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Review

Protein digestibility of soybean: how processing affects seed structure, protein and non-protein components

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Abstract
Protein digestibility is a key indicator of dietary protein quality because the amino acids present in a protein food may not be available to an organism for nutrition and health unless they are digested. In spite of being a good source of protein, Soybean seed has limited digestibility mainly in their whole form. In this paper, we highlight the factors that affect the digestibility of soybean proteins like the quantity, structure, and distribution of the kinetically stable proteins plus the anti-nutritional compounds in soybean seeds. Furthermore, factors such as seed coat thickness and composition, cellular integrity, and seed hydration can also impact the protein digestibility of soybeans. It was found that wet thermal treatments like cooking along with operations such as fermentation, grinding and germination have a more favourable effect on hydrolysis of soybean proteins than dry-heat treatments such as roasting. Also, all processing operations have the ability to reduce the anti-nutritive compounds to varying degrees, ensuring the safety and increased digestibility of the soybean. The current review exhibits the potential processing methods for facilitating mechanical disintegration and protein hydrolysis of soybean seeds. Hence, the insights gained from this review can be used to understand the mechanism by which various processing methods enhance the protein digestibility of soybean seeds. The findings of this review indicate the necessity to carefully adjust processing conditions to preserve nutritional quality, reduce anti-nutritional components to safe levels, and optimize both protein digestibility and palatability of whole soybean seeds.

Keywords Protein hydrolysis · Traditional processing · Legume · Anti-nutritional compound

Abbreviations

IVPD	In-vitro protein digestibility
7S	β-Conglycinin
11S	Glycinin
CIA	Chymotrypsin inhibitor activity
TIA	Trypsin inhibitor activity
HIA	Haemagglutinins inhibitor activity
BBI	Bowman-Birk inhibitor
KTI	Kunitz trypsin inhibitor
SAA	Sulfur-rich amino acid
SDS	Sodium dodecyl sulfate
KSP	Kinetically stable protein
mc	Moisture content

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ANC Anti-nutritional component
LAB Lactic acid bacteria

1 Introduction

The evaluation of protein quality in terms of digestibility is crucial to maintain and improve the health and well-being of human population especially in regions where food insecurity and protein energy malnutrition are common [1]. Protein digestibility is defined as the proportion of proteins that are hydrolyzed by digestive enzymes and have the potential to be absorbed by the body as amino acids or any other nitrogen compound [2]. Currently, legumes are one of the most widely embraced sustainable and plant based protein source [3]. They contribute to boosting overall system productivity by facilitating the diversification of crop rotations, while also replenishing soil nitrogen without relying on fertilizers [3]. They serve as an important source of low cost protein, especially for people who follow vegetarian or vegan, gluten-free or athletic diets [4, 5]. Apart from proteins, legumes are valued for their dietary fiber, complex carbohydrates, vitamins, minerals and active molecules with antioxidant properties. Some of the health benefits attributed to the consumption of legumes include their ability to regulate blood sugar levels, control weight and protect a spectrum of cardiovascular and chronic ailments [3]. The legume market is also projected to experience a Compound Annual Growth Rate of 5.3% to reach a value of USD 17.25 billion by the end of 2030 [6]. Therefore, legumes emerge as an ideal protein source when considering sustainability, affordability, health benefits, and market demand.

The soybean's distinctive chemical composition compared to other legumes makes it one of the most valuable and cost effective agricultural commodities [7]. It has high amount of protein (about 40 g per 100 g) compared to other legumes (20 to 30 g per 100 g) [7]. On a wet basis, the soybean contains around 35% protein, 17% oil, 31% carbohydrate, and 4.4% ash [7]. This makes the soybean protein, a comparable source to animal protein (meat, dairy and eggs) with no cholesterol and low saturated fatty acids. The worldwide consumption of soybean has been growing over the past 12 years due to its enormous benefits in human health such as improving bone health, reducing cholesterol and symptoms of menopause [8, 9].

Soybean, being a protein rich legume, is processed to produce products such as soy meal, soy protein isolates and concentrates. Soybean meal contains approximately 49% protein content whereas soy concentrate is about 70% and soy isolate around 90% protein. However, the digestibility of soybean in its whole or flour form is limited [10–13]. The digestibility of soybean proteins cannot be fully governed by a single factor. It is a combined effect of physical structure, properties of native protein and non-protein components and, also the moisture content of soybean seeds during processing. Both protein (like trypsin and chymotrypsin inhibitors) and non-protein fractions (like phytates and tannins) of soybean can act as an inhibitors to the proteolytic enzymes present in the human digestive system. The proteolytic enzymes aid in the splitting of high molecular weight proteins into small molecular weight peptides and amino acids [14]. The extent of protein hydrolysis is influenced by the method of processing applied before the ingestion of food. The processing treatments such as soaking, heating, germination, milling, roasting and fermentation involve mechanical, biological or thermal operations which can bring physicochemical and structural changes in soybean. In this review, we have discussed the changes occurring to soybean cellular structure, protein and non-protein fraction during the processing of soybean seeds (Fig. 1). Furthermore, our review will give crucial insights into how different processing techniques can affect the digestibility of soybean protein.

2 Effect of processing on soybean cell structure

Soybean seeds have a dynamic cellular structure in which starch disappears in the last phases of seed development [7]. The cells in the soybean cotyledon are mostly made up of protein bodies (approx. 5–8 µm size) in which distinct spherical oil bodies (approx. 0.2–0.5 µm size) are embedded [7] as shown in the schematic in Fig. 2b. An intricate network of structural proteins and polysaccharides makes up the cell wall of soybean cotyledons. The polysaccharides of soybean cotyledons cell wall consist of pectin (50–70% on cell wall weight), hemicellulose and cellulose [15]. A pectin-rich layer called the middle lamella adheres to the cotyledon cells of soybeans [15] as shown in the schematic in Fig. 2a.

The existence of an intact cell wall of soybean cotyledon cells can slow down (not an absolute barrier) the contact of intracellular proteins with digestive enzymes [16–18]. The processing operations like milling breaks up or damages the cellular structure of some intact soybean cells (Fig. 2c) resulting in the production of broken cells [18]. This can expose

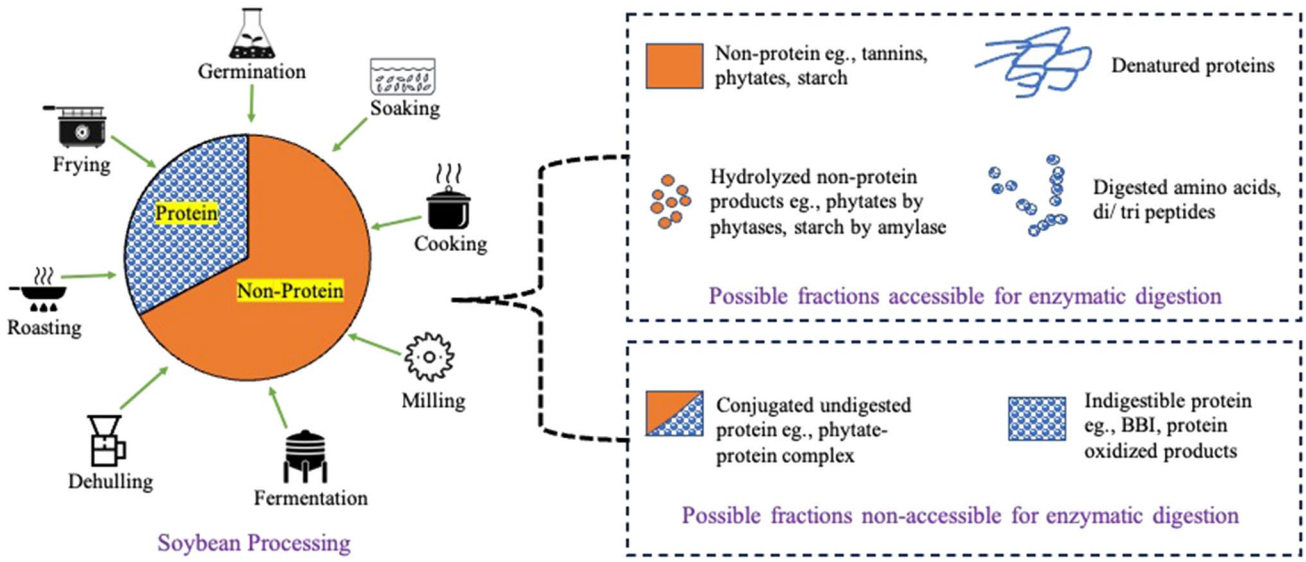

Fig. 1 Schematic representation of possible protein and non-protein fractions obtained after soybean processing

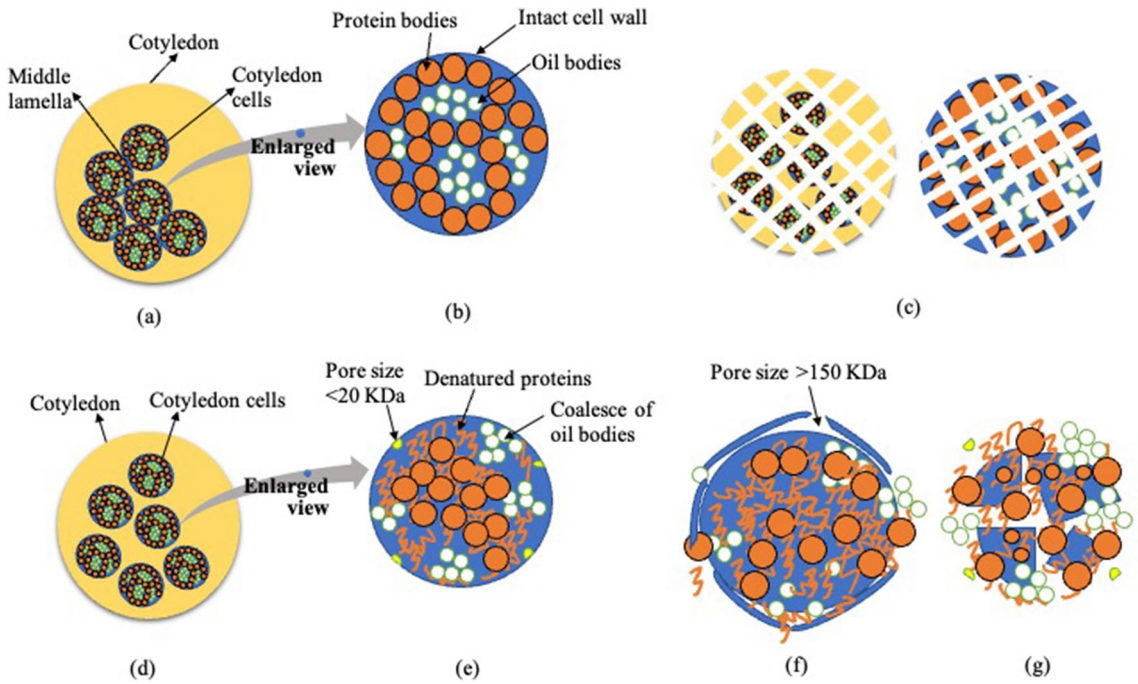

Fig. 2 Schematic representation of soybean cotyledon. **a** Soybean cotyledon and its cells cluster. **b** An individual cell of soybean cotyledon. **c** Fragmentation of cotyledons and their cells after milling. **d** Separation of cotyledon cells by cooking. **e** Denaturation of intracellular and structural cell wall proteins, coalesce of oil bodies towards cell wall and increase in cell wall porosity by cooking. **f** Increase in cell wall permeability due to digestion of structural cell wall proteins by small size enzymes like trypsin produced by fermentation. **g** Reduction of compactness of intracellular matrix due to hydrolysis of intracellular proteins

the intracellular proteins for proteolytic action in the human digestive system thus improving the protein digestibility [18]. The degree of protein hydrolysis increased by 8% when cooked for 30 min as flour compared to its intact soybean counterpart [19]. The same study [19] also stated that for both cooked cotyledons and flour, longer cooking periods (90 and 180 min) only slightly improved the protein digestibility and changed its physicochemical characteristics. However, the change observed during the milling of germinated seeds, followed by boiling for 30–180 min was found to be insignificant [19]. This implies that desirable protein digestibility can be obtained at an optimal cooking times when

preceded by a milling operation. However, if germination precedes the cooking process, additional operations such as milling may not have a significant impact. Milling and then boiling of soybean produce higher fraction of broken cells and better protein digestibility (about 30.7% more percentage change increase for 71–125 µm particle size) compared to same sized particles that undergo boiling and then milling [18]. This could be due to the increased resistance of the cell wall to fracturing after getting hydrated from boiling. This is corroborated by another study which observed that the rupture force did not correspond with the grinding characteristics at high moisture soybean seeds as at low moisture content seeds [20].

The cooking process increases the separation of cotyledon cells (Fig. 2d) due to partial solubilization of pectin that glued the adjacent cells together [17, 18]. It can also solubilize polysaccharides present in the cell wall of cotyledon cells because of the denaturation of structural proteins. This in turn impacts the cell wall structural integrity, increasing its permeability or porosity as shown in schematics in Fig. 2e. It has also been proposed that soaking in salt solution will remove divalent cations, especially (Ca^{2+} and Mg^{2+}) from pectin of the middle lamella. These modified pectates are believed to be more heat-labile and water-soluble, thus causing pectin solubilization and affecting cell wall permeability of cooked soybeans [15]. Soaking in acid or alkaline solution has also indicated enhanced seed coat permeability and thus increased rate of water absorption or hydration coefficient [21]. Though the separation of cotyledon cells has happened as a result of cooking operation, this is not sufficient to improve the digestibility of soybeans as the intracellular macronutrients in a cotyledon cell are still in a tightly packed environment (schematics in Fig. 2e) [19].

The processing methods such as germination and fermentation, can also disrupt cellular integrity by reducing the compactness of the intracellular environment which may act as an extra obstruction for the passage of digestive enzymes [17, 22]. They may produce enzymes such as proteases that can digest the structural proteins (present in the walls of cotyledon cells) or hydrolyze the seed proteins [23] moving towards the core of particles [17]. This further increases the cell wall permeability and decreases the compactness of intracellular components present in soybean seeds (schematics in Fig. 2f, g). The activation of several other native and non-native enzymes, such as lipases and phytases, throughout the processes of soaking, fermentation, and germination can also loosen the cellular structure of soybean. This, in turn, facilitates the action of proteases in breaking down proteins.

This shows that the breakdown of cell structure, separation of cells within cotyledons and solubilization of structural carbohydrates can disrupt the cellular integrity and permeability of soybean. Such modifications enhance the digestibility of soybean protein and can be brought up through operations like milling, cooking, soaking in salt/acid/alkaline solutions, fermentation and germination.

3 Effect of processing on soybean proteins and its properties

There are four fractions of proteins: albumins, globulins, prolamins and glutenins. Soybean, on a dry weight basis, includes 35–40% protein, of which, 90% consists mainly of two storage globulin proteins, approx. 35% β-conglycinin (7S) and 52% glycinin (11S) [24]. β-conglycinin (7S fraction) is a trimer with a molecular weight of approx. 180 kDa, while glycinin (11S fraction) is a hexamer with a molecular weight of around 360 kDa [25]. 7S consists of α-, α′- and β-subunits whereas 11S comprises acidic and basic polypeptides [26]. The enzymes inhibitors such as protease inhibitors (trypsin and chymotrypsin), lectins and α-amylase inhibitors represent a small fraction that belongs mainly to the albumin portion of soybean proteins [27, 28]. The compact globular structure of albumins is stabilized by numerous disulfide bonds, creating an intrinsic structural barrier that restricts enzyme access. Hence, it could be possible that the digestibility of protein decreases with an increase in albumins to globulins ratio [29, 30].

A study [31] reported that there is no correlation between the protein quantity and its digestibility. However, the retention of adequate amounts of nutritive proteins after processing is important to gain maximum benefits out of soybean. The increment in the protein content [14, 17–19, 23, 32–40] compared to the control can happen by leaching or losses of soluble solids other than proteins such as minerals, sugars, soluble fibres, phytates and raffinose during soaking, cooking [41] and dehulling. This will lead to the concentration of proteins present primarily in the cotyledons fraction of soybean. The utilization or breakdown of seed reserves for example, carbohydrates or lipids as a source of energy supply for forming sprouts during germination can also lead to a rise in soy protein levels. It can also be increased from the biosynthesis of proteins in the form of amino acids by endogenous and/or exogenous enzymes or simply due to the growth of microflora during the germination and fermentation period.

On the other side, the decrement in proteins [17, 18, 33, 37, 40] can take place because of more leaching of soluble protein into soaking and cooking water and from extensive milling (to obtain finely ground sample), as well as by thermal degradation of proteins from dry roasting. Studies that have compared roasting with germination and/or fermentation showed less or negative change in the protein value of soybean compared to the control [23, 33, 34, 36]. This shows that roasting is causing more degradation of soy proteins compared to germination and fermentation techniques. However in certain cases, the germination and fermentation of soybean have also reported less or negative change in the protein content compared to the control [17, 40]. This is usually caused by the involvement of pre and post-processing operations such as soaking, cooking and milling in fermented and germinated soybean.

The most researched anti-nutritional protein is trypsin inhibitors (TIs), which are serine protease inhibitors, that vary between 2–6 mg/g in soybean [42]. Kunitz trypsin inhibitor (KTI), about 1.4 g/kg and Bowman-Birk inhibitor (BBI), about 1.6 g/kg, are two typical low molecular weight proteins which fall under 2S fraction of soybean [25, 42]. KTI is heat labile and single-chain polypeptides of approx. 20 kDa with two disulfide bridges that exclusively inhibit the enzymatic action of trypsin and not chymotrypsin, whereas BBI is heat-stable and also single-chain polypeptides of approx. 8–10 kDa in size with seven disulfide bridges that block the activity of both trypsin and chymotrypsin simultaneously at independent binding sites [27, 43]. They can either attach to the active site of proteolytic enzymes or modify their structure, disrupting their catalytic activity. This interference can impede the digestion of soybean protein as it travels through the gastro-intestinal system. The inactivation of TI during thermal treatments may vary depending on the content of these two inhibitors in soybean, each of which has a distinct thermal stability [44]. We propose that the inhibitory activity of heat-stable BBI may also undergo augmentation when exposed to heat. However, a comprehensive investigation is required to explore this phenomenon thoroughly across various temperature and moisture conditions. Also, the genetic or physical elimination of TIs [45, 46] from soybean seed alone may not fully solve the problem of its low protein digestibility. Moreover, it is not advisable to eliminate these proteins, as they serve as secondary metabolites produced by plants to safeguard themselves against insect pests and pathogenic microorganisms in the early stages of plant growth and development. [47]. Furthermore, they have been found to possess properties that can combat inflammation, prevent cancer, and counteract obesity [48, 49].

The effect of various processing treatments on trypsin, chymotrypsin and hemagglutinins inhibitor activity, was studied by [28, 32–35, 50–52]. It was observed that all processing methods reduce the protease inhibitors to various degrees. The wet thermal treatments such as cooking, boiling, autoclaving alone or in combination soaking [19] have been found to have a more impact in inhibiting these enzyme inhibitors (TI- upto 99.4%, CI- upto 100% and HIA- upto 100%) than soaking (TI- upto 41.2%, CI- upto 6.7%, upto 84.1%), dehulling (TI- upto 17.3%), germination (TI- upto 69.4%), fermentation (TI- upto 94.6%, CIA- upto 100%) and dry thermal treatments like roasting (TI- upto 59.7%). The combination of germination and milling with cooking has caused upto 100% percentage reduction in TIA. The effect of prolonged storage and dehulling of soybeans on TIs was found to be insignificant [33, 37, 53]. This is probably attributed to the presence of a higher amount of TIs in cotyledons than in the hull portion of soybean. It is important to highlight here that methods used to assess TIA, CIA and HIA vary from study to study. Reported literature expressed the inhibition of an enzyme in percentage terms or as weight or enzyme units per quantity of protein or product. Thus, these reduction percentages are not comparable. Also, certain processes, like germination and fermentation, which include pre-treatments such as soaking and cooking, cumulatively contribute to the suppression of protease inhibitors. It was reported that thermal inactivation of TIs may result from processes involving deamidation, splitting of covalent bonds and modifications to disulfide bonds [54]. Chymotrypsin inhibitors are more susceptible to heat than TIs [28]. However, some residual protease inhibitory activity might be present due to heat-stable BBI or in cases where proper heating was not accomplished or when a compromise is needed between the negative effects of excessive heat on protein quality and the thermal destruction of inhibitors. The chronic ingestion or consumption of these low residual levels through a processed soybean diet is unlikely to cause any negative health impact on people and rather may have some pharmacological effects, as reported by many studies. However, some studies proposed that it can be of concern if they exert negative effect while passing through the gastrointestinal system. For example, TIs, lectins and undigested proteins can cause more excretion or hypersecretion of pancreatic serine proteases (trypsin and chymotrypsin which are made up of sulfur-rich amino acids (SAAs)) by removal of feedback inhibition of pancreatic secretion. Because of this hyperactive pancreas's impact, these SAAs are diverted from being used to make body tissue protein to making more of these enzymes, which are then lost in the faeces [11, 27]. Hence, substantial loss of exogenous and endogenous SAAs through faeces happened in the form of enzymes. Such views were constructed mainly by experimenting with animals like rats, chicks and mice [42] but should be investigated further in the human system. The correlation value between the amount of some soybean protease inhibitors and in-vitro protein digestibility (IVPD) of soybean is shown in Table 1. However, it should be noted

that this dynamic secretion of proteolytic enzymes in an in-vivo system and its impact on protein digestibility can not be observed in a static in vitro digestion system.

This overall means that the amount of anti-nutritive-proteins and non-proteins retained and leached out respectively or vice-versa determines the content of nutritive protein in processed soybean. The germination and fermentation processes (through biosynthesis) have more potential to increase the nutritive protein of soybean compared to roasting. The retention of soluble proteins in soybeans can be maximized through optimal soaking, cooking and milling conditions. In most of the studies, cooking resulted in a higher reduction of soybean protease inhibitors compared to other processing operations. However, efforts should be made to modify the structure of these anti-nutritive proteins (especially ones that are rich in SAAs) through processing. It should be done in a way that they no longer hamper the digestibility of soybean protein as well as provide therapeutic benefits.

3.1 Kinetic stability and denaturation of soybean proteins

The kinetic stability is one of the properties of proteins which can decrease their susceptibility to attack by proteolytic enzymes [61]. Kinetically stable proteins (KSPs) are those that have a very slow unfolding rate and exhibit resistance to proteolysis, and denaturing detergent, sodium dodecyl sulfate (SDS) [62, 63]. In a study [61], biodefense proteins (TIs and lectins) and two globulin like storage proteins (7S and 11S) in soybeans were reported to be hyper or kinetically stable. This stability can be the result of more oligomeric and beta-rich conformations (which give rigidity to the barrel structure of such proteins) than monomeric alpha helix-rich conformations in KSPs [61, 62]. The presence of these configurations i.e., alpha helix and beta sheet content can increase or decrease the digestibility of soybean [56, 61, 64], respectively (Table 1). The abundance of KSPs in soybean could be because of its growth in warmer climates, which may require more biodefense proteins to fight against relatively high biotic stress compared to cool season legumes [61]. The differences in the reported IVPD values in the literature may be closely associated with the phenomenon of kinetic stability of proteins.

Table 1 Correlation values between in-vitro protein digestibility of soybean and some factors affecting it

Parameters	Correlation values	References
Trypsin inhibitor	− 0.82[2]	[19, 44, 45]
	− 0.85	
	− 0.41	
Chymotrypsin inhibitor	− 0.72	[55]
Beta-sheet content	− 0.79[1]	[19, 56, 57]
	− 0.61[2]	
	− 0.98	
	− 0.63	
Alpha helix	0.53	[19]
Sulphydryl content	0.93[2]	[58]
	0.99[2]	
Carbonyl content	− 0.69	[47]
Protein solubility	− 0.58[1]	[47]
Water absorption capacity	0.45	[59]
Beta-turns	− 0.44	[19]
Random coils	0.78	[19]
Surface Hydrophobicity	0.88	[19, 60]
	0.98	
Soluble protein by SDS + Urea or Non-covalent interactions	0.59	[19]
Tannins	− 0.76	[44]
Saponins	− 0.46	[44]
Phytic acid/Phytates	− 0.77	[44]

[1]Only gastric enzyme

[2]Only intestinal enzyme (s)

Rest using gastrointestinal enzymes

For instance, the diverse biotic and abiotic stresses encountered by a plant during its growth can induce variations in the levels of KSPs in seeds, leading to differences in IVPD values of raw soybean. Moreover, if a sample is kept for more time for digestion or heating then it will be most likely that even the KSPs get denatured for proteolytic attack whereas shorter digestion or heating duration could lead to incomplete denaturation and thus lower digestibility. This hypothesis needs to be tested and validated with the proper experimental design. Hence, this kinetic stability characteristic of proteins, which nature has chosen for adaptation, protection and survival of organisms against harsh conditions, can be one of the significant factors for the reduced digestibility of soybean proteins [61, 63].

The heat treatment makes the legumes edible and palatable to humans. During heating, the denaturation or unfolding of proteins occurs which is essential for increasing the accessibility of susceptible sites to proteolysis and thus improving the protein digestibility. The complete denaturation of 7S and 11S soybean proteins was found when heated at 160 °C and 200 °C for 10.68% moisture content (mc); 145 °C and 185 °C for 29.70% mc; 130 °C and 160 °C for 46.29% mc; 115 °C and 140 °C for 62.05% mc respectively [57]. The inactivation of enzyme inhibitors during heating was also reported to be high in case of more hydrated legume seeds [28, 33] compared to dry conditions [65]. This means that a barrier against heat denaturation of protein can be provided by the restricted water present inside the soybean cotyledon cells. The soaking of legumes, which is usually a pre-treatment for most of the processes such as boiling, germination and fermentation and sometimes even for roasting, can hydrate the seeds and increase their moisture content. This results in speeding up the rate of protein denaturation at a lower temperature during thermal treatment. Now, the ability to imbibe the water by raw soybean seeds (increasing their moisture content) may be dependent on its waxy cuticle layer or/and the amount of hemicellulose mainly xylans in soybean seed coat [66]. Soybeans' cuticle layer can be thicker (making the seeds of soybean hard) if grown under hot and humid conditions compared to its growth in warmer and dry conditions [67]. However, these hard seeds can easily take up the water once this waxy cuticle is removed by processing operations that involve chemicals or mechanical changes [67]. The xylan content in the seed coat can also lead to the hardness of soybean seeds [66]. Some of the other factors that can affect the hydration property of raw soybean seeds are soaking conditions (soaking media type, time and temperature) and physical properties (seed/particle size, hull/seed coat thickness (if not removed), hull and/cotyledons porosity). The proportion of hard-to-cook seeds in a soybean sample can indirectly affect the IVPD values. This needs to be investigated in detail with different varieties of soybean.

Apart from low moisture seeds, the existence of an intact cell wall has also been reported to raise protein denaturation temperature by about 10% during cooking [19]. Though for significant TI denaturation or inactivation, heat treatment is a must as could be seen through SDS-PAGE [14, 68] and TIA [19, 33, 37, 45, 51, 53, 65, 69] results. But its reduction as stated by some studies [21, 28, 32–34, 51] can also be possible by its leaching in soaked water. Now this lowering of soybean TIs has happened as a result of soaking or some kind of post-processing treatments such as shade or oven or freeze drying given to it for further analysis is questionable. However, BBI is one such inhibitor which may be slowly inactivated [70] or may not be eliminated even by heating as detected in unfermented soy seeds [39]. It was observed to be gradually diminished by solid-state fermentation using L. plantarum [39] or by heating under alkaline [71] and rapidly inactivated in the presence of salt conditions [70].

The roasting like treatments which usually occur at higher temperatures cause moisture loss at a faster rate [72, 73]. This is because of the creation of a large number of capillaries and passable structures in soybean [72]. It was confirmed when a slightly porous structure of endosperm was obtained from roasted soybean sample as observed by its decreased bulk density [72]. Some studies have reported an increase or slight increase (3.2–15% percentage increase) in IVPD of roasted soybeans [23, 36, 72]. This might be due to the destruction of TIs, limited opening of protein structure and reduction in disulfide bonds with breakdown of starch [65, 72]. But, for very high temperatures, IVPD was found to be reduced [74] which could be the result of more evaporation of water [33, 73, 75], non-enzymatic browning reactions or burning of particles and thermal cross-linking of proteins or amino acids [74, 76]. The biological processes such as fermentation and germination can hydrolyze the proteins into smaller peptides with the help of endogenous and/or exogenous enzymes, increasing the protein digestibility.

Altogether this indicates that more KSPs, low moisture content, cellular or tissue integrity and intracellular matrix of soybean seeds have protective effects on soy proteins. Thus, preventing its denaturation and reducing the proteolysis. Hence, optimal thermal treatments are necessary to cause sensible denaturation of soybean proteins. The more the degree of hydration (or water to protein ratio), the lower will be the protein denaturation temperature and the higher will be its denaturation rate. This can be accomplished with soaking treatment followed by processes that may play a role in reducing the heat-stable BBI present in soybean such as heating (under alkaline/NaCl conditions) and fermentation. As per our knowledge, this sequence of operations has not been studied in depth to improve the protein digestibility of whole soybean seeds.

3.2 Oxidation of soybean proteins

The oxidation of proteins can also affect their degree of hydrolysis by digestive enzymes [77]. The digestibility was increased by lower levels of protein oxidation, while it decreased by greater levels [78]. However, no clear correlation between hydrolysis rate and the amount of protein carbonyls in soybean was found when the seeds were heat processed at 100 and 140 ^{o}C [77]. Heat treatments can turn molecular oxygen into reactive oxygen species (ROS), which can cause direct oxidation and fragmentation of proteins [78]. Since the soybean seeds with 10.68% and 29.70% mc required higher temperatures for 11S denaturation, they could form more ROS [57]. This may increase the oxidation of lipids and reducing sugars and can further accelerate the protein oxidation in soybean [57, 77, 79]. This was confirmed when carbonyl content (an indicator of the extent of protein oxidation) was compared between heated and controlled soybean samples with various mc [57]. The carbonyl content of heated samples with 10.68% mc increased to about 20-fold, 29.70% to fivefold, 46.29% to 1.5-fold and for 62.05% mc [57]. This more oxidative degradation was also observed in roasted and soaked + roasted soybean samples as measured by total chlorophyll content [33]. The correlation value between carbonyl content and IVPD of soybean is shown in Table 1.

Ambient or low temperature processing like soaking or mild thermal treatments can reduce the possibility of protein oxidation. So high mc and low oxidation rate together may have a favourable effect on protein digestibility of soybean than the dry-heat treatments such as roasting which usually happens at low moisture and high temperature conditions.

3.3 Aggregation and solubility of denatured soybean proteins

The solubility of proteins is an essential property that can be used as an indirect indicator of protein digestibility [65]. It has been reported that soluble proteins of soybeans may not be as resistant to enzymatic proteolysis as insoluble proteins [80]. The heating which causes denaturation and dissociation of the quaternary structure of proteins has a negative impact on the protein solubility [36, 81]. This is because of the unfolding of soybean globular proteins which exposed more hydrophobic residues (concentrated initially in the interior of the molecule) relative to hydrophilic groups on the external region of the molecule. Thus, giving rise to protein hydrophobicity and lowering protein solubility [65].

The re-assembling ability of unfolded polypeptides (caused by heat denaturation as discussed previously) and protein–protein interaction caused by surface charge variation lead to aggregation of proteins. These microstructural changes in proteins after heat processing were also examined by a study [65] using scanning electron micrographs (SEM) and particle size analysis. It was reported that when the denaturation temperature went up the solubility of protein aggregates significantly decreased in Tris–HCl buffer [57]. This might prevent further increase in surface hydrophobicity or may even lead to its reduction [19]. Limited increase in protein surface hydrophobicity during long heating times was noticed for both non-germinated and germinated samples [19]. This indicated that the degree of aggregation of soybean proteins is limited, which was also confirmed by the SDS-PAGE profiles [19].

During aggregation of proteins, there is a possibility for different intra- and intermolecular interactions, particularly disulfide bonds between amino acids that contain free thiol groups [33]. It has been reported that amino acid residues specific for protease action are localized differently in cross-linked and aggregated proteins. Such residues become less accessible to digestive enzymes, resulting in lowering the digestibility of soybean protein. However, the formation of protein aggregates can occur not only by disulfide bridges but also by non-covalent interactions. It has been found that accessibility of digestive enzymes will be more to the protein aggregates formed by non-covalent interactions, such as hydrogen bonds and hydrophobic interactions than those formed by disulfide interactions due to their less dense or loose structure [57]. It was revealed that an increase of β-sheet and β-turns caused protein aggregates to have a regular and compact structure, leading to reduced protein digestibility [19, 82] as shown in Table 1.

The denaturation or inactivation of KTI induced by heat resulted from its integration into protein aggregates via disulfide and/or non-covalent molecular interactions. However, this effect was found to be restricted in the case of BBI inactivation. NaCl fastens the formation of KTI aggregation and breaks one peptide bond of BBI [70]. The denaturation of both 7S and 11S at 46.29% and 62.05% mc soybean samples resulted in loose and unfolded insoluble aggregates formed by non-covalent interactions [57]. This was measured using Urea and SDS reagents, which disrupt non-covalent interactions and β-ME, a strong reducing agent, which cleaves disulfide bonds [83]. However, the insoluble protein aggregates formed at 200 °C with 10.68% and 185 °C with 29.70% mc soybean samples were not dissolved in the Urea, SDS and β-ME reagents [57]. This might have happened because of the change in the structure

of aggregates attributed to the protein oxidation at high denaturation temperature [84] or as a result of the maillard reaction [64], which not only produced a thiol oxidation product but also covalently bound to Schiff base and other oxidation products to create aggregates [79]. This was inferred from the observation when the soybean samples cooked at 200 °C for 10.68% mc and 185 °C for 29.70% mc produced brown precipitate after centrifugation, while other samples primarily produced white precipitate [57]. Another study [65] also obtained lower protein solubility due to the covering up of charged amino groups such as the epsilon-amino group of lysine while complexing with carbohydrates or reducing sugars.

During fermentation process of soaked and cooked soybeans, the pH can be reduced with the help of proteolytic enzymes produced by inoculated microflora. This further enhances the proteolytic activities, increasing the solubility of protein aggregates by unleashing smaller, more hydrophilic and solvated polypeptide units [36, 65, 85]. Such breakage of proteins to smaller chain polypeptides makes them easily digestible [38]. This study [65] showed the greater protein solubility of the enzyme-modified flour compared to autoclaved flour as its hydrolysis could have been fully completed as no specific bands were seen with SDS-PAGE profile. Other processes such as germination which took place without heating [86, 87] or combined with enzymatic (eg., alcalase) hydrolysis [87] could also bring positive modifications in the protein structure. The activity of native or endogenous proteases improves the soy protein solubility [87] and may prove to be advantageous for digestibility of the protein in gut.

This largely indicates that properties such as protein hydrophobicity and protein solubility will not give the full idea of the protein digestibility of soybean. This is because the less compact structure of low soluble protein aggregates (like random coils) formed by non-covalent interactions can still be susceptible to proteolytic enzymes [64] present in the human digestive system. The optimal heating conditions before and after fermentation and germination respectively along with adequate moisture percentage in soybean seeds can lead to the formation of desirable protein aggregates or their hydrolyzed products, thus improving its digestibility. The effect of heating (cooking or drying) on protein conformations, whether applied before or after fermentation/germination of soybean seeds should be investigated to a great extent.

4 Effect of processing on soybean non-protein components

The non-protein part of soybean comprises about 19% oil, 5% minerals and 35% carbohydrates of which 17% is dietary fibre [26]. Other components that are present in small amounts are phytic acid-related compounds, oligosaccharides, tannins, saponins, starch, vitamins and minerals. Many of these components play bio-defensive role by protecting plants from both biotic and abiotic stresses. In the present era, they are also bringing about potential as therapeutic agents: phytic acid, lowering the risk of cancer, heart issues, kidney stones, and blood sugar levels [88, 89]; oligosaccharides, having pre-biotic qualities and exhibiting anti-allergic, anti-obesity and anti-diabetic properties [90] and saponins, showing anti-inflammatory, antiviral, anti-diabetic, anticarcinogenic and anti-obesity effects [91]. Nevertheless, these components are regarded as anti-nutritional substances and may not be palatable for human consumption. They have the potential to disrupt the digestion of proteins and starches, as well as hinder the absorption of various macro and micro nutrients. The non-protein components such as tannins and phytic acid usually form complexes either by cross-linking with proteins (to limit their digestion) or protein digestive enzymes (to inhibit its activity). This results in reduced protein solubility and susceptibility to proteolytic attack in the gastro-intestinal tract. On the other hand, their reduction can create more space within the matrix, increasing the diffusion of enzymes and making them more likely to come in contact with intracellular proteins, thus improving the digestibility of soybean [22]. Figure 3 gives an overview of how processing operations selectively reduce/eliminate/change heat-sensitive and insensitive ANCs from different parts (hulls, cotyledons) of soybean, thus affecting the protein digestibility of soybean. The efficiency of eliminating ANCs from various regions of soybeans is influenced by the specific form(s) in which ANCs exist, their location within the seed, and the processing conditions applied such as soaking or cooking media/solvent kind (water/salt/acid/base), type (sodium carbonate/sodium bicarbonate/sodium sesquicarbonate), concentration and time–temperature combination, germination or fermentation time–temperature combination, starter culture, fermentation type (solid or liquid), microbial activity, moisture and oxygen levels etc. There could be a possibility of better removal of ANCs from smaller soybean seed sizes, thus enhancing its protein digestibility.

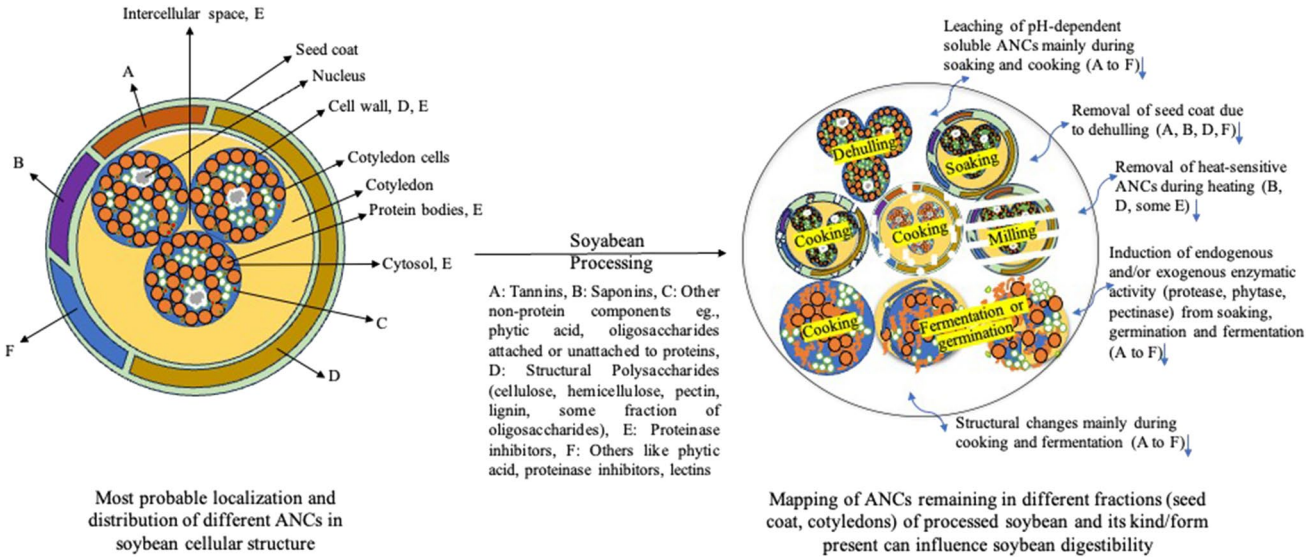

Fig. 3 Schematics of the effect of processing on anti-nutritional compounds of soybean seeds

4.1 Tannins

The mechanism of how tannins act and affect protein digestibility is reviewed in [11]. Briefly, tannins are naturally occurring polyphenolic substances that are water soluble and have a molecular weight of 0.5 to 3 kDa. There are two types of tannins, hydrolyzable and condensed. The hydrolyzable tannins can be easily broken down by acids, alkalis and enzymes to produce glucose, polyhydroxy alcohol, gallic acid or other closely related phenolic acids. Condensed tannins, also known as flavolans, are primarily polymerized products of flavan-3-ol (catechin) and flavan-3,4-diol, or a mixture of these, and they are marginally resistant to hydrolysis. The main polyphenols are condensed tannins, and hydrolyzable tannins are only found in trace quantities. Both kinds of dietary tannins are capable of forming complexes with proteins which can inhibit the activity of proteolytic digestive enzymes, thus decreasing the digestibility of proteins. They can also decrease the solubility of soybean proteins due to their hydrophobic coverage surface, impeding protein digestibility.

Tannins are concentrated mainly in hulls or seed coats of legumes and affect their colour. The dark-coloured legumes presumably have a higher concentration of tannins [92]. Tannins form water soluble and heat-stable complexes with proteins. The reduction in tannins was found by various processing operations [32, 34, 50, 51, 73]: dehulling (upto 48.4%), soaking (upto 93.3%), cooking (100%), fermentation (100%), germination (upto 54.8%), roasting (upto 96.7%). The reasons for their reduction could be the removal of hulls from dehulling, leaching from soaking and cooking and endogenous or exogenous tannase activity from germination and fermentation [50, 93]. An insignificant increase in condensed tannins was found by roasting soybean [34]. The correlation between the amount of tannins and IVPD of soybean is shown in Table 1.

4.2 Saponins

Saponins are naturally occurring triterpene plant glycosides with a carbohydrate component (mono/oligo-saccharides) joined to a lipid-soluble aglycone with a steroidal or triterpenoid structure [92]. They act as protective agents against insects [94]. They can interact with proteins creating complexes capable of suppressing protease activity found in the digestive system.

Saponins are mainly located in the seed coat of pulses. They are heat-sensitive and water-soluble but are not easily affected by dry thermal or non-hydrothermal methods such as roasting and baking [95]. The reduction in saponins has been reported by leaching or through its structural changes from soaking (upto 58.6%), cooking (upto 77.3%) [51], through reduced water solubility due to the removal of side groups of sugars from saponins structure assisted

by β-glucosidase induced from LAB during fermentation (3.5% change with respect to cooking) [39]. However, the effect of saponins on the protein digestibility of soybean is insignificant as shown in Table 1.

4.3 Phytates

Plants produce phytate by the sequential phosphorylation of inositol [11]. Thus, phytic acid or phytates are usually present in the form of inositol phosphate, of which myoinositol hexaphosphate is the most abundant and studied component [11, 92]. It forms complexes with proteins and inhibits the activities of digestive enzymes, such as pepsin, trypsin etc. Phytic acid, which carries a negative charge, can also chelate with positively charged mineral ions such as calcium, zinc, and magnesium [92]. These minerals are essential for activating certain proteolytic enzymes that play a crucial role in protein digestion. This may result in a decrease in the protein digestibility of soybean.

Phytic acid is a relatively heat-stable and water-soluble compound that is mainly located in aleurone's outer portion or the edible cotyledons. Thus, mechanical processing such as dehulling does not substantially reduce it (upto 7%) [33]. But it was found to be reduced by various processing operations [21, 23, 28, 32, 33, 39, 40, 51, 73]: soaking (upto 52.3%), cooking (upto 73.4%), fermentation (upto 64.3%), germination (upto 62.4%), roasting (upto 46.2%), frying (upto 75.4%). The reasons could be (a) water-soluble or pH-dependent leaching or hydrolysis during soaking, cooking, and fermentation (b) destruction by heat from boiling, autoclaving, frying, and roasting (c) poor extractability (d) its breakdown which involves successive dephosphorylation by the increased activity of native phytase during germination, fermentation and soaking [23, 35, 51, 86]. The reduction in phytates was also found due to the production of phytases by inoculated microflora eg., LAB during fermentation. The LAB either catalyzes the conversion of phytates to a variety of partially phosphorylated compounds such as inorganic orthophosphate [96] or it causes the release of phosphoric acids from phytic acid producing inositol. All of these have varying degrees of protein binding ability, but possibly lower than that of native phytates, leading to increased digestibility of soybean protein. Germination and fermentation were found to cause more reduction in phytic acid than autoclaving and roasting [23], thus improving protein digestibility. The correlation value between the amount of phytic acid and IVPD of soybean is shown in Table 1.

4.4 Non-starch polysaccharides

Soybean seeds typically contain around 7–8% hulls predominantly composed of fiber (63.8 to 81.2% total dietary fiber (TDF)). It is an indigestible component of food, made up of lignin (1–4%), oligosaccharides, enzyme-resistant starch and other structural polysaccharides in the cell wall (29–51%, cellulose, 10–25% hemicellulose, and 4–8% pectins [97]). The fibre can be associated with protein, protein inhibitors (trypsin inhibitors) and non-protein (phytic acids) parts reducing the digestibility of protein. It can also make the digestive juices more viscous [98] obstructing the activity of gastrointestinal enzymes. Soybean seeds with thicker hulls can reduce the accessibility of enzymes such as cellulases, xylanases, causing less degradation of cellulose and lignin in seed coat. This may decrease the protein digestibility of whole soybean. The total amount of dietary fibre is reported to be the least in fermented soybean seeds (80.4 g/kg) compared to raw (202.2 g/kg), germinated (177.3 g/kg), autoclaved (179.0 g/kg) and roasted (175.1 g/kg) seeds [23]. Another study [38] found that the fibre content decreases as the fermentation progresses. This is possibly caused due to the leaching of soluble polysaccharides during pre-processing or due to the production of enzymes such as cellulases, hemicellulases etc. by the inoculated microflora during fermentation. These enzymes assist in hydrolyzing and solubilizing the fibre, thus decreasing its content in food. The losses in fibre can also take place due to its mobilization during germination [15]. On the contrary, no significant change in fibre was observed in fermented flour compared to control [40]. Also, a higher amount of crude fibre is reported in germinated seed powder (about 10.5%) than the raw (8.5%), soaked (8.6%), dehulled (5.6%), and roasted soybean (8.4%) samples [33]. This could be due to the production of structural polysaccharides that alter the dietary fibre content in the cell wall during germination [33]. A separate study [17] found the lowest soluble dietary fibre (including carbohydrates) in boiled soybean cotyledon cells (16.2% average) compared to their raw (21%), germinated boiled (21.1%) and fermented boiled (23.2%) counterparts. This could be due to the leaching of soluble fibre like pectins into boiling media resulting in its reduction. We observe that the variation (within the same processing treatment) in the total and crude fibre content of soybean samples across different studies can occur due to the fibre analysis method, soybean variety, type of soybeans (unhulled, dehulled, or ground) used during processing or for further analysis. The dehulling process has the potential to significantly decrease the fiber content in soybean seeds. Milling and cooking operations can individually break down and disturb the intact cell walls of the seeds, rendering the fiber more readily available for enzymatic digestion.

Oligosaccharides such as raffinose, verbascose and stachyose are short chains of sugars with low-molecular weight, also known as α-galactosides or α-galactosyl derivatives of sucrose [99]. They have α-galactosidic bonds and are characterized by α1-6 links between galactose units. These bonds cannot be hydrolyzed by mucosal enzymes of the small intestine due to the lack of α-galactosidase enzyme. This makes the undigested oligosaccharides enter into the lower gut (colon) where they are fermented by gut bacteria and produce intestinal gas such as CO_2 or CH_4 [21, 92]. This causes digestive discomfort depending on the individual tolerance to oligosaccahrides levels. These oligosaccharides can also form complexes with proteins or some digestive proteases decreasing the digestion of proteins in human body. Their content decreases due to leaching during soaking (raffinose: upto 19.8%, stachyose: upto 22.8%) [21, 32, 35], cooking (upto 43.3%) [36], destruction by heat during roasting (– 52.9%) [36], frying (upto 68.1%) [21] and the production of α-galactosidase enzyme by inoculated microflora during germination, fermentation (upto 86.2%) [36, 100].

4.5 Starch

Starch is a complex carbohydrate consisting of glucose units that are linked together, and are primarily breakdown by enzymes like amylase, resulting in the production of simpler sugars like glucose. The amount of starch is very low in soybean (0.2–1%) [101] but, along with proteins it can make the environment of soybean cotyledon cells tightly packed. This may create an extra hurdle for the digestive enzymes to reach soybean proteins [18]. Many studies [17, 36, 38] reported that the fermentation caused the reduction of starch present in raw soybean. This is caused by the production of starch hydrolytic enzymes, for example, amylases (having an amylolytic activity) from inoculated microorganisms during fermentation. They can hydrolyze the starch into simple fermentable sugars [38]. In a separate study [34], germinated soybean was reported to have less amount of starch compared to raw, soaked and roasted soybean samples. This was attributed to starch hydrolysis which gives energy to the growing sprouts during germination. An increase in the starch content can also occur as a result of loss of other soluble components from soybean during processing treatments such as cooking.

The presence of phytic acid can also decrease starch digestibility and consequently, the protein digestibility. The gelatinization of starch granules may be required to improve the protein digestibility of soybean. In a study [75], heat treatments such as cooking and roasting were reported to cause the gelatinization of starch granules. In a different study [72] starch granules did not collapse, thus, preventing the diffusion of water and gelatinization of starch during high temperature and low moisture conditions. Overall, the digestion of starch seems to positively influence the protein digestibility of soybean.

Altogether, the processing of soybean seeds is useful in reducing or eliminating the smaller fractions of non-protein soybean such as tannins, phytic acid and saponins but with varying magnitudes. However, it must be noted that due to the wide diversity of approaches used by researchers to process the soybean, a comparison and statistical analysis of these reduction percentages (mentioned above) is challenging. Different studies may have used different soybean cultivars with varying moisture content; variable processing conditions (soaking/cooking media, time–temperature combination used during soaking, heating, germination, fermentation, natural or controlled fermentation with different inoculum type and amount); post-processing operations to prepare the sample for analysis (drying type, temperature and time, sieving with variable mesh size); storage conditions (time, temperature and relative humidity); particle size; analysis methods and also, a cumulative effect due to some pre-treatments eg., cooking and fermentation preceded by soaking and cooking respectively. Sometimes, the formation of certain complexes makes the extraction of these non-nutritive components difficult from the soybean matrix. Therefore, they remain undetected during certain chemical analyses leading to an overestimation of the reduction in the anti-nutritional compounds. Also, there are instances when the growth of probiotic strains, such as L. plantarum used during germination, have resulted in an over-production of anti-nutritive factors such as a rise in the amount of condensed tannins and the activity of digestive hydrolase or enzyme inhibitors [55]. However, other studies [40, 54, 55] have reported that the IVPD of soybean protein can possibly stay the same with a reduction in these anti-nutritive factors, or even improve with an increase in such factors. This is possibly because the processing induced positive changes in the structure and the proteins of soybean seeds that enable the digestive enzymes to easily access the intracellular proteins, dominate the unfavourable changes such as an increase in the anti-nutritive factors, thus, on the whole enhancing the IVPD of soybean [55]. Although an increment in the anti-nutritive compounds may not affect the IVPD of soybean, caution must be exercised as over-consumption of such compounds may have adverse effects on human health. The overall impact of ANCs could vary based on factors such as an individual's overall dietary composition, digestive physiology, health status, age, and mood.

5 Conclusion

This review provides valuable knowledge on modifications of structure and different components when soybean seeds undergo processing. This includes the changes in the soybean cell and protein structure, permeability and moisture content of seeds, intracellular matrix components etc. Such processing-related alterations have an impact on how soybean protein hydrolysis occurs inside an animal gut. The amount, structure and distribution of KSPs and ANCs (associated with season and other environmental conditions) in different fractions of soybean (seed coat, cell wall, protein bodies) before and after processing can determine the digestibility of soybean protein. The protein digestibility of whole soybean can be enhanced effectively by using a combination of optimal processing treatments such as milling and cooking; soaking, milling and cooking; soaking, cooking, fermentation and wet milling; soaking, germination and cooking etc. The frying was not investigated in available literature as extensively as the other processing operations. The promising combinations to process the soybean are germination combined with either fermentation or boiling, which needs to be investigated in detail for improvement of soybean protein digestibility. The consumption of ANCs is still controversial in terms of its beneficial and adverse effects on human and gut health. Hence, it is not essential to completely remove ANCs for increasing the digestibility of soybean. However, it is suggested that the processing conditions must be fine-tuned to retain the nutritional quality and bring the anti-nutritional components to safe levels while maximizing the protein digestibility and palatability of whole soybean.

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Declarations

Ethics approval and consent to participate Not applicable.

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