-value of 17.1 min was observed against a Z-value of 20.4°C for skim milk and fat milk, respectively. The study concluded that at a temperature of 90°C and above, the antimicrobial peptide P34 is heat stable and is not degraded at HTST or UHT treatments.

8. Applications of heat activation in dairy industries

8.1. Thermisation to improve the safety of raw milk cheese

According to the Centers for Disease Control and Prevention, unpasteurized milk products cause 840 times more illnesses and 45 times more hospitalizations in the United States than pasteurized milk products (Costard et al., 2017). Between 1993 and 2006, cheese was identified as the main cause of 27 foodborne disease outbreaks linked to fresh milk products in the United States (Langer et al., 2012). The safety of raw milk cheeses is determined by the microbial load of the milk, not through the effectiveness of the cheese-making process to inhibit the growth of microorganisms (Condron et al., 2009; Donnelly, 2018). The only exceptions are Raw milk cheeses with a curd-cooking process (such as pasta filata or Swiss varieties) & feta cheese (whose pH is decreased to 4.4 & curd is cooked to 48°C amid manufacture). While fresh milk cheeses may legally meet FDA requirements for 60 days of aging at greater or equal to 2°C, this duration has been shown to be inadequate in removing foodborne bacteria from several cheese varieties (Lindsay et al., 2021). As a result, several countries, including Food Standards Australia New Zealand, have mandated a maturing duration of 90 days for fresh milk cheese products (Condron et al., 2009). Thermisation has been used in the United States to promote food safety by lowering microbial count in fresh cheese milk. This stage ensures that the final product meets the raw milk cheese labeling requirements and is recommended by the FDA (Lindsay et al., 2021), &has been prescribed by Canadian regulators to enhance the microbiological quality of Gouda or Gouda-like cheeses (Boyd et al., 2021).

Shiga toxin-producing *E. coli (STEC)*&*L. monocytogenes* have indeed been identified as particularly high-risk pathogens in fresh milk cheeses, because of the extent of disease that is associated with each, in addition to their capacity to proliferate or survive amid the aging process of some cheese (Condron et al., 2009; Donnelly, 2018). STEC was found to be more resilient to naturally producing lactic acid bacteria in unpasteurized milk than *Salmonella species, S. aureus,* & *L. monocytogenes* by Montel et al. (2014), while Pereira et al. (2009) discovered that non-Shiga toxin-forming *E. coli* & *L. innocua* had been the most challenging contaminants to regulate by co-inoculation with LAB isolates obtained from fresh milk model cheese by Pereira et al. (2009). *L. monocytogenes* is more heat resistant than most other non-spore-forming infections (such

as STEC strains) (Doyle et al., 2001), thermisation techniques to diminish *L. monocytogenes* must also lessen STEC at equivalent or greater rates (Lindsay et al., 2021; Fernandes, 2009; Van-Asselt & Zwietering, 2006).

In the scientific literature, there is less research addressing STEC in milk liquid dairy as well as in other goods with an added constraint for cheese thermisation due to the incorporation of non-STEC general *E. coli* D-values in two previously published meta-analyses (Van-Asselt & Zwietering, 2006). Van-Asselt & Zwietering, 2006 developed predictive analytics that anticipated *E. coli* z-values of 6°C & 10.6°C, as well as D-values of 39 &113 s at 60°C, accordingly. A projected STEC Z-value of 4.5°C and D-value at 60°C of 115 s were revealed in a meta-analysis of 25 whole milks infected with STEC and warmed to temperatures of 55-65°C. (S. Engstrom, unpublished results).

8.2. Ultra-high temperature treatment of milk

The objective of the UHT process is to prevent spores & bacteria in milk so it can be stored at room temperature. UHT milk is a ' commercially sterile ' product because certain spores may withstand the thermal treatment and trigger deterioration. However, like storing at temperatures beyond 40°C, thermal abuse can induce spoilage. The thermal-treated milk is paced in aseptic conditions, and the biggest concern is microbial spore viability during the thermal processing rather than contamination after that. All vegetative cells would be inhibited at the processing temperatures employed. Various bacterial spores will also be inhibited, but those that do persist are known as high-heat-resistant spores.

These can be extracted by heating the sample to 100°C for 30 min & incubating the agar plates aerobically or anaerobically at 30°C for mesophilic spores or 55°C for thermophilic spores (ISO/IDF, 2009; Wehr & Frank, 2004). *G. stearothermophilus,* which withstand UHT &spores of *B.sporothermodurans,* which were first recovered from UHT milk (Lindsay et al., 2021), are normally typically of interest in terms of quality. These spores are found in small numbers in fresh milk and are often of interest in terms of quality conditions that favor their growth, such as high temperatures amid processing (Burgess et al., 2010). Certain strains could produce proteolytic activity, leading the UHT product to deteriorate (Pinto et al., 2018). Bacillus species that cause spoiling, such as *Paenibacillus lactis*, were identified from UHT milk. B. cereus spores do not withstand UHT heating, &deterioration of UHT products due to B. cereus proliferation is linked to post-heat re-contamination concerns (Scheldeman et al., 2004).

9. Impact of thermal treatments on milk and milk-based products

The dairy company employs a variety of methods & technology to enhance the storage life of the product. Thermal treatment of fluid dairy products for a deterministic temperature & time; acidification by addition of starter culture mostly in particular instance of cultured milk products like kefir, milk, or buttermilk; dehydration of milk concentrate to produce enough milk or skim milk powder; as well as the inclusion of preservatives like emulsifying salts, e.g., phosphates as well as citrates in shelf-stable processed cheeses are just a few examples of frequently used methods. The storage life of liquid milk products, for instance, is attributed to a number of elements, including thermal treatment (commonly called as pasteurization), the quality of the receiving raw milk, as well as other procedures, including microfiltration, filling conditions, temperature control, & packaging technologies. On the other hand, thermal treatment continues to be the most important criterion for assessing storage life (IDF, 2012). One of the most extensively utilized processing technology in the milk industry is thermal process. Its major goal is to eliminate both infectious & spoilage bacteria from the milk, ensuring that it is safe and also has a long storage life

9.1. Impact of thermal treatments on milk

Milk is either processed at high temperature (85°C for 2-s) in a short-time, flash process (72–75°C /15–30 s) in plate heaters, or at low temperature (63–66°C for at minimum 30–32min, including stirring) & subsequently cooled. Milk's pH was raised by pasteurization and afterward commercial sterilization, resulting in a reduction in acidity. Milk's fat, fat-soluble vitamins, carbs, & minerals are mostly unaltered by healing. The constituents most impacted by thermal treatment include proteins & water-soluble vitamins (Petrus et al., 2011). In contrast, Jose, Adriano, Bruna, Daniel, & de Souza, 2015 found that pasteurized & ultra-high temperature milk had identical protein & lactose levels to raw milk. Conventional heat processing techniques & drying appear to have minimal effect on lactose. Pasteurization itself seems to have little effect on milk's vitamin A & carotene levels. However, when combined with evaporation, like in the production of condensed milk, 20% of vitamin A is lost, whereas carotene is unaffected. Pasteurization and evaporation have little effect on the vitamin E content of milk, while drying & reconstitution can result in a 9% loss. During the thermal treatment of milk, no vitamin D seems to be lost. Thermal treatment has little effect on the B-complex vitamins, nicotinic acid, pyridoxine, riboflavin, biotin & pantothenic acid. However, the depletion of B1 (thiamin), B12, & C rise from 10 to 20% in pasteurisation & UHT processing to 90% in bottle sterilisation & evaporation (Sfakianakis & Tzia, 2014)

9.2. Impact of thermal treatments on Yoghurt

The acidity rate & incubation duration are affected by the thermal treatment used throughout yoghurt production. Consequently, whey protein denaturation is responsible for the shorter incubation time. The lesser the incubating period, hence more whey protein dissociation is caused. The amount of milk fat in yoghurt has a big impact on the incubation duration, viscosity, acetaldehyde, & acidity. Skim milk yoghurts showed reduced incubation rates, but higher viscosity, acetaldehyde, & lactic acid contents. In most cases, adding milk solids to yoghurt lengthens the incubation time (Hassan, Mudawi Abdelrahim, & Mustafa, 2014). It is worth emphasizing that the storage life of yoghurt products is usually determined by the quality & hygiene of the manufacturing & filling activities. For uncontrolled processes, the storage life of "fresh" yoghurt can be as short as a few weeks and even as long as 6 weeks or more for well-operated, ultraclean amenities (Bean & Roger, 2010). Rennet or other appropriate coagulating compounds are used to coagulate these materials. Cheese is produced in practically every country on the planet, because there are over 2000 different varieties (Shelke, Sabikhi, Khetra, & Ganguly, 2018)

9.3. Impact of thermal treatments on Cheese

Ricotta is a whey cheese that is soft, spreadable, & unripened. The yield, TS, & protein recovery of cow milk Ricotta cheese were all affected by the heating procedures & holding duration (Shelke, Sabikhi, Khetra, & Ganguly, 2018). According to Mohamed & Omer, 2010, heat treatment had no major impact on fat content. The cheese made using heat treated milk at 63°C for 30 min has the greatest fat content (21.953.37%).The raw milk cheese seemed to have the maximum protein level (23.851.60%), whereas the cheese made from heat treated milk at 63°C for 30 min would have the lowest (20.801.85%). Protein breakdown as a consequence of enhanced moisture owing to thermal treatment might explain the low protein concentration of heat-treated milk cheese. Even as thermal treatment temperature raised, the total solids content was reduced. Raw milk cheese had the highest ash concentration (3.700.55%), whereas cheese exposed to elevated temperatures at 63°C for 30 min seemed to have the lowest ash content (2.291.22%). Cheese prepared with raw milk had the maximum titratable acidity (0.550.09%), whereas cheese made from heat-treated milk at 63°C/30 had lowest (0.240.03%) (Jalilzadeh et al., 2015). But since cheese was

held at ambient temperature, the inherent microflora of raw milk was stimulated to create acidity as just a consequence of lactose fermentation, resulting in the greatest acidity of raw milk cheese (Coutouly et al., 2013).

10. Conclusions

Milk, which is regarded as an important food for people of all ages, carries some risks in addition to its beneficial effects. While milk is considered a superfood, it is not suitable for everyone. Although it is a nutrient powerhouse, one should be aware of the microflora that lives in it. Newer and more innovative technologies are being developed to eliminate the microorganisms while preserving the functional nutrients and enzymes. The action of various heat treatments can eliminate the microbial risks associated with dairy and dairy products. The same method could be used to achieve the enzyme degradation process, which might cause some changes in the milk enzymes, resulting in less milk spoilage. To design the thermal processing schedule, the thermal destruction rate of the test microorganisms must be established under typical containment circumstances so that an acceptable heating duration at a particular temperature may be determined. Thermal treatment kills microorganisms by coagulating microbial proteins and inactivating enzymes essential for their metabolism. Thermal denaturation of secondary and tertiary structures of macromolecular cellular systems caused microorganisms to decease.

Declaration of Competing Interest

There is no conflict of Interest between the authors and agreed to Publish in Food Chemistry Advances.

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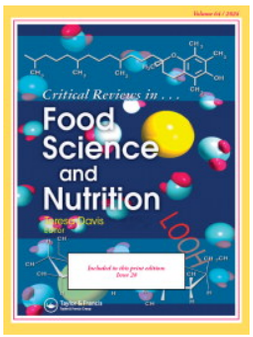

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REVIEW ARTICLE

Prospects on emerging eco-friendly and innovative technologies to add value to dry bean proteins

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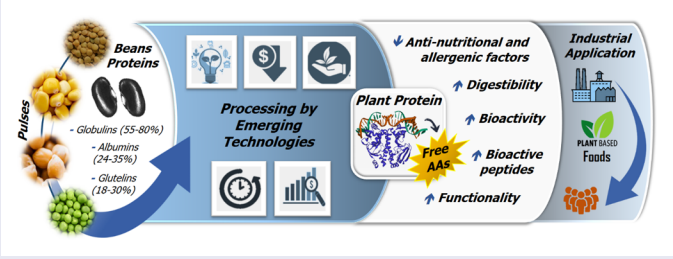
ABSTRACT

The world's growing population and evolving food habits have created a need for alternative plant protein sources, with pulses playing a crucial role as healthy staple foods. Dry beans are high-protein pulses rich in essential amino acids like lysine and bioactive peptides. They have gathered attention for their nutritional quality and potential health benefits concerning metabolic syndrome. This review highlights dry bean proteins' nutritional quality, health benefits, and limitations, focusing on recent eco-friendly emerging technologies for their obtaining and functionalization. Antinutritional factors (ANFs) in bean proteins can affect their *in vitro* protein digestibility (IVPD), and lectins have been identified as potential allergens. Recently, eco-friendly emerging technologies such as ultrasound, microwaves, subcritical fluids, high-hydrostatic pressure, enzyme technology, and dry fractionation methods have been explored for extracting and functionalizing dry bean proteins. These technologies have shown promise in reducing ANFs, improving IVPD, and modifying allergen epitopes. Additionally, they enhance the techno-functional properties of bean proteins, making them more soluble, emulsifying, foaming, and gel-forming, with enhanced water and oil-holding capacities. By utilizing emerging innovative technologies, protein recovery from dry beans and the development of protein isolates can meet the demand for alternative protein sources while being eco-friendly, safe, and efficient.

HIGHLIGHTS

- Dry beans are a source of lysine-rich proteins and high-quality AA for the diet.
- Physical treatments can reduce the ANFs of beans and increase protein digestibility.
- Eco-friendly technologies can treat, modify, extract, and separate bean proteins.
- Conformational changes with protein unfolding improve WHC, EA, and solubility.
- The combined use of emerging technologies allows for conveying advantages of each one.

GRAPHICAL ABSTRACT



KEYWORDS

Anti-nutritional factors; bioactive peptides; digestibility; emerging technologies; protein allergens; pulse proteins; techno-functional properties

Introduction

Pulses (beans, lentils, peas, and chickpeas) are low-lipid leguminous harvested for producing dry grains that are increasingly important in the international market (Gan et al. 2011; FAO 2017). The world's population growth trend (9.6 billion people projection by 2050) (United Nations (UN), 2019) points toward a substantially increased food demand, especially for protein, which requires alternative and sustainable sources. The vital protein content (21–26%) and amino acids (AAs) composition of pulses can offer many health benefits, such as controlling cholesterol levels and reducing the risk of cardiovascular and chronic diseases, cancer, and type-2 diabetes when consumed as part of a healthy diet (Roy, Boye, and Simpson 2010; FAO 2017).

Given the importance of pulses to human nutrition and health, the Food and Agriculture Organization (FAO) and General Assembly of the United Nations (UN) instituted on 10th of February World Pulses Day as a contribution to the

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UN Sustainable Development Goals and the FAO's Hand-in-Hand Initiative (FAO 2016). Pulses consumption as a staple food is therefore essential to attend to the nutritional needs of growing populations with limited access to animal protein, being powerful allies in combatting hunger and overcoming poverty, providing food security and nutritional health.

Dry beans refer to pulses belonging to the *Fabaceae* family, mainly common (*Phaseolus* genus), faba (*Vicia faba* L.), mung (*Vigna radiata* L.), and cowpea (*Vigna unguiculata* L. Walp) beans obtained after the normal processing steps (raw bean with an 8–10% moisture content). Different cultivars are cropped worldwide, and common beans are the most consumed varieties with high protein and lysine levels, making them an indispensable commodity due to their nutritional value. The main physical differences between these beans are size, shape, and color. The chemical composition of dry beans, nutrient distribution, and protein contents according to the grain species and cultivars are shown in Figure 1a, b.

In recent years, pulses have played a central role as alternative plant proteins to animal-derived proteins (Frias et al. 2011) since consumers are devoted to pursuing healthier eating habits. As new food trends emerge, the plant protein market becomes more competitive by including alternative plant protein sources to soy-derived products that might present allergenic effects on consumers. Considering the forecast estimates for the alternative protein market (US$15.6 billion as of 2026) (Shahbandeh 2021), diversifying plant protein sources by introducing highly nutritious and potentially functional proteins derived from pulses is a profitable branch of the protein market to be explored soon. According to McClements and Grossmann (2021), these alternative proteins must be healthy, digestible, bioavailable, and provide a balanced amino acid profile, which requires knowledge of plant-derived ingredients' molecular and physicochemical properties.

A bottleneck to food industries aimed to develop plant protein-derived products is identifying manufacturing processes to economically create large-scale plant-based foods with unique attributes that can replace animal protein (McClements and Grossmann 2021). Many techniques have been exploited to extract protein from beans. Usually, heating methods are commonly used; however, many disadvantages have been reported: large by-product quantities, high energy, and water consumption, and long process times (Chemat, Huma, and Khan 2011). In contrast, emerging methods (e.g., ultrasound, microwaves, pressurized liquid, high-hydrostatic pressure, and enzymatic processes) represent alternatives to improve the proteins' purity, yield, biological activity, and techno-functionality. In addition, they can be considered safe, efficient, and eco-friendly (Tiwari 2015).

The functionality of proteins directly interferes with their role and application in food products, and the lack of plant proteins with consistent functional attributes represents a major issue in the plant-based food sector (McClements and Grossmann 2021). The most important functional-technological properties of bean proteins include solubility, emulsifying and foaming properties, gel formation, and water or oil-holding capacities. Many studies have well-described these important properties of dry bean proteins (Sathe 2002; Sharan et al. 2021; Quintero-Quiroz et al. 2022;) that showed to depend on different molecular characteristics of the protein (such as molecular size and structure) and notwithstanding, the processing conditions (Shevkani et al. 2015). Moreover, the degree of alteration in the association and native state of proteins during isolation and purification by emerging technologies that affect their functionality must be evaluated (McClements and Grossmann 2021).

There is no up-to-date research relating dry bean proteins' potentialities, their main properties, benefits, and limitations, emphasizing the potentialities and limitations of emerging methods of extraction and functionalization. This review provides a summary of dry beans proteins' nutritional quality, biological properties, phytotherapeutic mechanisms, and allergenicity, with a special focus on recent trends involving non-conventional, eco-innovative, and sustainable technologies proposed to pre-treat, improve protein recovery

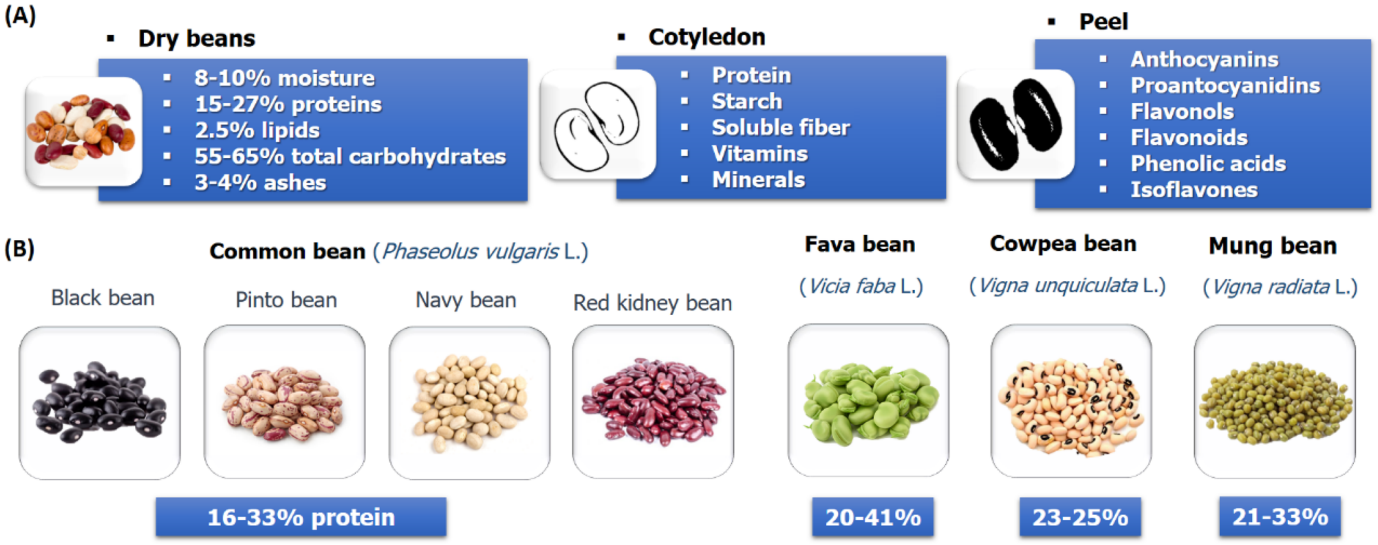

Figure 1. (a) Dry beans' proximate and chemical composition and (b) main common bean species as plant protein sources.

and potentialize techno-functional properties for further use of the high-valuable isolated nutrient. The literature search methodology included a comprehensive search in scientific databases, selecting relevant studies published in the last decades. For this, Boolean operators were adopted to compose relevant keywords for each topic and ensure a broad scope of coverage.

Bean proteins: classification and nutritional value

Pulse proteins classification and structural features of bean proteins

Albumins and globulins are the main pulse proteins, followed by smaller amounts of glutelins and prolamins (Adebowale et al. 2007), varying proportions according to the plant species and cultivars (Oomah et al. 2011). Albumins have a hydrophilic characteristic that makes the proteins soluble in water and englobe structural proteins and enzymes of relatively low-to-medium molecular weight (Boye, Aksay, et al. 2010), while globulins are marked by high molecular weight with a relatively hydrophobic surface that limits solubility in aqueous media (Boye, Aksay, et al. 2010).The physiological attributes of globulins and albumins from beans are attributed to the different AA compositions of their fractions. In general, protein from pulses has low contents of sulfur-containing AAs (SAA) and tryptophan and higher amounts of lysine than cereals (Koblitz 2011). Albumins and glutelins have higher contents of lysine and SAA, mainly cysteine and methionine, which are limiting AAs in pulse proteins (Mundi and Aluko 2012; Multari, Stewart, and Russell 2015). Globulins, in turn, present a higher proportion of aspartic acid/asparagine and glutamic acid/glutamine which are important storage of amino groups in the body (Vasconcelos et al. 2010). The presence of these AAs in the protein composition of dry beans tends to increase protein quality, adding nutritional value to the final product, which is an important factor when considering alternative plant proteins.

Bean proteins include proteins belonging to the vicilin and legumin families, in which phaseolin is the predominant protein in common beans. Due to its compact secondary structure, it shows high resistance to proteolysis in the gastrointestinal tract (Luna-Vital et al. 2015). The predominance of the β-sheet secondary structure is related to greater resistance to denaturation due to the reduced activity of proteases. Thus, beyond impairing the digestibility and availability of essential amino acids (EAAs), the structural stability of pulse proteins also affects the release of bioactive peptides in the body (Bessada, Barreira, and Oliveira 2019), often requiring a subsequent process to modify its structure and improve nutritional aspects.

Nutritional quality

Protein from dry beans can exert *in vitro* and *in vivo* biological and physiological activities, playing a determinant role in human nutrition. However, the nutritional quality of these plant proteins depends on the equilibrium in EAA composition, digestibility, and presence of anti-nutritional factors (ANFs) (Chéreau et al. 2016). The usefulness of any protein for nutrition purposes is governed by its digestibility. The proportion between protein fractions in beans (globulins, albumins, glutelins, and prolamins) strongly affects their nutritional quality due to the different digestibility (Vasconcelos et al. 2010; Sun-Waterhouse, Zhao, and Waterhouse 2014).

Protein digestibility (PD) expresses the percentage of ingested protein (or AA) that is hydrolyzed by digestive enzymes and absorbed by the digestive system relative to the total protein consumed (López et al. 2018). PD is greatly affected by the protein's chemical structure and can be determined by *in vitro* or *in vivo* methods. *In vitro* protein digestibility (IVPD) methods, as the multi-enzyme methodology developed by Pedersen and Eggum (1983) and Hsu et al. (1977), make use of specific enzymes (trypsin, α-chymotrypsin, and peptidase) to measure maximum hydrolysis rates that apply to a wide range of proteins with good correlations with *in vivo* PD. Although these methods do not consider biologically unavailable AAs, they are still useful for assessing protein nutritional quality (Aguilar et al. 2015). Notwithstanding, the digestibility of plant proteins is also affected by the presence of ANFs, which are unfavorable for protein digestion (Boye, Aksay, et al. 2010; Arribas et al. 2017; Bessada, Barreira, and Oliveira 2019). ANFs of dry beans are considered harmful to the quality and digestibility of plant proteins and therefore need to be inactivated by an appropriate process.

Anti-nutritional factors (ANFs)

ANFs are naturally occurring compounds in plant foods that directly hinder the nutritional value of plant proteins, lowering nutrient absorption or producing toxic effects (Sánchez-Chino et al. 2015). Unlike animal proteins, ANFs are one of the main limiting factors of plant proteins because they can decrease the bioavailability of EAAs, causing irritation and lesions to the gastrointestinal mucosa when ingested at high concentrations (Campos-Vega, Loarca-Piña, and Oomah 2010; Adeleye et al. 2020). In general, the knowledge of the main ANFs from dry beans helps to elucidate action mechanisms, their effects on the quality of plant proteins, and strategies to remove or inactivate them. The classification, definitions, action mechanisms, and physiological responses of bean ANFs are listed in Table 1.

ANFs can be categorized as protein and non-protein kinds (Muzquiz et al. 2012; Luo and Xie 2013; Rahate, Madhumita, and Prabhakar 2021) in which trypsin and chymotrypsin protease inhibitors are the main protein ANFs in pulses. Protein ANFs affect proteins' globular structure and are resistant to pepsin and the stomach pH. The reduced activity of digestive proteases creates insoluble complexes of proteins and minerals that decrease nutrients' bioavailability, solubility, and digestibility (Gilani, Cockell, and Sepehr 2005; Muzquiz et al. 2012; Gupta, Gangoliya, and Singh 2015). Lectins (agglutinins) are mentioned in the literature as

Table 1. Classification, definition, and overall characteristics of anti-nutritional factors of dry beans.

Type (main ANF)	Definition	Action mechanism	Overall physiological responses
Protein ANFs			
Lectins (Phytohemag-glutinin, PHA)	Glycoproteins can form complexes with carbohydrates and proteins, bind cells, or precipitate glycoconjugates	Interferes in the absorption of nutrients	Growth deficits, agglutination of red blood cells, and loss of epithelial resistance. *May cause acute gastroenteritis
Protease inhibitors (Trypsin and chymotrypsin inhibitors)	Proteins that act by inhibiting enzyme activity	Complexation with trypsin and chymotrypsin secreted by the pancreas. Impedes the action of proteolytic enzymes, reducing protein digestibility	Metabolic changes in the pancreas (increased enzyme secretion). Retention of nutrients (protein and sulfur-containing AA)
Non-protein ANFs			
Alkaloids	Natural amines with nitrogen-containing heterocyclic rings	Antipalatable effect (bitter taste). Affect protein digestibility	Central nervous system disturbance and digestive, reproductive, and immunological disorders
Glycosides (Saponins, α-galactosides, vicin, and convicin)	Compounds originated from the condensation of sugar and organic compound molecules	Decrease the digestibility and biological value of protein	Flatulence and intestinal gas production. It May cause diarrhea and abdominal pain. *Hemolytic anemia in individuals with G6PD deficiency
Phenolics (Tannins)	Compounds have an aromatic ring with one or more hydroxyl groups.	Decreases protein digestibility and AA availability. Reduce palatability (astringency). Complex/precipitate proteins from aqueous solutions	Inhibit digestive enzymes, decreasing the utilization of vitamins and minerals. *May cause damage to the mucous membrane of the digestive system or exert systemic toxic effects.
Phytates (Phytic acid)	Compounds derived from phytic acid can form chelators with divalent ions, forming soluble complexes resistant to digestion	Inhibit the action of digestive enzymes. Impair the absorption of nutrients in the small intestine. Reduce the bioavailability of minerals and proteins	Flatulence. Hinders nutrient absorption, *may trigger anemia and slow metabolism

*High dose intake.
ANFs: anti-nutritional factors; AA: amino acid; G6PD: glucose-6-phosphate dehydrogenase.

protein ANFs in dry beans. For example, phytohemagglutinin (PHA) in red beans and the high human consumption of lectins from raw grains or derived flours are related to adverse health effects (Gilani, Cockell, and Sepehr 2005; Jezierny, Mosenthin, and Bauer 2010; Liu et al. 2013; Gupta, Gangoliya, and Singh 2015).

Regarding non-protein ANFs (Table 1), phytates are a representative group present in dry beans cotyledons (0.7–1.5%) (Coelho et al. 2002), known as a potent metal chelator of mineral cations (Parmar et al. 2017). In turn, tannins are polyphenols concentrated in bean hulls, which can precipitate proteins, affecting PD and diminishing the nutritional value of pulse proteins. Moreover, the sensory properties of beans are impaired by the astringent properties of the bitter condensed tannins and the anti-palatable effect of saponins (plant glycosides) and alkaloids (Gilani, Cockell, and Sepehr 2005; Jezierny, Mosenthin, and Bauer 2010). In addition, the glycosides belonging to the amino pyrimidine derivatives, such as vicin and convicin, are generally present in faba beans (*Vicia faba* L.) (Jezierny, Mosenthin, and Bauer 2010). These glycosides can lead to hemolytic anemia (favism), a lethal disease triggered by insufficiency of the glucose-6-phosphate dehydrogenase (G6PD) enzyme that causes damage to the red blood cells (Jamalian and Ghorbani 2005; Muzquiz et al. 2012; Siah et al. 2012; Rahate, Madhumita, and Prabhakar 2021).

Pretreatments for IVPD increase and ANFs reduction

Dry beans have been submitted to many pretreatments to improve their IVPD, such as autoclaving, boiling, cooking, extrusion, and germination, to name a few. Table 2 compares IVPD for raw and processed cowpea, faba, mung, and common (kidney) beans. As expected, there is a significant improvement in the PD of processed beans compared to raw grain (Carvalho et al. 2012). High PD improvements for isolated proteins from cowpea (11.53% IVPD increase) and extruded proteins from common and faba beans (21.88 and 23.45% IVPD increase) can be found, respectively (Alonso, Aguirre, and Marzo 2000; Frota et al. 2017). The PD correction by thermal treatment occurs through the destruction of thermolabile compounds, deactivation of protease inhibitors, and protein denaturation (Luna-Vital et al. 2015). This mechanism may unfold the secondary and tertiary protein structures, thus increasing the superficial area and exposing sites for proteolysis (Bessada, Barreira, and Oliveira 2019).

Concerning ANFs, pulses can be submitted to various physical treatments to make them safer for consumption. The levels of many ANFs of raw and processed beans, as affected by each method and combination described in the literature, are also summarized in Table 2. The applied treatments affected the chemical composition of beans, with pronounced changes in the ANFs and, consequently, improvement in the nutritional quality of beans, markedly the IVPD, when assessed. For example, germination, microwave, and cooking (at ambient or high pressures) treatments showed to reduce contents of tannins, phytic acid, trypsin inhibitors, and lectin/hemagglutinin activity in cowpea, faba, kidney, and mung beans, improve the protein quality and increase IVPD (Mubarak 2005; Khattab, Arntfield, and Nyachoti 2009; Kalpanadevi and Mohan 2013; Luo and Xie 2013). Further, microwave and

Table 2. Influence of different treatments on dry beans' protein digestibility and anti-nutritional factor levels.

Bean type (cultivars)	Main treatments (conditions)	PD (%)	Phytic acid (mg/g dm)	TIA (TIU/mg)[1]	Tannins (mg/g dm)	Lectin or HA	Reference
Common bean (*Phaseolus vulgaris* L.)	Raw	50–67					Rezende et al. (2017)
	Raw		15.7–18.8			87.7–88.6 HU/mg	Shi, Arntfield, and Nickerson (2018)
	Soaking (4 h, 25 °C)		15.5–19.2			86.5–87.3 HU/mg	
	Cooking (95 °C, 1 h)		15.3–18.5			0.2 HU/mg	
	Raw	28.2–28.6	8.2–11.3	4.6			Batista, Prudêncio, and Fernandes (2010)
	Extrusion (150 rpm, 150 °C)	48.5–52.8	6.8–8.4	1.4			
	Raw	33.8–34.0	12.3–14.4		9.8–11		Rehman and Shah (2005)
	Soaked (4 °h, 25 °C) + cooking (100 °C)	63.6–64.1	9.3–11.0		7.4–8.0		
	Soaked (4 h, 25° C) + autoclaving (121 °C, 20 min)	24.2–24.9	7.4–8.8		6.0–6.4		
	Soaked (4 h, 25° C) + autoclaving (128 °C, 20 min)	22.8–24.6	6.4–7.4		5.7–6.3		
	Raw	70.6	11.0	19.5	2.3		Abd El-Hady and Habiba (2003)
	Extrusion (140 °C)	76.8–78.7	10.3–10.9	Nd	2.1–2.2		
	Extrusion (180 °C)	76.4–77.3	9.6–10.1	Nd	1.9–2.1		
	Soaking (30 °C, 16 h) + extrusion (140 °C)	78.0–79.2	10.0–10.4	Nd	1.8–1.9		
	Soaking + extrusion (180 °C)	77.3	9.5–9.8	Nd	1.7–1.8		
	Raw	68.1	15.9	4.5	3.6	49.3 HU/mg	Alonso, Aguirre, and Marzo (2000)
	Soaking (12 h, 30 °C)	71.4	15.0	4.3	2.7	49.3 HU/mg	
	Germination (72 h, 25 °C)	78.0	11.1	3.3	1.0	49.3 HU/mg	
	Extrusion (100 rpm, 0.4 kg/min)	83.0	12.6	0.05	0.6	0.2 HU/mg	
	Raw	70.5–78.0	11.6–11.9	3.1–3.6	26.7–67.1		Khattab, Arntfield, and Nyachoti (2009), Khattab and Arntfield (2009)
	Soaking (20 h)	75.9–83.2	6.1	2.5–3.1	3.7–27.5		
	Boiling (45 min)	87.4–94.1	4.9–5.0	0.0	0.2–21.1		
	Roasting (180 °C, 20 min)	64.9–72.9	7.1–7.4	0.0	5.6–61.3		
	Autoclaving (20 min)	78.9–86.1	3.5–3.8	0.0	1.3–19.5		
	Microwave (1200 W, 20 min)	81.7–88.6	4.4–5.3	0.0	2.2–33.9		
	Fermentation (24 h)	73.4–80.1	36.9–38.4	1.8–2.2	4.2–33.5		
	Raw	65.6–80.7	17.3–24.1	4.6–29.3	5.4–28.8	1.9–9.9 g/kg PHA	Shimelis and Rakshit (2007)
	Soaking (12 h)	68.9–84.7	14.0–19.9	4.2–27.5	4.0–21.9	1.9–9.9 g/kg PHA	
	Germination (24 h)	72.2–88.7	12.5–15.6	4.1–26.0	3.7–19.3	1.8–9.3 g/kg PHA	
	Germination (48 h)	76.1–87.1	2.2–9.1	3.9–24.6	1.4–5.8	1.7–8.5 g/kg PHA	
	Germination (72 h)	70.9–84.0	1.4–5.1	3.9–24.9	1.2–7.2	1.6–8.2 g/kg PHA	
	Germination (96 h)	66.9–81.5	0.7–5.1	3.9–24.6	1.3–7.2	1.6–8.2 g/kg PHA	
	Cooking (97 °C, 35 min) + soaking	73.5–90.3	6.7–8.7	2.5–12.6	1.6–12.1	Nd	
	Autoclaving (121 °C, 30 min) + soaking	74.8–92.8	6.6–8.4	Nd	1.3–12.8	Nd	
	Germination (72 h) + autoclaving	80.1–92.4	Nd	Nd	Nd	Nd	
	Raw	69.1	1.13	18.0			Linsberger-Martin et al. (2013)
	HHP (100 MPa, 60 min, 60 °C)	67.9	1.01	2.9			
	HHP (100 MPa, 30 min, 60 °C)	68.8	1.15	14.0			
	HHP (600 MPa, 60 min, 60 °C)	75.1	1.08	2.9			
	Cooking (100 °C, 80 min) + soaking (24 h)		0.59	Nd			
	Cooking (100 °C, 75 min)		0.88	Nd			
	Extrusion (400–600 rpm, 45–55 kg/h)	55.3–82.2					Nosworthy et al. (2018)
	Baking (165–190 °C, 35 min)	53.2–78.5					
	Cooking (100 °C, 25–35 min) + soaking (16 h)	49.8–82.4					
Cowpea bean (*Vigna unguiculata* and *Vigna sinensis* L.)	Raw	68.7–72.0					Teka et al. (2020)
	Raw	81.6–82.3	8.0–9.8	1.6–2.4	22.6–26.1		Khattab, Arntfield, and Nyachoti (2009), Khattab and Arntfield (2009)
	Soaking (22 h)	86.7–87.4	4.6–5.5	1.3–2.1	5.8–14.7		
	Boiling (35 min)	97.2–98.1	3.5–4.3	0.0	3.3–6.3		
	Roasting (180 °C, 15 min)	76.6–77.6	5.0–6.2	0.0	1.2–15.7		
	Autoclaving (20 min)	89.7–90.3	2.8–3.1	0.0	1.6–8.4		
	Microwave (1200 W, 15 min)	92.2–92.8	2.9–3.8	0.0	1.6–12.9		
	Fermentation (24 h)	84.3–85.1	2.6–3.2	0.9–1.3	5.5–17.6		
	Raw	71.3	3.9	26.5	3.8		Kalpanadevi and Mohan (2013)
	Soaking (12 h)	73.2	2.7	24.3	2.8		
	Germination (30 °C, 96 h)	73.5	0.2	21.6	0.9		
	Soaking (12 h) + cooking (100 °C, 30 min)	78.5	1.3	15.3	0.8		
	Soaking (12 h) + autoclaving (30 min)	80.1	1.1	Nd	0.6		
	Germination (30 °C, 96 h) + autoclaving	84.9	Nd	Nd	Nd		

(*Continued*)

Table 2. Continued.

Bean type (cultivars)	Main treatments (conditions)	PD (%)	Phytic acid (mg/g dm)	TIA (TIU/mg)[1]	Tannins (mg/g dm)	Lectin or HA	Reference
Faba bean (*Vicia faba* L.)	Raw		1.1–12.8	1.2–23.1		0.8–3.2 HU/mg	Mayer Labba, Frøkiær, and Sandberg (2021)
	Raw	75.3	3.9	0.7	3.9		Ruckmangathan et al. (2022)
	Conventional (alkaline medium)	82.1	1.2	0.2	1.4		
	Raw		9.7				Rosa-Sibakov et al. (2018)
	Soaking (2 h)		8.2				
	Phytase (2 U activity, 55 °C,2 h)		4.9				
	Phytase (10 U activity, 55 °C,2 h		1.8				
	Fermentation (24 h)		8.8				
	Raw		19.7			5.5 HU/mg	Shi, Arntfield, and Nickerson (2018)
	Soaking (4 h, 25 °C)		19.4			5.5 HU/mg	
	Cooking (95 °C, 1 h)		15.9			0.1 HU/mg	
	Raw	75.4	6.4	2.3	4.9		Abd El-Hady and Habiba (2003)
	Extrusion (140 °C)	78.2–78.4	6.3–6.9	Nd	4.3–4.4		
	Extrusion (180 °C)	78.4–78.6	6.0–6.2	Nd	3.9–4.3		
	Soaking (30 °C, 16 h) + extrusion (140 °C)	79.6–80.1	5.1–5.5	Nd	3.7–4.3		
	Soaking + extrusion (180 °C)	78.1–78.9	4.8–5.3	Nd	3.6–3.9		
	Raw	51.8–61.2	6.5–7.2		3.1–4.1		Osman et al. (2014)
	Gamma irradiation (0.5–1.0 kGy)	51.2–70.9	4.8–9.0		2.0–2.4		
	Cooking (100 °C) +soaking	70.9–79.9	5.4–6.1		2,7		
	Raw	70.8	21.7	3.1	1.9	74.5 HU/mg	Alonso, Aguirre, and Marzo (2000)
	Soaking (12 h, 30 °C)	71.3	14.6	2.9	1.0	74.5 HU/mg	
	Germination (72 h, 25 °C)	78.1	8.5	2.2	0.8	74.5 HU/mg	
	Extrusion (100 rpm, 0.4 kg/min)	87.4	15.9	0.4	0.9	0.2 HU/mg	
	Raw	72.6–73.3	8.4–8.6	2.5–2.9 mg/g	5.6–6.4	28–56	Luo and Xie (2013)
	Soaking (12 h)	73.6–73.9	8.5–8.7	2.7–3.4 mg/g	5.5–6.4	28–56 U	
	Soaking (48 h)	74.3–74.5	8.9–9.2	2.7–3.2 mg/g	6.1–7.2	28–56 U	
	Microwave (6 min)	75.8–74.5	9.0	1.2–1.7 mg/g	4.6–6.4	7.0 U	
	Microwave + soaking	75.3–82.3	6.7–9.1	1.9–2.2 mg/g	2.8–4.3	7.0 U	
	Cooking (100 °C, 30 min) + soaking	79.8–85.1	6.1–9.2	1.2–2.0 mg/g	2.6–4.2	0.0–4.0 U	
	Autoclaving (121 °C, 20 min)	76.3–78.6	8.9–9.3	0.5–1.2 mg/g	4.7–6.5	0.0–4.0 U	
	Autoclaving + soaking	79.6–85.3	5.1–9.2	0.7–1.9 mg/g	2.2–4.0	0.0 U	
Mung bean (*Vigna radiate* L. and *Phaseolus aureus*)	Raw	78.9	6.3	16.5	3.5	2.6 HU/mg	El-Adawy et al. (2003)
	Germination (72 h)	81.3	5.3	12.9	2.8	0.4 HU/mg	
	Germination (120 h)	81.8	4.9	13.5	3.2	0.4 HU/mg	
	Raw	80.2	5.8	15.8	3.3	2.7 HU/mg	Mubarak (2005)
	Soaking (12 h)	87.4	4.3	13.3	2.0	1.4 HU/mg	
	Germination (72 h) + soaking	89.1	4.0	12.3	1.9	0.6 HU/mg	
	Boiling (100 °C, 90 min)	87.8	4.2	0.0	1.8	0.0	
	Autoclaving (121 °C, 35 min)	88.7	4.4	0.0	1.6	0.0	
	Microwave (2450 MHz, 15 min)	88.2	4.3	0.0	1.2	0.0	
	Raw	37.9	1.3	15.5 TIU/mg	2.4		Prachansuwan et al. (2020)
	Soaking (7 h)	48.7	0.7	Nd	0.9		

Note: PD: protein digestibility; TIA: trypsin inhibitor activity; TIU: trypsin inhibitor units; HA: hemagglutinating activity; PHA: phytohemagglutinin; HU: one unit of HA; U: units; HHP: high hydrostatic pressure; dm: dry matter; Nd: not detectable.

high-pressure cooking methods completely inactivated the trypsin inhibitor activity and hemagglutinin (Mubarak 2005; Khattab, Arntfield, and Nyachoti 2009). Likely, high-temperature-pressure cooking (HTPC) and extrusion reduced ANFs quickly upon limited exposure to high temperatures (Abd El-Hady and Habiba 2003; Boye, Aksay, et al. 2010) and thus prevented detriment in the biological activity of bean proteins, in addition to making pulses consumption safer for health.

Biological activity and health benefits of bioactive peptides from beans

Protein from beans is a known source of nutrients and bioactive compounds for the diet, specifically bioactive peptides – sequences from 2 to 20 AAs that offer nutritional and physiological benefits when consumed (Chalamaiah et al. 2019). These AAs sequences are inactive as part of the protein molecule; however, they are released during protein hydrolysis in the digestive tract (Korhonen and Pihlanto 2006; Hou et al. 2017) and become bioactive. Some pulse proteins exhibit bioactivity directly upon consuming them as part of whole grains. In contrast, others must be hydrolyzed by digestive, fermentative, or hydrolytic processes (by specific proteases) to release bioactive peptides (Rui et al. 2012; Garcia-Mora, Peñas, et al. 2015).

The antioxidant, anticancer, and antihypertensive potentials are commonplace for the bioactivity of protein hydrolysates (Luna-Vital et al. 2015) that can be explored when aiming for functional food and protein-rich applications. The uptake of dry beans has been related to a significant reduction of the factors linked to metabolic syndrome and cardiovascular diseases (Ma et al. 2005; Zhu, Jiang, and Thompson 2012; Luna-Vital et al. 2015; Hou et al. 2019) and largely attributed to prevention of chronic and degenerative diseases (Hayat et al. 2014). The association of proteins and peptides with reducing metabolic disorders is conditioned to inhibiting key enzymes related to the biological process. After digestion, released bioactive peptides are

capable of inhibiting the angiotensin-converting enzyme (ACE) involved with hypertension (ACE inhibitory activity) (Awika and Duodu 2017; Yi-Shen, Shuai, and FitzGerald 2018; Hou et al. 2019) and the catalytic activity of enzymes related to cholesterol solubilization into micelles, revealing another beneficial aspect for the prevent cardiovascular diseases (Ashraf et al. 2020).

Concerning the anticancer activity of beans bioactives, the antiproliferative action on colorectal tumoral cells of bioactives from dry beans (Xu, Yuan, and Chang 2007; Campos-Vega et al. 2013; Luo and Xie 2013; Hayat et al. 2014; Frota et al. 2015; Luna-Vital et al. 2015) and protease inhibitors on reduce pre-stomach tumors and hepatocellular carcinoma (Fei Fang et al. 2011) are being investigated. The antitumor responses of bioactives from faba bean occur *via* selective inhibition of factors involved in the invasive growth of cancer cells (i.e., chymotrypsin or trypsin inhibitors). Besides, faba bean has been used to treat Parkinson's disease due to the high content of the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) (Rabey et al. 1992; Hornykiewicz 2002; Fei Fang et al. 2011; Rahate, Madhumita, and Prabhakar 2021).

The bioactivity of peptides can undergo alterations due to structural modifications affecting the bioavailability of AAs and peptides. Thermal treatments commonly used to process plant proteins affect protein stability by interfering with the concentration and nature of the peptides released from hydrolysis (Ashraf et al. 2020) since heat favors protein denaturation during enzymatic hydrolysis and, consequently, facilitates the release of bioactive peptides (Stanciuc et al. 2010; Ashraf et al. 2020; Zhang and Romero 2020) such as ACE-inhibitor peptides of common beans (Rui et al. 2012). In addition, for a beneficial health effect, bioactive peptides need to reach target sites crossing the gut barrier and resist the action of digestive enzymes, which will depend on the protein structure. Further studies need to compile applications of bean protein hydrolysates *in vivo* and the physiological responses related to health maintenance. The beneficial health effect of bean protein will also depend on other factors associated with suppressing the allergenic reaction of the immunological system.

Allergenicity of bean proteins

Allergen response and allergens of dry beans

Food allergy results from a specific immune response caused by exposure to a determined food component. They occur primarily through allergen-specific immunoglobulin-E (IgE)-mediated type I anaphylactic reactions, including systemic, gastrointestinal, and mucosal immune system symptoms (He et al. 2021). An increasing prevalence of food allergies has been reported worldwide in the last decade (Sicherer 2011; Bessada, Barreira, and Oliveira 2019), mainly related to soybean and peanut as main plant allergens. On the other hand, proteins from pulses such as beans, peas, lentils, and chickpeas do not account for priority allergens, reinforcing pulses as safe alternative sources of protein isolates and concentrates.

Although anaphylaxis is a very uncommon reaction to pulses consumption, allergic responses arising from IgE-mediated hypersensibility reactions were identified. The usual symptoms include gastrointestinal (nausea, vomiting, diarrhea, and abdominal distention and bloating), respiratory (rhinitis), and cutaneous (urticaria and angioedema) alterations (San Ireneo et al. 2000; Popp et al. 2020; Sackesen et al. 2020; He et al. 2021). Pulses present structurally homologous proteins; they can share common allergen epitopes (San Ireneo et al. 2000; Lu et al. 2020), which are the antigen subunits in proteins recognized by IgE antibodies. Therefore, pulses allergenicity should not be disregarded (Verma et al. 2013; Bessada, Barreira, and Oliveira 2019).

A way of determining the allergic potential of a protein is based on its stability in processing (thermal resistance) and digestion (protease- and acid-resistance) (Cochrane et al. 2009). The major plant protein allergens produce pepsin-resistant fragments that trigger the allergenic response in the organism (Verma et al. 2013). For example, the beans lectins are the main proteins that induce potentially allergic reactions (Kumar et al. 2013; He et al. 2021). The allergenic mechanism triggered by beans lectins is complex and include mixed allergic reactions, IgE-mediated or not (Wang et al. 2021). He, Sun, et al. (2018) highlighted that lectins from black beans showed high homology with erythroglutinating phytohemagglutinin (PHA-E), increasing its allergenic potential as a hemagglutinating agent.

Processes to reduce the allergenicity of bean proteins

Many thermal and chemical (e.g., glycosylation, PEGylation, and pH adjustment) processes were investigated as tools to minimize the allergenicity of beans. These methods promote the structural alterations in proteins and conformational epitopes that modify the sensitivity to food allergens. Kasera et al. (2012) combined boiling and gamma irradiation treatments to significantly reduce the allergen potential of soluble and insoluble proteins from kidney beans, black gram, and peanuts, verified by the diminished IgE binding to protein compared to raw grain antigens. He, Sun, et al. (2018) and Yang et al. (2018) confirmed the reduced allergenicity of protein isolates from black beans by the PEGlyation method showing lower hemagglutination activity, while the unfolding of protein in low pH reduced the immunogenicity of lectin (Zhao et al. 2019; He et al. 2021). Other non-thermal approaches (i.e., high-pressure, ultrasound, and enzyme technology) revealed up-and-coming results reducing antigenic-related properties of various plant protein sources, as presented in Table 3.

The allergenic response of protein ingestion depends on the bonding between antibodies and protein antigens. The applied pretreatment and processing methods aim for the irreversible removal of allergens from plant proteins, making them unrecognizable to the immunological system. High hydrostatic pressure implies conformational changes to the native protein that affect epitopes, making them inaccessible to IgE (Li et al. 2012, 2018). The sonication by ultrasound treatment provides physical disintegration with disruption or

Table 3. Usage of emerging technologies for allergenicity reduction and protein recovery from many plant sources.

Source	Process (conditions)	Main observations	Reference
Protein allergenicity factors			
Mung bean protein hydrolysate (MBPH)	EH (*Alc*: 50 °C, E/S 100:1; *Pan*: 37 °C, E/S 100:1; *Fla* + *Pan* (1:1): 50 °C, E/S 1:1)	MBPH with minimal toxicity (< 30%) and high antiallergic activity: *Fla* + *Pan* (135.6%), *Alc* (121.7%)> *Pan* (80.3%) of β-hexosaminidase inhibition	Budseekoad et al. (2019)
Peanut protein (PNP)	HHP + TT (600 MPa, 55–75 °C, 10 min)	IgE inhibition: decrease/elimination of NP allergen immunoreactivity	Long et al. (2016)
	US + SET (50 Hz, 1 h, E/S 0.25%, 1–4 h)	SET US-trypsin-α-chymotrypsin reduced the immunoreactivity of the peanut allergens, Ara h 1 (99.9%) and Ara h 2 (98.2%), with decreased IgE binding of PNP. The combination of treatments cleaves peptide bonds: peptides with lower allergenicity	Li et al. (2013)
Soy protein isolate (SPI)	HHP (300 MPa, 15 min)	Change in allergenic epitope conformation: reduced SPI allergenicity (45.5–48.6%)	Li et al. 2012, (2018)
	EH + HPP (50 °C, 15 min, >300 MPa, *Fla*, pH 8.0, E/S 0.5%)	HPP: the unfolding of proteins and epitopes more susceptible to proteases. HPP (>300 MPa) + EH increased the proteolytic activity of *Fla*, reducing immunoreactivity of the main SPI allergen (Gly m5)	Meinlschmidt et al. (2017)
Soybean sprout protein (SSP)	US (300 W, 40 kHz, 30 min)	IgE binding capacity (51.4%) and reduced (98.8%) trypsin inhibitor content. US altered the major soybean allergens during germination: peptides and AA from SSP with low allergenicity	Yang et al. (2015)
Walnut protein (WAP)	HHP/TT (650 MPa, 100 °C, 15 min)	Reduced nut allergen immunoreactivity: WAP IgE binding reduced up to 86.4%	Yang et al. (2017)
Protein recovery			
Pomegranate seeds waste	PLE (alkaline buffer, 170 °C, 12 min); HIFU (alkaline buffer + 1.5% SDS, 20% wave amplitude, 5 min) and *(HIFU + NADES + PLE)	PLE recovered more protein (15.3%) with higher TPC (14.2 mg GAE/g) than HIFU (10.2% and 3.1 mg GAE/g, respectively). Higher antioxidant and hypocholesterolemic activities in PLE due to synergism between phenolics and proteins/peptides. Using NADES in sequential extraction*, a recovery of 23.8% protein was obtained	Guzmán-Lorite, Marina, and García 2022a, (2022b)
Ora-pro-Nobis leaves	SWE (10.5 MPa, 185 °C, 15 min)	Increased yield and protein recovery (3 times more) than the conventional extraction (30 C, 45 min)	Torres et al. (2022)
Brewer's spent grain (BSG)	PLE (50% ethanol, 155 °C, 17 min)	Higher protein and TPC content (50% ethanol, 155 °C, 17 min) than UAE-alkaline (15 min, 60 °C). BSG hydrolysates with greater *in vitro* antihypertensive capacity and antioxidant activity	González-García, Marina, and García (2021)
Rice bran	SWE (220 °C, 30 min)	Higher protein, AA, and antioxidant content (200 °C, 30 min). Thermal degradation with increasing temperature (220 °C)	Sereewatthanawut et al. (2008)
Peanut	MAE (725 W, 8 min) and UAE (25 kHz, 15 min)	MAE: increased yield (77%) and protein purity (~100%). Improved WAI, FS, EA, and IVPD; UAE: increased yield (136%) and protein purity (86%)	Ochoa-Rivas et al. (2017)
Pomegranate peel	PLE (103 bar, 70% ethanol, 120 °C, 12 min) and HIFU (ChCl: AA DES, 11 min, 60% amplitude)	High antioxidant and hypocholesterolemic capacities are mainly associated with PLE-coextracted polyphenols. High antihypertensive capacity using DES-HIFU	Hernández-Corroto et al. (2020)
Rice bran	MAE (1000 W, 90 s, 0,89 g/10 mL)	Increased yield (1.54 times) with greater protein extraction capacity (33.18%) in a shorter period	Phongthai, Lim, and Rawdkuen (2016)
Soybean	MAE (60,1 °C, 12,6 mL/g, 30 min)	Higher protein yield until reaching the optimum point. Decreased yield with further increased temperature and solid–liquid ratio	Choi et al. (2006)
Soybean meal	SWE (120 °C, 20 min) + EAE (protease)	SWE + EAE: Higher protein quality with a large amount of hydrophobic AA. Protein purity decreased with prolonged hydrolysis	Lu et al. (2016)

ANFs: anti-nutritional factors; EA: emulsifying activity; FS: foam stability; IgE: immunoglobulin-E; IVPD: *in vitro* protein digestibility; Gly m5: β-conglycinin; TPC: total phenolic compounds; WAI: water absorption index; Treatments and extraction methods: enzyme hydrolysis (EH); enzymes: alcalase (*Alc*), Flavourzyme© (*Fla*) and pancreatin (*Pan*); high hydrostatic pressure (HHP); sequential enzyme treatment (SET); high-pressure processing (HPP); thermal treatment (TT); ultrasound (US); enzymatic assisted extraction (EAE); microwave-assisted extraction (MAE); pressurized liquid extraction (PLE); high intensity focused ultrasounds (HIFU); Choline chloride: Acetic acid (ChCl: AA); natural deep eutectic solvents (NADES); deep eutectic solvents (DES); subcritical water extraction (SWE); ultrasound-assisted extraction (UAE).

elimination of epitopes (Li et al. 2013; Yang et al. 2015). Enzymatic cleavage can inactivate the potential binding site for the antibodies, eliminating the allergenic factor (Wu et al. 2016). Furthermore, studies have shown that biological processes such as fermentation and enzymatic hydrolysis promise an approach to reducing allergenicity in plant matrices such as soybeans, peanuts, and wheat (X. Dong, Wang, and Raghavan 2021; Pi, Fu et al. 2021; Pi, Sun, et al. 2021; Rui et al. 2019; H. Yang et al. 2020).

Fermentation can remove allergens through proteolysis and acid-mediated denaturation, glycosylation, Maillard reactions, or solvent dissolution (Pi et al., 2019), significantly reducing allergens and immunoreactivity. Hydrolysis during fermentation modifies or destroys linear and conformational epitopes (Rahaman, Vasiljevic, and Ramchandran 2016; Dong, Wang, and Raghavan 2021). Specific fermentation techniques, such as long-time fermentation (X. Dong, Wang, and Raghavan 2021), co-culture fermentation (Fu et al. 2021), and fermentation with specific strains like *Lactobacillus plantarum* (Rui et al. 2019) or *Bacillus natto* (Pi, Fu, et al. 2021) have shown promising results in reducing allergenicity of food proteins. The structural modifications in proteins, characterized by a decrease in α-helices and conformational changes, significantly reduce allergenic potential. Overall, a combination of physical and biological processes can contribute to a complete proteolysis and allergenicity reduction of plant proteins to a safe level (Meinlschmidt et al. 2017). However, the fermentation processes' potential in deactivating dry bean allergens has not been explored.

The effects of processing methods on the allergenicity of these "new proteins," in the case of dry beans, must be continuously investigated. Clinical trials should help to unveil the main mechanisms responsible for allergenic responses in protein-sensitive groups. The suitability of processing methods to the molecular properties of plant proteins can minimize the food allergen elicitation threshold and, hence, incorporate pulse proteins into new hypoallergenic foods to ensure food safety.

Technologies for recovering and functionalization of dry bean proteins

To recover protein from beans, wet (solvent) and dry (no solvent) methods can be applied, and many (conventional or emerging) technologies can be adapted to obtain, purify and concentrate protein into a food-grade quality product (Pojić, Mišan, and Tiwari 2018). Based on the protein content of the final product, protein flour (up to 65% protein content), concentrates (65–90%), and isolates (more than 90%) can be obtained (Oreopoulou and Tzia 2007; Gençdağ, Görgüç, and Yılmaz 2021) regardless of the extraction technique used.

To obtain protein isolates or concentrates, the matrix's non-protein components (e.g., starch, fiber, minerals, and sugar) must be separated from the protein-rich fraction. Protein recovery will therefore consist of three stages: (i) protein extraction: using saline, alkali, or acid medium at pH far from the isoelectric point; (ii) protein precipitation: isoelectric precipitation of protein by adjusting the pH (Boye, Aksay, et al. 2010) (pH ~4.5: isoelectric point of bean protein) when acid or alkaline extraction approaches are used, followed by separation of the protein-rich fraction by centrifugation. For saline extraction, the dissociation of the protein supernatant from salt ions may be performed by ultrafiltration or diafiltration membranes (Hadnađev et al. 2018); and (iii): drying of the precipitated protein for solvent removal.

Appropriate protein extraction from pulses is conditioned to many operational factors (temperature, pH, solvent, solvent-to-feed ratio, particle size), as well as protein properties, such as molecular weight, isoelectric point, and solubility (Nadar, Rao, and Rathod 2018; Pojić, Mišan, and Tiwari 2018). Furthermore, the extraction method should be able to release cellular protein by cell wall rupture for a complete recovery. As previously discussed, conventional and emerging technologies under optimized conditions can overcome limitations concerning ANFs (Table 2) or allergens (Table 3), obtaining structurally modified proteins and high-quality products that can implement foodstuffs with nutritional, functional, and biological benefits.

Conventional methods for protein recovery

Bean proteins are conventionally obtained by methods involving several steps, namely the heating-stirring extraction (HSE) with pH adjustment of the medium. The long-used process, patented by Anson and Pader (1957), represents a simple approach to protein extraction. It entails solubilizing the ground material in an alkaline medium (acid or saline medium can be employed) under stirring for approximately one hour. However, these conventional methods often result in lower protein extraction yields due to protein degradation. Various factors, including extraction time, temperature, and solvent pH, can influence the protein yield (M. Kumar et al. 2021).

Using acid solvents may imply inefficient cell wall degradation and permeation of the cellulose interstices and impairs protein diffusion to the extracting medium (Pojić, Mišan, and Tiwari 2018). Alkaline solvents, in turn, are commonly used because plant proteins exhibit better solubility at high pH. However, alkaline treatment can lead to undesirable reactions, such as protein degradation, which negatively impacts digestibility and results in amino acid loss (Xia et al. 2012). Balancing alkali strength and protein extraction efficiency might mitigate conventional methodologies' drawbacks (Kumar et al. 2021).

Temperature also plays a key role in extraction, enhancing mass transfer (Gençdağ, Görgüç, and Yılmaz 2021) and stabilizing protein structure during alkaline extraction (Kumar et al. 2021). However, high extraction temperatures can potentially damage heat-labile proteins and cause chemical degradation. Furthermore, conventional methods have limitations such as excessive water, energy, and chemical, making them less efficient. Therefore, alternative emerging extraction methods are continually sought to overcome these disadvantages. Table 4 summarizes the advantages and limitations of conventional and emerging technologies for obtaining plant protein.

Table 4. Comparison between the conventional method and emerging technologies for protein extraction.

Conventional protein extraction by heating and stirring (HSE)				
General remark		Advantages	Disadvantages	Main parameters
Applying heat and mechanical agitation helps break down the cell structure, facilitating the release of proteins into the aqueous solution		Simplicity, it does not require complex equipment.	Protein degradation by prolonged exposure to heat and stirring results in losing its functional properties. High solvent (acid, alkaline, salt), water, and energy consumption	Temperature and stirring intensity)
Eco-innovative emerging technologies to assist protein extraction *General remark:* A physical disturbance is applied to the plant matrix or protein fraction favoring protein release or its modification *Advantages:* Reduced use of solvents, "greener" and more sustainable processes, fewer by-products, milder conditions, and higher purity *Limitations:* Limited applicability due to lack of scale-up and economic viability studies, moderate energy input, and operational costs				
Method	General remark	Advantages	Limitations	Main parameters
Ultrasound	Acoustic cavitation of the solvent by US waves disrupts the plant matrix	Less temperature gradient, fast, easy, and low-cost method	High power/intensity can denature and alter protein structure without losing functionality	Frequency, power/intensity, and equipment setup
Microwave	Electromagnetic radiation energy causes the rapid heating of the material	Convenience, last energy propagation, less solvent use, extraction time, and by-products	Risk of prolonged heating, low penetration of MW, and need for optimization for energy efficiency	Time, temperature, power, and dielectric properties of the plant material
Subcritical fluids	A pressurized fluid with enhanced solvent power flows through the plant matrix	Extraction is expedited more efficiently than processes at atmospheric pressure, tunability of solvent power, and use of green solvents	Higher instrumentation and implementation costs need further processing steps to eliminate solvent and extract collection	Pressure, temperature, solvent flow, extraction time
High-hydrostatic pressure	The material is subjected to high pressures (>100 MPa) at hydrostatic or micro fluidization modes	Non-thermal and less energy-demanding process, advantageous protein structural changes, and reduced allergens	Irreversible denaturation and aggregation affect the techno-functional properties of proteins, which have a high cost to operate at an industrial scale	Pressure, process time
Enzyme processes	Use of diverse enzymes to disrupt and modify different substrates in the vegetal matrix, including protein	Products obtained are of high nutritional quality and adequate for human consumption	Long extraction times, high-cost enzymes, and difficulty in controlling the bioprocess	Enzyme type, pH, temperature, incubation time, solvent: substrate ratio
Dry fractionation methods	Separation of protein fractions from milled grain by density (air classification) or electric charge (electrostatic separation) differences	Higher energy efficiency and less environmental impact than wet (solvent) methods	Less purity of protein fractions, low ANF reduction, and risk of particle agglomeration	Particles size and homogeneity, the density of charges

Emerging eco-friendly technologies: wet processes

The most common emerging extraction methods of protein from diverse types of alternative plant sources in the literature include microwave- (MW), ultrasound- (US), enzymatic processes (EP), high-hydrostatic pressure (HHP), subcritical fluid (SF), in addition to dry fractionation based techniques (air classification and electrostatic separation) as illustrated in Figure 2.

Overall these tools apply a physical disturbance to the plant matrix under mild process conditions, allowing higher protein yields in lower extraction times, with improvement in the digestibility and nutritional quality of proteins (Pojić, Mišan, and Tiwari 2018; Gençdağ, Görgüç, and Yılmaz 2021). In addition, there are only a few reports on utilizing these emerging technologies for processing and improving the quality of dry bean proteins, as listed in Table 5.

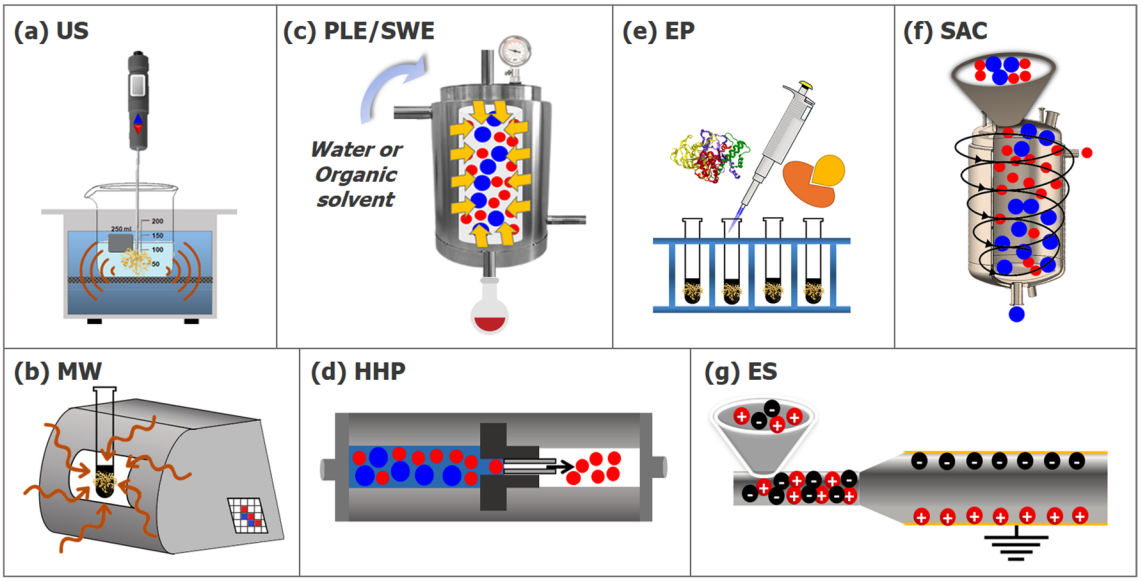

Figure 2. Schematic representation of the main emerging technologies applied to recover or functionalize plant proteins. (a) Ultrasound; (b) Microwave; (c) Pressurized liquid extraction and Subcritical water extraction; (d) High-hydrostatic pressure; (e) Enzyme processes; (f) Separation by air classification; (g) Electrostatic separation.

Therefore, the technologies mentioned above and their main effects on pulse and bean proteins will be addressed to suggest suitable alternatives for recovering these plant proteins.

Ultrasound (US)

The ultrasound-assisted extraction (UAE) or treatment by ultrasound (US) is based on the acoustic cavitation phenomena produced by sound waves in the 20 kHz to 1 GHz frequency range (Cheeke 2002). Cavitation creates high temperature and pressure points in the solvent, implying the formation of gas bubbles that collapse. The rapid implosion of the bubbles results in many physical transformations and stress into the matrix (microchannels, microjets, shock waves, turbulence, and intense shear force) with enough energy to disrupt the vegetal cell wall, thus facilitating solvent percolation in the matrix and increasing mass transfer (Tiwari 2015; Ochoa-Rivas et al. 2017). Therefore, US treatment efficiency depends on parameters such as the frequency, amplitude, ultrasonic power or intensity, and equipment (ultrasonic bath or probe, Figure 2a). Nevertheless, the inherent parameters of the extraction media (solvent) are also fundamental for a high protein recovery (Chemat et al. 2017).

Table 3 lists some research on the application of US/UAE to recover and reduce allergenic factors of various types of plant protein. Moreover, Table 5 depicts several works employing US technology to modulate the structure and improve the techno-functional properties of bean protein. The great interest in UAE applied to plant protein extraction is because it provides high yield and purity, with less thermal gradient than the traditional methods, and can alter protein structure without excessive heat treatments. It is a fast, easy-to-handle, and more energy-efficient eco-friendly method that allows a more selective extraction with lower solvent consumption and extraction times. It requires a low capital expenditure for installation and maintenance compared to other emerging technologies (Tiwari 2015; O'Sullivan et al. 2017; Rahman and Lamsal 2021).

Several studies have demonstrated the effectiveness of ultrasound-assisted extraction (UAE) when combined with different technologies or used as a pretreatment in protein extraction (Tiwari 2015; Chemat et al. 2017; Gençdağ, Görgüç, and Yılmaz 2021; Rahman and Lamsal 2021). Chemat et al. (2017) reviewed the synergistic effects of UAE with emerging extraction methods such as microwave, enzymes, and supercritical fluid, resulting in increased extraction efficiency and high-quality extracts. Furthermore, UAE significantly enhances extraction efficiency, leading to higher protein yields and improved physicochemical, functional, and nutritional properties of plant proteins compared to conventional methods (Rahman and Lamsal 2021). In a more recent study, Gençdağ, Görgüç, and Yılmaz (2021) discussed how ultrasound pretreatment improves the recovery of plant proteins by breaking down cellular structures, facilitating protein release, and increasing the solubility, thus leading to higher yields. The literature supports ultrasound technology in various applications, including protein extraction and structural and functional modification, offering opportunities for innovation and sustainable resource utilization.

The US can also affect hydrogen bonds and electrostatic interparticle repulsions of proteins, enhancing the recovery efficiency in a reduced time with less formation of unstable protein aggregates. Dong et al. (2011) reported that the UAE method resulted in higher efficiency in recovering proteins from rapeseed than the traditional alkaline extraction method. The diffusion of intense shear forces can fragment proteins into smaller ones, resulting in considerable changes in their molecular structure and functionality without changing their primary structure (Jiang et al. 2014; Hu et al. 2015;

Table 5. Emerging technologies used for extraction and functionalizing bean proteins.

Bean type	Process conditions	Main results	References
Ultrasound (US)			
Black beans	20 kHz, (0, 150, 300, 450 W), 12 and 24 min	Increased free–SH group content and improved EC, FC, WHC, solubility, and gel strength (300 W). Formation of large aggregates (450 W): impaired functional properties of BBPI	Li et al. (2020)
	37 kHz, 320 W, 20 min, pH 9.0	Improved WHC, EC, and increased protein yield (61.7%)	Quintero-Quiroz et al. (2022)
	20 kHz, (0, 150, 300, 450 W), 12 and 24 min	Increased H_0 and solubility of BBPI dispersions. Increased power: formation of macromolecule aggregates, decreased H_0, and solubility proteins	Jiang et al. (2014)
Bean (var. Ganxet)	40 kHz, 250 W, 30 and 60 min, NaOH 0.4 M	High protein recovery (78.7%), extraction yield (37.9%), and percentage of solubilized material (54.6%). Improved functional properties (WHC, OHC, FC, EC)	Lafarga et al. (2018)
Faba bean	20 kHz, 1200 W, 10 and 20 min	Increased H_0, FC, and solubility. Greater stability, smaller particle size, and reduced free–SH content	Alavi, Chen, and Emam-Djomeh (2021)
	20 kHz, 750 W, 17 min	Improved foamability and solubility, however, decreased protein digestibility	Martínez-Velasco et al. (2018)
Red bean	20 kHz, 750 W, 2.5 and 4.5 W/cm^3, 5 min	Change in native protein structure with increased extraction yield (16.4%) and free–SH content: partial protein unfolding	Byanju et al. (2020)
Red bean-lutein complex	20 kHz, 120, 240, 360 W, 10 and 20 min	Increased EAI and ESI with reduced particle size, higher surface charge, and H_0, in addition to highest antioxidant activity (240 W, 10 min): the US-induced unfolding of protein molecules. Increase US power (360 W) and/or time (20 min): aggregate formation and reduced antioxidant activity	Zhao et al. (2022)
Mung bean	20 kHz, (114, 222, 330, 438, 546 W), 20 min	US promoted higher antioxidant activity in mung bean protein hydrolysate (maximum value at 546 W) with increased aromatic AA content and exposure to hydrophobic groups	Liu et al. (2022)
Microwave (MW)			
Common bean	2450 MHz, 15 and 20 min	TIA inactivation: 97% (15 min); 100% (20 min)	Jourdan, Noreña, and Brandelli (2007)
	2450 MHz, 3 min, 800 W	TA, PA, and TIA reduced: increased IVPD (85.7%). However, there was a decrease in protein solubility. Prolonged heating may have caused Maillard's reaction	Shimelis and Rakshit (2005)
Cowpea bean	1200 W, 15 min	Decreased TA, PA, and TIA content. Increased EAA and SAA content. Improved protein quality and increased IVPD (92.2–98.8%)	Khattab, Arntfield, and Nyachoti (2009), Khattab and Arntfield (2009)
Faba bean	900 W, 1000 J/g	Decreased TIA (70–75%) and IVPD (76.1–78.2%) increased. Decreased protein solubility with increasing energy (2000 J/g)	Pysz et al. (2012)
Mung bean	2450 MHz, 15 min	Reduction of TA (62.1%) and PA (26%) content. Inactivation of TIA and HE. Improved nutritional quality, IVPD (88.2%), and decreased cooking time	Mubarak (2005)
Enzyme processes (EP)			
Carioca bean	Bromelain (pH 7.0, 55 °C)	Lower molecular weight protein fractions with higher free AA content. Improved functional properties (foaming, emulsion properties, and stability with increased solubility at basic pH)	Los et al. (2020)
Navy, black, and small red bean	Alcalase/Flavourzyme© (AF, pH 8.0, 50 °C) or Alcalase/papain (AP, pH 6.5, 60 °C)	Increase in the DH with thermal treatment, except for black beans (AF). Hydrolysates with higher ACE inhibitory activity (AP) and resistance after gastrointestinal hydrolysis with preserved bioactivity	Rui et al. (2012)

(*Continued*)

Table 5. Continued.

Bean type	Process conditions	Main results	References
Pinto bean	Protamex© (pH 6.5–7.5, 1 h, 50 °C)	Obtaining peptide fractions (<3 kDa) with bioactive properties: high antioxidant activity (pH 7.5) and α-amylase inhibition (pH 6.5)	Ngoh and Gan (2016)
	Alcalase and Savinase (pH 8.0, 50 °C)	High proteolytic efficiency, the concentration of small peptides (<3 kDa) with antioxidant and antihypertensive activity (Alcalase, 120 min). High total phenolic content and ACE inhibition (Savinase, 90 min)	Garcia-Mora, Peñas, et al. (2015)
Pinto Durango and Negro beans	Alcalase (pH 8.0, 50 °C) or bromelain (pH 7.0, 45 °C)	Antioxidant properties and inhibited diabetes-related enzymes: fractions <1 kDa with greater inhibition of α-amylase (Pinto Durango-bromelain), α-glucosidase and DPP-IV (Pinto Durango-Alcalase)	Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano (2015)
High-pressure processing (HPP)			
Red kidney bean	200, 400, and 600 MPa, 15 min	Increased WPC, EAI, ESI (400 MPa), and FC reduced (>200 MPa). High potential of KBPI as a functional and nutritional ingredient in foods	Ahmed et al. (2018)
	200, 400, and 600 MPa, 20 min	Improved EAI, ESI, and KBPI solubility (400 MPa). Decrease in trypsin digestibility (>200 MPa, 120 min)	Yin et al. (2008)
Red kidney bean - PHA	50, 150, 250, 350, and 450 MPa	Increased yield of soluble proteins and reduced PHA hemagglutination activity (250–450 MPa)	Liu et al. (2013)
Pinto bean	150, 300, 450, and 600 MPa for 5, 10, and 15 min	Increased EAI (<450 MPa, 15 min) and ESI. Decreased protein solubility and WHC (>300 MPa, >5 min).	Lin and Fernández-Fraguas (2020)
White bean	100 and 600 MPa, 30 and 60 min	Decreased oligosaccharides, phytic acid content, and trypsin inhibitor. Increased IVPD (600 MPa, 60 min)	Linsberger-Martin et al. (2013)
Kidney bean	300–600 MPa, 15 min + Alcalase (0.5–1.0% E/S)	Increased Alcalase activity, % DH, WHC, FC, EAI, and ESI (300 MPa). Higher antioxidant potential of KBPI (300 MPa, 1% Alcalase)	Al-Ruwaih et al. (2019)

ACE: angiotensin I-converting enzyme; AA: amino acid; DH: degree of hydrolysis; DPP-IV: dipeptidyl peptidase IV. BBPI: black bean protein isolate; US: ultrasound; WHC: water holding capacity; OHC: oil holding capacity; FC: foaming capacity; EC: emulsifying capacity; H_0: surface hydrophobicity; –SH: sulfhydryl. KBPI: kidney bean protein isolate; WHC: water holding capacity; EAI: emulsifying activity index; ESI: emulsifying stability index; FC: foaming capacity; PHA: phytohemagglutinin; IVPD: *in vitro* protein digestibility; % DH: degree of hydrolysis, TIA: trypsin inhibitor activity, TA: tannins.

Quintero-Quiroz et al. 2022). ; According to Jiang et al. (2014) and Li et al. (2020), the application of US (300 W) reduced the dispersions of black bean protein isolate (BBPI) into smaller aggregates with higher solubility, due to an increase in surface hydrophobicity and enhanced water-protein interaction by cavitation forces. UAE (320 W) also caused the unfolding of the secondary protein structure of black beans (Quintero-Quiroz et al. 2022), which increased hydrogen bonds, disulfide bridges, hydrophobic and electrostatic interactions, in addition to an improvement in the emulsifying activity, and water-holding capacity (WHC) of proteins (Table 5).

On the other hand, a power increase in ultrasound (450 W) led to the formation of a macromolecular network of aggregates through non-covalent interactions, resulting in undesirable techno-functional properties of BBPI (Jiang et al. 2014; Li et al. 2020). Using high-intensity US results in protein denaturation and modification of the secondary structure with exposure of amino groups favoring the Maillard's reaction (Byanju et al. 2020; Quintero-Quiroz et al. 2022). US at 750 W increased the free –SH content by cleavage of disulfide bonds in red bean protein (Byanju et al. 2020), whereas UAE (1200 W) in an alkaline medium (pH 11) had the opposite effect decreasing the free –SH groups and leading to the formation of S-S bonds in faba bean protein. As a result, the bean proteins presented more stable emulsions with smaller particle sizes (Alavi, Chen, and Emam-Djomeh 2021). Even though US technology offers several advantages as a simple and low-cost extraction method, one of the biggest challenges to its implementation at the pilot scale is the different results in protein yield obtained at the lab scale in batch and continuous ultrasonication mode (Rahman and Lamsal 2021). Therefore, operational parameter optimization is required to properly apply US for bean protein extraction since process performance is affected by the equipment setup, extraction conditions, and substrate.

Microwave (MW)

Microwave (MW) employs radiation energy induced by electromagnetic waves at frequencies from 0.3 to 300 GHz (Villanueva et al. 2018). The MW mechanisms englobe the ionic conduction and dipole rotation, and the interaction between microwaves and the food matrix depends on their dielectric properties (Divekar et al. 2017; Villanueva et al. 2018). Water molecules of substrate absorb microwaves that

convert it into thermal energy; the fast heating of the material raises internal pressure until the vegetal cell wall ruptures. The disruption of cellular structure facilitates solvent penetration into the food matrix and contributes to higher extraction yields (Roselló-Soto et al. 2015). Microwave-assisted extraction (MAE) presents many advantages over conventional thermal treatments: faster heating, energy propagation with high efficiency, less solvent consumption, and lower extraction times (Villanueva et al. 2018; Suhag et al. 2021). Furthermore, despite requiring increased investment for its implementation, microwave operational expenditure is lower than conventional heating methods, and capital is returned quickly (Suhag et al. 2021).

MAE technology (Figure 2b) has been widely employed in obtaining and treating bioactive compounds and plant proteins mainly due to its convenience, efficiency, safety, and cost-benefit (Phongthai, Lim, and Rawdkuen 2016; Ochoa-Rivas et al. 2017). Some studies pointed out that the use of MW in the processing of common, faba, mung, and cowpea beans provided inactivation and reduction of ANFs (lectin, tannins, phytic acid, and trypsin inhibitor activity) (Mubarak 2005; Jourdan, Noreña, and Brandelli 2007; Khattab, Arntfield, and Nyachoti 2009; Luo and Xie 2013) (Table 2). Microwaves can easily degrade ANFs in a mechanism that involves hydrolysis of peptide bonds, deamination, and destruction of sulfhydryl groups (S-H) or disulfide bonds (S-S) (Suhag et al. 2021). An increase in IVPD values is also related to ANFs degradation and unfolding of protein structure, making it more susceptible to proteolysis. Nonetheless, an improvement in functional (such as fat absorption index, emulsifying, and foaming capacities) and sensory properties of pulses protein has been associated with the application of microwaves (Ashraf et al. 2012; Jiang et al. 2016; Divekar et al. 2017; Pojić, Mišan, and Tiwari 2018). Furthermore, the integration of MAE and other technologies can promote structural modifications in the protein, inducing peptides extraction (Bandyopadhyay, Chakraborty, and Barman 2012; Ye and Li 2012) and improving protein recovery and allergens detection (Amponsah and Nayak 2016; Ochoa-Rivas et al. 2017).

Many researchers applied microwave technology for protein recovery from diverse plant sources, as shown in Table 3. Regarding different bean varieties, Tables 1 and 3 consider data concerning the extraction and treatment of ANFs from bean protein with MAE at optimized conditions, respectively. In these studies, treatment time and temperature are the first variables investigated in MAE. The use of MW heating for a prolonged time caused the denaturation of faba bean proteins, negatively affecting the solubility (Jiang et al. 2016). MW power also strongly affects MAE efficiency, which controls the thermal energy conversion and hastens the equilibrium rate. A gradual increase in the power at high temperatures allows for higher extraction yields; however, it implicates less selectivity (Kumar et al. 2021). Low-to-moderate microwave power (~1000 W) is adequate for treating plant protein. Microwave is a promising technology for protein extraction since it affects the conformational structure of the protein, improves nutritional and techno-functional properties, and enables high protein yields due to the cell disruption effect.

MAE and UAE are described as economically suitable technologies for use at an industrial scale, most because of efficacy in terms of energy usage when put into perspective with other emerging technologies (Ochoa-Rivas et al. 2017; Pojić, Mišan, and Tiwari 2018). Otherwise, some of the challenges toward MAE application at the industrial scale lie in the process scale-up; although it seems viable to operate in continuous mode at the pilot scale (Terigar et al. 2011; Ho et al. 2015), the lack of experimental MAE data for bean proteins narrows such applications. Other limiting issues needing further investigation include foods' different dielectric properties and microwaves' low penetration into bulk solids (Suhag et al. 2021).

Subcritical fluid extraction (PLE and SWE)

Extractions employing pressurized subcritical fluids (below their critical point) receive various acronyms; the most known are pressurized liquid extraction (PLE) and subcritical-water extraction (SWE) when an organic solvent or water is used as the extraction media, respectively (Figure 2c). In general, the principle of this emerging technology is to conduct extractions at elevated pressure and moderate to high temperatures in a short time so that the extracting fluid remains in the liquid state above its normal boiling point (Zielinski et al. 2021). The temperature increase under high pressures simultaneously lowers the solvent density and the superficial tension while it increases diffusion rates; therefore, there is an increased solvent penetration into the matrix and solubilization of solutes with less solvent consumption and better overall extraction performance (Xi 2017; Gençdağ, Görgüç, and Yılmaz 2021; Zielinski et al. 2021). Compared to processes held at atmospheric pressure, techniques such as SWE are faster and more effective (Herrero et al. 2015).

SWE is a "more environmentally-friend" high-pressure technology used to extract and hydrolyze protein and recover carbohydrates, lipids, and functional phenolic compounds (Marcet et al. 2016). The dielectric constant of water (ε) is a considerably influential property of SWE. With a temperature increment to 250 °C, ε drops from ~80 to 27, a polarity value close to that of acetone at ambient conditions, allowing hydrophobic substances to dissolve. The catalytic action of subcritical water makes it possible to depolymerize polysaccharides into oligomers and monomeric units as well as to generate smaller soluble protein fractions and AAs (Álvarez-Viñas et al. 2021; Gençdağ, Görgüç, and Yılmaz 2021), therefore dismissing the use of chemical catalysts.

Proteolysis by pressurized fluids is an alternative to obtaining peptides and AAs, sometimes employed as a pretreatment step for subsequent enzymatic hydrolysis (Ziero et al. 2020). Sereewatthanawut et al. (2008) used SWE as a hydrolysis method to obtain higher protein and AAs recovery from deoiled rice bran with antioxidant properties than alkali hydrolysis. They attributed these results to the ionization constant of water at elevated temperatures. According to Lu et al. (2016), SWE can promote an equilibrium of the

hydrophobic sites on the molecular surface due to the protein's unfolding and structural rearrangement, which favors solubility. However, SWE associated with EAE, heat, and enzyme action may induce protein aggregate formation due to exposure to hydrophobic groups during hydrolysis (Lu et al. 2016). Despite the great potentiality of SWE to obtain plant protein hydrolysates (Table 3), there is no report in the literature employing SWE for extracting protein from beans that concerns our knowledge.

Similarly, PLE has been largely employed to extract phenolic compounds and antioxidants from pulses, such as anthocyanins from black beans (Teixeira et al. 2021), but, to date, there is no study reporting the utilization of PLE to obtain protein from beans, even though encouraging results were found for others vegetable matrices (Table 3). For instance, plant protein hydrolysates obtained by PLE showed high antioxidant capacity, anti-hypertensive and hypo-cholesterolemic activities attributed to the co-extraction of phenolics, and increased release of ACE inhibitor peptides (Hernández-Corroto et al. 2020; González-García, Marina, and García 2021; Guzmán-Lorite, Marina, and García 2022a). The study by Ziero et al. (2020) discusses the influence of operational parameters on protein hydrolysis and obtained properties through sub- and supercritical extraction technologies, relating the advances in SWE processes and highlighting the importance of connecting academia with industries to make upscaling viable.

High-hydrostatic pressure (HHP)

HHP, also known as high-pressure processing (HPP), is a non-thermal emerging technology employed as a safe and effective alternative for protein modification (Rastogi et al. 2007; Linsberger-Martin et al. 2013; Sun-Waterhouse, Zhao, and Waterhouse 2014; Li et al. 2018). HHP is based on controlling time and temperature at high pressures (up to 1000 MPa) inside high-pressure vessels (Figure 2d). Unlike thermal treatments, HHP does not affect covalent bonds such as crosslinks within macromolecules. However, it induces changes in the conformational structure of proteins since high pressures compress internal cavities and weakens non-covalent bonds tending to alter S-S linking, intermolecular, intramolecular, and solvent–protein bonds (Sun-Waterhouse, Zhao, and Waterhouse 2014). Alteration in proteins surface activity is another recognized feature of HHP, which is associated with the ratio of hydrophobic and hydrophilic groups, related to the conformational changes induced by the unfolding of protein structure (C. Liu et al. 2013; Lin and Fernández-Fraguas 2020).

Contrary to extraction with pressurized fluids (PLE/SWE), HHP has been widely applied in obtaining or treating protein from beans, as shown in Table 5. Pressures between 100 and 400 MPa tend to cause reversible alterations, such as the dissociation of protein-protein complexes, while pressures above 500 MPa cause irreversible denaturation (Rastogi et al. 2007). Moreover, proteins' secondary and tertiary structures are generally modified at pressures above 200 MPa, which may cause denaturation, aggregation, or gelling, while the degree of surface hydrophobicity (H_0) is increased (Yin et al. 2008; C. Liu et al. 2013; Ahmed et al. 2018). Therefore, a decrease in the solubility and WHC in HHP-treated protein can correlate to a rapid denaturation with the formation of high molecular weight aggregates due to the exposure of hydrophobic groups.

Changes to the tertiary structure, presence of hydrophobic regions, and free–SH groups after HHP are important attributes that provide the functionality to plant proteins, especially emulsifying activity (EA). Kidney bean protein isolates (KBPI) had EA, and solubility improved at 400 MPa, probably due to the gradual unfolding by high pressure with forming S-S bonds of free –SH groups. These phenomena allow the turning of insoluble aggregates into low molecular weight soluble ones enabling an EA increase. However, higher pressures (600 MPa) seem to impair the emulsion-forming capability of KBPI (Yin et al. 2008; Ahmed et al. 2018).

HHP also explored the reduction of allergens and ANFs in beans, such as PHA (Table 3). Liu et al. (2013) observed proteins with distinct intensity bands in the high molecular weight portion owing to an aggregation effect at 450 MPa. The pressure rise causes expansion, rearrangement, and aggregation of the protein structure of PHA. The secondary structure modification and its presumable denaturation at elevated pressure resulted in less hemagglutination activity of PHA (Liu et al. 2013). HHP (600 MPa) also reduced the trypsin activity inhibition and the phytic acid content of white beans, besides affecting IVPD (Linsberger-Martin et al. 2013).

Moreover, HPP seems to ease the enzymatic hydrolysis process of peptide bonds by proteases, given the promoted conformational changes with more exposure of peptides, molecular interactions, and susceptibility of cleavage sites in a shorter reaction time under pressurized conditions. HPP-assisted hydrolysis (200–300 MPa) resulted in improved bioactive peptides with better high antioxidant activity (Al-Ruwaih et al. 2019) and ACE inhibition (Garcia-Mora, Peñas, et al. 2015). The evidenced benefits of applying high pressures to treat plant protein underline the prospects of this growing emerging technology, especially in the food industry. As well as other emerging technologies discussed above, the high capital expenditure of large-scale high-pressure processes is one of its biggest drawbacks. However, according to Wang et al. (2016), the average costs of HPP can be lower than those of thermal processing depending on applied conditions since the latter demands more time and energy to impart heat to food. Furthermore, owing to the development of new high-pressure equipment and the constant exploration of this technology, HPP holds optimistic expectations of being absorbed by the market.

Enzyme processing (EP)

Enzymes, due to their specificity, can be used to extract proteins (Figure 2e) by breaking down the plant cell wall through enzymatic hydrolysis (EH), thus increasing extraction yields by releasing the protein linked to the plant polymeric matrix (Sari, Bruins, and Sanders 2013; Pojić, Mišan, and Tiwari 2018). Although EH has higher costs than chemical

hydrolysis, it is an eco-friendly technique that shows many advantages in producing commercial hydrolysates on a large scale. Proteolysis can increase protein hydrophilicity and convert proteins into high-quality peptides of desired sizes under mild conditions and short reaction times (Liceaga and Hall 2019; Zheng et al. 2019). Protein hydrolysates obtained by enzyme-assisted extraction processing (EAE) showed high solubility, thermal stability, and oxidation resistance. In addition, they have higher emulsifying properties and prevent undesirable compounds (Ngoh and Gan 2016; Nadar, Rao, and Rathod 2018; Pojić, Mišan, and Tiwari 2018; Los et al. 2020).

However, some inconvenience of EAE arises from this complex method, requiring a precise process control to obtain protein hydrolysates (Ngoh and Gan 2016; Pojić, Mišan, and Tiwari 2018). In addition, EH, with a high degree of hydrolysis, can also release peptides with high non-polar AA content, resulting in a bitter taste in protein hydrolysates. The chosen enzyme interferes in the AA sequence of the peptides, which are responsible for the functional, nutritional, and bioactive properties of hydrolysates. Each enzyme class has specialized catalytic sites that provide substrate specificity and direct its cellular location to cleave different peptide bonds (Liceaga and Hall 2019). Commercial proteases such as alcalase, Flavourzyme©, Protamex©, chymotrypsin, trypsin, pepsin, pancreatin, and papain, have been widely employed for hydrolysates production from bean proteins (Rui et al. 2012; Rui and Barbana 2012; Aluko 2015; Ngoh and Gan 2016), as depicted in Table 5.

Enzymatic processes can also destroy IgE-reactive peptides, decreasing plant protein allergenicity and allowing allergic individuals or those with nutrient malabsorption to consume protein hydrolysates (Liceaga and Hall 2019; Schlegel et al. 2020). EAE can improve the hydrolytic reactions and increase extraction yield (Lopes Junior et al. 2010), enhancing bean proteins' functionality and bioactive properties. Bioactive peptides obtained by EAE from bean proteins showed antioxidant capacity and inhibitory activities against α-amylase (Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano 2015; Ngoh and Gan 2016) and ACE when alcalase and savinase (Patricia Garcia-Mora, Peñas, et al. 2015) and sequential hydrolysis by alcalase/Flavourzyme© or alcalase/papain (Rui et al. 2012) were applied. In this sense, alcalase has been described as an effective enzyme for obtaining bioactive peptides with anti-hypertensive properties. Torruco-Uco et al. (2009) reported that bean hydrolysates produced with alcalase showed high content of hydrophobic AA residues that favor ACE inhibitory activity, potentiating their application in foods or drugs to attenuate arterial hypertension symptoms.

Hydrolysate functionality depends on the extent of hydrolysis and process parameters (i.e., pH, temperature, enzyme-to-substrate ratio) used. Therefore, it is fundamental to define the suitable conditions for the protein of interest to preserve its functionality and biological value (Evangelho et al. 2017; Liceaga and Hall 2019). An alternative to improve the process is to apply different techniques to increase extraction efficiency. The successful association of US as a pretreatment or during EAE is related to the structural alterations and breakage of covalent bonds. US treatment increases protein solubility and efficiency of enzymatic hydrolysis with higher exposure to hydrolytic sites for the enzyme to access. This synergic effect in the protein conformation enables the production of protein hydrolysates rich in bioactive peptides (Ozuna et al. 2015; Jain and Anal 2016) and widens the possibilities of using different emerging technologies as complementary methods that take advantage of personal benefits from each technique.

Other emerging technologies applied to plant proteins

Similarly to MW, radio frequency (RF) is a dielectric heating treatment that employs electromagnetic waves (1–300 MHz), in which energy can penetrate more easily into the matrix compared to MW (Ling, Ouyang, and Wang 2019a). Various reports have shown RF's effects on protein's physicochemical properties from multiple sources. RF heating as a stabilization treatment altered the secondary structure and improved functional properties like emulsifying capacity of rice bran protein isolate (Ling, Ouyang, and Wang 2019a, 2019b) and the hydrophobicity of soy protein isolate (Guo et al. 2017). Regarding dry beans, RF has been successfully adopted for batch disinfestation of black and mung beans (Song et al. 2020; Carbajal-Padilla et al. 2022), and, as far as we know, no results were found regarding the recovery of bean proteins.

The use of high-intensity (10–80 kV/cm) pulsed electric fields (HIPEF) leads to the fracture of cell membranes and enhances protein extraction yields due to the increased permeability that facilitates the transport of cellular content to the extracting medium (Eze et al. 2022; Rajpurohit and Li 2023), including protein. However, a research gap exists concerning enhanced protein extraction from pulses (Eze et al. 2022) and dry beans. For example, mung bean protein isolate treated by HIPEF resulted in enhanced techno-functional properties (solubility, surface hydrophobicity, and emulsifying capacity) with significant structural changes (Gulzar et al. 2023). Similarly, moderate PEF in alkaline pH promoted the unfolding of SPI structure that substantially contributed to higher solubility, surface hydrophobicity, emulsifying, and foaming properties (Wang et al. 2023). PEF was also employed to enhance common bean hydration as an alternative to soaking (Devkota et al. 2022).

Emerging eco-friendly technologies: dry processes

Dry fractionation methods are attractive to produce proteins that do not require a high degree of purity. These methods include milling and sieving, electrostatic separation, and air classification. They are applied to low-fat pulses (e.g., beans, peas, and lentils) (Schutyser and van der Goot 2011; Pelgrom, Boom, and Schutyser 2015b; Tabtabaei et al. 2016a; Pojić, Mišan, and Tiwari 2018; Saldanha do Carmo et al. 2020), in the obtaining of pulse-protein rich flours, whereas starch granules are detached from protein matrix during milling and are separated by a sequential fractionation step.

Air classification

Separation by the air classification method (Figure 2f) splits the light protein fraction (~1–10 µm) from the starch fraction (~15–40 µm) by size and density differences as the flour is submitted to a spiral air stream (Boye, Aksay, et al. 2010). Separation efficiency is linked to the milling process, so the particles must be small and non-aggregated to facilitate movement and fraction separation through the airflow. The fine fraction's protein content depends on the initial protein, the dispersibility of the flour, and the cutoff point. While coarsely ground particles tend to result in aggregation of protein and starch, excessive grinding can lead to contamination of the protein fraction by damaged starch fragments of similar size. Thus, rotary air classifiers with a classificatory wheel or rotor are often employed to obtain protein of suitable powders size (Schutyser and van der Goot 2011; Pelgrom et al. 2013; Pelgrom, Boom, and Schutyser 2015b; Fernando 2021).

Studies employing combined dry fractionation methods (milling followed by air classification) showed promising results in obtaining protein-rich products from beans (Pelgrom, Boom, and Schutyser 2015b; Saldanha do Carmo et al. 2020; Vogelsang-O'Dwyer et al. 2020; Zhu et al. 2020). Zhu et al. (2020) reported values for the separation efficiency (84.0%), purity (63.2%), and yield in the protein-rich fraction (31.9%) for mung bean, with the protein concentrate from dry fractionation resulting in higher antioxidant capacity than the one isolated by alkaline extraction (Zhu et al. 2020). The protein-rich flour from faba beans resulted in better techno-functional properties like solubility, foaming, and gelling abilities compared to the protein isolate from acid extraction (Vogelsang-O'Dwyer et al. 2020). However, dry methods have some limitations regarding an inefficient separation process of particles with similar size and density (Pelgrom, Boom, and Schutyser 2015b; Tabtabaei et al. 2016b), lower protein content, difficulty in eliminating ANFs, and low IVPD. On the other hand, life cycle assessment emphasized that these methods have less environmental impact mainly due to the limited use of water and higher energy consumption of solvent-based methods (Vogelsang-O'Dwyer et al. 2020).

Electrostatic separation

Electrostatic separation (ES) is another dry method that consists of positively or negatively charged particles and further dividing them under an external electrical field (Figure 2g). The mechanism is based on the contact between two materials that transfer electrons from one to another (Schutyser and van der Goot 2011; Fernando 2021), having triboelectrification as the most appropriate charging mechanism for ES. It can occur by ions or electrons transfer through particle movement in a tribocharging medium (e.g., a fluidized or vibratory bed, a pneumatically transported particle flux) (Tabtabaei et al. 2016a; Pojić, Mišan, and Tiwari 2018). The protein- and carbohydrate-rich particles separation occurs based on the magnitude of their electrical charges since triboelectrification of proteins is much larger than that of carbohydrates due to the presence of functional groups in AA residues that are ionizable such as amino and carboxyl (including terminal -N and -C) groups (Pelgrom, Boom, and Schutyser 2015b; Tabtabaei et al. 2016a). Several factors affect the performance of protein enrichment by ES (charging rate, transporting gas flow and velocity, length of tribocharger tube, and plate tension, to name a few), and ideal process conditions prompt a high charge density and stronger electrostatic attraction forces of the proteins (Tabtabaei et al. 2016a).

As application examples, the protein content of navy bean flour increased from 23.8 to 47.2% using ES (Jafari et al. 2016; Tabtabaei et al. 2016a, 2016b). Besides, Jafari et al. (2016) observed that this technology preserved the native structure of proteins and hence the AA distribution in bean flour in the face of a considerable SAA loss of isolates from acid precipitation. As previously discussed, the process under acidic conditions alters protein structure giving rise to denaturation and aggregation of high molecular weight proteins. The use of dry fractionation methods such as air classification and ES are advantageous because they dismiss chemical solvents and maintain protein structure thus preserving their functional characteristics. However, when different compounds have similar charges, ES becomes limited in terms of the purity of the protein concentrate, and pendant particles may be influenced by gravitational force before they are drawn by the electrodes, which explains lower ES yields when compared to wet processes (Assatory et al. 2019; Fernando 2021).

In an attempt to increase plant protein yield and purity, combining separation techniques based on different physical driving forces is a recurring topic in processing pulse proteins. A two-step approach employing air classification followed by ES enabled the obtaining of peas protein concentrate with a high yield, purity, and a higher protein recovery compared to the fraction obtained from single-step milling and air classification (Xing et al. 2020). Pea and lupine protein fractions adhered to fiber and became more accessible with ES post-treatment (Pelgrom et al. 2015). Most recently, a hybrid dry and wet fractionation process allowed a 92% protein recovery from faba bean cotyledons (Dumoulin et al. 2021) and increased the pea protein yield (63%) (Pelgrom, Boom, and Schutyser 2015a). In addition, the combined process achieved lower energy consumption and water use (~5.5 times lower) than traditional one-step wet extractions (Pelgrom, Boom, and Schutyser 2015a; Dumoulin et al. 2021). Therefore, the combined approach is an ongoing strategy to improve plant protein isolates' purity, recovery, and concentration without jeopardizing the energy efficiency of dry fractionation methods that can extend to the processing of bean proteins.

Conclusions and future outlook

The increasing world population and consumer concerns with a healthful diet and awareness of the beneficial effects of pulses stimulate the consumption of dry beans, justifying the expanding number of recent studies focusing on nutritional, functional, and bioactive properties associated with

introducing bean proteins into the diet. There is also an increasing demand for non-allergenic plant-based food that is nutritious and safe for consumption. However, the biggest drawback for the efficient substitution of animal protein lies in the nutritional quality limitations of plant proteins regarding their digestibility, EAA profile, and presence of ANFs.

In the last decade, a considerable advance was achieved concerning eco-friendly and innovative emerging technologies that have been applied to diverse plant materials and proved effective techniques in several steps in plant protein processing. Although this scenario for bean proteins remains partially explored to date, the obtained results are encouraging regarding overcoming the major issues in this review related to bean protein's digestibility, allergenicity, ANFs, and extraction yield. The use of emerging technologies over conventional heating protein extraction methods surpasses most related technical disadvantages and generally attends to sustainability and green chemistry principles in addition to the less environmental impact. Even though solvents have the GRAS status, some challenges include minimizing their use in wet methods and improving protein purity in dry fractionation methods.

The reviewed studies showed that a switch from thermal pretreatments to processes that promote preferentially non-thermal physical alterations to the plant matrix and the (isolated) protein (e.g., conformational changes that favor proteolysis) to be overall beneficial for protein functionality digestibility and extraction yield. These studies demonstrated that processes at optimized conditions (combined or isolated) promoted an improvement in the techno-functional and nutritional quality of protein subjected to US, MW, EP, and high pressures (PLE, SWE, HHP) treatments, mainly due to the partial unfolding in the protein structure that facilitates the inactivation and reduction of ANFs (trypsin inhibitors, phytates, and beans lectin) and modification of allergen epitopes. Enzyme processes are a suitable emerging technique to obtain hydrolysates at controlled reaction conditions, such as bioactive peptides with a high biological value for human health (antioxidant, anti-hypertensive, and hypocholesterolemic activities). Moreover, high-pressure extraction with subcritical fluids (PLE, SWE) is also very promising, although there are no studies regarding bean proteins.

In this context, research on the impact of different processes on the overall quality of bean proteins remains restricted; the action mechanisms of each emerging technology and their influence on protein peptides from beans at the molecular level need further investigation. Likewise, clinical trials evaluate humans' dose-response to utilize bean proteins in hypoallergenic foods safely. These approaches can substantially stimulate bean protein-based product development and contribute to supplying the market demand for alternative plant-derived proteins. Notwithstanding, the proper process implementation at the industrial level requires scale-up and economic viability studies regarding the utilization of resources, which are relatively scarce, especially for considerably "new" raw materials.

Overall, integrating different emerging technologies in the various processing steps of plant protein from dry beans should guarantee food safety for obtained products, as demonstrated for diverse sources of plant proteins other than beans and pulses. Therefore, implementing and optimizing the discussed emerging technologies will soon compose new alternatives for the potential development of plant protein derived from pulses, particularly dry beans – a potential but a limitedly explored source of alternative protein.

Authors' Contributions

Renata F. Teixeira: conceptualization, methodology, investigation, data curation, writing -original draft preparation, review, and editing; Clóvis A. Balbinot Filho: resources, data curation, writing - review, and editing; Débora de Oliveira: methodology, writing - review, and editing, supervision; Acácio A. F. Zielinski: methodology, writing - review, editing, supervision.

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