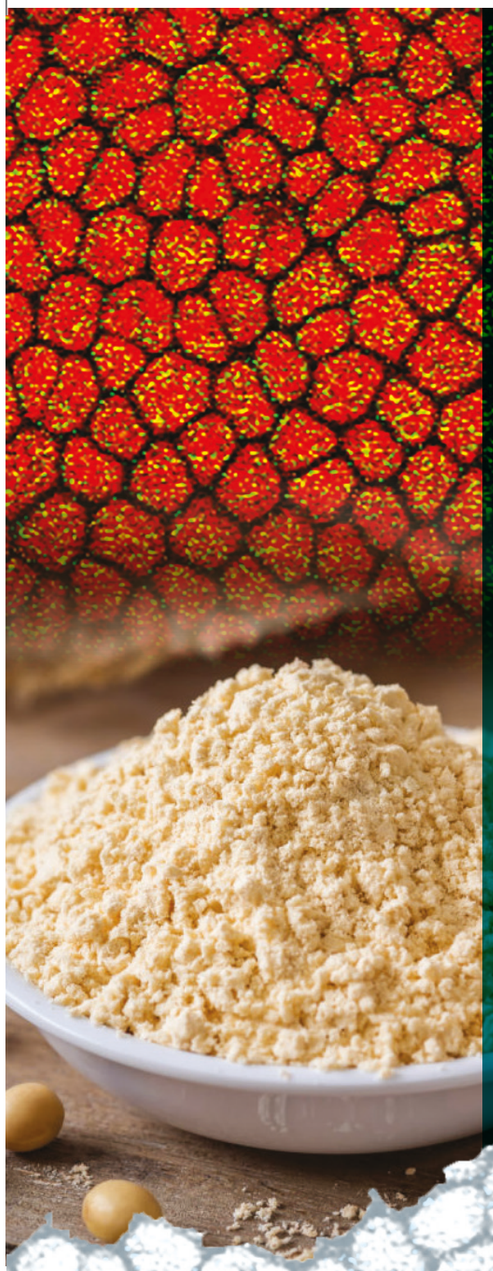
Effect of cell integrity on soybean protein digestion and fermentation: an *in vitro* study Mostafa Zahir 2021



Effect of cell integrity on soybean protein digestion and fermentation:

an *in vitro* study

Mostafa Zahir

Propositions:

1- Plant food structure is the main factor in determining how nutrients are digested and utilized in the human body. (this thesis)

2- Food processing is an indispensable tool to modulate digestive barriers in plant-based foods and improve their protein digestibility.
(this thesis)

3- Poor quality of education in developing countries should be a worldwide concern.

4- Hosting scientists and their findings in daily TV news is the key to engaging a broader public in their activities.

5- A good idea cannot become commercially feasible unless it gains public support.

6- Sustainable farming helps to build economic resilience.

7- When women play a significant role in the peace process, peace becomes more durable, more sustainable, and more inclusive *(Peter-Derrek Ambassador of the Netherlands to Yemen (Oct 25, 2020), the Group of Nine Coalition and UN Women virtual met with the Ambassador of Kingdom of Netherlands to Yemen).*

Propositions belonging to the thesis, entitled

Effect of cell integrity on soybean protein digestion and fermentation: an *in vitro* study

Mostafa Zahir

Wageningen, 26th of February 2021

Effect of cell integrity on soybean protein digestion and fermentation: an *in vitro* study

Mostafa Zahir

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Effect of cell integrity on soybean protein digestion and fermentation: an *in vitro* study

Mostafa Zahir

Thesis

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To my wife Amat Al-salam, and my kids Karim and Jannat

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Chapter 1

General introduction and thesis outline

1.1. Sustainable food protein supply

Currently, food production and natural resource depletion, and environmental degradation are on a collision course [1]. It is argued that the intersection of human and ecosystem health is the major challenge to align future protein supply and demand as shown schematically in Fig. 1.1 [2]. From the land-use perspective, it has been estimated that the land required to raise animals for producing animal protein is 6–17 times greater than the land required for producing plant proteins for human nutrition [1, 3]. The large-scale extension of pasture land for animal protein production at the expense of forests is highly problematic concerning biodiversity loss [3]. The conversion of forests into agricultural lands also has negative consequences on greenhouse gases emission that are tightly associated with climate change [4, 5]. Therefore, shifting towards plant-based protein at the global level would slow down the depletion of resources, land use, biodiversity loss, and climate change [6, 7].

Legumes form an important part of a healthy, balanced diet and have an important role in providing sustainable and healthy dietary protein [8, 9]. Intervention studies have demonstrated the positive effects of high plant protein diets in the treatment and management of obesity, related metabolic disorders, and chronic illnesses [4, 9-11]. For example, one study which tracked 3083 participants through the use of 2 non-consecutive 24-hour dietary recalls, found that plant protein intake was inversely associated with body mass index and waist circumference. Similarly, an eleven-year follow-up study that investigated the macronutrient intake of 469,339 participants concerning the risk of urothelial cell carcinoma found that a 2% increase in the consumption of plant proteins was associated with a 23% decreased risk for developing urothelial cell carcinoma. In contrast, a 3% increase in animal protein consumption was associated with a 15% increased risk for cancer [12]. However, in epidemiological studies, it is not possible to separate the health benefits of plant protein as opposed to the health benefits of other plant dietary components, since plant diet foods often contain different dietary components with favorable health effects [2, 9].

Besides, plant-based protein is deemed to be preferable from animal welfare and ethical perspectives [7, 13]. Society is more and more concerned about animal suffering and welfare in the farming industry. Some consumers have become strongly opposed to the killing of animals, the consumption of meat, and become vegetarians or vegans [14]. It is also argued that animal welfare and ethical reasons could serve as an underlying reason to position plant-based protein as a desirable option from a sustainability perspective [7, 13]. Overall, in the foreseeable future, the growing demand for plant proteins due to sustainability and health reasons turns the focus on their bioavailability for human digestion [15].

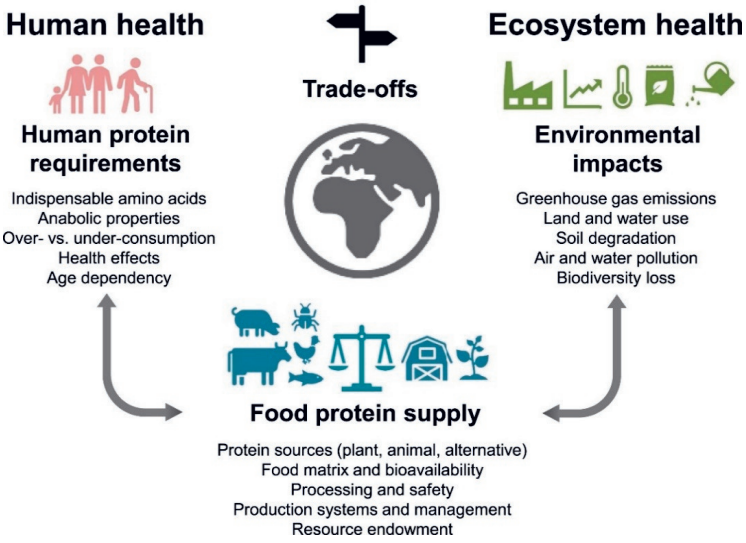

Fig.1.1. Sustainable food protein supply at the intersection of human and ecosystem health [2].

1.2. Digestion of dietary protein in the human gastrointestinal tract

In the stomach, dietary proteins are denatured by the acid and partially hydrolyzed by gastric pepsin, the main proteolytic enzyme found in the human gastric juice [16-18]. Pepsin has broad specificity, and its hydrolysis of food products are fairly large polypeptides, few smaller peptides, and few amino acids [18]. The second phase of protein digestion takes place in the lumen of the small intestine and carry out by pancreatic enzymes. The digestion of protein in the small intestine is a crucial phase due to the specificity of proteolytic enzymes produced by the pancreas, such as trypsin, and chymotrypsin. In the small intestine, proteins are hydrolyzed into small oligopeptides and free amino acids [19, 20]. However, dietary proteins are not equally digestible in the small intestine, and some proteins can survive intact or partially intact before reaching the large intestine. Fig.1.2 shows a schematic representation of protein digestion in the small intestine.

Proteins, peptides, and amino acids that escape the digestion and absorption in the small intestine and reach the large intestine are metabolized by the microbiota via multiple pathways. First, they undergo proteolysis by proteases and peptidases that are secreted by the resident microbiota. The amino acids and peptides (the hydrolysis products) can be then taken up by the bacteria cells in which they undergo different fates of catabolism according to differences in physiological conditions [21-24]. The amino acids metabolism by the gut bacterial produces harmful metabolites compounds such as branched-chain fatty acids (BCFA), ammonia, and hydrogen sulfide [24, 25].

However, amino acids utilized by gut microorganisms contribute to approximately 30% of SCFAs production [26, 27]. SCFAs are an important energy source for colonocytes and exert beneficial effects on the host physiology such as lowering the pH of the colon, growth inhibition of pathogenic organisms, and maintenance of normal bowel structure and function [21, 24, 28-31]. Besides, fermentation of aromatic amino acids produces other bioactive end products such as phenol and indole [25, 32]. Fig.1.3 shows a schematic representation of the bacterial metabolism of proteins in the gut [21].

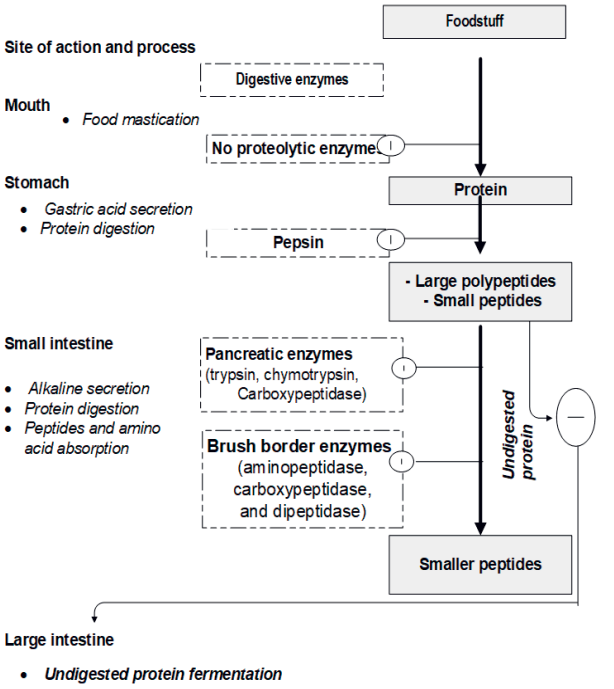

Fig.1.2. Schematic representation of protein digestion in the small intestine [33].

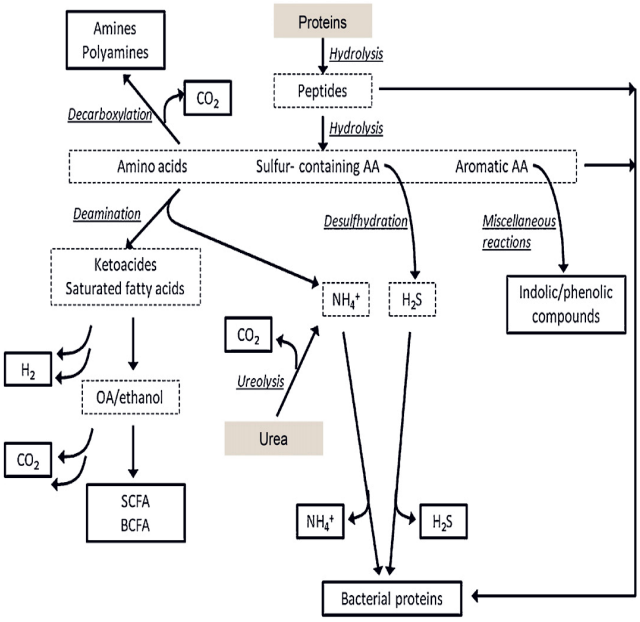

Fig.1.3. Schematic representation of protein metabolism pathways by gut: ■ substrate; ⬚ : intermediary metabolite: ▭ end product [21].

1.3. Factors affecting plant protein digestibility

The digestibility of protein is defined as the proportion of ingested proteins that are hydrolyzed in the small intestine by proteolytic enzymes into amino acids made available for absorption [34, 35]. In comparison with protein from animal sources, proteins from plant sources are characterized by low digestibility [34, 36]. The low digestibility of plant proteins has been attributed to their limited susceptibility to enzyme hydrolysis which is caused by the structural properties of their proteins, the presence of protease inhibitors, and the natural encapsulation of intracellular proteins within the rigid cell walls [37-42].

1.3.1. The effect of protein structural properties

The structural properties of proteins have been recognized as important factors affecting the digestibility of protein [34, 43] In comparison with the structure of proteins of animal origin, the secondary structure of plant proteins is characterized by a high content in β-sheet conformation and a relatively low amount in α-helix [43, 44]. Evidence from *in vitro* studies conducted with cereals, legumes, chicken, and milk products found an inverse correlation between the β-sheet

content of protein and in vitro protein digestibility values [44]. Proteins with a high content of β-sheet have been found to present high resistance to proteolysis [44, 45]. The tertiary structure of plant proteins has also been found to present high resistance to enzymatic hydrolysis. For example, *in-vitro* studies have shown that that the low digestibility of major storage protein, (phaseolin) in kidney beans is attributed to the closely tertiary structure which prevents protease enzymes from reaching the internal sites of hydrolysis [46, 47]. However, plant protein susceptibility to proteolysis can be improved by proper heat treatment due to the unfolding of the tertiary structure as a consequence of protein denaturation [15, 48]. It has been observed that the susceptibility of phaseolin to proteolysis is increased drastically by 80–90 % after heat treatment [47, 49].

1.3.2. The effect of protease inhibitors

Protease inhibitors are naturally found in plant proteins. The types of proteinase inhibitors found in legumes proteins are Bowman-Birk inhibitor and Kunitz inhibitor [38]. Bowman-Birk inhibitor hinders the activity of both chymotrypsin and trypsin, whereas, in the case of Kunitz inhibitor, it only inhibits trypsin [50]. The amount of trypsin inhibitors of different legume seeds ranged from negligible in lupine to very high in soybean [51]. Studies have shown that the presence of high levels of trypsin inhibitors in soybeans and kidney beans causes a substantial reduction in protein digestibility [52]. The trypsin inhibitors have long been recognized not only as an antinutritional component that adversely affects the biological activity of trypsin and chymotrypsin but also as a component that limits pancreatic enzyme secretion in animals [51, 53]. Despite the adverse effect of protease inhibitors on protein digestibility, their effects are eliminated/inactivated during heat treatment in most cases [54, 55]. The thermal inactivation of protease inhibitors is corroborated by several studies to be an important treatment to improve plant protein digestibility [54-58].

1.3.3. The effect of cell wall encapsulation

In nature, edible plant foods are present in highly complex and diverse structures which can be classified into two major groups, namely fleshy structures and encapsulated embryos (see Fig. 1.4). The fleshy plants (e.g., tubers, fruits, and vegetables) consist of groups of cells that retain water and are bonded together at the cell walls. The encapsulated embryos of plants such as grains and legumes contain less water in their intercellular environment that are densely packed with starch, proteins, and fat, within the cells encapsulated by cell walls and assembled into discrete packets [59].

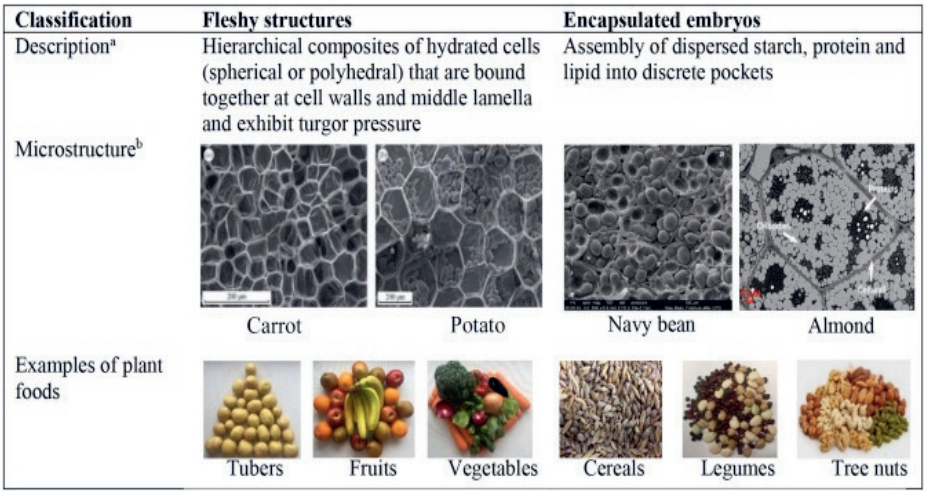

Fig. 1.4. Classification of plant foods in nature into two broad categories; fleshy structures (e.g., tubers, fruits, and vegetables) and encapsulated embryos(e.g., cereals, legumes, and nuts) [60].

The plant cell wall consists of three layers, namely the middle lamella, the primary cell wall, and the secondary cell wall (see Fig. 1.5). The middle lamella is the outer layer that is shared between adjacent cells and composed of pectic compounds and proteins. The primary cell wall is the second layer that is located next to the middle lamella and consisted of pectin, cellulose, and hemicellulose. The secondary cell wall, which is closest to the plasma membrane and rarely present in edible plant foods cells (fruits, vegetables, cereals, and legumes), composes of cellulose, hemicelluloses, and lignin [61-64].

The primary cell wall, which is the main element of the cell wall in plant foods, forms the structural base of the skeleton of the plant foods [64]. Among plant foods, two types of primary cell walls are identified based on the polysaccharide compositions of the wall. Type I primary cell wall is found in dicotyledonous plants such as fruits and vegetables, and non-gramineous monocotyledonous plants. On the other hand, Type II primary cell wall is found in Gramineae such as cereals and grasses. The most abundant polysaccharides in Type I cell walls are pectic polysaccharides and xyloglucans, while, little pectin content and much cellulose and arabinoxylan are found in Type II [65, 66].

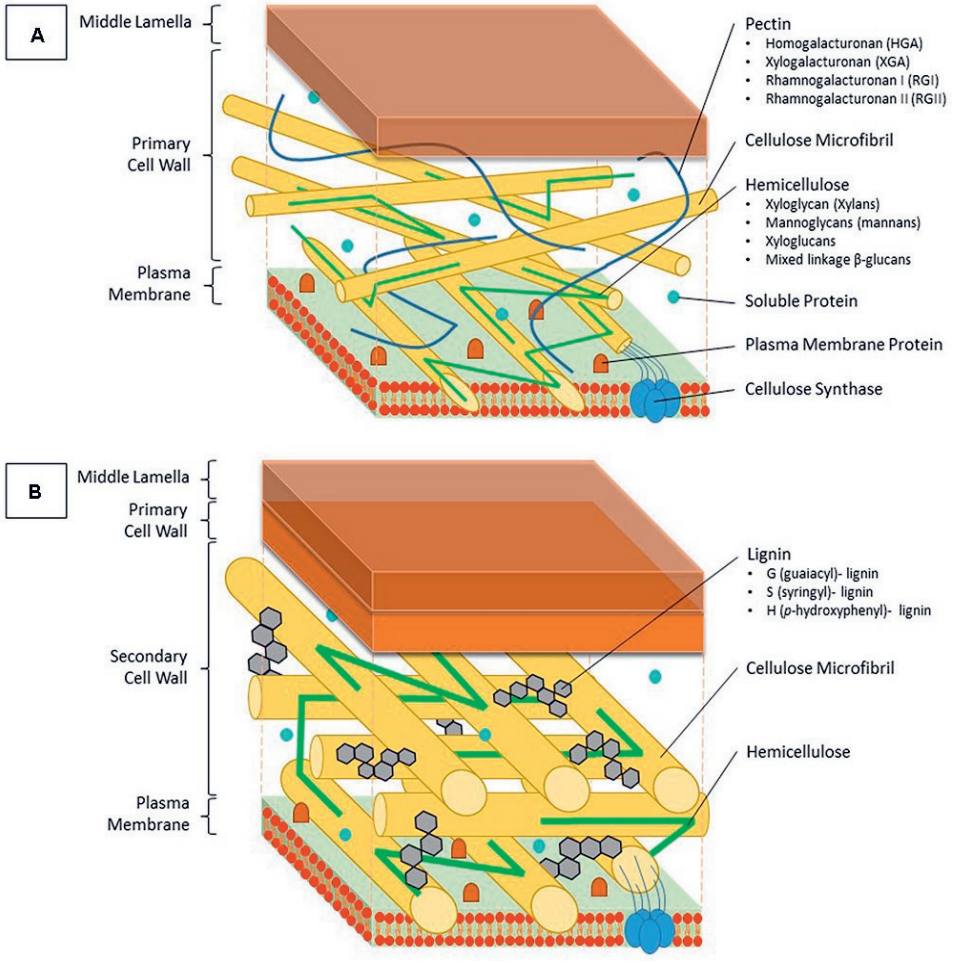

Fig. 1.5. Structure and composition of the primary cell wall (A), and the secondary cell wall (B) [64].

The presence of pectic polysaccharides within the primary cell walls of plant cells is thought to confer a high resistance on them to breakage when subjected to physical disruption (i.e., mastication or mechanical force) after cooking. For example, cotyledon cells of legumes, which contain a higher amount of pectic polysaccharides within their cell walls tend to separate rather than fracture when a mechanical force is applied after cooking (Fig.1.6) [45, 67-70]. It has been suggested that such behavior is mainly attributed to pectin thermo-solubilization within the middle lamella and the primary cell walls. Consequently, this the separation of individual cells whilst maintaining their cellular structural integrity during the application of mechanical force [71].

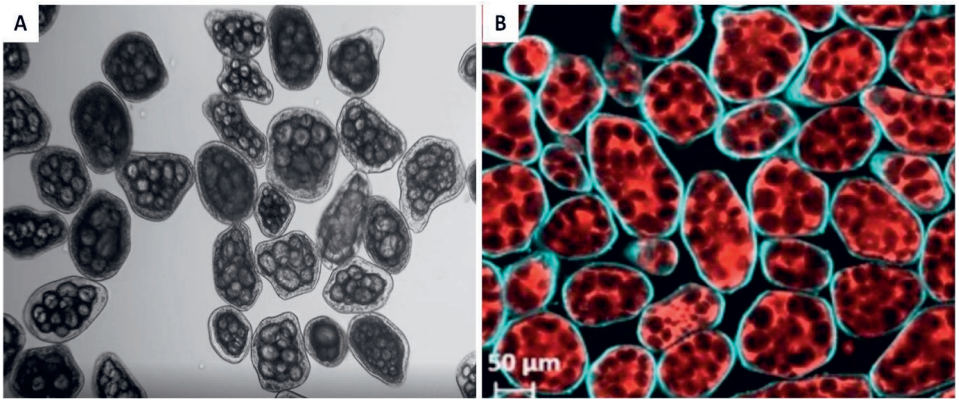

Fig.1.6. Representative light photomicrographs of intact cells isolated from navy bean cotyledon (A) [70] and Confocal laser scanning microscopy image of isolated intact cells from cooked red kidney cotyledon cells (B) [45]. In (image B), the cell wall is highlighted in the light blue stain. Starch granules (black dots) are entrapped by a protein matrix stained in red. Both protein matrix and starch granules are encapsulated within a cell wall.

Because the human digestive enzymes are unable to hydrolyze plant cell wall polysaccharides, the presence of intact plant cell walls that encapsulate macromolecules (starch, lipid, and protein) can act as structural barriers to impede digestive enzymes diffusion upon digestion. Consequently, this thus limiting the hydrolysis of macromolecules. *In vitro* digestion studies conducted on different plant tissues have reported that the occurrence of cell wall intactness during digestion limits the diffusion of the digestive enzymes (i.e., pancreatic α-amylase, and lipase) inside the cellular space, thereby delaying the hydrolysis of macronutrients (e.g., starch, fat, and proteins) [6, 45, 67-69, 72-76]. Evidence from an earlier study [42] highlights the role of cell wall encapsulation in hindering the digestion of macronutrients in plant tissues (Fig. 1.7). Fig.1.7 showed that intracellular nutrients (i.e., lipid, and protein) in fractured cells had been fully digested while those nutrients in structurally intact cells showed no signs of digestion. Indeed, it would be expected that alterations in the cell wall porosity and /or integrity would facilitate the diffusion of digestive enzymes, thereby improve macronutrient digestion. In this sense, all foods processes that could induce cell wall porosity or completely disrupt the cell wall becomes essential for protein digestion and absorption in the upper gastrointestinal tract of humans.

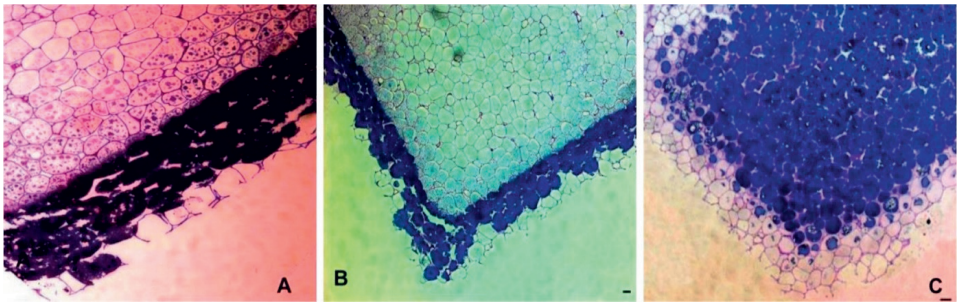

Fig.1.7. Light microscopy images of sections of almond cubes (2 mm) after in vitro or in vivo digestion [42]. Image A shows a section after 3 hours of in vitro gastro-duodenal digestion. It is clear in this image that the nutrients of the cells in the first cellular layer (fractured cells) have been digested. The cell walls and intracellular nutrients are still intact in the underlying cells. Image B shows a section after 3.5 hours of digestion in humans while image C shows a section after 12 hours of digestion in humans. The release of nutrients underneath the fractured surface at about three to five layers is clear in image c.

1.4. Knowledge gap and research objectives

It is well recognized that physical encapsulation of macronutrients by intact cell walls have the potential to modulate the extent of macronutrient intestinal digestion and colonic fermentation. Accumulating evidence elucidating the mechanisms by which intact cell walls in plant-based foods limit the diffusion of α-amylase [45, 67-69, 73, 77-79], and lipase [74, 80, 81] during intestinal digestion, thus limiting the digestion of starch and lipids contained within intact plant tissues. However, compared with what is known about the starch and lipid digestibility contained within the intact plant cellular structure, much less is known about the effect of cellular structure on protein digestion and fermentation. Moreover, the relationship between protein digestibility and particle size reduction, which closely reflects food processing and human mastication and may alter cell wall integrity, has received little attention.

There is an accumulation of evidence in the literature demonstrating that fermented or germinated legumes are superior in their protein digestibility compared to their unfermented or ungerminated counterparts. This is due to the activation of endogenous enzymes that degrade antinutritional factors such as trypsin inhibitors. Because fermentation and germination are cheap and much practiced around the world, they have become of particular interest. Thus knowledge linking germination, and fermentation to plant cell wall porosity and protein digestibility are of importance, especially in populations where there is a need for improved protein nutrition.

The main research objective of this thesis is to elucidate the role of plant cell wall structure in modulating protein digestion and fermentation in plant tissues, with special attention to the net contribution of food processing. The following sub-objectives were formulated to achieve the main objective:

a. Determine the fate of cell wall integrity after boiling and mashing treatments, and how protein can respond differently to the hydrolysis process during digestion, depending on the level of cell integrity in particles of different size and isolated intact cells.
b. Examine the effect of food processing conditions (boiling alone or in combination with germination and fermentation) in modulating cell wall porosity and protein digestibility.
c. Identify the net contribution of cellular integrity during cooking in regulating protein physicochemical changes and how that could affect the extent of protein digestibility.
d. Investigate the role of cellular integrity and heat treatment in controlling legume proteins colonic fermentation.

1.5. Soybean protein as a study model

Soybean (Glycine max) cotyledons have been selected as a plant food model for studying protein digestion and fermentation. Mainly because of their high protein content (>40% on a dry weight basis) located within cotyledon cells. Fig.1.8 highlights the organization of the microstructure of soybean cotyledon cells, and shows how soybean proteins are organized in protein bodies surrounded by oil bodies, and encapsulated by rigid cell walls [82, 83]. Typically, soybeans are cooked and consumed as whole cotyledons. In such conditions, it is generally assumed that the rigid cell walls encapsulating the intercellular protein may reduce the accessibility of digestive enzymes, leading to limited protein digestibility and utilization by the human body. Thus, it is pertinent to consider the role of cell wall integrity in soybean protein digestion and colonic fermentation since soybean protein contains all the essential amino acids necessary for human nutrition [84-87]. Furthermore, soybeans are consumed worldwide, for example, in most Asian countries, soybean is regularly consumed as a staple food and in various products such as soy flour, miso, soy sauces, natto, tempeh, tofu, soy sprouts, and soy milk [88].

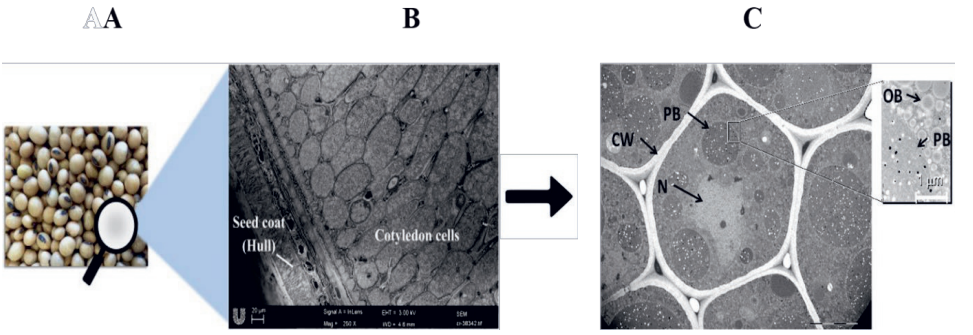

Fig.1.8. A= Image of soybeans (about 5 mm in size), B= Scanning electron microscopy (SEM) image of a pre-soaked soybean (B), and SEM image of dry soybean cells. [83] and C= Transmission Electron Microscopy (TEM) image of soybean cotyledon cell cross-section. PB, protein body; CW, cell wall; N, cell nucleus; OB, oil body [89].

1.6. Thesis outline

In this thesis, we hypothesized that the prerequisite for plant protein digestion from intact tissues in the human body is to fully disrupt cellular structure barriers in plant cells before and/or during digestion. This is because cell wall integrity can hinder the access of digestive enzymes to the intracellular space of intact plant cells, thus limiting macronutrient digestibility. A systematic approach to assess the effect of cellular structure on protein digestibility and how it possible to use food processing as a tool to modulate this effect has been investigated in this thesis. A schematic overview representation of the thesis outline is presented in Fig.1-9.

In **Chapter 2,** food breakdown and size reduction that takes place during oral processing were simulated by mechanical force and sieving procedure. The cellular structure disintegration (cellular integrity) as a result of particle size reduction and the cooking procedure was visualized using confocal microscopic. Furthermore, the relationship between cell wall integrity of soybean tissues (i.e., particle sizes and intact cells) and protein digestibility was determined. Besides, the modulating effect of the simultaneous digestion of intercellular oil on intercellular protein digestibility was assessed as the digestion process of macronutrients in plant tissue is known as a cooperative process and that efficient hydrolysis of one substrate may be affected by the simultaneous hydrolysis of another.

The cell wall porosity and permeability of plant tissue is a critical factor in limiting digestive enzymes diffusion to intracellular nutrients and thus delay nutrients digestion. Therefore, in

Chapter 3, cell wall porosity and permeability of boiled soybean were measured at the microscopic level. Furthermore, the potential role of other food processes (e.g., germination and fermentation), as well as the potential role of protease enzymes used during *in vitro* digestion in modulating cell porosity, and permeability of cells of cooked soybean cotyledons were investigated.

As the protein physicochemical properties determine the chemical accessibility of protease enzymes to protein and protein digestibility, the role of cellular integrity in controlling the protein physicochemical changes during cooking was studied in **Chapter 4**. Also, the potential role of germination in limiting the effect of cellular integrity on protein physicochemical changes during cooking was investigated, as the cellular integrity, and protein physicochemical properties could be altered by other food processes **(Chapter 4)**.

To get insights into the role of cellular structure/ food processing in steering colonic protein fermentation, a batch fermentation study using different raw and cooked soybean tissue was conducted and described in **chapter 5.**

In Chapter 6, the main findings of all the chapters are summarized and integrated. The implications of these findings in relation to soybean protein digestibility and its utilization by the human body are discussed. Finally, a general discussion of the methods used in this thesis, and further research directions were proposed.

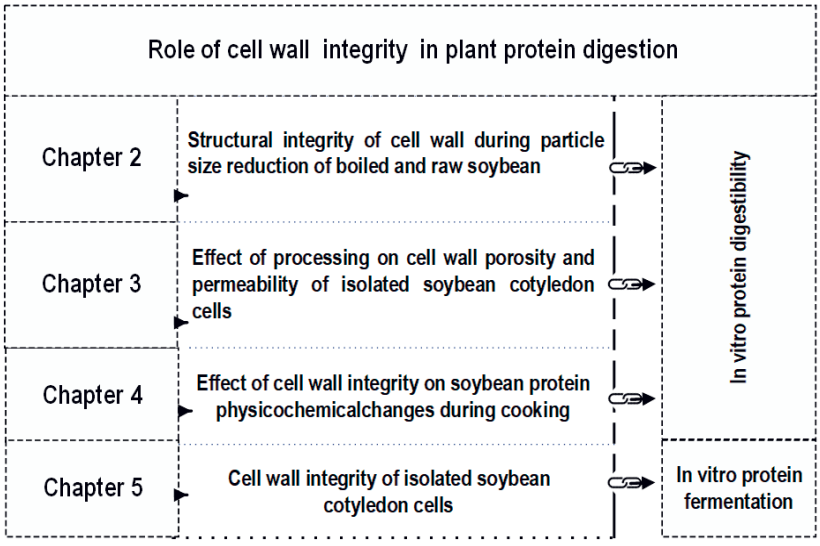

Fig.1.9. Schematic overview of the thesis.

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Chapter 1

2

Chapter 2

Food matrix and processing modulate *in vitro* protein digestibility in soybeans

This chapter is based on:

Mostafa Zahir, Vincenzo Fogliano, Edoardo Capuano. Food matrix and processing modulate in vitro protein digestibility in soybeans. Food Function., 2018, 9, 6326

Abstract

Soybeans represent the largest source of plant proteins on the planet but their proteins are associated with low digestibility. Although several studies addressed the limiting factors affecting the rate and extent of soy protein digestion, the net effect of the food matrix, especially of an intact cell wall, has been poorly investigated so far. The purpose of the present study was to examine the relationship between the cell-matrix and protein hydrolysis during simulated *in vitro* digestion of soybean particles of different sizes prepared from unheated and boiled cotyledons. In addition, intact cells were isolated from unheated and autoclaved cotyledons and then digested with and without lipase inhibitors to understand the impact of an intact cell wall and the presence of oil bodies on soybean protein digestibility. Protein digestibility was the highest in the particles prepared after boiling of previously milled cotyledons compared to particles of the same size obtained by milling previously cooked cotyledons as well as uncooked cotyledons. Protein digestibility in isolated intact cells was lower than that of extracted proteins regardless of the thermal load applied whereas inhibition of pancreatic lipase reduces protein digestibility only slightly. The data indicated that the cell wall could contribute to limit protein digestion in soybean tissues; however, it is not an absolute barrier to pancreatic proteases. An accurate design of the milling and cooking process could be instrumental to modulate the digestion kinetics of soybean proteins.

2.1. Introduction

There is nowadays an increasing demand for a sustainable supply of plant protein and soybeans, being the major source of plant protein in the human diet, greatly contribute to meet this demand [1, 2]. The structural features of soybean cotyledons and the way soybeans are cooked and consumed may influence protein digestibility [3]. The cell wall of soybean cotyledons, like that found in other legumes, is mainly composed of pectin [4], which are affected by the storage conditions and become less degradable upon cooking [5]. Consequently, legume cotyledon cells are able to maintain their intact structure when the legumes are cooked as whole cotyledons [5, 6].

Recent studies have provided evidence that the presence of an intact cell within plant tissues during digestion restricts the access of digestive enzymes and the hydrolysis of intracellular starch in navy beans [7] and red kidney beans [8], as well as intracellular lipid digestion in almond [9] and hazelnut [10]. These studies observed an increase in starch and lipid hydrolysis when the cell wall structure is damaged by mechanical or enzymatic treatments either before or after cooking. Despite the modulating role of the cell wall being very much investigated for starch and lipid digestion, much less is known about the barrier effect of the cell wall on plant protein digestion [11]. An additional structural feature that is thought to play a critical role in macronutrient digestibility is the interaction with other macromolecules. Indeed, a recent study has shown that the digestibility of each lipid, starch, and gluten in wheat flour is affected by the interaction with the remaining components [12]. In a tightly packed environment such as legume cotyledon cells, the presence of protein and starch/lipids may represent an additional barrier for the diffusion of digestive enzymes to their substrate. Another recent report has demonstrated that the efficient hydrolysis of starch is strongly affected by the simultaneous hydrolysis of protein in kidney beans [8]. Soybean seeds own a unique cellular structure in which starch disappears in the final stages of seed maturation, and soybean cotyledon cells are mostly constituted of protein bodies immersed in a lipid matrix of individual oil bodies [13].

Preparations from plant foods having different particle sizes have been widely used in *in vitro* digestion studies, especially to understand the behaviour of plant tissues during mastication and its implications on nutrients digestibility. The macronutrients in plant foods may be digested to different extents, depending on the degree of particle size reduction, cell rupturing or disruption within the particle and crowded cellular environment [12, 14]. Investigation of protein digestibility in legumes with different particle sizes and which have undergone different cooking and mashing

procedures could elucidate the rate-limiting factors in legume protein digestion. The aim of this study was, therefore, to monitor the fate of the soybean cell wall and intracellular matrix during different cooking and mashing procedures as well as during digestion to understand the role of the cell-matrix in protein bioavailability in soybeans.

2.2. Materials and methods

2.2.1. Materials and reagents

Soybean seeds were obtained from a local windmill (De Vlijt, Wageningen, The Netherlands) and stored at room temperature. Ethylenediaminetetraacetic acid (EDTA), porcine pepsin (P6887, 3.200–4.500 U mg−1 protein), pancreatin (P1750, 4X USP), porcine bile extract (B8631), Pefabloc® SC, orlistat (≥98%, solid), sodium dodecyl sulfate (SDS), O-phthaldialdehyde (OPA), DL-dithiothreitol (DTT), L-serine, rhodamine B, BODIPY 505/515 and calcofluor white were purchased from Sigma-Aldrich Ltd (St Louis, MO, USA). Trichloroacetic acid (CAS 76-03-9) and disodium tetraborate decahydrate (CAS 1303-96-4) were bought from Merck & Co. (Darmstadt, Germany). All chemicals used for the simulated digestive fluids were of analytical grade and were obtained from Sigma Aldrich or Merck.

2.2.2. Preparation of samples

2.2.2.1. Preparation of soybean particles

Soybean seeds were soaked in 3 parts of ice-chilled water overnight and the hulls were removed manually. The dehulled soybean cotyledons were then placed in laboratory bottles and distilled water was added (1 : 3 w: v). The mixture was then autoclaved at 121 °C for 10 min or boiled at 100 °C either for 3.5 h or 1 h using a water bath. Both autoclaving and boiling for 3.5 h resulted in substantial cell separation as shown in Fig. 2. 1a, b, and c, so boiling for 1 h was used to produce cooked particles (Fig. 2. 1d). To determine the behaviour of cotyledon cells when a mechanical force is applied either before or after cooking and its implications on protein digestibility, one part of the dehulled cotyledons was boiled first and then mashed; this sample will be hereafter indicated as BM particles (boiled-then-mashed particles). The second part of the dehulled cotyledons was first mashed and then boiled; this sample will be hereafter indicated as MB particles (mashed-then-boiled particles). An additional sample was included as control where no heating treatment was used; this sample will be hereafter indicated as RM particles (raw-mashed particles). Each paste of BM, MB, and RM was loaded on a stack of sieves with 5 aperture sizes: 1000–2000, 425–1000, 250–425, 125–250, and 71–125 μm, and rinsed with water (wet sieving). The collected

materials were stored at 5 °C until used for further characterization and *in vitro* digestion experiments.

2.2.2.2. Preparation of intact cells of soybean cotyledons.

Intact cells of cooked cotyledons (IC-CC) were isolated following the method of Dhital et al *[15]*. with some modifications. Briefly, the soybean seeds were soaked and dehulled as previously described in section 2.2.1. The dehulled cotyledons were autoclaved at 121 °C for 10 min instead of boiling at 100 °C for 1 hour to induce cell separation whilst facilitating intact cell isolation. The cotyledons were gently mashed and sieved as described in section 2.2.1. Confocal microscopy observations confirmed that the material, which passed through a sieve of 125 µm but was retained on a sieve of 71 µm, mainly contained free intact cells. The isolated intact cell fraction was then dispersed in sodium azide solution (0.02%). For isolation of intact cells of uncooked cotyledons (IC-UCC), soybean whole seeds were kept suspended in a solution containing 3.8% EDTA (pH 10) and 0.02% sodium azide at 37 °C for 2 days with gentle stirring by using a magnetic stirrer [16]. The separated seed coat was removed and then the cotyledons were mashed and sieved to obtain isolated raw intact cells. The collected fraction in the size range of 71–125 µm was also examined by confocal microscopy in order to confirm the isolation of intact cells. Both IC-CC and IC-UCC were stored at 5 °C and subjected to *in vitro* digestion within 24 h of their isolation.

2.2.2.3. Preparation of extracted soybean protein

Protein extraction from uncooked and cooked soybean cotyledons was performed according to Wang et al [17]. with minor modifications. In short, ground cotyledons were suspended in Milli-Q water at a 1: 10 (w/v) ratio. The pH of the mixture was adjusted to 8.5 with 2 N NaOH and kept under constant stirring at room temperature for 30 min. The dispersion was then centrifuged at 14 000g, at 15 °C for 30 min and the insoluble part was then discarded. The protein in the separated supernatant was precipitated at pH 4.5 with 2 N HCl and stored at 4 °C for 60 min. The refrigerated supernatant was then subjected to centrifugation at 14 000g, at 4 °C for 30 min to reduce the protein solubility in the whey. The isolated curd was neutralized at pH 7 using NaOH and stored at −20 °C until use.

2.2.3. Determination of protein content, moisture content and final dry content adjustment

The total protein content was determined in triplicate based on a dry basis with the Dumas method using a Flash EA 1112 NC analyser (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's protocol. A protein conversion factor of 6.25 × N was used to calculate the protein content [18]. To determine the moisture content of the samples, the standard oven drying method at 105 °C for 24 h according to Suthar *and Das [19]* was used. The final dry matter content was adjusted to 25% (w/w) prior to the digestion experiment by means of oven drying at 45 °C.

2.2.4. In vitro protein digestion

The *in vitro* digestion for the determination of protein hydrolysis was performed for all sample preparations in duplicates according to the harmonized INFOGEST protocol [20] except that the electrolyte solutions were prepared following the modified version of Mat et al [21]. This involved replacing NaHCO3 with NaCl to maintain the pH of the sample mixture stable at 7.0 during the intestinal phase. The digestion procedure was composed of an oral phase, a gastric phase, and an intestinal phase. Based on a final digestion volume of 40 ml, 5 g of the sample was mixed with 5 mL of pre-warmed simulated salivary fluid (SSF) without salivary α-amylase to begin the oral phase. The mixture was incubated at 37 °C for 2 min and then subjected to gastric phase digestion by mixing with 10 mL of warmed simulated gastric fluid (SGF) containing 1.6 mL of freshly prepared porcine pepsin stock solution of 25 000 U mL−1 and the pH of the mixture was adjusted to 3.0 with HCl prior to incubation for 2 hours. The intestinal digestion phase was started by adding the gastric digest to 20 mL of pre-warmed simulated intestinal fluid (SIF) containing 2.5 mL fresh bile (160 mM) and 5.0 mL of an 800 U mL−1 pancreatin stock solution in order to achieve a trypsin activity of 100 U mL−1 in the final mixture. The pH was then raised to 7.0 with 1 M NaOH and the mixture was incubated for 2 h at 37 °C. The digestion was performed in a laboratory incubator with constant mixing using a rotator shaker. Samples (1 mL) of the digesta were taken at 0, 30, 60, and 120 min of intestinal digestion. To stop the enzymatic activity the protease inhibitor Pefabloc® (5 mM) was added to the obtained sample and the mixture was stored at −20 °C until further analysis. Blank digestion was performed by using a mixture of simulated digestion fluids and the same concentration of pancreatin and bile but the 5 g of sample was replaced with Milli-Q water.

2.2.5. Inhibition of lipase

In a separate experiment, to study the effect of the interactions between lipids and proteins on protein digestibility, pancreatic lipase was inhibited before starting the intestinal digestion phase. Briefly, 5 µg of a lipase inhibitor (orlistat) was added for each mg of pancreatin used to prepare pancreatin solution and then incubated for 25–30 min to allow the inhibition of pancreatic lipase prior to undertaking simulated intestinal digestion as described by Bhattarai et al [12]. The use of orlistat in pancreatic digestion to inhibit lipase has been reported previously [22, 23].

2.2.6. Separation of free amino acids and peptides

Free amino acids and peptides from the digested samples were prepared by precipitating intact and undigested protein by trichloroacetic acid addition [24]. In brief, 0.83 mL of 5% TCA was mixed with 0.5 mL of the digested sample followed by centrifugation at 10 000g for 30 min at room temperature. The supernatants were then filtered by using a 0.45 µm syringe filter (25 mm diameter, 0.45 µm pore size PVDF membrane).

2.2.7. Determination of degree of hydrolysis (DH%)

The concentration of free amino groups released after *in vitro* digestion was measured by the O-phthaldialdehyde (OPA) spectrophotometric assay according to the method of Nielsen et al [25]. with minor modifications. In brief, the OPA solution was freshly prepared for every experiment as follows: 7.620 g disodium tetraborate decahydrate was completely solubilized in 150 mL of Milli-Q water. Then, 160 mg o-phthaldialdehyde was dissolved in 4 mL ethanol and added to the above solution. Finally, 200 mg of sodium dodecyl sulfate (SDS) and 176 mg of 99% dithiothreitol (DTT) were dispersed in the solution and the volume was made up to 200 mL with Milli-Q water and further mixed. Then the solution was covered with aluminium foil to prevent the development of color that could influence the subsequent measurements. To determine the absorption value, 3 mL of the OPA reagent was added to 400 µL of each of the tested samples (blank, standard solutions, and digested supernatants) and the solution was mixed for 5 s using a vortex mixer. The reaction mixture was then allowed to stand for exactly 2 min at room temperature before the measurement was taken at 340 nm. The free amino group concentration of *in vitro* digested and acid hydrolysis samples were determined with reference to a calibration curve constructed using

L-serine (12.5–100 mg L−1) prepared in phosphate buffer whereas the degree of protein hydrolysis (DH %) was calculated according to the method of Schasteen et al *[26]*. using Eqn (1):

$$DH\% = \frac{NH_2\ (final) - NH_2\ (initial)}{NH_2\ (acid) - NH_2\ (initial)} \text{ x } 100 \qquad \text{equation 1}$$

where NH2 (final) is the concentration of free amino groups in the hydrolysate of the digested sample, NH2 (initial) is the concentration of free amino groups in the undigested sample (at time 0 of digestion), and NH2 (acid) is the total content of free amino groups in the sample completely hydrolyzed in 6 N HCl at 110 °C for 24 h.

2.2.8. Confocal microscopy

Based on the fluorochrome binding affinity to cell microstructures, the fluorescent dyes calcofluor white, rhodamine B, and BODIPY 505/515 were used to stain the cell wall, protein bodies, and oil bodies respectively [27-29]. The three dyes were diluted with demi water to a final concentration of 0.002 wt% for calcofluor white and 0.001% for rhodamine B and BODIPY 505/515. For slide preparation 30 µl of the dye mixture was added to 30 µl of the homogenized sample previously placed in a glass slide. Samples were visualized through the use of a confocal scanning laser microscope (CLSM) type 510 (Zeiss, Oberkochen, Germany) using a 405 nm blue/violet diode laser for calcofluor white, a 543 nm HeNe laser for rhodamine B, and a 488 nm argon laser for BODIPY. All images were acquired using a 10/20 EC Plan-Neofluar/0.5 A lens and analysed with ZEN blue edition (Carl Zeiss Microscopy).

2.2.9. Statistical analysis

The amounts of free amino groups were measured in triplicate for each sample and the data were presented as means and standard deviation of three replicates using Microsoft Office Excel 2016. A linearized form of limited exponential kinetics (Eqn (2)) has been used for the interpretation of the *in vitro* digestibility data.

$$\frac{\mathrm{DH}\%_t - \mathrm{DH}\%_\infty}{\mathrm{DH}\%_0 - \mathrm{DH}\%_\infty} = kt \qquad \text{equation 2}$$

where DH%0 is the DH% at the beginning of the intestinal phase; DH%∞ is the limiting value for DH% taken as 100%; DH%t is the DH% at time t and k is the rate constant for protein digestion.

2.3. Results

2.3.1. The impact of particle size reduction and cooking conditions on the protein content of soybeans

The protein content of soybean particles of different sizes is presented in Table 2.1. Particles of smaller size contained much fewer proteins in the RM preparation as compared to the BM and MB preparations having the same size. More specifically, in the MB samples, the smallest particles have the same protein content as the biggest ones, while for the BM samples only a limited decrease is observed. The loss in protein content as a function of milling has been reported for decreasing the size of wheat flour, [30] and it is in line with the large decrease found in the RM samples of our study.

Table 2.1. Protein content (%) on a dry basis of soybean particles of different size (mean ± standard deviation, n = 3). RM= particles from raw soybean, BM = particles obtained from boiling and then mashing of soybean, MB= particles obtained from mashing, and then boiling of soybean.

Sieve sizes (µm)	*Treatments*		
	RM	BM	MB
1000-2000 µm	42.03 ± 0.85	42.11 + 1.02	41.82 + 1.26
425-1000 µm	40.02 + 2.52	42.33 + 0.53	34.47 + 1.27
250-425 µm	28.41 + 0.33	38.15 +1.34	33.06 + 0.70
125-250 µm	20.84 + 1.02	35.74 + 0.55	30.57 + 0.65
71-125 µm	12.56 + 1.10	32.84 + 0.76	40.84 0.35

2.3.2. Microscopic characteristics of soybean cotyledons after autoclaving and long and short cooking time

To optimize the procedure to obtain particles of different sizes and intact cells in appreciable yield, preliminary experiments were carried out where the soybeans were subjected to thermal treatments of variable severity. The microscopy images in Fig. 2.1 clearly indicates that soybean cotyledon cells preserved their intactness despite prolonged cooking times, and the extent of cell separation has an inverse relationship with cooking times. For this reason, we decided to apply relatively milder cooking (boiling at 100 °C for 1 h) to produce the soybean particles of different sizes with an appreciable yield of cells. To isolate intact cells, we found it more convenient to use a relatively more severe thermal treatment (at 121 °C for 10 min), which reduced the intensity of the grinding step as well as the fraction of broken cells while increasing the yield of intact cells.

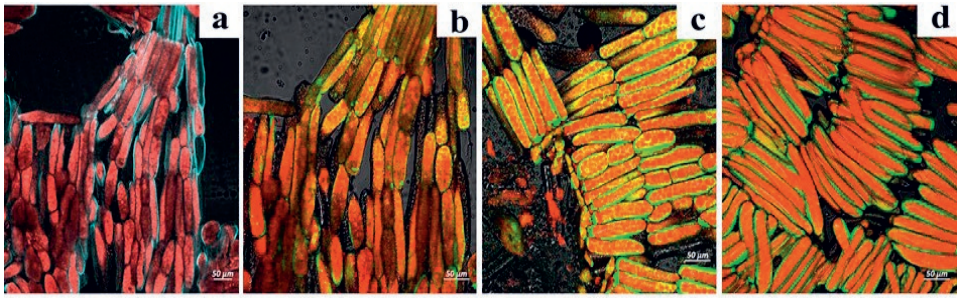

Fig. 2.1. Confocal laser scanning micrographs of soybean cotyledons. Autoclaved at 121 °C for 10 min (a and b); boiled at 100 °C for 3.5 h (c); and boiled at 100 °C for 1 h (d). Cell wall (depicted in blue) only in (a), protein bodies (depicted in red), and oil bodies (depicted in green) were stained with calcofluor white, rhodamine B, and BODIPY 505/515 respectively. These micrographs clearly indicated that soybean cotyledon cells preserved their intactness despite different/prolonged cooking times, and cell separation has an inverse relationship with cooking time.

3.3.3. Microscopic observation of the particle size before and after digestion

In Fig.2.2 representative micrographs of soybean particles of selected sizes before *in vitro* digestion are shown. The micrographs of RM particles (panels a1 and a2) show that most particle cells remained physically intact after milling and tightly adherent to each other. Nevertheless, ruptured cells can be seen on the surface and the core of the particles. Likewise, observation of BM particles (panels b1 and b2) reveals that the particles contained mainly intact cells; also the strong adhesion between cells is apparent but to a lesser extent compared to RM particles. The micrograph of BM particles shows that damaged cells are mostly observed on the particle surface. MB preparations of particles (panels c1 and c2) show more ruptured cells, both at the surface and the cell layers immediately beneath, compared to both RM and BM particles. In the MB samples, most of the cells are seemingly emptied and only a few still retain proteins and lipids in apparently intact cells in the core of the particles. On the other hand, intact protein bodies can be identified within the cells for both BM particles (Fig.2.2 b1 and b2) and RM particles with a size range of 125–250 µm (Fig.2.2 a).

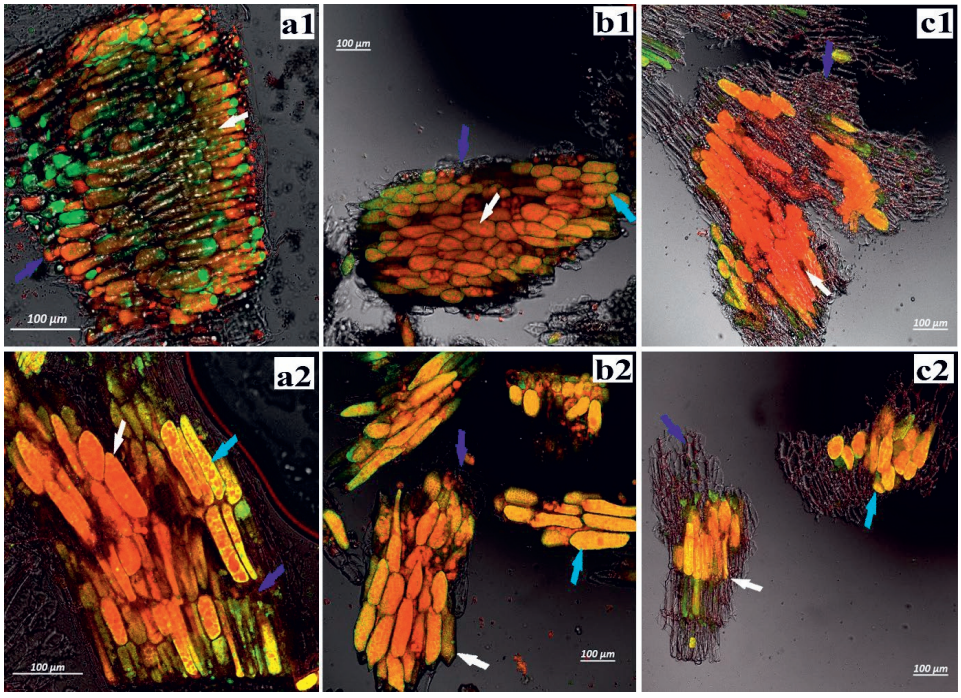

Fig. 2.2. Confocal laser scanning micrographs of soybean particles before digestion; Panels a1 and a2 = particles prepared from raw-milled cotyledons (RM particles); panels b1 and b2 = particles prepared from boiled-and-then-mashed cotyledons (BM particles); panels c1 and c2 = particles prepared from mashed-and-then-boiled cotyledons (MB particles). Top panels (a1, b1, and c1) display particles with a size range of 250–425 µm. Bottom panels (a2, b2, and c2) display particles with a size range of 125–250 µm. Protein bodies and oil bodies were stained with rhodamine B (red) and BODIPY 505/515 (green/yellow) respectively. The intact cells in all images are indicated by the white arrows whereas the ruptured cells are indicated by the blue arrows. In panels b1, a2, b2, and c2, the light blue arrows indicate the uniformly distributed protein bodies and oil bodies.

In Fig.2.3, representative micrographs of soybean particles after *in vitro* enzymatic digestion are shown. The images clearly showed that cells of the tissues retained their physical structure; however, a substantial amount of intracellular proteins was digested from ruptured cells. Oil bodies had coalesced and are concentrated at the inner face of the cell wall of structurally intact cells from cooked preparations (Fig. 2.3 b and c).

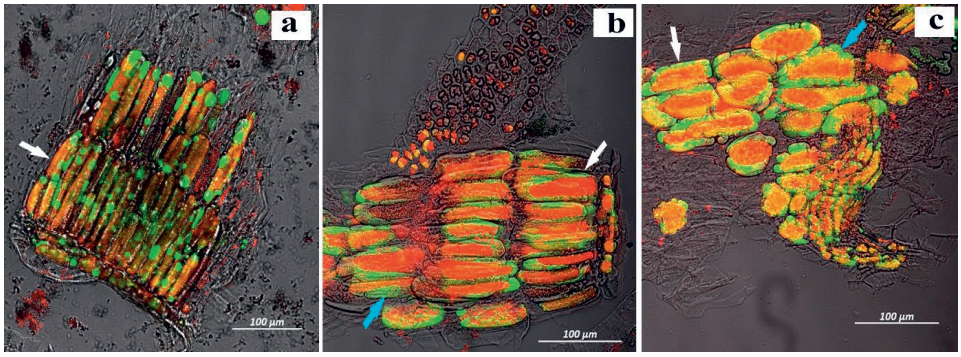

Fig.2.3. Confocal laser scanning micrographs of soybean particles (125–250 µm) after *in vitro* digestion; Panel a = particles prepared from raw milled cotyledons (RM particles); panel b = particles prepared from boiled-and-then-mashed cotyledons (BM particles); panel c = particles prepared from mashed-and-then-boiled cotyledons (MB particles). Protein bodies and oil bodies were stained with rhodamine B (red) and BODIPY 505/515 (green/yellow) respectively. The intact cells in all images are indicated by the white arrows whereas the light blue arrows indicate the coalesced oil bodies in panels b and c.

2.3.4. Microscopic observation of isolated intact cells before and after digestion

The micrographs of intact cells of cooked cotyledons (IC-CC) and intact cells of uncooked cotyledons (IC-UCC) before and after digestion are shown in Fig. 2. 4. The IC-CC and IC-UCC appear to be cylindrical/elongated in shape with an average length of 100–150 µm and a diameter of 10–40 µm; see Fig. 2 .4 (panels a1 and b1). The micrographs of IC-CC before digestion show clear evidence of physically intact cells compared to the micrographs of IC-UCC. In Fig. 2. 4 a1, it can be seen that the intracellular proteins and lipid bodies are uniformly distributed within IC-CC and IC-UCC, which is in line with what can be observed in Fig.2.1(b and c) and 2(a2, b1, b2, and c2). However, it appears that in IC-CC, oil bodies (stained in green) tend to coalesce and move toward the periphery of cells (Fig.2.4, panel a1), as also observed in digested particles (Fig.2.3, panels b, and c). The microscopy images of the digested IC-CC (Fig.2.4, panels a2 and a2i) indicated a variable level of digestion of intracellular proteins, with most of the cells partially digested and a few cells either completely full or completely emptied. The observation of the image clearly suggests that the more severe the cell wall damage, the more completely emptied the cell after digestion. Not surprisingly, the micrographs of IC-UCC (Fig.2.4, panels b2 and b2i) show that the cells maintained their structural integrity after digestion with negligible digestion of the intracellular material.

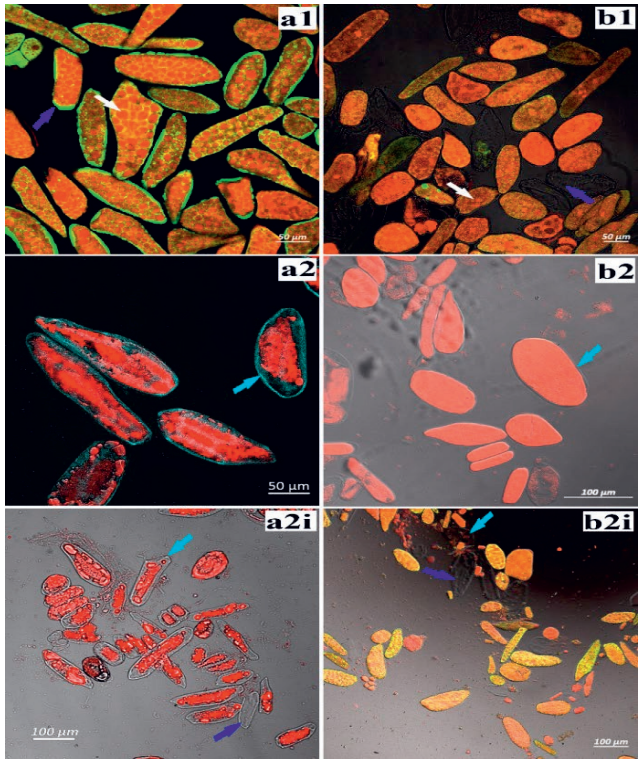

Fig. 2. 4. Confocal laser scanning micrographs of intact cells of cooked soybean cotyledons (IC- CSC) and intact cells of uncooked soybean cotyledons (IC-UCSC) both before and after *in vitro* digestion; panel a1 = IC-CSC before *in vitro* digestion; panels a2 and a2i = IC-CSC (higher and lower magnification respectively) after *in vitro* digestion; panel b1 = IC-UCSC before *in vitro* digestion; panels b2 and b2i = IC-UCSC (higher and lower magnification respectively) after *in vitro* digestion. Cell wall, protein bodies, and oil bodies were stained with calcofluor white (blue), rhodamine B (red), and BODIPY (green/yellow, depending on the extent of staining) respectively. Intact protein bodies in panels a1 and b1 are indicated by the white arrows. The broken cells in panels b1, a2i, and b2i and the coalesced oil bodies in panel a1 are indicated by the blue arrows. The light blue arrows indicate the digested area in panels a2, a2i, b2, and b2i.

2.3.5. *In vitro* protein digestibility of soybean fractions

Protein digestibility, measured by the OPA method and expressed as the degree of protein hydrolysis (DH%), is presented in Fig. 2. 5. In general, a trend towards an increase in DH% as the particle size decreases is evident regardless of the treatment applied to soybeans. In particular, the fractions with a size higher than 425 µm have a very low protein digestibility (DH% < 10%) at the end of intestinal digestion, whereas the DH% values, increased with a decrease in particle size reaching values of 29.5, 38.4, and 48.0% for particles of the size range of 71–125 µm of RM, BM and MB preparations respectively. The differences in DH% between the BM and MB fractions

of the same size range clearly indicated the importance of the way in which cotyledons were cooked. For all the particle sizes, the MB fractions have the highest DH% value, but substantially lower than the DH% value of the extracted protein (89.80%) as shown in the bottom right panel of Fig.2.5.

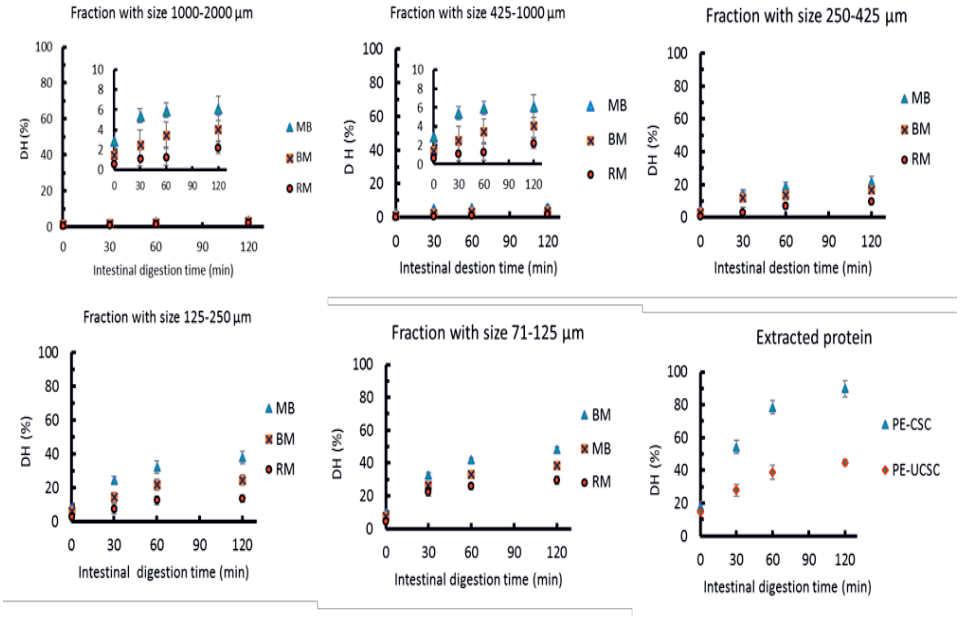

Fig. 2.5. Degree of protein hydrolysis (DH%) during the duodenal digestion of soybean fractions; RM = particles prepared from raw-milled cotyledons; BM = particles prepared from boiled-and- then-mashed cotyledons; MB = particles prepared from mashed-and-then-boiled cotyledons; PE-CSC = protein extracted from cooked soybean cotyledons; PE-UCSC = protein extracted from uncooked soybean cotyledons. For particles with size 1000–2000 µm and 425–1000 µm, a zoom-in is provided in the inserts. Data are reported as mean ± SD of two digestions.

2.3.6. *In vitro* protein digestibility of intact cells with and without the addition of a lipase inhibitor

The protein hydrolysis (DH%) of intact cells of cooked cotyledons (IC-CC) and intact cells of uncooked cotyledons (IC-UCC) with and without the use of a lipase inhibitor is presented in Fig. 2.6. Fig.2.6 shows that DH% in IC-CC is 63.66%. This is higher than the DH% of MB and BM particles of comparable size (48.0% and 38.4%) and IC-UCC (37.17%). When a lipase inhibitor was used with pancreatin to prevent the simultaneous hydrolysis of lipids, a slight decrease in the DH% of IC-CC from 63.66% to 57.90% was observed. Comparatively, a significant decrease was observed in DH% of IC-UCC from 37.17% to 29.00%.

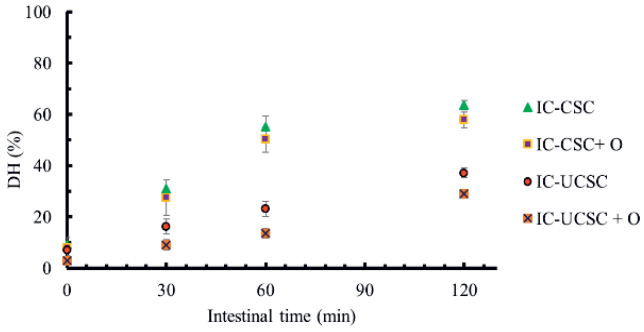

Fig. 2.6. Degree of protein hydrolysis (DH %) during the duodenal digestion of intact cells of cooked cotyledons and intact cells of uncooked cotyledons with and without the use of a lipase inhibitor; IC-CSC = intact cells of cooked soybean cotyledons; IC-UCSC = intact cells of uncooked soybean cotyledons; IC-CSC + O = intact cells of cooked soybean cotyledons digested with the use of orlistat (lipase inhibitor); IC-UCSC + O = intact cells of uncooked soybean cotyledons digested with the use of orlistat (lipase inhibitor. Data are reported as mean ± SD of two digestions.

2.4. Discussion

It is well recognized that in plant-based foods, the direct contact between intracellular macronutrients and digestive enzymes can be hindered by the presence of an intact cell wall [11]. Based on the confocal laser scanning micrographs of various particle sizes presented in Fig. 2.2, most cells of soybean particles underlying the fractured surface retained their intact structure despite cooking and digestion. The intact cell wall barrier is able to slow down or completely prevent the access of proteases inside the cell, therefore limiting intracellular protein hydrolysis. This phenomenon can be clearly quantified by the rate and extent of protein hydrolysis reported in Fig.2.5.

The microscopy images are shown in Fig.2.1 provide insights into the behaviour of cotyledon cells and their microstructures during cooking and milling and subsequently the fate of intercellular proteins under *in vitro* digestion conditions. When cotyledons are milled before boiling (MB particles), the strong “glue” between adjacent cells made up of pectin-rich middle lamellae would affect the mechanical breakdown and result in a relatively higher fraction of broken cells on the particle surface (see Fig.2.2, panels c1 and c2). Interestingly, when intact cotyledons are boiled before mashing (BM samples), the solubilization of pectin in the middle lamella would reduce the cell-cell adhesion force, which would produce a relatively higher fraction of intact cells on the

particle surface upon milling (Fig.2.2, panels b1 and b2). Furthermore, it was observed that the softening of soybean cotyledons after cooking is positively affected by the cooking method/time and correlated with cell separation as shown in Fig.2.1. Thus when cotyledons boiled at 100 °C for 1 hour were used as the starting material to produce intact cells, an extensive physical strength was needed during mashing compared to autoclaved cotyledons. This resulted in a substantial amount of broken cells during sample preparation (images not shown). For this reason, it was decided to cook soybeans at 120 °C for 10 min in an autoclave, which was found to be more effective for the isolation of intact cells compared to boiling at 100 °C for 1 hour. From a nutritional perspective, the positive correlation between heat treatment and degree of cell separation upon mechanical stress has important consequences on the size distribution of bolus particles and thus on protein digestibility as will be discussed below. However, extended cooking time or cooking under pressure caused the oil bodies inside the cell to coalesce and migrate towards the inner face of the cell wall (Fig.2.1b and c; Fig.2.4, panel a1). A similar finding has been reported by Kasai et al [31]. In contrast, the coalescence of oil bodies was not observed in intact cells isolated from uncooked material (Fig.2.4 b1). Coalescence of oil in intact cells isolated from autoclaved soybeans may have been facilitated by the denaturation of the protein layer surrounding individual oil bodies.

As shown in Fig. 2.5. protein digestibility is determined by the presence of an intact cell wall. The DH% of the extracted protein generated from soybeans (where no physical barrier exists that can hinder the contact between the pancreatic protease and protein substrates) was 41% higher compared to the DH% in the heated intact cells (Fig.2.6) where an intact cell wall was preserved. The values of *in vitro* protein digestibility reported in our study are in good agreement with an early report [32], which observed a decrease in *in vitro* protein digestion ranging from 58% to 72% in some legume preparations that were thought to contain intact cells. An increased protein digestibility ranging from 77% to 89% was associated with the disruption of legume cells. The results of our present study suggest that proteases can trespass the cell wall of intact cells prepared from autoclaved soybean cotyledons and improve the hydrolysis of intracellular proteins up to 63.66% (Fig.2.6). This value was higher when compared with values reported in another study [33] for the protein digestibility of intact cells obtained from boiled legumes (e.g. chickpeas, kidney beans, peas, and mung beans). The adopted thermal treatment in our study is likely to be one of the reasons behind the increase in protein digestibility.

The inverse relationship between particle size and protein DH% is the key highlight in Fig.2.5. The same trend was reported for cowpea protein hydrolysis by Tinus et al [34]. who suggested that the milling condition is one of the most important factors regulating the level of protein digestion. The increase in protein digestibility with reduced particle size can be explained by the fact that smaller particles will expose relatively more surface than bigger particles per unit weight. Since the cells on the surface of the particles are damaged by mechanical treatment, most cells are likely to be ruptured in smaller particles; thus the intracellular proteins are more susceptible to come in contact with digestive enzymes. The higher loss of proteins from smaller RM particles can also be seen from the protein content data reported in Table 2. 1. The DH% values reported in our research for intact soybean cells provide evidence that an intact cell wall can contribute to limit protein digestion in plant tissues but it is not an absolute barrier to pancreatic proteases. Proteases can trespass the cell wall in soybeans and digest proteins locked within cell walls like those placed in the core of particles beneath the layer of damaged cells on the surface. It is worth noting that the kinetics of digestion of proteins locked within intact cells will be diffusion-limited, i.e. limited by the time needed for the digestive enzymes to progress towards the core of the particles [35, 36]. This may represent an additional explanation for the slower kinetics of protein hydrolysis observed in bigger particles.

Interestingly, heat treatment increases the rate and extent of protein digestion. This is, per se, not surprising and can be explained by a combination of the following mechanisms: (1) increased porosity of the cell wall to digestive enzymes due to solubilization of pectin, (2) thermal inactivation of trypsin inhibitors that are present in soybeans [37] and (3) heat-induced protein denaturation [38]. The latter can increase the susceptibility of protein to pancreatic protease [38-40], because of the exposure of peptidic bonds normally hidden in the hydrophobic core of globular proteins. Solubilization of pectin from type I cell walls is well known [41] and changes in pectin structure have been suggested to modulate starch digestibility in potato [42]. However, the magnitude of the effect of pectin solubilization on enzyme diffusion within legume cells and consequently on protein digestion has not been deeply investigated yet. The adopted experimental design, which demonstrated the effect of heat treatment, was substantially different depending on whether it was applied before or after milling. Protein digestibility was higher in milled and then boiled soybean particles compared to particles of matching size that have been first boiled and then milled. The most probable explanation for this is related to pectin solubilization from the cell wall after cooking and the effect it has in determining the fraction of intact and broken cells after grinding which is much higher when cooked samples are milled [43].

Apart from the barrier effect of an intact cell wall, cellular integrity may contribute to modulate macronutrient digestion also by providing a relatively compact intracellular environment which may represent an additional barrier for the diffusion of digestive enzymes. In addition, molecular interaction between macromolecules may interfere with their digestion and this may be more so in a “crowded cellular environment”. In raw soybeans, protein bodies are evenly distributed within a matrix of oil bodies, whereas coalescence of oil bodies and accumulation towards the periphery of the cells are evident after thermal processing. We hypothesized that the presence of lipids would affect the digestion of proteins, especially in cooked soybeans where coalesced lipids seem to form a protective layer at the inner face of the cell wall. We have therefore used orlistat, which is a known lipase inhibitor[22, 23], to study the interactions between lipids and proteins in raw and cooked soybeans. Contrary to our expectations, the effect of intact or coalesced oil bodies on protein hydrolysis was higher for intact cells of uncooked cotyledons (IC-UCC) compared to intact cells from cooked cotyledons (IC-CC) (Fig. 2. 6), presumably because of the loss of the compact native microstructural organization of oil and protein bodies as a result of heating. However, the effect of lipids on protein digestion in soybeans is rather limited and substantially smaller than that observed in other matrices and for other macronutrient pairs such as the effect of proteins on starch digestion observed in kidney beans [8].

Usually, protein digestion follows typical exponential kinetics which is reported also for starch and is justified by the decrease in the digestion rate due to substrate depletion as digestion proceeds [44]. Since substrate depletion is not complete or digestion is monitored by the increase over time of a hydrolysis product, the protein (and starch) digestion kinetic data are commonly described and fitted by limited exponential kinetics. Notwithstanding the few data that were used to generate the kinetic rate analysis, the kinetic plots in Fig. 2. 7 indicated a more complex behaviour during protein digestion in our samples. If limited exponential kinetics applies, a semilogarithmic plot of ln((DH%t − DH%∞)/(DH%0 − DH%∞) as a function of time should produce a straight line (see eqn (2)), which is the case for particles with a size range of 1000–2000 μm, extracted proteins, and isolated cells. However, this was not the case for particle size smaller than the size range of 425–1000 μm. A reduction in the rate constant for protein hydrolysis can be noted in those samples represented by a curve with upward concavity. We suggest that this behaviour is explained by the existence of two distinct fractions of proteins in a particle with sizes in the range less than 425–1000 μm: a fraction of proteins released from damaged/ broken cells that are freely accessible to digestive enzymes and a fraction of proteins that are still locked within intact cells. The first fraction is digested at a faster rate and accounts for the relatively higher slope of the plot at an early stage

of digestion whereas the second is digested at a slower rate and explains the smaller slope at a later stage of digestion. In other words, the upward concavity that is observed in particles with a size less than 425–1000 µm is the result of more complex kinetics which is better described by a double exponential. This hypothesis is corroborated by the fact that similar behaviour has been already described for starch digestion kinetics in wheat and chickpea particles [45]. Interestingly a monophasic behaviour is observed under the two extreme conditions, bigger particles and extracted protein; however this is due to two opposite reasons. In large particles of 1000–2000 µm size, the fraction of "locked" proteins is by far more abundant than the small fraction of proteins from the relatively small particle surface which explains the substantially monophasic behaviour in protein digestibility in those samples. Similarly, a monophasic behaviour is observed in the extracted protein and intact cells, where no locked protein (in the extracted protein) or free proteins (in isolated cells) are present.

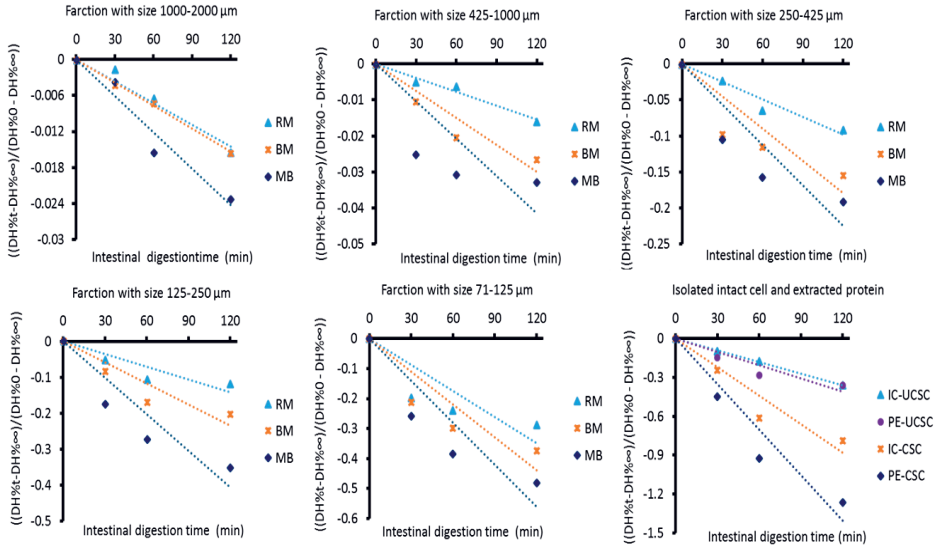

Fig. 2.7 Semi-logarithmic plot of DH% against digestion time for different soybean fraction sizes; RM = particles prepared from raw-milled cotyledons; BM = particles prepared from boiled-and-then-mashed cotyledons; MB = particles prepared from mashed-and-then-boiled cotyledons; IC-CSC = intact cells of cooked soybean cotyledons; IC-UCSC = intact cells of uncooked soybean cotyledons; PE-CSC = protein extracted from cooked soybean cotyledons; and PE-UCSC = protein extracted from uncooked soybean cotyledons. Data are reported as means of two digestions. Standard deviations have been omitted for clarity.

2.5. *Conclusion*s

The results of the present study indicated that the rate and extent of digestion of soybean proteins depend on the fraction of broken cells wherein proteins are freely accessible to pancreatic proteases. This is determined in turn by the mechanical forces applied to the sample (i.e. particle size produced) and the way soybeans are treated, e.g. whether milled before or after cooking. In any case, cooking had a large, positive impact on protein digestibility, likely by increasing protein denaturation, including that of protease inhibitors and possibly modifying protein-lipid interactions in the cell matrix. These results improved the knowledge about the rate-limiting factors in soybean protein digestion and suggested that tailored processing strategies can be used depending on consumer needs. For instance, a cooking design in which grinding seeds is applied before boiling could be employed as a sustainable technique to reduce the longer cooking time and the firewood energy consumption for the use of legumes in developing countries [46]. On the other hand, cooking of intact legume seeds should be preferred in a product aimed at the slow release of energy and amino acids and delivery of nutrients to the microbiota [47]. The results of this study also indicated that more in-depth studies of the effect of pectin solubilization, cell wall permeability, and oil coalescence could provide extra knowledge to improve the digestibility of soybean proteins.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Chapter 2

3

Chapter 3

Effect of soybean processing on cell wall porosity and protein digestibility

This chapter is based on:

Mostafa Zahir, Vincenzo Fogliano, Edoardo Capuano. Effect of soybean processing on cell wall porosity and protein digestibility. Food Function., 2020, 11, 285.

Abstract

Apart from the presence of antinutritional factors, digestibility of soybean proteins is limited in intact cells by cell wall permeability to proteolytic enzymes. Food processing may modulate cell wall permeability and hence the accessibility of protease enzymes to intracellular proteins. In this study, soybeans were processed in various ways, e.g. cooking applied alone or with either germination or fermentation processes, and the modification in cell wall permeability was investigated using confocal microscopy to visualize the penetration of FITC-dextran probes into isolated cells/cell clusters. Diffusion of fluorescently labelled trypsin into cells and cell clusters was also monitored. Microscopy observations showed that fermentation and germination as well as proteolytic enzymes increase the permeability of boiled soybean cotyledon cells. The diffusion of trypsin into all the isolated cells was observed at an early stage of simulated in vitro digestion, whereas diffusion into cell clusters was delayed due to a bigger size and limited permeability of cell clusters. A modest, although significant, increase in protein digestibility was observed when boiling was combined with fermentation or germination likely due to pre-digestion of storage proteins and inactivation of trypsin inhibitors. This study highlights the positive role of fermentation and germination in improving protein digestibility in soybeans but overall suggests that cell wall permeability to trypsin plays a minor role in the extent of protein digestion of intact soybean cells.

3.1. Introduction

Legumes are important sources of proteins in human diets, but their bioavailability is often lower than that of animal proteins. An efficient protein utilization by humans is an essential pre-requisite for protein nutritional quality and even a strategy to fulfil the growing global demands for proteins, especially in developing countries [1-3]. Soybeans are one of the major sources of plant proteins [1]. It is well known that the digestibility of soybean proteins is affected by the presence of trypsin inhibitors [4] but recently the role of the structural integrity of cell walls has emerged. An intact cell wall is thought to act as a barrier reducing the accessibility of the digestive proteases to the intracellular proteins [5].

Heat treatment is the main process applied to soybeans to make them edible. However, due to the substantial amount of energy required, long thermal processing (namely >3 hours) is an unaffordable technique, especially in some developing countries [2, 6]. It has been postulated that the combination of heat treatment and relatively inexpensive treatments such as fermentation or germination may enhance the bioaccessibility of soybean proteins during digestion. The improved digestibility is attributed to “pre-digestion” of storage proteins into smaller peptides and inactivation of trypsin inhibitors [7-9].

The natural porosity of the cell wall can be greatly enhanced by thermal treatments but also modulated by milder processing like germination or fermentation. During cooking, legume cotyledon cells tend to separate as a result of the partial solubilization of pectin within the middle lamella that glued adjacent cells. This in turn affects the structural integrity of cell walls [10]. A major change in the cell wall structure is predicted when other food processes (i.e. fermentation or germination) are applied to legumes. Previous studies have suggested that both microorganisms and microbial proteolytic and carbohydrate-degrading enzymes during fermentation may cause cell wall polysaccharide degradation or solubilisation, and thus modify their architecture [11, 12]. In the same manner, other studies have pointed out that cell wall polysaccharides are mobilized during the germination process as a result of the metabolic reactions naturally occurring [13, 14].

There is very limited information about the net impact of cooking and the combined effect of heating and fermentation/germination on the changes in plant cell wall porosity and permeability. In particular, it is not known if and how it is possible to use the combination of cooking and fermentation/germination to modulate the rate of protease enzyme diffusion through intact cell walls and plant tissues. Therefore, in this study, we examined the changes in the permeability of

cell walls of processed soybean cotyledon cells after the boiling process alone or in combination with other food processes (i.e. fermentation or germination) or after an in vitro digestion process using FITC-dextran probes (20 kDa, 40 kDa, 70 kDa, and 150 kDa). We further investigated the diffusion of fluorescently labelled trypsin into cells in processed and digested soybeans. The changes in protein digestibility as affected by the applied processing procedures were also explored and correlated with the changes in cell wall permeability.

3.2. Materials and methods

3.2.1. Materials

Dried soybean seeds were purchased from De Vlijt (Wageningen, The Netherlands) and stored at room temperature.

Amyloglucosidase (3300 U mL−1) and an integrated Total Dietary Fiber Assay Kit (K-RINTDF 09/18) were purchased from Megazyme (K-TSTA, Megazyme, Co. Wicklow, Ireland). Pectinase from Aspergillus niger (P2736), fluorescein isothiocyanate conjugate tagged dextran (FITC-dextran) of four different molecular weights (20, 40, 70, and 150 kDa), calcofluor white, rhodamine B, bodipy 505/515, Nile red, trypsin (porcine pancreas 1000–2000 U mg−1), α-chymotrypsin (bovine pancreas ≥40 U mg−1), pepsin (porcine gastric mucose 3200–4500 U mg−1), pancreatin (P1750, 4X USP), Porcine bile extract (B8631), pefabloc® SC, sodium dodecyl sulfate (SDS), O-phthaldialdehyde (OPA), DL-dithiothreitol (DTT), and L-serine were purchased from Sigma-Aldrich, (USA). Trichloroacetic acid (CAS 76-03-9) and disodium tetraborate decahydrate (CAS 1303-96-4) were bought from Merck & Co. (Darmstadt, Germany). An Alexa Fluor™ 488 Protein Labeling Kit (A10235) was purchased from Invitrogen (Carlsbad, CA, USA). NuPAGE® 4–12% Bis-Tris Gels, NuPAGE® LDS sample buffer (4× concentrated), reducing agent (10× concentrated), MES running buffer, and Mark 12 Unstained Standard for SDS-PAGE analysis were provided by Thermo fisher scientific (Van Allen Way Carlsbad, CA, USA). Other chemicals used in this study were of reagent grade.

3.2.2. Sample preparation

3.2.2.1. Thermal processing of soybean cotyledon

Dried soybean seeds (100 g) were soaked for 12 hours in 300 mL of tap water at room temperature to simulate soybean domestic preparation. The excess water after soaking was discarded and the seed coat was removed manually along with embryo. The dehulled cotyledons were prepared in fresh tap water (1 : 3 w: v) in laboratory bottles and boiled at 100 °C for 3.5 hours using a water

bath. Our previous work has shown that a prolonged cooking time is necessary to induce cell separation whilst facilitating intact cell isolation from soybeans [5].

3.2.2.2. Preparation of cotyledon cell clusters and isolated intact cells

The procedure of isolation of cotyledon cell clusters and intact cells was adapted from the method reported by Dhital et al [15]. and described in detail in our previous work [5]. Briefly, the boiled cotyledons were gently mashed using a mortar and pestle and subsequently subjected to wet sieving. The fraction of particles with a particle size range of 180–315 μm was obtained and used as the cell cluster sample. The confocal microscopy observations confirmed that the fraction in the size range of 45–71 μm is mainly constituted of free intact cells; this fraction will represent the boiled cotyledon intact cells hereafter indicated as BC.

3.2.2.3. Soaking in salt and pectinase treatment

Soaking in salt and pectinase treatment served as comparative models for inducing cell wall permeability. Soaking in salt was carried out as described in section 2.1.1 replacing the water with a solution containing 0.5% NaHCO3, and 2.5% K2CO3, (w/v). Pectinase treatment was carried out as follows: a set of cell clusters of soaked and cooked cotyledon in water (10 g) was mixed with 5 mL acetate buffer (pH 5.5) and treated with 200 μL of pectinase for 2 h at 40 °C under constant agitation. The pectinase from Aspergillus niger used here contains mainly pectintranseliminase, polygalacturonase, and pectinesterase and small amounts of hemicellulases and cellulases (information from the manufacturer).

3.2.2.4. Cotyledon cell fermentation

Intact cells of cotyledon previously boiled (BC) were fermented to prepare a boiled-fermented cell sample. The fermentation was carried out with the method described by Silva, et al [16]. with some modifications. Two hundred mg of dried commercial baker's yeast (Saccharomyces cerevisiae) was mixed with 30 g of BC (~75% moisture). The sample was then fermented at 40 °C for 60 h in a laboratory incubator. The fermented sample was washed thoroughly with water and the soluble material was removed through filtration using Whatman® glass-fiber and the remaining pellet was immediately used for the diffusion experiment or dried in an oven at 60 °C for 2–4 h before use in the digestion experiment. The drying step allowed normalizing the protein content of the sample before the simulated digestion and avoided the underestimation of the

contribution of the fermentation process in protein solubility or pre-hydrolysis. This sample will be hereafter indicated as boiled cells fermented (BCF).

3.2.2.5. Germination and cell isolation

Dried seeds were washed in running tap water and disinfected with 0.07% sodium hypochlorite before soaking in tap water (1 : 3 w: v) for 6 h. The excess soaking water was then drained off and the seeds were placed on a paper in trays. Samples were germinated for 4.5 days at 27 °C in darkness using a laboratory incubator. Water was spread on the seeds once daily to provide a moist atmosphere during sprouting. The germinated seeds that have the same length of sprouts were carefully collected to ensure a homogeneous sample, the sprouts and seed coats were removed and the cotyledons were then washed thoroughly with water. The cleaned cotyledons were boiled at 100 °C for 3.5 hours followed by mashing and sieving as described above in section 2.2.1 to obtain isolated intact cells of germinated–boiled cotyledons (referred to as GBC).

3.2.3. BC, BCF, and GBC chemical composition analysis

Prior to the analysis of the chemical composition, samples were subjected to intense milling to break down the cell structure and to avoid underestimation of the macronutrient content. Samples were analysed in triplicate for moisture, insoluble and soluble dietary fibre (IDF), lipid, protein, starch, and ash contents using standard analytical methods. The moisture content for all samples was determined based on a standard oven drying method at 105 °C for 24 h. The insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) including carbohydrate were approximately estimated by an enzymatic-gravimetric method using the rapid integrated total dietary fibre assay kit (AOAC Method 2017.16). Total lipid determination was performed with automatic Soxhlet using hexane as a solvent. The protein content was analyzed based on the
Dumas method using a Flash EA 1112 NC analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's protocol. For the determination of total ash, the AOAC method, 2000 was employed. Starch content was determined by a Total Starch Assay Procedure (amyloglucosidase/α-amylase method, Megazyme Inc, Bray, Ireland).

3.2.4. BC, BCF, and GBC microstructural characterisation

Microstructural characteristics of BC, BCF, and GBC cells were visualized using a confocal scanning laser microscope (CLSM) type 510 (Zeiss, Oberkochen, Germany) according to the procedure reported in detail in our previous study [5].

3.2.5. Microscopy observation of dextran probe permeation in processed soybean cells

Fluorescein isothiocyanate dextrans (FITC-dextran) of four molecular weights 20, 40, 70 and 150 kDa were dissolved in phosphate-buffered saline (PBS) at pH ~ 7.0 to a final concentration of 2 mg mL^{-1}. One mL of each FITC-dextran solution was added to 100 mg of suspension of the freshly prepared sample (cells clusters/isolated cells) in microcentrifuge tubes. The microcentrifuge tubes were covered with aluminium foil and then were incubated at 37 °C in a laboratory incubator with constant mixing for 4 h to simulate the gastric and duodenal digestion time. The cell pellet (~30 µg) of each sample was then taken at the end of incubation and homogenized with 30 µL of rhodamine B (0.001% v/v in deionized water) and then placed on a glass slide. The penetration of FITC-dextran into the cells was visualized using CLSM. All imaging was performed with a 30 mW argon ion laser at 6.0 v (40%) power with excitation of 488 nm for FITC dextrans, and a 1 Mw HeNe laser at 30% power with excitation of 543 nm for detecting protein bodies stained with rhodamine B. Images were acquired using 20× (N.A. 0.5) objective lenses and analysed with ZEN blue 2.3 edition software.

3.2.6. Cell wall porosity evaluation during the food digestion process

To investigate whether the simultaneous presence of proteases would facilitate intracellular diffusion of the molecular probes, trypsin and chymotrypsin and dextran probes were simultaneously added to the mixture of the aqueous medium and the boiled cotyledon cells (the control cells sample) during the dextran diffusion experiment. Enzyme solutions were prepared as recommended in the INFOGEST protocol [17]. The diffusion experiment was carried out only for 2 h to simulate the time of intestinal digestion as described in section 2.5.

3.2.7. Microscopy observation of pancreatic trypsin diffusion

3.2.7.1 Fluorescent trypsin labelling

Trypsin from porcine pancreas (2 mg mL^{-1}) was labelled with Alexa Fluor® 488 reactive dye according to the manufacturer's instructions (Life Technologies, #A10235). Labelled trypsin with dye was separated and purified from unincorporated dye using the provided column and the coloured fraction was collected. The degree of labelled trypsin was determined before storage at −20 °C until its use.

3.2.7.2 Diffusion experiment and microscopy observation

Fifty mg of suspension of a freshly prepared sample (cell cluster/intact cells) was immersed in 250 µL of simulated intestinal fluid. The pH of the suspended sample was normalized to be at 7.0 with

1 M NaOH. Fifty µL of labelled trypsin (0.57 mg mL^{-1}) was then added to the mixture and incubated at 37 °C under constant agitation for 30, 60, and 120 min (individual microcentrifuge tubes were used per each time point). After each time point, the sample was immediately subjected to filtration through Whatman® glass-fibre filter and the remaining pellet of cells was washed with deionized water several times before CLSM visualization. For slide preparation, Nile red that dissolved in methanol (1 mg mL^{-1}) was used to stain and localize oil bodies. Thirty µL of the solution dye was added to 30 µL of sample suspension and homogenized before placed on the slide. The penetration of the labelled trypsin into cells was visualized using CLSM. The excitation wavelength of a 30 mW argon ion laser was set at 488 nm emission at 6.0 v (40%) power to detect labelled trypsin and the 1 mW HeNe laser at 30% power was set at 543 nm excitation to detect oil bodies that were stained with Nile red. The flour of particle size 180–315 µm that was obtained from boiled soybean cotyledon at 100 °C for 30 min was set as a control sample.

3.2.8. In vitro protein digestion

In vitro protein digestion experiments were performed following the recommendation of the harmonized COST INFOGEST protocol [17] as described in our previous study [5]. In brief, the freshly prepared cotyledon cells (BC, BCF, and GBC) were previously dried as described in section 2.2.4 to normalise the protein content and then subjected to gastric and intestinal simulated digestion. Samples were first mixed with the simulated salivary fluid (SSF – without adding salivary α-amylase). The mixture of the sample and SSF fluid was combined with simulated gastric fluids containing pepsin solution. The pH of the mixture was adjusted to 3.0 and incubated at 37 °C. After 2 h incubation for the gastric phase, the gastric chyme was mixed with simulated intestinal fluid (SIF) containing fresh bile and pancreatin solution. The intestinal digestion phase was started by adjusting the pH to 7.0 and incubated at 37 °C for 2 h. An aliquot (500 µL) was taken at 30, 60, 90 and 120 min of intestinal digestion and mixed with 8 µL of protease inhibitor Pefabloc® (5 mM) to stop enzyme activity before subsequent storage at −20 °C until further analysis.

3.2.9. Free α-amino groups released (NH2) and quantification of protein digestion

Peptides and amino acids were separated from larger proteins through the use of TCA precipitation followed by a filtration step before the reaction with o-phthaldialdehyde (OPA) as described previously in ref. [5, 18]. The free α-amino group content in the TCA-supernatant was determined based on the o-phthaldialdehyde (OPA) method [19] as described in detail in our previous study.5 Briefly, the OPA solution (200 mL) containing 7.620 g disodium tetraborate

decahydrate, 160 mg O-phthaldialdehyde, sodium dodecyl sulfate (SDS) and 176 mg of 99% dithiothreitol (DTT) was freshly prepared. The reaction between α-amino groups released in the TCA-supernatant and OPA reagent was started by mixing 400 µL of each of the tested samples with 3 mL of the OPA reagent. The reaction mixture was allowed to stand for exactly 2 min before determining the absorbance of the adduct at 340 nm. The free amino group concentrations in the in vitro digested sample (NH2 (final)), completely hydrolysed sample in 6 N HCl at 110 °C for 24 h (NH2 (acid)) and in the undigested samples (NH2 (initial) that correspond to the oral phase (2 min) were determined with reference to a calibration curve constructed using L-serine (12.5–100 mg L^{-1}). The degree of protein hydrolysis (DH%) was estimated according to the following equation:

$$DH\% = \frac{NH_2\ (final) - NH_2\ (initial)}{NH_2\ (acid) - NH_2\ (initial)} \quad \text{x } 100$$

3.2.10. SDS-PAGE analysis of protein profiles

Protein was first extracted from undigested and digested cells (BC, BCF, and GBC) through an extraction buffer according to Xia et al [20] with some modifications. Sixteen mL of extraction buffer composed of SDS 1% (w/v), 25 mM phosphate-buffered saline (PBS) and 100 mM NaCl, (pH 7) was mixed with four grams of cells pellet. Subsequently, the mixture was homogenized and then kept under constant sonication using an ultra turrax (t 25 basic IKA WERKE) for 10 min in an ice bath followed by filtration through Whatman® glass-fibre. The insoluble fraction was discarded and the protein content in all supernatants was determined based on the Dumas method. Protein concentrations were normalized to be 10 mg mL^{-1} in each sample before SDS-PAGE analysis. SDS-PAGE analysis was performed under reducing conditions in an XCell SureLock™ Mini-Cell (Invitrogen Life Technologies) using a 4–12% polyacrylamide NuPAGE Novex Bis-Tris 15-well. Samples (2 µL) were diluted with 5 µL NuPAGE® LDS buffer (4× concentrated), 2 µL a reducing agent (10× concentrated) and MilliQ water (15 µL). The mixture was then mixed and subsequently heated (70 °C) for 10 min. Samples (10 µL) were then loaded onto the well. Mark 12 Unstained Standard (Invitrogen) was used as a protein reference for molecular weight. Electrophoretic separation was run in MES buffer that contained 0.5 mL of NuPAGE™ Antioxidant at 60 V for the first 20 min and then at 100 V until the end of the run. Afterwards, the gel was separated from the plastic plate and rinsed in deionized water before staining with Coomassie Brilliant BlueG-250 solution for ~2 h. The gel was then fixed in washing buffer (10% absolute ethanol and 7.5% glacial

acetic acid) for ~2 h and the protein profiles were analysed by using Image Lab software TM (Bio-Rad Laboratories, California, USA).

3.2.11. Statistical analysis

The data of the chemical composition analysis and DH% (at each time of intestinal digestion) are presented as mean ± standard deviation (SD) of at least three replicates. Statistical significance between samples was assessed by repeated-measures analysis of variance (ANOVA) using IBM SPSS statistics 25 link (NY:IBM Corp). Significant differences ($p < 0.05$) of means were determined by the Tukey test.

3.3. Results

3.3.1. Effect of processing on dextran probe diffusion in soybean cells

3.3.1.1. Soaking solution and pectinase treatment

Pectin solubilisation is a vital factor in determining cell wall porosity of soybeans [12]. Hence a preliminary experiment was carried out to monitor the diffusion of the molecular probes in boiled samples previously soaked in a salt solution or previously treated with pectinase. These samples were expected to become more permeable to the probes compared to samples previously soaked in water. Soaking in a salt solution has been reported to increase bean softening [21]. Beneficial effects of using salts soaking before cooking have been postulated to remove divalent cations, particularly (Ca^{2+} and Mg^{2}+) from pectin of the middle lamella. The altered pectates are thought to be more water-soluble and heat-labile; thus soaking in salt may influence cell wall permeability of cooked beans as a result of pectin solubilisation [22]. Fig. 3.1 highlights the combined effect of soaking solution (water/salt) with boiling treatment as well as the effect of additional pectinase treatment on cell wall permeability. It is evident in Fig.3.1I and II that boiled samples previously soaked in water or soaked in a salt solution were impermeable to 20 kDa dextran, except for a weak appearance of fluorescence for isolated cells of the boiled sample soaked in a salt solution. 20 kDa dextran, on the other hand, was able to penetrate both cell clusters and isolated cells after additional enzymatic treatment with pectinase as presented in Fig.3.1III. Compared to 20 kDa dextran, both cell clusters and isolated cells of pectinase-treated samples show very limited permeability to 70 kDa dextran (SI–Fig.3.1i). The appearance of dextran probe diffusion into the cells of pectinase-treated samples is also noteworthy although the apparent intact cell wall was visualized by calcofluor stain (light blue). This suggests that the dextran probe was able to penetrate pectinase-treated cells via pores that occurred as a result of pectinase treatment rather

than mechanical damage of the cell wall structure which might have occurred as a result of sample preparation.

3.3.1.2 Fermentation and germination

Fig.3.2 demonstrates that dextrans of molecular sizes 20, 40, 70 and 150 kDa were able to penetrate soybean cells when fermentation or germination was employed as an additional process to boiling. The cell morphology images in SI–Fig.3.3 show distinctive differences between the cell wall of soybean cells prepared after a combination of fermentation or germination with boiling (SI–Fig.3.3b1 and c1) compared to BC (SI– Fig.3.3a1), possibly due to chemical modifications induced by the additional treatment. Furthermore, compared to the tightly-packed environment of intracellular macronutrients that could be observed in BC (SI – Fig.3. 3a2), the intracellular space adjacent to cell walls in both BCF and GBC was degraded (SI–Fig.3. 3b and c). In addition, the morphology of GBC showed that the native microstructure organization of intracellular macronutrients is lost and the size of protein bodies becomes smaller. The presence of starch granules was identified only for GBC using iodine solution for staining and light microscopy observation (data not shown). No purple-black staining was seen when BC or BCF was examined.

Table 3.1 highlights the changes in the chemical compositions of the extracellular and intracellular matrix of isolated cells upon soybean processing. A remarkable change in the protein content of samples was observed: BC contained 47.6% protein compared to 41.6% found in RF. In contrast, BCF showed a decrease in protein content from 47.6% to 40.7%. The same trend was observed for the GBC sample, where the protein content was 37.7%. The difference among the means of the protein content of samples was statistically significant ($p < 0.05$). The lipid content was higher in BCF (25.6%) and GBC (27.3%) compared to BC (24.8%) and RF (21.5%). The lipid contents of samples were also statistically different ($p < 0.05$). As expected, processing soybean and cell isolation decreased the contents of IDF and SDF, but there was no significant difference between different isolated cells. Another remarkable change in the chemical composition is the significant difference in starch content: in line with the microscopy observation, GBC contained much more starch (4.2%) as compared to the RF (0.8%). Starch content of dry soybean seeds at maturity sharply declines to <1% on a dry basis [23]. The ash contents showed no significant difference between the isolated cells and raw sample, and negligible changes were observed among the BC, BCF and GBC.

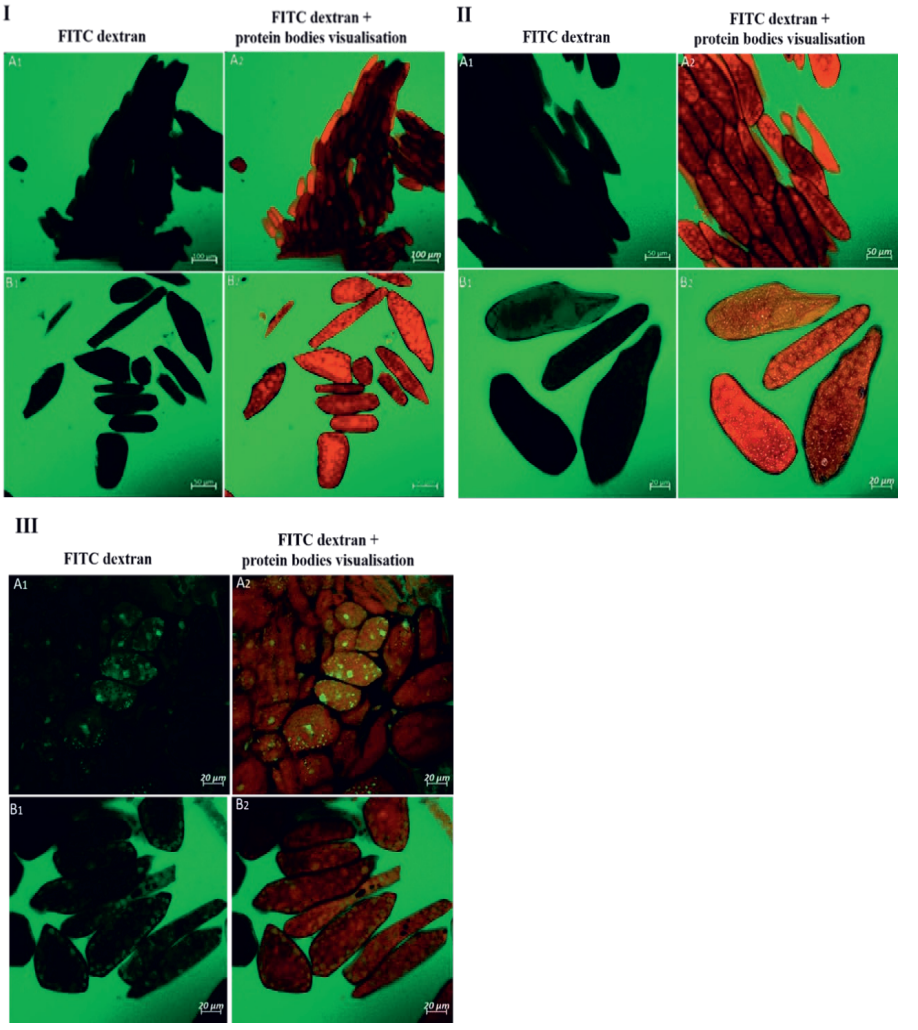

Fig.3.1. Confocal micrographs of FITC-dextran 20 kDa (visualized in green) permeation into the cell cluster and isolated cells; I: cells or clusters from cotyledon boiled for 3.5 h and soaked in water; II: cells or clusters from cotyledon boiled for 3.5 h and soaked in salt, III: cells or clusters from cotyledon boiled for 3.5 h and soaked in water and then treated with pectinase. Protein bodies were stained with rhodamine B (visualized in red) and used for the simultaneous visualisation of the dextran and the protein bodies within the intercellular matrix.

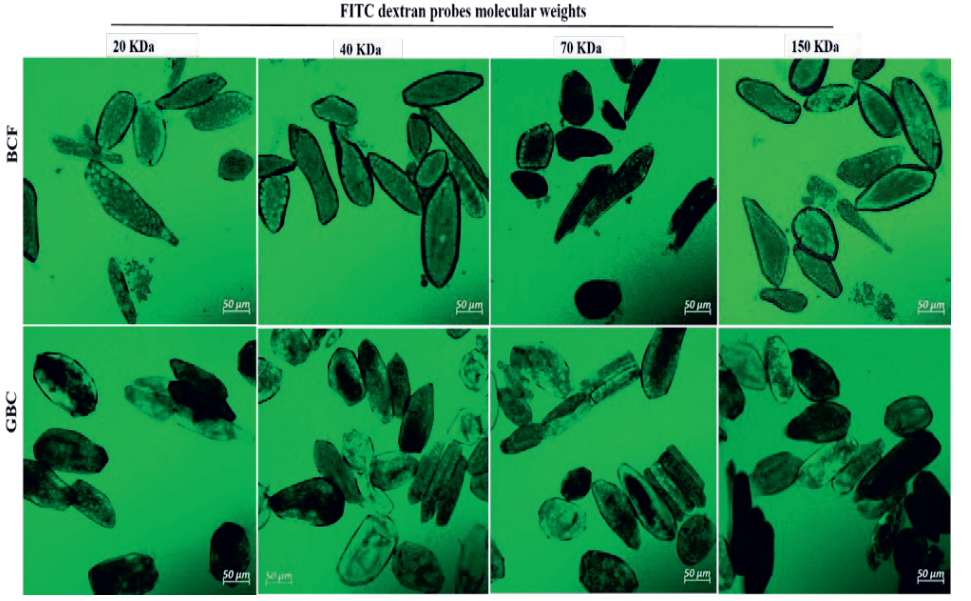

Fig.3.2. Confocal micrographs of FITC-dextran 20, 40, 70, and 150 kDa (visualized in green) permeation into the cell of fermented boiled cotyledons cells BCF (top panels) and cells of germinated–boiled cotyledons GBC (bottom panels).

Table 3.1: Chemical composition (%) of different soybean samples

Samples	Protein	Lipid	Starch	IDF	SDF/CHO	Ash
RF	41.6 ± 0.7 [a]	21.5 ± 0.9 [a]	0.8 ± 0.06 [a]	9.7 ± 0.3 [a]	21.0 ± 0.9 [a]	5.4 ± 0.6 [a]
BC	47.6 ± 0.9 [b]	24.8± 0.6 [b]	0.7 ± 0.02 [a]	7.3 ± 0.4 [b]	16.2 ± 0.6 [b]	3.3 ± 0.3 [b]
BCF	40.7 ± 1.9 [a]	25.6 ± 0.4 [b]	0.5 ± 0.08 [b]	6.6 ± 0.5 [b]	23.2 ± 1.2 [a]	3.3 ± 0.1[b]
GBC	37.7 ± 1.5 [c]	27.3 ± 0.3 [c]	4.2 ± 0.18 [c]	6.7 ± 0.5 [b]	21.1 ± 0.4 [a]	2.8± 0.3 [b]

Values are expressed on a dry basis. Values marked with different letters in each column are significantly different ($p < 0.05$). Raw soybean cotyledon flour (RF), boiled cotyledons cells (BC), fermented boiled cotyledons cells (BCF), germinated - boiled cotyledons cells (GBC), insoluble dietary fibre (IDF) and soluble dietary fibre including carbohydrate (SDF/CHO).

3.3.2. Effect of the digestion process on dextran probe diffusion in soybean cells

Fig.3.3 shows the influence of trypsin and chymotrypsin in modulating soybean cell permeability to the molecular probes. As shown in Fig.3.3, the addition of trypsin and chymotrypsin to the aqueous medium during dextran probe diffusion induced an extensive permeability of dextrans of molecular sizes 20, 40, 70 and 150 kDa for BC cells which were impermeable to dextran diffusion

(Fig.3.1I) in the absence of proteases. Thus, the results in Fig.3.3 suggest that protease enzymes facilitate the penetration of the molecular probes within the cells.

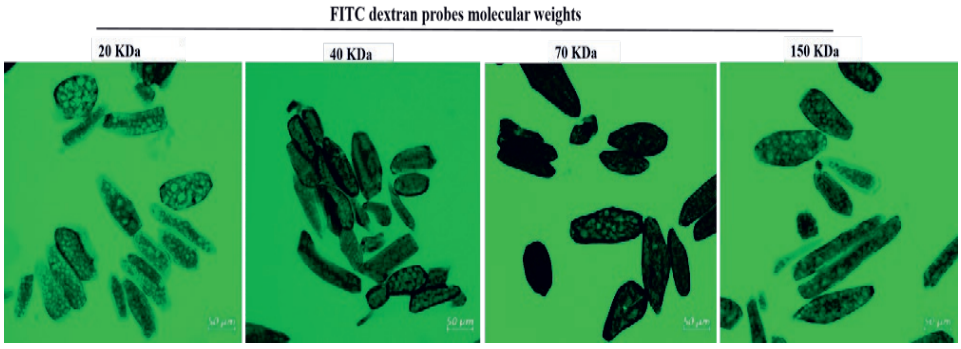

Fig.3.3. Confocal micrographs of FITC-dextran probes (visualized in green) permeation into the cell of boiled cotyledons when simultaneously treated with trypsin and chymotrypsin.

3.3.3. Diffusion of labelled trypsin into soybean cells

Whereas the diffusivities of dextran probes of variable size were used as a tool to characterize cell wall porosity, the key question remains of whether digestive enzymes having a similar size to dextran probes are able to trespass the cell wall barrier. Fig.3.4 shows the passage of labelled trypsin into cell clusters (Fig.3. 4A) and isolated cells (Fig.3. 4B). It is evident from Fig.3.4A that trypsin penetration into the cell cluster was not observed within the first 30 minutes of incubation (Fig.3. 4AI), but some fluorescence accumulation was clearly seen in the space between adjacent cells of the cluster starting from 60 minutes of incubation (Fig.3. 4AII). After 2 h of incubation, labelled trypsin is found in the intracellular environment of the cell cluster (Fig.3. 4AIII). By contrast, a substantial amount of fluorescence is visible as early as after 30 minutes of incubation of the isolated cells with labelled trypsin regardless of the treatment applied (Fig.3. 4B).

3.3.4. Kinetics of protein hydrolysis

Fig.3.5 shows the values of protein digestibility in BC, BCF and GBC expressed as the degree of hydrolysis (DH%). The samples showed different kinetics of protein hydrolysis during digestion and the differences in DH% were observed at 0, and 30 minutes of intestinal digestion. After 120 min of gastric digestion, which corresponds to time zero of intestinal digestion, the DH% in BCF was 3-fold greater ($p < 0.05$) when compared with DH% observed for BC and GBC. As it is well known that the hydrolysis of proteins by pepsin is very limited [24], the higher DH% of BCF in the gastric phase demonstrates the role of the fermentation process in “pre-digesting” proteins into smaller peptides and eventually leading to more digestible proteins in the gastric phase. BC and

GBC exhibited more or less the same rate of proteolysis at initial stages of intestinal digestion; however, a significantly higher DH% was observed after 60 min and 90 min in GBC compared to BC ($P < 0.05$). The observed differences in DH% at the end of the intestinal digestion were small but statistically significant ($p < 0.05$) among samples, suggesting that either germination or fermentation could be employed along with heat treatment for improving the rate and extent of soybean protein digestion.

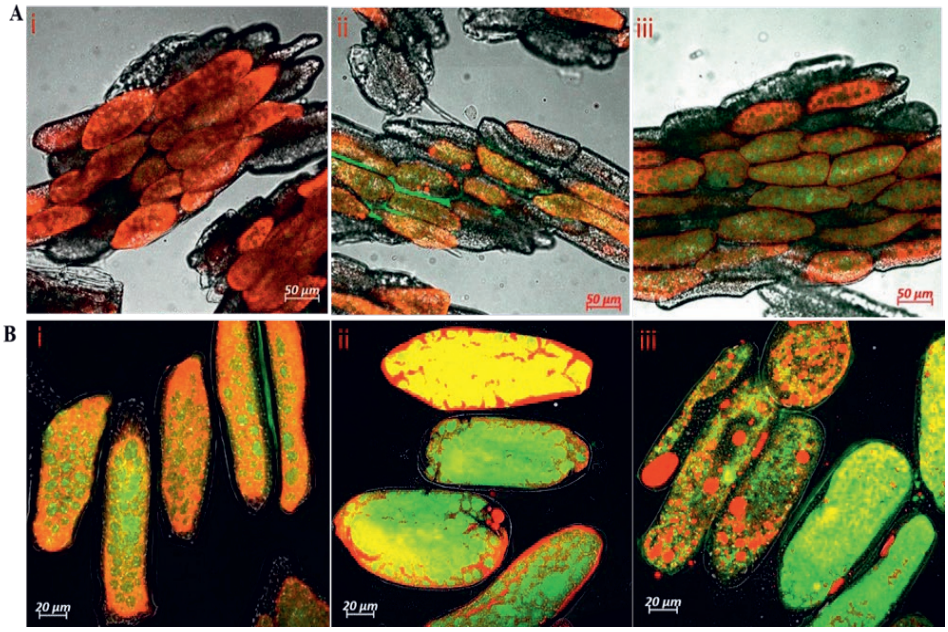

Fig.3.4. Confocal laser scanning observation of the diffusion of trypsin labelled with Alexa Fluor® 488 into processed soybean tissues. Top panels (A): diffusion of labelled trypsin over 2 h into cell clusters obtained from cotyledons boiled for 30 min. Images were taken at 30 min (i), 60 min (ii), and 120 min (iii). Bottom panels (B): labelled trypsin diffusion into boiled cotyledon cells (i), fermented boiled cotyledon cells (ii) and cells of germinated–boiled cotyledons (iii). Images were taken after 30 min of incubation. Labelled trypsin is displayed with green colour; oil bodies were stained with Nile red and are displayed with red colour inside the cells. Oil staining was used for the simultaneous visualisation of the labelled trypsin and the oil bodies within the intercellular matrix.

3.3.5. Protein profiles before and after in vitro digestion

Fig.3.6. shows the protein profiles of the isolated cells before and after in vitro digestion in comparison with the protein profiles of the raw sample. As expected, the raw sample (lane 1) showed a typical protein profile made up of several polypeptides in the molecular weight range of 10 to 140 kDa [25]. The observed bands with estimated molecular weights between 90 and 92 kDa had been described as lipoxygenase isoforms 1, 2, and 3 [26]. The bands appearing at

approximately 83.2, 72.4, and 48.4 kDa, probably corresponding to α, α′, and β subunits of β-conglycinin, and the bands appearing around 36.5 and 21.5 kDa might belong to acidic and basic subunits of glycinin [27]. The protein band patterns of isolated cells before digestion indicated that the intensity of major bands was generally reduced as an effect of the process applied. This may be due to the molecular degradation of the major storage proteins, (β-conglycinin and glycinin) that has taken place during boiling, fermentation or germination as seen in lanes 3–5. Regardless of the general disappearance of major bands in the isolated cells, the bands around 38.5 kDa remained visible only for BC, whereas the bands at approximately 21 and 24 kDa were observed for both BC (lane 3) and GBC (lane 5) but with slightly different intensity. The band of molecular weights around 21 kDa was previously identified as trypsin inhibitor subunits [28, 29]. Moreover, the increased intensities of oligopeptides with molecular weight <14 kDa in BCF (lane 4) could be the product of partial protein hydrolysis by proteolytic enzymes produced during fermentation. The new bands at 26 kDa in GBC might originate from the proteolysis of soybean storage proteins during germination [26].

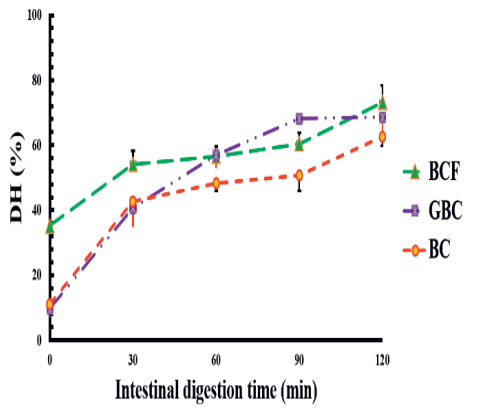

Fig. 3.5. Degree of protein hydrolysis (DH%) of isolated soybean cells during duodenal digestion BC: boiled cotyledon cells, BCF: fermentation boiled cotyledon cells; GBC: cells of germinated and boiled cotyledons.

Noteworthy differences were found between protein profiles of cells before and after digestion. The intensities of the oligopeptides with molecular weight <17 kDa are increased for the remaining pellet of digested samples. Furthermore, new bands having molecular weights ~24, 36, 38, and 52 kDa were also observed. These bands probably correspond to incomplete protein degradation during digestion or to the digestive enzymes used during in vitro digestion. The band around 36

kDa, for instance, might belong to pepsin, while the bands 51–54 kDa, 38 kDa and 23–27 kDa might correspond to pancreatin enzymes including trypsin, amylase, lipase, ribonuclease, and protease [29, 30]. Nonetheless, the protein profiles of the remaining pellet of cells after digestion (lane 6–8), showed no visible differences between BC, BCF, and GBC.

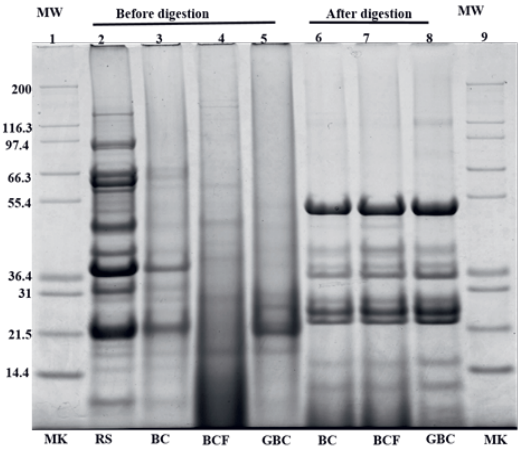

Fig. 3.6. SDS-page protein profile of isolated cells before and after in vitro digestion compared to the raw soybean protein profile (RS). Lane 1 and 9 (MK): standard molecular markers, lane 2: raw soybean (RS); lane 3: boiled cotyledon cell (BC); lane 4: fermented boiled cotyledon cells (BCF); lane 5: cells germinated and boiled cotyledons (GBC); lane 6–7: undigested pellet of cells after in vitro digestion of BC, BCF and GBC, respectively.

3.4. Discussion

An intact cellular structure has been identified as a critical factor affecting the rate and extent of soybean protein digestion, presumably as a result of the barrier effect exerted by an intact cell wall in limiting the passage of digestive enzymes [5]. Industrial and domestic processing used to make soybeans edible may modulate cell wall permeability and thus the diffusion of digestive enzymes through cell walls. The results of this study showed the effects of processing techniques on cell wall permeability in soybean cotyledon cells and how these changes may contribute to the extent of protein digestibility.

3.4.1. Food processing induce soybean cell wall porosity and permeability

The primary cell wall of soybeans is a complex structure formed by a network of polysaccharides and structural proteins. Soybean cell wall polysaccharides are mainly composed of pectin (50–

70% on cell wall weight), hemicellulose and cellulose. The amount of protein represents 2.1% of the water unextractable solids of isolated cell wall material [12, 31]. The adhesion between soybean cells is ensured by a pectin-rich layer known as middle lamella. Consequently, degradation or solubilisation of cell wall pectin is expected to alter the cell wall permeability [10, 12]. A striking confirmation of this hypothesis was obtained by the increase in cell wall permeability of soybean cells to FTIC-dextran probes observed after the treatment with pectinase (Fig.3.1IIIA and SI–Fig.3.1). A more modest increase can be observed after soaking in salt which is known to solubilize the pectates of the middle lamella [22]. Apparently cooking soybean cotyledons previously soaked in water or salt solution had no significant effect on 20 kDa dextran diffusion inside the soybean cells (Fig.3.1I). Cells of boiled soybeans were impermeable to 20 kDa dextran, and the pore size of boiled soybean cells was therefore estimated to be below 20 kDa. This is below the cell wall pore size reported for raw and roasted almonds [32], selected commercial pulses [33], and cooked kidney beans treated with acid/alkali [34]. Almonds and commercial pulse cells were reported to be permeable to 20 kDa probes, while acid/alkali-treated kidney beans showed permeability to a probe of 150 kDa. SI– Fig.3.2 suggests, however, that the limited permeability of boiled cells is likely the result of a relatively short exposure time to the probe and that penetration of dextrans of molecular weight 20 and 70 kDa into boiled cells is eventually achieved after 24 h exposure.

Compared to the limited permeability of boiled cells, the extensive permeability of fermented and germinated soybean cells to dextrans of high molecular sizes showed in Fig.3.2 is most likely due to partial degradation/solubilisation of pectin and other polymers of the cell wall that could have occurred during fermentation and germination. The apparent physical damage or changes in the cell wall structure (less efficiency of calcofluor white staining) reported in SI – Fig.3.3 panels b and c provided further evidence of such cell wall modification. It has been reported that soybean germination mobilizes fibre fractions and results in losses of the primary cell wall components [12], and the primary cell wall can also be affected by the fermentation process and becomes more soluble [9].

Despite the fact that the cell wall is the primary barrier for the passage of dextrans into cells, the relatively compact intracellular environment may serve as an additional barrier for probe permeability and delays its diffusion in intact plant food cells. The limited permeability of 20 kDa dextran to densely packed cells of BC as shown in Fig.3.1IIB and the extensive permeability of

dextran of molecular sizes 20, 40, 70 and 150 kDa for loosely packed cells of BCF and GCB samples as illustrated in Fig.3. 2 appear to support this conclusion. A previous study has reported that the loosely packed cells of acid/alkali-treated potato showed extensive permeability to probes of 150 kDa [34].

3.4.2. Food digestion process modulates cell permeability

Germination and fermentation of soybeans both increased cell wall permeability to molecular probes (Fig.3.2). A similar effect was found when protease enzymes were added during the probe diffusion experiment (Fig.3.3). The role of digestive enzymes, especially trypsin and chymotrypsin, during the digestion process in facilitating probe penetration within intact cells was demonstrated for the first time in the present study by CLSM visualisation. Although the exact mechanism of this effect is still unknown and is under investigation, we hypothesize that the main cause may be the hydrolysis of intracellular proteins providing space for probe penetration in the cells. However, we cannot rule out the possibility that the digestion of structural proteins from the cell wall might have increased cell wall permeability. There is evidence that the denaturation of structural cell wall proteins as an effect of the thermal treatment (as occurring in our samples as an effect of boiling) may facilitate their removal from the cell wall and this, in turn, facilitates the solubilization of cell wall polysaccharides [35]. This may increase indirectly the permeability of the cell wall. Additionally, an early study reported that digestion of nitrogen associated with cell walls is possible during passage through the gastrointestinal tract of monogastric animals [36]. On the other hand, however, the soybean cell wall is relatively poor in proteins [31].

3.4.3. Soybean cell permeability and pancreatic trypsin diffusion

Despite differences observed in cell wall permeability to dextran probes between BC and BFC and GBC, experiments with labelled trypsin, which has a radius of gyration similar to 20 kDa probes, showed different behaviour. The diffusion of labelled trypsin within plant cells is reported in this study for the first time, after similar investigations on the diffusion kinetics of amylase [37], and lipase [32]. In fact, labelled trypsin rapidly accumulated in all the isolated cells regardless of the process applied, with substantial fluorescence observed inside the cells already after 30 minutes of incubation. This behaviour is different compared to pancreatic amylase which is reported to be strongly hindered by intact cell walls [38, 39]. This difference may be explained by the smaller size of trypsin compared to other digestive enzymes or by hypothesizing that trypsin can “eat its way” through the cell wall or, most likely, the intracellular space as evidenced in Fig.3.3. Notably, trypsin seems to be evenly distributed within the isolated cells with no specific accumulation on

the cell wall material as reported e.g. for amylase. It is likely that boiling had also increased the permeability of cells to trypsin but a comparison with raw cells was not possible because the procedure of cell isolation requires a heating treatment. A previous study has shown an increase in labelled pancreatic α-amylase diffusion into isolated cells of the common bean upon long thermal processing times [37]. However, Fig.3. 4A shows that penetration of labelled trypsin within cell clusters obtained after boiling soybean tissues for 30 minutes (i.e. much milder treatment compared to the 3.5 hours boiling necessary for cell isolation) is slower compared to isolated cells and fluorescence starts to appear only after 60 minutes of incubation. This is likely because the limited pectin solubilisation offered a stronger barrier to trypsin.

3.4.4. Fermentation and germination processes enhance protein digestibility in soybean

Data of Fig.3.5 on proteins DH% showed that the large increase in permeability of isolated cells to trypsin only results in a modest, albeit significant, increase in protein digestibility in, BCF and GBC compared to BC. The lowest estimated DH% values were observed for BC cells which are the least permeable to trypsin. Clearly, the kinetics of protein hydrolysis does not only depend on cell permeability. BC cells were tightly packed with intracellular compounds as could be seen in SI– Fig.3.3a, whereas BCF cells and GBC cells showed a loosely packed intracellular environment due to a combination of fermentation and germination processes with heating treatment (SI– Fig. 3. 3b and c). This is in line with our previous study [5] where it was shown that the tightly packed environment of intracellular space can contribute to limiting the protein digestibility in raw and cooked soybean cells.

Factors such as the presence of trypsin inhibitors, level of protein denaturation, and inherent susceptibility to protease hydrolysis can also be responsible for limited protein digestibility [40, 41]. The appearance of oligopeptides with molecular weight <14 kDa for BCF (Fig.3. 6 lane 3), which is the likely result of storage proteins hydrolysis to smaller peptides by the action of endogenous enzymes during fermentation, was associated with higher protein digestibility after gastric digestion of BFC compared to the other two samples. The results from the SDS-page in Fig.3.5 suggest that boiling reduced the intensity of the band of molecular weight around 21 kDa which corresponds to trypsin inhibitor subunits (Fig.3.6. lane 1 compared to lanes 2, 3 and 4) and thus its partial inactivation. This may have contributed to the slow rate of protein digestion at initial stages of intestinal digestion of BC and GBC. However, the same band disappeared after fermentation likely through hydrolysis during fermentation. Regardless of the similar appearance of the 21 kDa band for GBC and BC, a faster rate of protein hydrolysis was observed for GBC

compared to BC. The relatively small size of protein bodies as could be seen for GBC in SI– Fig. 3. 2c might represent a favourable condition for faster hydrolysis of proteins in the intestinal phase. Previous studies have also shown that food processing such as fermentation and germination improves the overall protein digestibility in soybeans [8, 9, 42]. Here we show that boiling alone or combined with fermentation and germination increases cell permeability to trypsin and this effect may contribute to increasing protein digestibility together with protein pre-digestion during fermentation, or inactivation of trypsin inhibitors.

3.5. Conclusions

The present study demonstrates the influence of soybean processing on cell permeability to molecular probes and trypsin. In particular, we provided evidence that the combination of heat treatment with germination and/or fermentation can modify the permeability of soybean cells. In addition, we have demonstrated for the first time that the soybean cells became more permeable during the digestion process, perhaps as a result of cell wall protein hydrolysis or, more likely, through the digestion of intracellular proteins. Despite increasing cell permeability to trypsin, fermentation and germination only limitedly increase protein digestibility in intact soybean cells and this increase is partially due to pre-digestion or intracellular proteins, including trypsin inhibitors. On the other hand, the bigger size of large cell clusters delays the diffusion of trypsin inside the cells. This observation contributes to explaining the inverse relationship between the particle size and protein digestibility in soybeans previously reported [5].

Conflicts of interest

The authors declare no conflict of interests.

Acknowledgements

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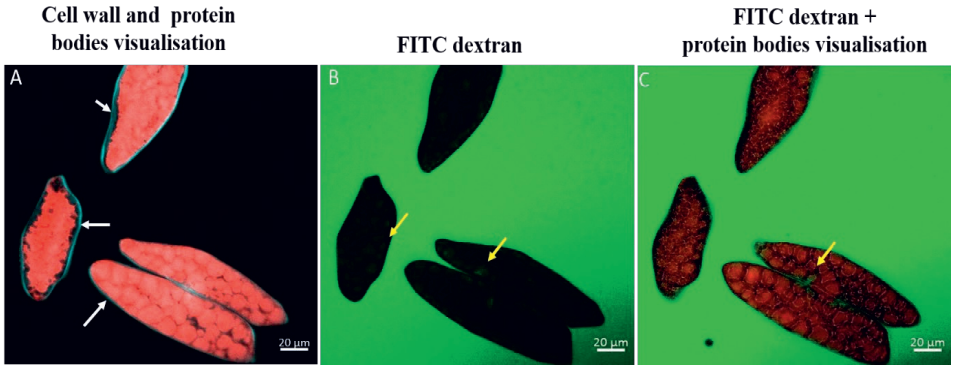
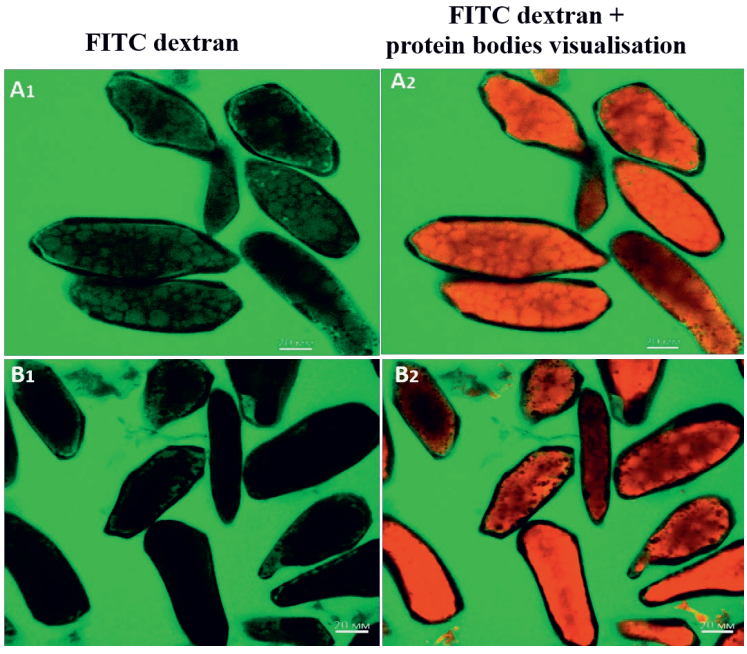
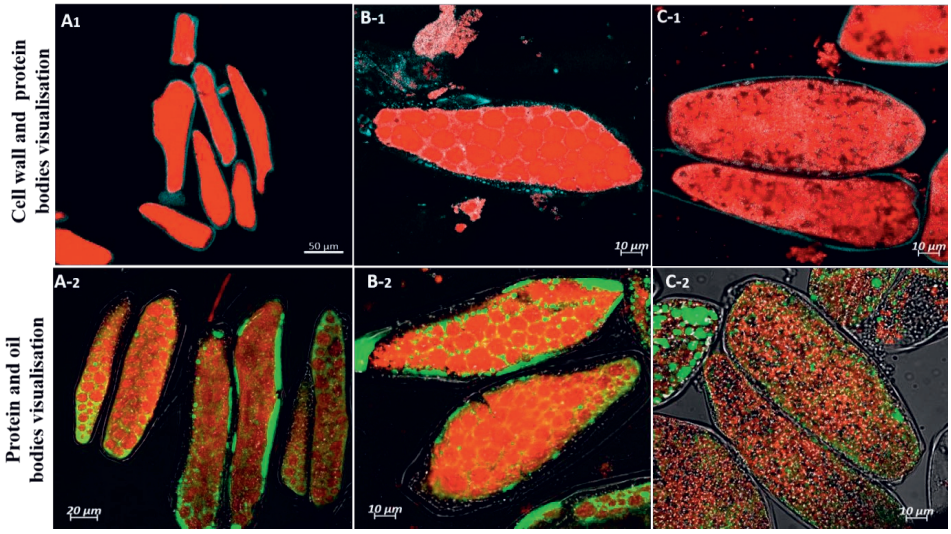
Supporting information



SI -Fig.3.1. Confocal micrographs of FITC-dextran 70 kDa (visualized in green) permeation into isolated cells obtained from soaked cotyledons boiled in water for 3.5 h and then treated with pectinase. Cell wall was stained with Calcofluor white and visible in blue only in (A); Protein bodies were stained with Rhodamine B and visible in red. The intact cells in panel A are indicated by the white arrows whereas the weak fluorescence of FITC-dextran 70 kDa within intact cells is indicated by the yellow arrows.



SI -Fig.3.2. Confocal micrographs of FITC-dextran 20 and 70 kDa (green colour) permeation into isolated cells of cotyledons boiled for 3.5 h after 24 h exposure to the probe. FITC dextran molecular weight 20 kDa is shown in top panels A1, and A2 whereas 70 kDa is shown in bottom panels B1, and B2.



SI -Fig.3.3. Confocal laser scanning micrographs of isolated cells; A = cells isolated from boiled cotyledons (BC), B = fermented cells of boiled cotyledons (BCF) and C = cells isolated from boiled cotyledons previously subjected to germination (GBC). Cell wall, protein bodies and oil bodies were stained with Calcofluor white (blue), Rhodamine B (red) and Bodipy 505/515 (green) respectively.

Chapter 4

Soybean germination limits the role of cell wall integrity in controlling protein physicochemical changes during cooking and improves protein digestibility

This chapter is based on:

Mostafa Zahir, Vincenzo Fogliano, Edoardo Capuano. Soybean germination limits the role of cell wall integrity in controlling protein physicochemical changes during cooking and improves protein digestibility. Under revision on Food Research International.

Abstract

Previous studies showed that *in vitro* digestibility of proteins in cooked beans is modulated by heat treatment and that the effect may be different whether proteins are heated in intact cells or in a bean flour. In this study, we investigated the role of soybean cellular integrity in the heat-induced changes of some physicochemical properties of protein and its relationship with *in vitro* protein digestibility. For this, soybean cotyledons and flour were boiled at 100 °C for varying times (30, 90, or 180 min). After grinding, the level of trypsin inhibitors, protein aggregation, surface hydrophobicity, secondary structure, and *in vitro* digestibility were studied. In separated experiments, the effect of germination in combination with boiling was also explored. The results showed that, in non-germinated samples, preservation of an intact cell increases the thermal stability of proteins, as well as a higher level of residual trypsin inhibitor activity, and contributed to limit protein digestion. However, the effect of cellular integrity on digestibility was not observed in germinated samples. Differences in cooking times resulted in limited improvement in the protein digestibility and slight changes in protein properties for both boiled cotyledons and flour of germinated and non-germinated soybean. Germination induced distinct changes in some physicochemical properties of raw soybean proteins and increased protein digestibility of cooked soybean. This work provides extra knowledge of the role of cellular integrity on protein properties in plant foods and suggests that germination or grinding before cooking may increase protein digestibility.

Keywords: Soybean, cotyledons, flour, cellular integrity, boiling, protein physicochemical properties, protein digestion

4.1. Introduction

Globally, the consumption of plant-based proteins has gained popularity in recent decades due to the increasing demand for sustainable and healthy foods. Soybean is one of the major sources of plant-based protein for human consumption [1], but plant protein utilization by humans is still too low compared to that of animal proteins [2]. Knowledge of the correlation between soybean processing and protein digestibility can be applied to maximize utilization by humans. In previous studies [3, 4] we have shown that the cellular integrity of soybean and processing methods lead to large differences in soybean protein digestibility. In particular, we demonstrated that protein digestibility in soybean particles obtained by first milling and then boiling was higher than protein digestibility of soybean particles of the same size that were first boiled and then milled. This difference is mainly driven by a different degree of tissue integrity being the fraction of broken cells higher when the soybean was first milled and then boiled. Another recent study has indicated that the protein conformational change of bean proteins is different according to whether they are heated inside intact cells (i.e. in the crowded bean intracellular environment) or in a flour (i.e. open environment due to the loss of cellular integrity) [5]. Data of that paper showed that differences in protein secondary structure may, at least partially, explain the higher digestibility of bean proteins heated in a flour.

The cellular structure and intercellular matrix may have a protective effect on proteins preventing the heat-induced conformational changes. Differential scanning calorimetry studies have shown a difference in protein denaturation temperature between soft and hard beans [6, 7]. The authors attributed the thermal stability of hard beans proteins to differences in the cell wall composition which would limit cell swelling, starch gelatinization, and protein denaturation. However, there is very limited information about the role of cell integrity in controlling the protein physicochemical changes during cooking. In particular, it is not known if the protein can respond differently to the hydrolysis process during digestion, depending on whether the thermal treatment occurred in a tightly packed intracellular environment (intact cotyledon) or in a relatively open environment (flour). Comparison of the protein physicochemical properties and digestibility in both boiled germinated and non-germinated soybean can be used to determine the extent to which the cell-matrix may affect the way proteins respond to heat treatments and consequently affect its digestion. Germination is indeed known to produce a change in the cell wall architecture and the cytoplasmic matrix which may modulate the effect of the cell-matrix on heat-induced changes in proteins properties and digestibility [4, 8, 9]. These insights might further lead to the possibility of re-design soybean processing to enhance the nutritional utilization of soybean proteins.

In this paper we aim to understand the influence of cotyledon cell integrity during boiling treatment on protein physicochemical changes and its impact on protein digestibility, and if it is possible to use the germination process to modulate this effect. Thermal properties of protein, level of trypsin inhibitors, protein aggregation, surface hydrophobicity, and secondary structure were investigated to observe protein physicochemical changes that might have occurred as a result of different processing conditions. *In vitro* digestibility of protein was also determined to assess the relationship between soybean processing and its protein digestibility.

4.2. Materials and methods

4.2.1. Materials

Dried soybean seeds were purchased from De Vlijt (Wageningen, The Netherlands) and stored at room temperature.

Porcine pepsin (P6887, 4.3.200-4.500 U/mg protein), pancreatin (P1750, 4X USP), Porcine bile extract (B8631), pefabloc® SC, sodium dodecyl sulfate (SDS), o-phthaldialdehyde (OPA), DL-dithiothreitol (DTT), L-serine, trypsin (bovine), N-α-benzoyl-DL-arginine-pnitroanilide hydrochloride (DL-BAPA), and bromophenol blue (BPB) were purchased from Sigma-Aldrich, USA. Trichloroacetic acid (CAS 76-03-9) and disodium tetraborate decahydrate (CAS 1303-96-4) were bought from Merck & Co. (Darmstadt, Germany). NuPAGE® 4–12% Bis-Tris Gels, NuPAGE® LDS sample buffer (4 x concentrated), MES running buffer, and Mark 12 Unstained Standard for SDS-PAGE analysis were provided by Thermo fisher scientific (Van Allen Way Carlsbad, CA, USA). Other chemicals used in this study were of analytical grade.

4.2.2. Samples preparation and heat treatment

Dried soybean seeds (100 g) were soaked in tap water at a 1:3 (w/v) ratio and kept at 5 °C for 12 h. The excess water after soaking was discarded and the seed coat was removed manually. Dehulled soybean cotyledons were cooked as whole cotyledons or as fine flour of size range 32–45 µm obtained by intensive milling and sieving. The cooking was conducted in a water bath at 100 °C for varying times (30, 90, or 180 min) to evaluate the effects of heat treatment time. Boiled cotyledons and control samples (uncooked cotyledons) were ground for 5-10 min using Retsch Cryo Mill (Retsch Technology GmbH, Haan, Germany) sieved to obtain a fine flour of the same size of the flour that was heated (32–45 µm). Samples were then dried at 40 °C until a moisture content of 10% was achieved and total protein content was estimated by the Dumas combustion method according to Zahir et al (2018,2020) [3, 4]. Dried samples were stored until further analysis.

In a separate experiment, the effect of germination was investigated. For this, dried seeds were germinated following the procedure described in detail in our previous work [4]. Briefly, dried seeds were disinfected with 0.07% sodium hypochlorite solution and soaked in tap water for 6 h prior germination process that was continued for 4 days at 27 °C under darkness. The sprouts and seeds coats of germinated seeds were removed and the germinated cotyledons were boiled and prepared using the same conditions as described for non-germinated soybean samples. An overview of the experimental plan is given in Fig.4.1.

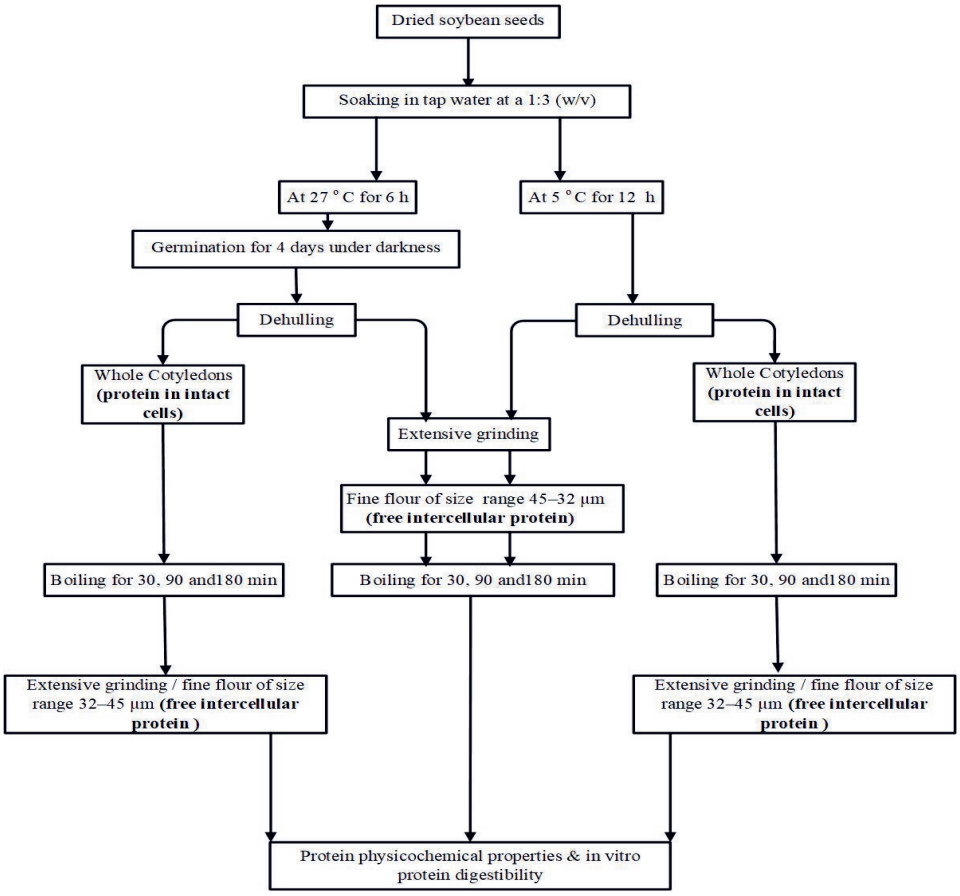


Fig. 4.1. Scheme for preparing samples for in vitro protein digestion and protein physicochemical properties analysis.

4.2.3. In vitro digestion and protein hydrolysis quantification

The digestion experiments were carried out based on the recommendations of the Infogest consortium [10, 11]. In brief, a mixture of sample flour (size 32–45 µm) and ultrapure water was prepared to normalize the protein content in all samples before in vitro digestion process. The sample mixture was suspended in a simulated salivary fluid (with no salivary α-amylase) for 2 min. Subsequently simulated gastric fluid (containing pepsin) was incorporated and the pH of the mixture was adjusted to 3 and incubated at 37 °C with constant mixing for 2 h. Following the gastric phase, the gastric chyme was combined with simulated intestinal fluid containing fresh bile and pancreatin solution. The pH of the mixture was then adjusted to 7 and incubated at 37 °C for 2 h. Aliquots of the digestion mixture (1 mL) were taken at different time points during simulated intestinal digestion. The enzymes' activity was stopped using 5% trichloroacetic acid (TCA) and samples were stored at −20 °C until further analysis. The concentration of free amino groups was determined by OPA method as described by [12] with minor modifications as described by Zahir et al., [3, 4]. The degree of protein hydrolysis (DH%) was quantified based on the concentration of free amino groups in enzymatically hydrolyzed samples, non-hydrolyzed samples, and completely hydrolyzed samples in 6 N HCl at 110 °C for 24 h as detailed elsewhere [3, 4].

4.2.4. Proteins thermal properties

Thermal properties of soybean protein were determined by the use of a differential scanning calorimeter (Perkin Elmer DSC with stainless steel large volume cups) in triplicate. For this, soybean particle size range 2000-3000 µm and fraction with size range 32–45 µm were used to simulate encapsulated protein and free protein samples respectively. Fifty mg of prepared sample (~50% moisture) was weighed into a DSC pan and the pan was then hermetically sealed and equilibrated at 25 °C for 5 h at room temperature. The thermograms were obtained at a heating rate of 10 °C min^{-1} from 25 °C to 160 °C. A sealed empty pan was used as a reference. The onset peak temperature (T onset), the end peak temperature (T end), the temperature at the peak maximum (Tp), which is generally used to indicate the temperature of denaturation [13] and the enthalpy of denaturation (ΔH, J/g dry matter) were obtained from thermogram analysis using the PyrisTM operation software (PerkinElmer, Ltd., United Kingdom).

4.2.5. Trypsin inhibitor activity

The trypsin inhibitor activity in fine flour with size 32–45 µm prepared as described in 4.2.2 section was assessed according to AACC Method 22–40.01 (AACCI, 2009) and the modified procedure proposed by [14] with slight modifications. In short, one gram of sample was mixed with 50 mL of

10 mM NaOH (pH 8.4) and kept under constant stirring at room temperature for 3 h to extract trypsin inhibitor. The dispersion was then centrifuged at 2000 g, for 5 min. One mL of soluble/ extracted part was diluted to achieve the concentration of trypsin inhibitor expected to cause 30–70% inhibition. To start the enzymatic and colorimetric reaction, trypsin (bovine, 2 mL) and N-α-benzoyl-DL-arginine-pnitroanilide hydrochloride (DL-BAPA) (5 mL) and 2 mL diluted extracted sample was mixed well and then incubated at 37 °C for 10 min. Each sample test was repeated at least three times. For the control sample, the extracted sample was replaced by ultrapure water. The enzymatic reaction was stopped by adding 1 mL of acetic acid (30%). The samples were then centrifuged at 2500 g for 10 min and their absorbances were determined at 410 nm. The trypsin inhibitor units (TIU) per mg sample was calculated based on the following equation:

$$\text{TIU/mg sample} = \frac{\{[(A_{410R} - A_{410RB}) - (A_{410S} - A_{410SB}) \times 100]\ \text{mL diluted soy extract}\}}{(\text{mg sample mL}^{-1}\ \text{diluted soybean extract used for the assay})}$$

Where ($A_{410\ R} - A_{410RB}$) = A_{410CR}, corrected reference reading of A_{410}, ($A_{410S} - A_{410SB}$) = A_{410CS}, corrected sample reading of A_{410}, ($A_{410CR} - A_{410CS}$)/ A_{410CR} should be in the range of 0.30 to 0.70 that is 30-70% of trypsin inhibition by given dilute soy extract.

4.2.6. Protein surface hydrophobicity

Protein surface hydrophobicity of samples was determined based on the interaction between hydrophobic chromophore bromophenol blue (BPB) and protein according to [15]. Sample (fine flour with size 32–45 µm prepared as described in 4.2.2 section) containing ~5 mg protein was dispersed in 1 mL of 20 mM phosphate buffer (pH 6.0). The mixture was combined with 200 µL of 1 mg/mL BPB (in ultrapure water) and mixed well. The tube contained 1 mL of phosphate buffer and 200 µL of 1 mg/mL BPB was used as a reference sample. Both sample and reference were kept under constant stirring at room temperature for 10 min. The dispersion was then centrifuged at 2000g for 15 min and diluted 10 fold with phosphate buffer. The absorbance of the diluted supernatant was measured at 595 nm against a blank phosphate buffer. The amount of BPB bound was estimated by the following equation:

$$Bound\ BPB\ (\mu g) = 200\ \mu g\ x\ \frac{(A595\ Control\ -\ A595\ Sample)}{A595\ Control}$$

Where A595 was the absorbance at 595 nm.

4.2.7. Protein aggregation

Soybean protein aggregation formed upon cooking was monitored using gel electrophoresis under non-reducing conditions. The method used was adapted from previous studies [16, 17] with some medications. Sample of fine flour size ranges (32–45 µm) was combined with NuPAGE® LDS buffer (4× concentrated), and ultrapure water in a ratio 5/5/10 (*w*/*v*/*v*). The mixture was kept under stirring for 10 min and then (10 µl) was taken and loaded onto the gel wells. Mark 12 Unstained Standard (Invitrogen) was used as MW marker. Gels were run at 120 V for ~1.5 h using MES buffer and stained with Coomassie Brilliant Blue R-250 for ~2 h. Afterward, gels were then fixed in washing buffer (10% absolute ethanol and 7.5% glacial acetic acid) and the gels images were acquired using Lab software TM (Bio-Radd Laboratories, California, USA).

4.2.8. Protein Secondary Structure

Protein secondary structure was studied using the Fourier transform infrared (FTIR) spectroscopy technique. Fine flour samples with size range 32–45 µm prepared as described in 4.2.2 section were used for FT-IR analysis. The spectra of the samples were analyzed in the wave number range from 400 to 4000 cm^{1} with 32 scans in a Bruker Tensor 27 (Bruker Optics, Ettlingen, Germany). The secondary structure of soybean protein that is commonly based on the amide I band (1,600–1,700 cm−1) was analyzed using Origin lab® software (Northampton, MA, USA) and following the method of Rovaline-Cordova et at [5] with some modifications. Briefly, before the curve fitting a straight baseline correction was performed in the region (1,600–1,700 cm−1). The identified peaks with the amide I (1700–1600 cm^{-1}) were assigned to their particular substructure of protein secondary structure according to the assignments of [18]. The relative composition of each secondary structure component was estimated by dividing the individual component peak area on the total area, obtained as a result of the calculations of the area of the entire peaks.

4.2.9. Statistical analysis

The data are presented as mean ± standard deviation (SD) of at least three replicates. The significant differences in means were determined at the $P < 0.05$ level using IBM SPSS statistics 25 link (NY: IBM Corp). The difference between DH% at each digestion time point, trypsin inhibitor units, and the amount of bound BPB were assessed by two-way ANOVA. The factors tested were boiling time, and soybean structure (cotyledons and flour), as well as their interaction (boiling time x structure set), independently for boiled samples of non-germinated and germinated samples. The statistical difference between boiled non-germinated and germinated samples was

determined by Student's paired t-test. The difference in the values of each thermal protein property among samples was assessed by ANOVA followed by Tukey's test. Pearson correlation was used to measure the association between the means of protein physicochemical and means of in vitro digestibility of final products (120 min of intestinal digestion) at a significance level of 0.05.

4.3. Results

4.3.1. In vitro protein digestibility

In vitro protein digestibility, expressed as a degree of hydrolysis (DH%), at different duodenal digestion time points, is presented in Fig.4.2. Fig.4.2- Panel A clearly shows that protein digestibility increased after cooking. The increase in DH% due to the differences in boiling times and /or in the level of integrity of boiled soybean (cotyledons and flour) was modest. However, we found statistical differences in DH% values among non-germinated samples ($P < 0.01$) for all intestinal digestion time points. At the end of the intestinal digestion, the DH% in the flour samples was significantly higher than the DH% in the cotyledon samples ($P \leq 0.01$). The effect of the boiling time was also significant ($P \leq 0.01$), but due to the interaction, the trend was different in flour compared to the intact cotyledon. Protein digestibility of raw soybean was markedly increased after germination. For the germinated samples, significant differences in DH% were found for all intestinal digestion time points, except for 120 min of duodenal digestion. Student's paired t-test showed that there was a significantly higher DH% in germinated compared to non-germinated samples ($P \leq 0.01$) at each intestinal time point. The data in Fig.4.2, Panel A shows that in non-germinated samples the thermal treatment applied to the flour induces a small but significant increase in protein digestibility compared to when the same treatment is applied to the intact cotyledon. The data in Fig.4.2 Panel B demonstrated that the combination of a mild treatment such as germination and a short thermal treatment significantly improved protein digestion in soybean.

4.3.2. Thermal properties of encapsulated and free proteins of soybean

Monitoring of thermal denaturation of soybean protein using DSC technique usually requires soybean flour or isolated soybean protein as substrate materials. In this study, it was observed that the denaturation temperatures (Td) of soybean protein fractions 7S and 11S were higher for particles of 2000-3000 µm compared to the fine particle of 32-45 µm where the proteins were free of any physical boundary (see Table 4.1). The same trend was observed for germinated soybean, although the difference between big particles and fine particles was negligible, (see Table 4.1). Statistically significant differences were observed between big particles and fine particles for both

non-germinated and germinated soybean for each thermodynamic property investigated, indicating that the cell wall barriers may delay the heat-induced denaturation of protein when pac **A** side the intact cells. **B**

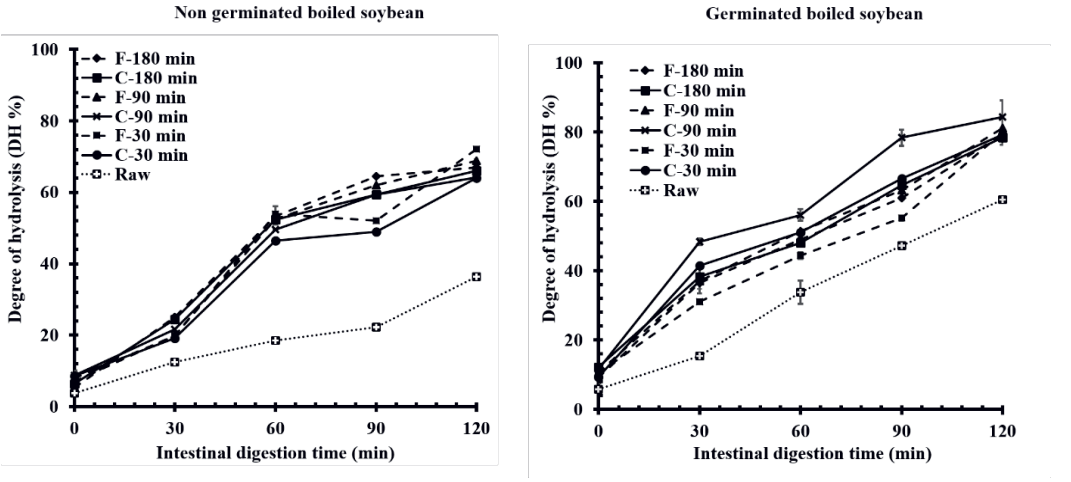


Fig. 4.2. DH% during in vitro intestinal digestion of soybean flour (32–45 μm) of raw and boiled cotyledon (C) and flour (F) for 30, 90, or 180 min. **A** = DH % in flour samples prepared from non-germinated soybean, **B** = DH % in flour samples prepared from germinated soybean.

Table 4.1: Comparison of thermal properties of big particles (2000-3000 μm) and fine particle (32-45 μm) prepared from non-germinated and germinated soybeans.

	Samples	7S				11S			
		T onset (°C)	T end (°C)	T d (°C)	ΔH (J/g)	T onset (°C)	T end (°C)	T d (°C)	ΔH (J/g)
Non-germinated Soybean	Particles (2000-3000 μm)	79.96 ± 1.3 ^{a}	87.02 ± 0.7^{a}	82.75 ± 0.9^{a}	0.16 ± 0.1^{c}	100.84 ± 0.6a	109.17 ± 0.4^{a}	104.65 ± 0.45^{a}	1.24 ± 0.22^{a}
	Particles (32-45 μm)	72.36 ± 1.5 ^{bc}	79.95 ± 1.9^{b}	76.76 ± 1.8^{b}	0.24 ± 0.1^{b}	91.4 ± 2.8b	99.52 ± 1.42^{d}	95.62 ± 0.31^{d}	0.26 ± 0.12^{b}
Germinated Soybean	Particles (2000-3000 μm)	76.95 ± 0.8 ^{a}	83.18 ± 1.7^{a}	80.19 ± 1.1^{ab}	0.102 ± 0.06^{d}	95.4 ± 0.1a	104.4 ± 0.5^{ce}	99.90 ± 0.5^{be}	0.65 ± 0.05^{b}
	Particles (32-45 μm)	72.04 ± 0.5 ^{bc}	80.73 ± 3.2^{c}	76.96 ± 2.1^{bc}	0.36 ± 0.2^{a}	96.1 ± 2.2a	104.7 ± 0.68^{be}	98.95 ± 0.70^{ce}	0.66 ± 0.50^{b}

Data are expressed as mean ± standard deviation of three replicates and analysed with ANOVA followed by Tukey's test). Column data with different letters are significantly different ($P < 0.05$.

4.3.3. Trypsin inhibitor activity

Thermal inactivation of trypsin inhibitor in non-germinated and germinated soybean is shown in Fig.4.3. Cooking either as a whole cotyledon or as flour resulted in substantial inactivation of the trypsin inhibitors. It is quite clear from Fig.4.3a and 3b, that ~ 90% inactivation has been achieved during the first 30 min of cooking. The effect of cooking time, cellular integrity, and their interaction were all significant for both germinated and not germinated samples ($p \leq 0.01$). A significant reduction in trypsin inhibitor activity (44 %) was observed in raw samples after the germination process (P <0.001) (see Fig.4.3.b). Statistically significant differences were observed between boiled samples of non-germinated and germinated soybean at each cooking time ($p \leq 0.03$).

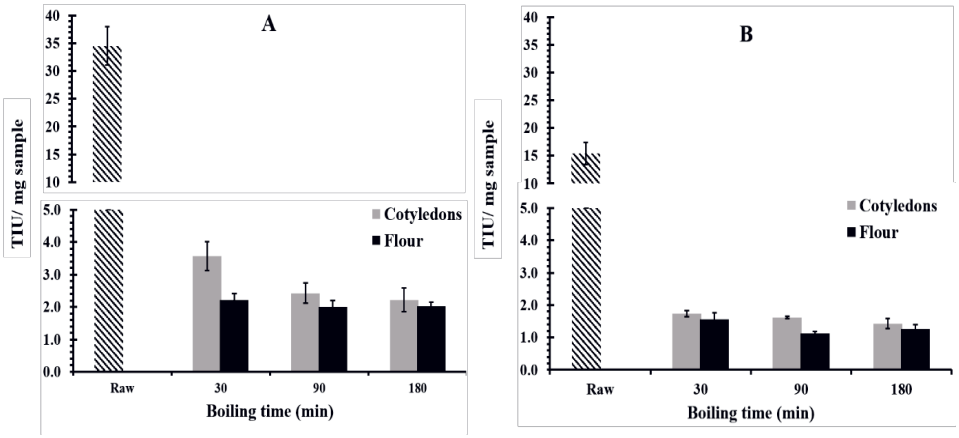


Fig. 4.3. The trypsin inhibitor units in raw and boiled soybean for varying times (30, 90, or 180 min) and different structures (intact cotyledons or flour); non-germinated soybean (A-left panel), and germinated soybean (B-right panel).

4.3.4. Protein aggregation

Fig.4.4 shows non-reducing SDS-PAGE images of non-germinated and germinated soybeans boiled for 30,90 180 min as whole cotyledons or fine flour. The smearing on the top of SDS PAGE gel is seemingly high-MW protein aggregates formed by S-S bridging and other covalent cross-linking. This is because it is possible that the SDS-PAGE condition, although under non-reducing condition, could damage the non-covalent interaction between proteins. Using native PAGE which maintains both the proteins' secondary structure and native charge density, could be the best technique to know the aggregation state of a protein [19, 20]. In the non-reducing SDS-PAGE condition used in our study, all samples seemingly formed protein aggregation due to the

processes applied. The highest intensities of the smearing on the top of SDS PAGE gel were detected for non-germinated flour boiled for 30 and 90 min (image A, Lanes 6-7), whereas the lowest intensities were detected for germinated soybean samples that were boiled either as cotyledons and flour (image B). However, we observed the formation of aggregates also in raw samples (Lanes 2 in both images) likely as a result of extensive grinding and drying for ~ 18 h at 40 ^{O}C. It has been shown that the longest soaking time of soybean (16 h) may partially cause protein denaturation or structural rearrangement [21]. The extensive grinding with the drying process may have led to protein aggregation in all samples. The SDS-PAGE profile based on MW distribution of protein show clear differences between uncooked (raw) non-germinated soybean sample (Fig.4.4, Panel a) and raw germinated samples (Fig.4.4, Panel b). The raw sample (lane 1- Panel a) show a typical profile of soybean proteins is made up of several polypeptides in the molecular weight range of 10 to 140 kDa. The band of molecular weights around 21 kDa corresponding to the trypsin inhibitor subunit was detected in both raw and germinated soybean (Lane 2 in both panels A and B). The new bands appearing at approximately 26 kDa (Lane 2 in panel B) might originate from the proteolysis of soybean storage proteins during germination [4, 22]. The intensity of major bands was gradually reduced after boiling (image a_Lanes 3-8) while those subunits were disappeared after combining germination and boiling treatment (image b - Lanes 3-8).

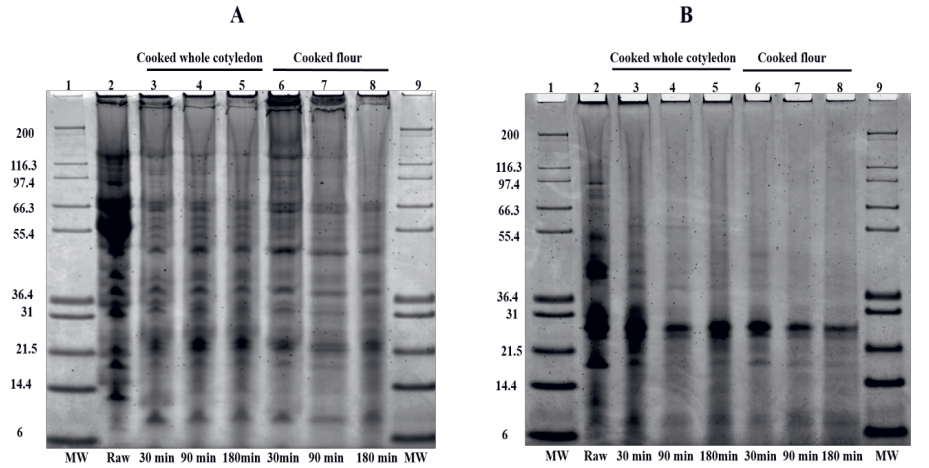


Fig.4.4. Non-reducing SDS-PAGE images of non-germinated and germinated-boiled soybean. Panel A: non-germinated boiled soybean either as cotyledons and as flour for 30, 90, or 180 min; Panel B: germinated boiled soybean at the same condition as in (a).

4.3.5. Protein Surface hydrophobicity

Fig.4.5 shows protein surface hydrophobicity that is expressed as the amount of hydrophobic chromophore bromophenol blue (BPB) that is bound to the hydrophobic domains of the proteins. Fig.4.5 shows a significant increase in the amount of bound BPB after boiling and germination. The amount of bound BPB detected for raw soybean (control sample) was approximately 50% lower compared to the value of its germinated counterpart and the non-germinated boiled for 30 min. the effect of cooking time, cellular integrity, and their interaction were all significant in germinated samples ($P < 0.01$), whereas only the effect of cooking time was significant in non-germinated samples ($P < 0.01$).

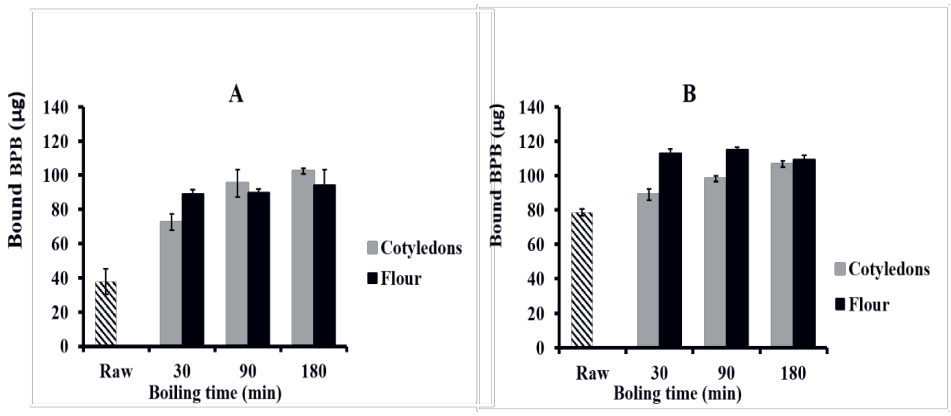


Fig.4.5. Protein surface hydrophobicity of non-germinated (A) and germinated soybean (B) boiled for varying times (30, 90, or 180 min) as the whole cotyledon form or as flour form. Raw samples of non-germinated and germinated soybean were used as control samples.

4.3.6. Protein secondary structure changes

The curve-fitting of amide I bands obtained in FTIR spectra (see S1-Fig.4.1) of cooked non-germinated and germinated soybean allowed to identify peaks within amide I (1700–1600 cm^{-1}). The relative contents of the secondary structures are presented in Table 4.2. Compared with raw samples, the random coil, α-helix and β-turn contents of boiling-treated samples increased while the content of β-sheets decreased. Increasing cooking time of both germinated and non-germinated cotyledon and the respective flours resulted in modest changes, suggesting very limited changes in protein secondary structure between intact cotyledons and flour cooked for varying times (30, 90, or 180 min). No clear trend was observed when boiled samples of non-germinated and germinated soybean are compared. Comparing uncooked samples, germinated

one showed the lowest content of β-sheet and the highest contents of the random coil, α-helix, and β-turn.

Table 4.2. Relative content of different secondary structures in soybean; cooked as whole cotyledon (BC) or cooked as flour (BF).

A- Non-germinated -boiled samples

Amide I band components	Wavenumber (cm^{-1})	Raw	Boiling time					
			30 min		90 min		180 min	
			BC	BF	BC	BF	BC	BF
A1	1618	8.0	10.0	5.1	6.8	6.8	5.0	3.5
β-sheet	1610-1640 1670-1680	37.4	25.6	20.6	20.5	24.4	21.2	25.8
Random coil	1640-1650	7.1	13.5	18.4	15.1	18.5	20.2	17.1
α-helix	1650-1660	15.0	17.1	27.5	24.8	21.3	23.5	24.3
Turn	1660-1670 1680-1700	24.2	27.3	24.3	27.1	24.0	25.8	25.9
A2	1682	8.3	6.5	4.1	5.6	5.0	4.4	3.3

B- Germinated -boiled samples

Amide I band components	Wavenumber (cm^{-1})	Raw	Boiling time:					
			30 min		90 min		180 min	
			BC	BF	BC	BF	BC	BF
A1	1618	4.8	6.3	2.2	6.8	5.0	4.7	4.1
β-sheet	1610-1640 1670-1680	25.0	25.7	24.4	20.5	26.6	20.5	27.6
Random coil	1640-1650	19.7	16.7	23.4	19.8	20.6	26.9	20.6
α-helix	1650-1660	20.1	21.2	25.0	26.1	20.7	25.0	17.6
Turn	1660-1670 1680-1700	24.8	25.0	20.2	22.4	23.9	19.1	23.3
A2	1682	5.6	5.0	4.9	4.3	3.2	3.8	6.7

4.3. Discussion

Recent studies provided evidence that the intracellular proteins entrapped within the soybean cotyledon cells are digested more slowly than proteins that are free from any cellular barriers [3, 4]. We hypothesized that the presence of an intact cellular structure during thermal treatment may also modulate the change in the intracellular protein conformation and hence protein digestibility. The current study highlights how the physicochemical properties of soybean proteins were differently changed during boiling depending on whether the thermal treatment occurred in a crowded intracellular environment (intact cotyledon) or in a relatively free space environment (flour), and how the combination of germination and heat processes could contribute to the extent of proteins digestibility. The most important finding from this study is that the structural integrity of the soybean tissue and the chemical environment surrounding proteins during the heat treatment has a limited effect on protein intestinal digestibility but only in non-germinated samples (see Fig.4. 2). Our initial hypothesis, i.e. that structural integrity of the soybean tissue and the chemical environment surrounding proteins would produce differential conformational changes influencing protein digestibility was therefore partially proven. The reason for the lack of effect observed in germinated samples might be that in those samples the intracellular material was less tightly packed [4] and therefore less effective on protein unfolding. The other interesting observation was that also cooking time had a limited effect on protein digestibility. The effect of thermal treatment on protein digestibility is far from trivial and strongly depends on the complex interplay between protein denaturation (which increases digestibility) and aggregation/cross-linking (which decreases digestibility). In legumes, thermal treatments are often reported to increase protein digestibility [3, 23, 24] but the modulating effect of varying cooking time is not clear or neglected. Here we show that thermal treatment increases protein digestibility but a limited improvement is achieved by prolonging the boiling from 30 min to 180 min in non-germinated samples. This is in line with Torres et al., [24] who reported that extending the autoclaving from 5 to 20 min does not improve the protein digestibility of legumes. Similarly, Another study found no advantage in improving the in vitro protein digestibility when selected legumes were autoclaved at 121 °C for 10, 20, 40, 60, 90 min, and autoclaving for 10 min showed the highest protein digestibility [25] Similarly, no linear relationship between heat intensity and protein digestibility observed when beans were boiled for 25, 30, 35, 40 and 45 min [26]. Finally, we confirmed that germination has a positive effect on protein digestibility, possibly because of the pre-digestion of proteins due to endogenous enzymes, including trypsin inhibitors, which was less abundant in germinated samples regardless of the physical structure and the cooking time compared to non-germinated

samples (see Fig.4.3). Interestingly, a substantial DH% was observed for raw germinated soybean which might be directly related to the substantial reduction of trypsin inhibitors concentration.

This observed behaviour reflects very well the protein digestibility scores and it is paralleled by the observed changes in the physicochemical properties of soybean proteins. As a general observation, we found that the structural integrity of the plant matrix and the cooking intensity have a limited impact on the protein structural features while germination produced some distinctive changes.

In line with our initial hypothesis, the thermal properties of soybean proteins are affected by the mechanical disruption of cotyledon cells or seed germination. The denaturation temperature of soybean proteins is lower when soybean cotyledon cells were previously disrupted (flour) before DSC analysis (Table 4.1) compared to soybean particles that have little or no physical damage to the cells. The observed decreases in protein denaturation temperature of soybean flour may have been facilitated by the ability of proteins of soybean flour to better absorb water from the matrix thus becoming more susceptible to denaturation. An early study reported that the denaturation temperatures of isolated soybean protein shifted to lower temperatures when the water/protein ratio was higher and indeed the water uptake can play a pivotal role in the denaturation rate [27, 28]. Proteins encapsulated by intact cell walls are thought to absorb less water. Limited water availability inside cotyledon cells/ intercellular protein has been reported to have protective effects on beans protein denaturation during thermal treatment [7]. On top of that, studies on macromolecular crowding in fundamental biology show that the thermodynamic properties of intracellular proteins are different from free proteins. This also results in protection towards denaturation [29]. In the case of flour germinated soybean, there was no considerable difference in protein denaturation temperatures of 7S and 11S between big particles and fine particles compared to what was observed in non-germinated soybean. The increase in soybean cell wall porosity, the reduction in the tightly packed intracellular environment, and the change in protein macrostructure caused by the germination process [3, 4] may facilitate the ability of proteins to absorb water from the matrix. This highlights the importance of sample preparation and the potential role of cell wall structure in limiting protein denaturation. However, in normal boiling conditions, water availability is unlikely to be a limiting factor and the complete protein denaturation may occur during cooking. In fact, the lack of the typical DSC endothermic peak of protein denaturation for previously boiled soybean samples suggested that proteins were already completely denatured after 30 minutes of boiling (data not shown). These observations are in good

agreement with the complete denaturation detected for commercial soybean isolate that underwent thermal processing before isolation [30, 31].

All in all, the difference in thermal properties for proteins within or outside intact cells is unlikely to produce differences in the denaturation level of proteins after normal cooking. This also applies to trypsin inhibitors which is one of the primary factors adversely affecting the soybean protein digestibility [32]. The low residual trypsin inhibitory activity found in soybean either boiled as whole cotyledons or as flour (Fig.4.3) provides an indication that most of the proteins were completely denatured which is consistent with previous literature [14, 33-35]. In Fig.4.3, besides the obvious reduction in TIU with cooking times, it is clear that the thermal inactivation of trypsin inhibitor activity was influenced by the structural integrity of boiled soybean, which is in line with the observation that proteins are slightly less denatured when heated within the cellular structure. Germination contributes to reducing trypsin inhibitor activity, which becomes especially relevant at shorter cooking times. Fig.4.3 shows a substantial difference in trypsin inhibitor between non-germinated and germinated soybean at the raw state and at the short boiling time (30 min).

Because the relationship between heating times and physicochemical properties of protein towards digestion is complex and by no means unidirectional, we have studied the impact of heating load on protein aggregation, surface hydrophobicity, and secondary structure. From SDS page profiles reported in Fig.4.4, it can be noticed the presence of protein aggregation formed by S-S bridging and other covalent cross-linking upon heating in cooked soybean flour, as evidenced by the formation of high molecular weights protein that they fail to enter the separation gel. However, these aggregates were already present in the raw samples and the effect of thermal processing could not be fully appreciated. An early study has demonstrated that the thermal treatment of soybean protein caused the dissociation of the subunits of both β-conglycinin 7S and glycinin 11S which subsequently interacted with each other, forming soluble aggregates [36]. Other studies reported aggregation of soybean protein to form high molecular weights proteins and that does not permit their entry into the separating gel [36, 37]. However, no new band was detected in the cooked samples compare to the raw ones. The intensity of the aggregates formed upon heating is less visible in germinated samples a good indication that protein of germinated soybean was partially hydrolyzed.

Protein denaturation and dissociation of the quaternary structure lead to protein unfolding which increases surface hydrophobicity [38]. In the present study, protein surface hydrophobicity was drastically increased after germination/cooking (Fig.4.5). In contrast, the increase in protein surface hydrophobicity upon long thermal processing times was limited for both non-germinated

and germinated samples. Wang, Li, Jiang, Qi and Zhou [38] reported that surface hydrophobicity of isolated soybean protein first increased with the heat treatment but the formation of protein aggregates from dissociated and denatured polypeptides may limit further increase in surface hydrophobicity or even produce a decrease in it. This was observed in both flour and intact cotyledon, with significant differences in the hydrophobicity only in germinated samples.

The germination process instead was associated with an increase in protein surface hydrophobicity. This is not surprising as the amount of protein surface hydrophobicity was already reported to be proportional to the growth of hypocotyls length [39]. The authors suggested that changes in protein conformation during germination due to the action of endogenous proteases may increases surface hydrophobicity.

Previous studies have shown that heat treatment induced an increase in the content of α- helix and β-turn structure, and a decrease in the content of β-sheet structures [38, 40]. However, Zhang and co-workers found that the secondary structure contents (α-helix, β-sheet, and random coils) of soybean protein isolated did not change significantly after heat treatment [41]. In our study, boiled non-germinated cotyledon and both cooked germinated cotyledons and flour were accompanied by an increase in contents of the random coil, α-helix, and β-turn and a decrease in β-sheet conformation as shown in Table 4.2. Other studies showed a high amount of the β-sheet structure might partially limit the access of proteolytic enzymes [42-44]. Similarly, less organized secondary structures like random coils may increase the susceptibility of proteins to proteases [43]. In our work, we see changes in the relative contributions of each secondary structure induced by the heat treatment and germination. The effect of heat treatment was more intense in non-germinated samples (reduction of β-sheets, an increase of random coils) and less in germinated samples. Germination per se induced an increase in the random coils. However, we could not identify any specific trend in the distribution of secondary structures that may explain the difference in *in vitro* digestibility reported in Fig.4.2.

To find insight into the protein physicochemical factors that most affect in vitro digestibility at the end of intestinal digestion, a correlation analysis was performed and it is shown in S1-Table.1. As shown in S1-Table.1, protein digestibility was inversely related to trypsin inhibitor (r = -0.851 P= 0.001), β-sheet (*r* =-.626, *P* = 0.017) and β-turn (-0.442, *P* = 0.113*)*. On the other hand, protein digestibility was linearly related to the surface hydrophobicity (*r* = 0.876 , *P* = 0.001) random coil (*r* = 0.782 *P* = 0.001) and between α-helix (*r* = 0.530, *P* = 0.051) (see Table 1S for details). This correlation analysis basically confirms that the denaturation of proteins, including trypsin inhibitors and the modification of the secondary structures, may all affect protein digestibility. However, none

of the factors has a very strong correlation with DH%, so none alone can fully explain DH%. The example of TIU is indicative when e.g. germinated raw and cooked non-germinated samples are compared.

4.5. Conclusions

Altogether our data suggested that soybean proteins in flour are more susceptible to thermal denaturation compare to the same proteins present inside intact cells. This was in line with the differences in protein physicochemical properties between boiled soybean cotyledons and flour. This difference results in significant changes in the *in vitro* digestibility only in non-germinated samples. Germination produced more marked changes in protein properties and resulted in a significantly higher *in vitro* digestibility. Interestingly, the combination of the germination process with short boiling treatment resulted in major protein physiochemical changes leading to significant improvements in protein digestion. These results provide extra knowledge about the role cell walls intactness in protein physiochemical changes and digestion suggesting germination or grinding soybean seeds before heating treatment could be employed as a sustainable technique to improve the digestibility of soybean proteins.

Conflicts of interest

The authors declare no conflict of interest.

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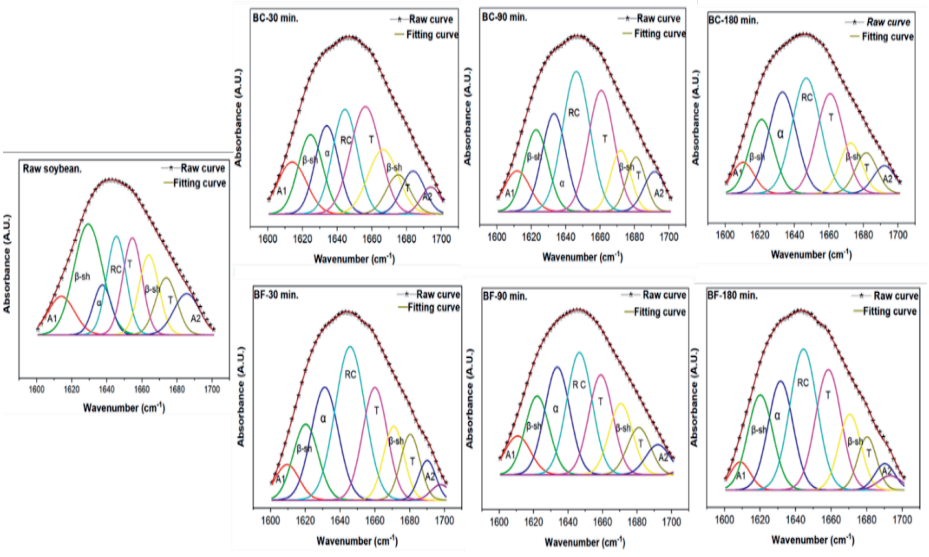
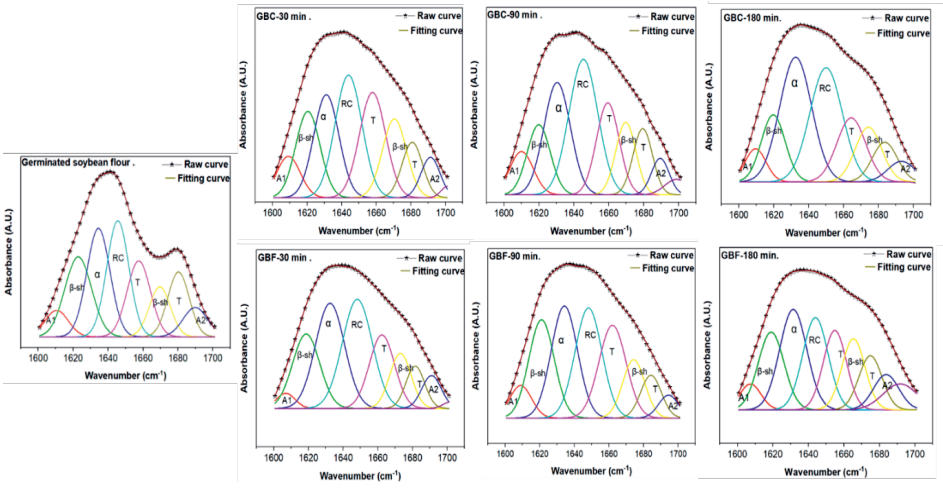
Supporting information

A



B



S1-Fig.4.1 Original FTIR spectra (upper) and the curve-fitted individual component bands (bottom) in amide I (1700–1600 cm–1) region. A: non-germinated; B : germinated soybean boiled as intact cotyledon or as flour for (30,90,180 min).

			Trypsin units inhibited	Protein surface hydrophobicity	Secondary structures components				In vitro protein digestibility at 120 min
					β-sheet	Random coil	α-helix	Turn	
Trypsin units inhibited		Pearson Correlation	1.00	-.842**	.771**	-.657*	-.587*	0.08591	-.851**
		Sig. (2-tailed)		0.00016	0.00124	0.01064	0.02718	0.77028	0.00011
Protein surface hydrophobicity		Pearson Correlation	-.842**	1.00	-.639*	.853**	.563*	-0.394	.876**
		Sig. (2-tailed)	0.00		0.014	0.000	0.036	0.163	0.000
Secondary structures components	β-sheet	Pearson Correlation	.771**	-.639*	1.00	-.652*	-.824**	0.107	-.626*
		Sig. (2-tailed)	0.00	0.014		0.012	0.000	0.715	0.017
	Random coil	Pearson Correlation	-.657*	.853**	-.652*	1.00	.562*	-.662**	.782**
		Sig. (2-tailed)	0.01	0.000	0.012		0.036	0.010	0.001
	α-helix	Pearson Correlation	-.587*	.563*	-.824**	.562*	1.00	-0.285	0.530
		Sig. (2-tailed)	0.03	0.036	0.000	0.036		0.323	0.051
	Turn	Pearson Correlation	0.09	-0.394	0.107	-.662**	-0.285	1.00	-0.442
		Sig. (2-tailed)	0.77	0.163	0.715	0.010	0.323		0.113
In vitro protein digestibility at 120 min		Pearson Correlation	-.851**	.876**	-.626*	.782**	0.530	-0.442	1.00
		Sig. (2-tailed)	0.00	0.000	0.017	0.001	0.051	0.113	

1-S -Table 4.1. Correlations evaluated between the means of protein physicochemical and means of in vitro digestibility of final products (120 min of intestinal digestion) of raw and boiled soybean.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

5

Chapter 5

The role of plant cell wall integrity and thermal treatment in modulating in vitro protein colonic fermentation

This chapter is based on:

Mostafa Zahir, Vincenzo Fogliano, Edoardo Capuano. The role of plant cell wall integrity and thermal treatment in modulating in vitro protein colonic fermentation. Manuscript to be submitted

Abstract

Proteins in whole plant foods are enclosed within the cells and therefore the retention of cellular entrapment to the end of the small intestine may limit the availability of protein for colonic microbial fermentation. In this study, the influence of cellular integrity on in vitro protein fermentation was studied by using intact and broken cells that were prepared from soybean cotyledon. Furthermore, raw and heat-treated soybean proteins without any cell wall were also studied. The time course of gas, short-chain fatty acids (SCFAs), branched-chain fatty acids (BCFAs), and ammonia produced from all samples during in vitro fermentation by human fecal inocula were monitored. The fermentation time course for intact cell samples either undigested or the one that was recovered after in vitro protein digestion was lower than their broken cell counterparts as judged by, SCFAs, BCFAs, and ammonia production. The marked differences in SCFAs concentration among samples may suggest that the main differences are produced by the difference in the structural integrity and the difference in carbohydrates content. The broken cells that were recovered after in vitro protein digestion and have the highest content of carbohydrates showed the highest SCFAs concentration. Differences in fermentability in terms of BCFAs and ammonia production was observed between raw proteins and heat-treated proteins, with the highest values for heat-treated proteins. This suggests that heating treatment may play a role in the availability of proteins to colonic microbiota. In conclusion, this study suggests that cell structure and cell composition may modulate soybean protein colonic fermentation.

5.1. Introduction

In humans and animals, protein flowing into the colon may come from bacterial cells, endogenous proteins, and undigested dietary proteins [1, 2]. The relative contribution from the latter depends on the host digestive capacity, the amount of protein in the diet, and its digestibility [3-5]. Dietary proteins that reach the colon are potentially prone to be metabolized by the resident microbiota. Microbial breakdown of protein results in amino acids which can be either utilized for the synthesis of bacterial cell components or catabolized through different pathways [6, 7]. The main pathway of amino acid fermentation in the colon is deamination, and the end products of this pathway are short-chain fatty acids (SCFAs), branched-chain fatty acids (BCFAs), and ammonia [6, 8, 9]. Apart from the fact that SCFAs are an important energy source for colonocytes and exert beneficial effects on the host physiology [10, 11] BCFAs and ammonia are potentially harmful to host health [8, 12, 13]. Thus modulating the flow of dietary protein from the small intestine into the colon or the access of colonic microbiota to the proteins might be relevant for reducing the concentration of these deleterious metabolites and their harmful effects [14].

Supplementing the diet with fermentable carbohydrates such as resistant starch and fibers has been shown to decrease the abundance of protein fermentation products [15, 16]. This is because fermentable carbohydrates are the preferred carbon source for microbes [17]. However, studies have been shown that protein fermentation predominates in the distal parts of the colon and attributed that to the depletion of the fermentable carbohydrate [17-19]. In this respect, dietary strategies that target a reduction in the accessibility of carbohydrates to colonic microbiota, and therefore shaping their fermentability in the transverse and descending colon and transferring a part of them towards the distal colon, may have benefits in reducing protein fermentation in the distal colon.

Recently, It has been shown that the isolated polysaccharides of cell walls are fermented faster compared to when they occur in complex supramolecular assembly as cell walls [20]. Thus, preserving the natural structure of cell walls in plant foods could be a viable approach to limit colonic fermentation of intracellular protein. The studies regarding the fermentation of protein contained within an intact plant matrix are still in their infancy and deserve further examination. Therefore, this study aims at understanding the role of the structural integrity of soybean cells in modulating the fermentation pattern of protein using an in-vitro batch fermentation model.

5.2. Materials and methods

5.2.1. Materials

Dried soybean seeds were purchased from De Vlijt (Wageningen, The Netherlands) and stored at room temperature. All other reagents were of analytical grade and purchased from Sigma Aldrich (St. Louis MO, USA) unless stated otherwise.

5.2.2. Samples preparation

5.2.2.1. Intact cell isolation

Intact cells of soybean cotyledons were isolated according to [21, 22] with some modifications. Briefly, soybean seeds (100 g) were soaked in 300 ml of tap water and placed in the refrigerator at 5 °C for 12 hours. The excess water after soaking was discarded and the seed coat was removed manually. The dehulled cotyledons were combined with fresh tap water in a ratio of 1:3 (w/v), placed in laboratory bottles, and boiled at 100 °C for 3.5 h using a boiling water bath. The boiled cotyledons were gently mashed using a mortar and pestle and subsequently subjected to wet sieving for fractionation using different aperture sizes. The fraction of the size range of 45–71 µm was collected and assessed in terms of cell integrity by the use of light microscopy. The light microscopy observations confirmed that the fraction of the size range of 45–71 µm is mainly constituted of free intact cells. The dry matter content of the isolated intact cell (IC) was adjusted to 25 % (w/w) before both in vitro digestion as well as before in vitro fermentation experiments using oven drying at 45 °C.

5.2.2.2. Modification of cell integrity structure (breaking cells (BC))

Mechanical damage of walls of isolated intact cells was carried out with the method described by Dhital, et al., [23] with some modifications. In short, 10 g of intact cells (25 % w/w dry matter) was mixed with 20 ml deionized water and place in a laboratory glass bottle. The mixture was kept under constant stirring using a magnetic stirrer for 36 h at 1500 rpm. Light microscopy was used for visual inspection of cell breakage. The dry matter content of BC was re-adjusted to 25 % (w/w) again before the in vitro digestion and in vitro fermentation experiments.

5.2.2.3. Soybean protein isolation

Isolated protein was extracted from uncooked soybean according to [24] with minor modifications. In short, dried soybean seeds were soaked and dehulled as described in 5.2.2.1 section. Dehulled soybean cotyledons were milled using 6875D Freezer/Mill machine to obtain fine flour. The fine flour was suspended in Milli-Q water at a 1:10 (w/v) ratio and the pH of the mixture was adjusted to 8.5 with 2 N NaOH. The mixture was then kept under constant stirring

at room temperature for 30 min and subsequently centrifuged at 14 000g, at 15 °C for 30 min. The supernatant was collected and then left for precipitation at pH 4.5, and 4 °C. After 1 h precipitation, the mixture was subjected to centrifugation at 14 000g, at 4 °C for 30 min. The supernatant was discarded. and precipitated protein curd was collected. The isolated protein was neutralized at pH 7 using NaOH before freeze-dried. This sample will be hereafter indicated as raw isolated soybean protein (RCP).

For preparing heated isolated soybean protein, a portion of RCP was suspended in Milli-Q water at a 1:10 (w/v) and boiled at 100 °C for 10 min using a boiling water bath; this sample will be hereafter indicated as HCP.

5.2.3. In vitro protein digestion

Gastric and intestinal in-vitro digestion experiments were carried out based on the recommendations of the INFOGEST consortium [25, 26]. In brief, a 10 gram of IC or BC (25 % w/w dry matter) was combined with 10 ml of simulated gastric fluid (containing pepsin). Subsequently, the pH of the mixture was adjusted to 3 with HCl and incubated at 37 ºC with constant mixing for 2 h. The gastric chyme was combined with simulated intestinal fluid containing trypsin (100 U/mL), chymotrypsin (25 U/mL). The pH was adjusted to 7 with NaOH and incubated at 37 ºC for 2 h. The incubation was performed under constant agitation throughout the whole experiment using a laboratory rotator. After 2 h intestinal digestion, the activity of the enzyme was stopped by subjected the samples to heat treatment (85°C) for 10 min. Samples were then were centrifuged at 4000 x g, 4 oC for 30 min, and pellets were dried using oven drying at 45 ˚C until achieving a dry content of 25 % (w/w). The remainder of protein left in the pellet after digestion was determined according to Dumas method. The dried pellet of digested IC hereafter named (DIC) and the dried pellet digested BC referred to as DBC hereafter. Both DIC and DBC were stored at -20 ºC until in vitro fermentation experiment.

5.2.4. Determination of moisture content and protein content

Samples were analyzed for their dry matter, protein, lipid, carbohydrate, and ash contents using standard analytical methods as described in our previous work [21]. The dry matter was determined in triplicate by the standard oven drying method at 105 C for 24 h. This dry material was then used to determine total protein, lipid, and ash in triplicate. The total protein content was determined using Dumas method. Lipid determination was performed with automatic Soxhlet using hexane as a solvent. As for the ash determination, the AOAC method, 2000 was employed. The carbohydrate content of the samples was estimated by difference.

5.2.5. In vitro batch fermentation and sampling

In vitro batch fermentation of samples by human fecal microbiota was performed as described by Van Den Abbeele, *et al* [27] with some modifications. In brief, 4 grams of sample (25 % w/w dry matter) was combined with 16 ml sterilized demi-water. The suspension of the sample was subsequently filled in sterilized penicillin bottles containing 43 mL sterilized colon growth medium (5.22 g/L K_2HPO_4, 16.32 g/L $KH2PO_4$, 2 g/L $NaHCO_3$, 2 g/L yeast extract, 2 g/L peptone, 1 g/L mucin, 0.5 g/L L-cysteine HCL, and 2 mL/L tween-80). The bottles containing sample and growth medium were closed with rubber caps and made anaerobic by flushing with nitrogen. Fresh human fecal samples were donated by two healthy donors aging from 30 – 40 years and were prepared separately in a phosphate buffer (8.8 g/L $K2HPO_4$, 6.8 g/L $H2PO_4$, and 0.1 g/L sodium thioglucolate). In a separate fermentation experiment using one collected fecal sample, the fermentation was initiated by injected 7 mL of fecal inoculum into each penicillin bottle of different samples. For each donor, two replicates were assigned to each different time point (6 h, 24 h, 48 h, and 72 h) of fermentation. Immediately after fecal inoculum injection, bottles were incubated in an incubator at 37°C with continuous shaking. At the end of each time point throughout fermentation, the volume of cumulative gas during fermentation was measured by the use of a manometer and a graduated syringe according to the method of Xie, Zhuqing, et al [28]. The bottles were then opened and plunged into ice-chilled water for 20 min to stop the bacterial activity. The fermented samples were transferred to centrifuge tubes and centrifuged at 2000 x g for 5 min. The supernatants were separated from the pellets and both were stored at -20 °C until further analysis. Blank fermentation bottles were performed for all samples, by mixing sample content with growth medium and fecal inoculum. These mixtures were not subjected to any incubation time and immediately centrifuged and stored at -20 °C until further analysis. These samples correspond to time point zero in the measurement of the samples. Besides, two control experiments were conducted for each fecal donor: in bottles (i) sample was replaced by phosphate buffer, in bottles (ii) both sample and growth medium was replaced by phosphate buffer.

5.2.6. Cell structure changes

Changes in IC and BC microstructure during fermentation were visualized using a confocal scanning laser microscope (CLSM)type 510 (Zeiss, Oberkochen, Germany) according to the procedure reported in detail in our previous study [22]. Before slide preparations, IC and BC pellets separated from the fractionation medium were washed several times with water to separate the bacteria, which might be stuck samples tissues.

5.2.7. SCFAs and BCFAs analysis

Straight-chain and branched-chain SCFAs analysis was performed as previously described [19, 29]. Briefly, 500 µL of supernatant collected after fermentation was thawed and combined with 250 µL of an internal standard (2-ethylbutyric acid in 0.3 M HC and 0.9 M oxalic acid). The mixtures of fermented supernatant and internal standard were centrifuged (9000 x g, 5min, 4°C), and filtered (15mm 0.*2µm* RC filter). The resulting supernatants were placed in a gas chromatography (GC) vial for GC analysis. The measurement of SCFAs was performed using GC coupled with a flame-ionization detector (GC-FID, Shimadzu, Kyoto, Japan). The carrier gas was nitrogen and the temperature of the injector and detector were 100 and 250°C respectively. The ratio between SCFAs and BCFAs was calculated according to Warren, et al [30]and hereafter named BCR.

5.2.8. Ammonia determination

Ammonia was measured using the Megazyme Rapid Ammonia Assay Kit (PC: K-AMIAR; Megazyme International, Wicklow, Ireland) according to the manufacturer's instructions. Before performing the analysis, I mL of supernatants collected at different time points throughout fermentation were thawed, centrifuged (9000 x g, 5min, 4°C), and filtered (15mm 0.2µm RC filter).

5.2.9. Statistical analysis.

Data were expressed as mean and standard deviations of at least triplicate measurements. Statistical analysis was performed using IBM SPSS statistics 25 link (NY: IBM Corp). Differences were assessed by ANOVA followed by Tukey's test. A value of $P < 0.05$ was considered statistically significant.

5.2.10. Curve fitting and estimation of fractional gas and ammonia production rate

A fractional conversion model (Eq. (1)) has been used for the interpretation of gas and ammonia production data and for investigating the effects of cell integrity and heat treatment on parameter estimates.

$$A_t = A_{max} \times \left(1 - exp^{(-kt)}\right) \qquad (1)$$

where **t** is time (h), ***exp*** is the exponential function, **k** is the fractional rate of gas or ammonia production (/h), $\mathbf{A}_{max}$ is the theoretical maximum production of gas (mL) or ammonia (mmol) after the asymptote is reached, and $\mathbf{A}_t$ is ammonia produced at time **t** (gas_(mL)) or (ammonia_(mmol)),

Curves fitting are shown in Fig.S.I-1 and 2 and the estimated parameters (**A_{max}** and **k)** are as presented in Tables 2 and 4 for gas and ammonia respectively. Statistical significance ($P < 0.05$) between estimated parameters within each pair of samples was evaluated based on the method developed by Julious [31] using the confidence intervals around individual means of the parameter estimates.

5.3. Results and discussion

5.3.1. Gas production during in vitro fecal fermentation

The degree of fermentation is usually measured in terms of gas production, fermentation products, and substrates degradation. During in vitro batch fermentation, measuring the cumulative gas production provide a quantitative estimation of substrate fermentability. The amounts of gas produced at various stages during fermentation can be a rapid indicator of fermentation kinetics of different substrates [32]. Fig. 5-1 shows the kinetics of gas production of different pairs of samples during the fermentation time course. In all samples, gas produced accumulated in the system for 24 hours and then tended to a plateau. However, no significant differences ($p < 0.05$) in the amount of gas production between undigested cell samples (IC and BC) were observed (Fig.5.1-A). The amount of gas produced from both DIC and DBC, i.e. cells that were pre-digested before fermentation, was also not significantly different ($p < 0.05$), although DIC produced a lower amount of gas compared to DBC. Furthermore, fractional gas production rate (k) and maximum gas production (A_{max}) which were obtained from the fractional conversion model (Table 3) were not influenced ($P > 0.05$) by cell structure damage for both undigested cells (IC, and BC) and digested cells (DIC, and DBC). This provides further evidence that the rate and extent of gas production during in vitro fermentation did not influence by the difference in soybean cell structure. As such, this result is in contrast with the conventional opinion, i.e. that the kinetics rate of gas production during fermentation is increased by compromising plant cell integrity [33-35]. The lack of a significant association between cell structure damage and gas production during in vitro fermentation found in the current study can be attributed to the absence of rapidly fermentable carbohydrates among the intracellular components of soybean cells, particularly starch in our soybean samples. The starch content of dry soybean seeds at maturity sharply declines to <1% on a dry basis [21, 36]. A recent study demonstrated the *in vitro* fermentation of starch resulted in significantly higher gas production as opposed to *in vitro* fermentation of isolated cell walls [35].

As for the pair of samples of heat-treated protein (HCP) and raw protein (RCP), no statistically significant differences in the amount of gas produced throughout the entire fermentation period were observed, except for the point of 48 h. When looking at the amount of gas production among the three pairs of samples (Fig.5.1), the pair of samples DBC and DIC (Fig. 5.1-B) that

has higher amounts of carbohydrate compared to other sample pairs (see Table 2) showed the highest gas production. An early study has shown that the ratio of protein to the carbohydrate of foods/diet is a determinant factor in the fermentation kinetics and protein contributed less than fermentable carbohydrates to the total gas produced during fermentation [31].

Table 5.1: Chemical composition (%) on a dry basis of samples.

Chemical composition (% DM)	Samples					
	Pair samples (A)		Pair samples (B)		Pair samples (C)	
	Undigested intact cells (IC)	Undigested broken cells (BC)	Predigested intact cells (DIC)	Predigested broken cells (BC)	Raw concentrated protein (RCP)	Heated concentrated protein (HCP)
Protein	48.5 ± 1.2	48.5 ± 1.2	23.28 ± 0.7	16.0 ± 0.5	77.3± 3.4	77.3± 3.4
Lipid	23.6 ± 0. 9	23.6 ± 0. 9	35.2 ± 1.4	38.5 ± 0. 9	NA	NA
Carbohydrates	24.8± 1.2	24.8± 1.2	36.9± 1.7	40.4 ± 2.5	NA	NA
Ash	3.1 ± 0.3	3.1 ± 0.3	4.6 ± 0.6	5.1 ± 0.5	NA	NA

Note: BC contains the same chemical composition as the corresponding intact cells, and were not assayed separately. Likewise, HCP contains the same protein as in RCP. NA: not analysed.

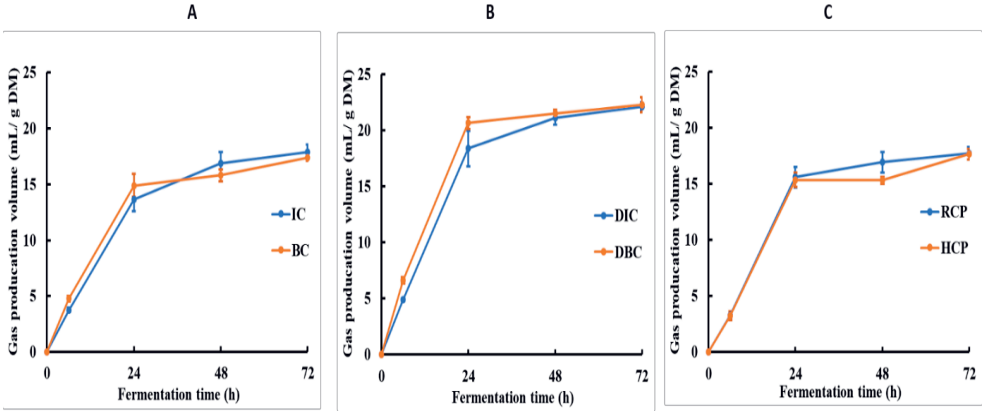


Fig. 5-1. Gas production during *in vitro* fecal fermentation time course of samples; A= pair samples of intact cells (IC), broken cells (BC), B = pair samples of pre-digested intact cells (DIC), pre-digested broken cells (DBC), C = pair samples of raw concentrated protein (RCP), and heat-treated concentrated protein (HCP).

Table 5.2. The fitted parameters of gas production profiles.

	Samples					
	Pair samples (A)		Pair samples (B)		Pair samples (C)	
	IC	BC	DIC	DBC	RCP	HCP
A_{max} (mmol)	18.64 ± 0.83 [a]	17.29 ± 0.83 [a]	22.83 ± 1.27 [a]	22.69 ± 1.14 [a]	18.36 ± 1.56 [a]	17.63 ± 1.74 [a]
k (h)	0.05 ± 0.01 [a]	0.07 ± 0.01 [a]	0.06 ± 0.01 [b]	0.08 ± 0.01 [a]	0.06 ± 0.02[a]	0.07 ± 0.02 [a]

A max, theoretical maximum ammonia production (mmol); k, fractional ammonia production (/h) Different letters indicate significant differences ($p < 0.05$) in k and A_{max} between samples in each pair. A= pair samples of intact cells (IC), broken cells (BC), B = pair samples of pre-digested intact cells (DIC), pre-digested broken cells (DBC), C = pair samples of raw concentrated protein (RCP), and heat-treated concentrated protein (HCP).

5.3.2 Characterization of structural changes during in vitro fecal fermentation

Fig.5.2. shows CLSM micrographs of IC and BC during *in vitro* fermentation time course (6, 24, 48, 72 h). It could be seen that IC (Fig. 5-2 - top panel) maintained their cellular structural integrity upon to 48 h of *in vitro* fecal fermentation. This indicates that the cellular structure of soybean cells has the potential to resist prolonged fermentation treatment. The resistance of plant cell structure to degradation during in vitro fermentation has already been reported for carrot cell clusters [33], kidney bean cells [37], and pinto beans cells [35] when those plant tissues were fermented in an *in vitro* fermentation model. Nevertheless, in our study, fractured cells were observed after the prolonged fermentation (72 h), indicating that cell walls had been partially utilized at the later stage (72 h) of fermentation. *In vitro* fermentation studies conducted with pea, and mungbean using porcine fecal inoculum suggested that the 95 ^{o}C treated cell walls in intact cells may not hinder the fermentation of the encapsulated starch [38]. Using SHIME® model colonic fermentation, a study conducted with intact cells compared to damaged cells of kidney bean showed that the low availability of starch to colonic microbiota, which caused by cell wall integrity of the intact cell, direct the colonic microbiota towards cell wall utilization, where damaged cells starch was highly utilized by colonic microbiota [19]. Data of this study showed differences in monosaccharide composition and starch content after 3 and 12 days of colonic fermentation of intact cells and damaged cells of kidney bean.

CLSM micrographs of fermented BC (Fig. 5-2 - bottom panel) show a qualitative impression of the degradation of the cellular contents during in-vitro fermentation. It is important to point out that, despite the availability of cell contents in BC due to the cell wall damage, residues of cell contents are still visible even at the end of fermentation (72 h). This finding goes in accordance

to what has already been reported for kidney bean broken cells [37] and mungbean broken cells [38].

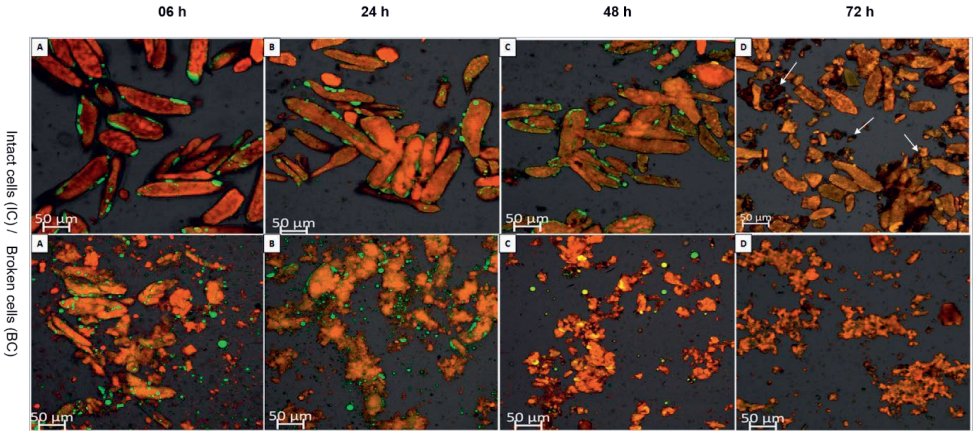


Fig.5.2. Confocal laser scanning micrographs of IC (intact cells isolated from soybean cotyledon - top panel) and BC (broken cells of soybean cotyledon - bottom panel) at 6, 24, 48, and 72 h of in-vitro fermentation. Protein bodies and oil bodies were stained with rhodamine B (red) and BODIPY 505/515 (green/yellow) respectively. For all samples, micrographs were taken using a 20x magnification lens. Fractured cells in image D- top panel are indicated by the white arrows.

5.3.3. SCFAs and BCFAs production during in vitro fecal fermentation

Table 3 shows the concentration and percentage of SCFAs (acetic, propionic, and butyric) and BCFAs (isobutyric, and isovaleric) produced at different time points of *in vitro* fermentation of different pairs of samples. Marked differences were found when comparing the total amounts of SCFAs generated throughout the whole fermentation period between the pair samples of IC and BC with the highest values observed for BC, except for the point of 24 h. Nevertheless, it is important to mention that the statistically significant differences at $p < 0.05$ were only observed at 6 h and 72 h. The values of SCFAs that were produced at the intermediate fermentation times (24 h and 48 h) of IC and BC showed no statistically significant differences. When comparing the pair samples DIC and DBC (the collected samples after *in vitro* protein digestion), a significant increase ($p < 0.05$) in the SCFAs production could be observed for DBC especially at 6 h, and 48 h of fermentation. In general, SCFAs are the end products of carbohydrate fermentation by the colonic microbiota [36], however, they can be produced, to a smaller extent, during the fermentation of amino acids by reductive deamination [2, 37]. This may explain why pair samples of RCP and HCP, which have the highest protein content (Table 5-1), have such low SCFAs production when compared to the other pairs of samples (IC and BC) and (DIC and DBC). In our study, despite that all samples display differences in the total concentration of SCFAs, SCFAs patterns follow the order acetate > propionate > butyric in all

samples. Various population survey data showed that the fecal SCFA production pattern is in the order of acetate > propionate > butyrate [39, 40].

Table 5-2 also represents the branched-chain ratio (BCR) which is identified as an indicator of protein fermentation [35] As for the BCFAs production during the fermentation time course, IC showed a lower BCFAs production ($p < 0.05$) compared to BC. Identical trends were observed when comparing pair samples (DIC and DBC). This suggests that differences in cell integrity and matrix could contribute to lower BCFAs production during protein colonic fermentation of plant foods. When comparing BCFAs production between heat-treated protein (HCP) to raw protein (RCP), it was not surprising to observe that heating treatment has significant effects on BCFAs production during the fermentation time course. it is generally accepted that heat treatment induces conformational changes in protein structure, which in turn, increases the protein susceptibility to proteolytic enzymes during intestinal digestion and fermentation at large intestinal [41, 42].

Interestingly, it was observed that for BC, the value of BCR was relatively higher than its IC counterpart, particularly at 24 and 48 h of the fermentation time course. This could be related to the quantity of protein available for bacteria during the early stages of fermentation. Therefore, isolated protein samples (RCP and HCP) which are devoid of cellular structures and are higher in protein content compared to the other pairs of samples (IC and BC) and (DIC, and DBC) showed the highest values of BCR (≤ 0.12).

Table 5.3. Concentration and Percentage of SCFAs and BCFAs produced during the in vitro fermentation of samples; intact cells (IC), broken cells (BC), pre-digested intact cells (DIC), pre-digested broken cells (DBC), raw isolated protein (RCP), and heated isolated protein (HCP).

Sample		Time (h)	*SCFAs (mmol/ g 1DM)			Total SCFAs	**BCFAs (mmol/ g 1DM)		Total BCFAs	% of total SCFAs			***BCR
			Acetic	Propionic	Butyric		Isobutyric	Isovaleric		Acetic	Propionic	Butyric	
Samples pair (A)	IC	6	0.30 ± 0.03^{b}	0.14 ± 0.01^{b}	0.02 ± 0.00^{a}	0.46 ±0.02^{b}	0.01 ± 0.001^{a}	0.000 ± 0.0000^{b}	0.01 ± 0.0007^{b}	66.34	29.92	3.74	0.03
		24	0.77 ± 0.02^{a}	0.36 ± 0.04^{a}	0.04 ± 0.00^{b}	1.17 ±0.03^{a}	0.01 ± 0.001^{b}	0.000 ± 0.0001^{b}	0.01 ± 0.001^{b}	65.90	30.91	3.19	0.01
		48	0.78 ± 0.02^{a}	0.37 ± 0.01^{a}	0.09 ± 0.01^{a}	1.24 ±0.03^{a}	0.03 ± 0.002^{b}	0.011 ± 0.0014^{b}	0.04 ± 0.003^{b}	62.55	30.02	7.43	0.03
		72	1.02 ± 0.03^{b}	0.59 ± 0.04^{d}	0.18 ± 0.01^{a}	1.78 ±.05^{b}	0.07 ± 0.000^{b}	0.013 ± 0.0014^{b}	0.08 ± 0.001^{a}	57.15	33.01	9.84	0.05
	BC	6	0.54 ± 0.02^{a}	0.23 ± 0.00^{a}	0.02 ± 0.00^{a}	0.79 ±0.02^{a}	0.01 ± 0.001^{a}	0.002 ± 0.0002^{a}	0.02 ± 0.002^{a}	68.74	28.48	2.77	0.02
		24	0.71 ± 0.02^{a}	0.34 ± 0.01^{a}	0.10 ± 0.00^{a}	1.15 ±0.03^{a}	0.03 ± 0.001^{a}	0.009 ± 0.0004^{a}	0.04 ± 0.002^{a}	61.57	29.74	8.69	0.04
		48	0.76 ± 0.02^{a}	0.41 ± 0.01^{a}	0.10 ± 0.00^{a}	1.27 ±0.03^{a}	0.04 ± 0.002^{a}	0.017 ± 0.0008^{a}	0.06 ± 0.002^{a}	59.93	31.96	8.12	0.04
		72	1.33 ± 0.10^{a}	0.78 ± 0.02^{b}	0.17 ± 0.00^{a}	2.29 ±0.11^{a}	0.06 ± 0.002^{a}	0.021 ± 0.0003^{a}	0.08 ± 0.002^{a}	58.17	34.32	7.51	0.03
Samples pair (B)	DIC	6	0.63 ± 0.02^{b}	0.22 ± 0.02 ^{b}	0.03 ± 0.00^{a}	0.88 ±0.02^{b}	0.01 ± 0.002^{a}	0.002 ± 0.0002^{a}	0.02 ± 0.001^{b}	71.78	24.84	3.38	0.02
		24	1.47 ± 0.04^{b}	0.53 ± 0.01^{a}	0.08 ± 0.00^{b}	2.08 ±0.04^{a}	0.02 ± 0.001^{a}	0.003 ± 0.0003^{a}	0.02 ± 0.001^{b}	70.78	25.29	3.94	0.01
		48	1.75 ± 0.03^{b}	0.86 ± 0.02^{a}	0.10 ± 0.01^{a}	2.71 ±0.02^{b}	0.03 ± 0.001^{a}	0.011 ± 0.0014^{a}	0.04 ± 0.002^{a}	64.65	31.82	3.54	0.01
		72	1.84 ± 0.04^{a}	0.91 ± 0.01^{a}	0.15 ± 0.01^{b}	2.90 ±0.02^{a}	0.04 ± 0.002^{a}	0.011 ± 0.0004^{a}	0.05 ± 0.002^{b}	63.45	31.33	5.22	0.02
	DBC	6	0.70 ± 0.02^{a}	0.30 ± 0.01 ^{a}	0.04 ± 0.00^{a}	1.04 ±0.02^{a}	0.02 ± 0.002^{a}	0.002 ± 0.0002^{a}	0.02 ± 0.002^{a}	67.31	29.24	3.45	0.02
		24	1.40 ± 0.14^{a}	0.71 ± 0.02 ^{a}	0.05 ± 0.00^{a}	2.16 ±0.15^{a}	0.02 ± 0.001^{a}	0.003 ± 0.0002^{a}	0.02 ± 0.002^{a}	64.98	32.75	2.27	0.01
		48	1.95 ± 0.11^{a}	0.89 ± 0.02^{a}	0.09 ± 0.00^{a}	2.93 ±0.11^{a}	0.03 ± 0.001^{a}	0.006 ± 0.0004^{a}	0.03 ± 0.002^{b}	66.45	30.39	3.16	0.01
		72	2.00 ± 0.15^{a}	0.89 ± 0.02^{a}	0.18 ± 0.01^{a}	3.08 ±0.14^{a}	0.04 ± 0.002^{a}	0.011 ± 0.0005^{a}	0.06 ± 0.001^{a}	65.12	29.05	5.83	0.02
Samples pair (C)	RCP	6	0.21 ± 0.01^{a}	0.05 ± 0.00 ^{b}	0.01 ± 0.00^{a}	0.28 ±0.01^{b}	0.02 ± 0.002^{a}	0.002 ± 0.0002^{a}	0.02 ± 0.002^{b}	75.55	19.21	5.24	0.09
		24	0.29 ± 0.05^{a}	0.22 ± 0.01^{b}	0.02 ± 0.00^{a}	0.53 ±0.04^{a}	0.05 ± 0.002^{b}	0.012 ± 0.0009^{a}	0.06 ±.002^{b}	54.95	41.97	3.08	0.12
		48	0.62 ± 0.01^{b}	0.33 ± 0.00^{b}	0.02 ± 0.00^{a}	0.97 ±0.01^{b}	0.05 ± 0.001^{b}	0.019 ± 0.0019^{a}	0.07 ± 0.003^{b}	64.06	33.59	2.35	0.07
		72	1.07 ± 0.04^{a}	0.57 ± 0.01^{b}	0.03 ± 0.00^{b}	1.67 ±0.03^{a}	0.10 ± 0.007^{a}	0.023 ± 0.0017^{a}	0.12 ± 0.006^{b}	63.99	34.36	1.64	0.07
	HCP	6	0.34 ± 0.03a	0.11 ± 0.01^{a}	0.01 ± 0.00^{a}	0.46 ±0.04^{a}	0.01 ± 0.001^{a}	0.002 ± 0.0002^{a}	0.02 ± 0.002^{a}	73.58	23.48	2.94	0.03
		24	0.73 ± 0.01^{a}	0.62 ± 0.03 ^{a}	0.02 ± 0.00^{a}	1.37 ±0.03^{a}	0.07 ± 0.001^{a}	0.012 ± 0.0005^{a}	0.08 ± 0.002^{a}	53.26	45.07	1.67	0.06
		48	0.81 ± 0.01^{a}	0.60 ± 0.01^{a}	0.03 ± 0.00^{a}	1.44 ±0.01^{a}	0.07 ± 0.002^{a}	0.022 ± 0.0010^{a}	0.09 ± 0.002^{a}	56.33	41.76	1.91	0.07
		72	1.13 ± 0.10^{a}	0.69 ± 0.02^{a}	0.06 ± 0.00^{a}	1.88 ±0.01^{a}	0.10 ± 0.003^{a}	0.026 ± 0.0026^{a}	0.13 ± 0.003^{a}	60.06	36.71	3.23	0.07

Data are expressed as means ± standard deviation of four replicates of two donors/in vitro fermentation experiments. The different letters branched-chain fatty acids, Different letters indicate significant differences ($p < 0.05$) in acid concentration between samples in each pair. * SCFAs = short-chain fatty acids, ** BCFAs = *** BCR = the ratio between SCFAs and BCFAs.

5.3.4. Ammonia production during in vitro fecal fermentation

Ammonia produced at different time points of *in vitro* fermentation of different pairs of samples is shown in Fig. 5.3. It is evident from Fig. 5.3-a that the concentration of ammonia is relatively lower in IC compared to BC. One way ANOVA revealed significant ($P < 0.01$) effects of cell integrity on ammonia production during IC and BC fermentation. This result is in agreement with data from a previous *in vitro* fermentation study that used intact cells and broken cells of different legumes (e.g. pea and mungbean) and reported a reduction in ammonia production due to the structural integrity of cells [38]. The kinetic parameters of ammonia production from an intact cell (IC) and broken cell (BC) in our study (Table 2) showed that cell wall integrity decreases the extent of ammonia production. The theoretical maximum estimated from the curve fitting of the ammonia production (Fig. S.5.I.1) was 9.46 mmol /g for IC (Table 5.4), which was lower ($P < 0.01$) than that obtained for BC (13.2 mmol /g DM). The cell structure, however, has no significant effect on rate constant (k). It seems that the lower availability of cell wall materials, as it is the case for the intact cell, and the absence of fermentable carbohydrates within intracellular nutrients (e.g. starch) of soybean cells directed bacterial to utilize protein as the energy source, resulting in the same ammonia production rate for IC and BC, although the extent of ammonia production vary between both samples. When comparing DIC and DBC, that have a different residue of protein as a result of previously *in vitro* protein digestion, a significant difference ($P < 0.01$) in rate constants (k) was observed. DBC showed the higher rate ammonia production (0.157 mmol /h) compared to DIC (0.067 mmol /h). A possible reason is that both cell structural damage and partially protein hydrolysis result in higher protein accessibility and eventually rapid protein utilization by fecal bacteria. The lack of a significant difference in the extent of ammonia production (A max) observed between DIC and DBC is likely to be due to protein depletion of DBC. It could be seen in Fig.5.3.b that the ammonia production in DBC kept slightly stable after 24 h of fermentation. Thus, it could be inferred that both cell structure and cell composition could modulate protein colonic fermentation.

As for the effect of heat treatment (Fig.5.4-B), heat-treated protein (HCP) showed a significant increase in ammonia concentrations compared to raw protein (RCP) but only during the early stages of fermentation (0-24h). This could indicate that bacteria might have used more protein for metabolic energy at the latter stages of fermentation (48-72h), which results in equal ammonium production for both HCP and RCP. This because HCP and RCP contain mainly proteins (see table 5-1). It has shown that in the absence or the depletion of a favourable energy source for gut bacteria such as carbohydrates during the fermentation process, gut bacteria tend to utilize more protein for metabolic energy, resulting in higher ammonium and BCFA production during fermentation [6, 43, 44].

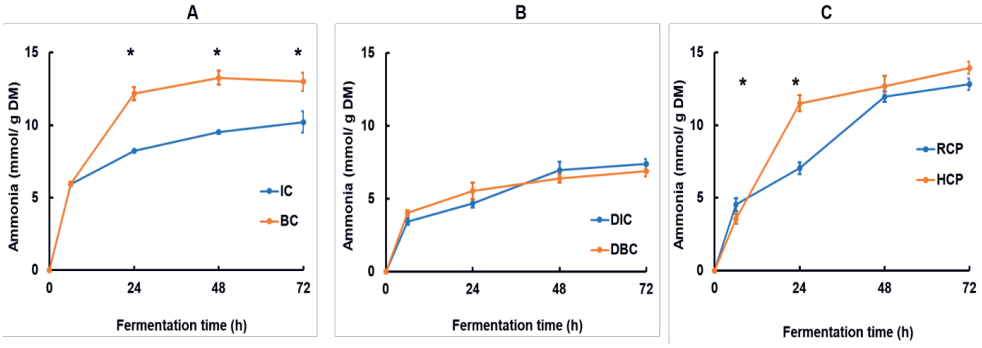


Fig.5.3. Ammonia production during in vitro fecal fermentation time course of samples; A= pair samples of intact cells (IC), broken cells (BC), B = pair samples of pre-digested intact cells (DIC), pre-digested broken cells (DBC), C = pair samples of raw concentrated protein (RCP), and heat-treated concentrated protein (HCP). Data are expressed as mean of two donors ± SEM, with *p < 0.05,

Table 5.4. The fitted parameters of ammonia production profiles.

	Samples					
	Pair samples (A)		Pair samples (B)		Pair samples (C)	
	IC	BC	DIC	DBC	RCP	HCP
A max (mmol)	9.46 ± 0.45 [b]	13.21 ± 0.12 [a]	7.13 ± 0.77[a]	6.36 ± 0.32 [a]	13.70 ± 1.91 [a]	13.99 ± 0.64 [a]
k (h)	0.154 ± 0.04 [a]	0.102 ± 0.00 [a]	0.067 ± 0.03 [b]	0.157 ± 0.04 [a]	0.038 ± 0.01[a]	0.061 ± 0.01 [a]

A max, theoretical maximum ammonia production (mmol); k, fractional ammonia production (/h). Different letters indicate significant differences ($p < 0.05$) in k and A_{max} between samples in each pair. A= pair samples of intact cells (IC), broken cells (BC), B = pair samples of pre-digested intact cells (DIC), pre-digested broken cells (DBC), C = pair samples of raw concentrated protein (RCP), and heat-treated concentrated protein (HCP).

5.4. Conclusions

The current in vitro fermentation study showed that the structural integrity and the chemical composition of plant tissues, as well as the heat treatment, may modulate in vitro fermentability of soybean plant protein. Higher BCFAs and ammonia production were associated with cell wall damage, the presence of protein throughout fermentation, and the application of heat treatment. Furthermore, the occurrence of cell structure damage during intestinal digestion increases protein digestibility. This modulated the supply of dietary components into the large intestine, particularly the ratio of protein to dietary fiber, and SCFAs production. Our results indicate that the food structure and composition, as well as the heat treatment, may be used as strategies to modulate protein colonic fermentation. However, further studies are needed to confirm these results and to expose the changes in the microbiota composition which may occur as a result of different protein fermentability.

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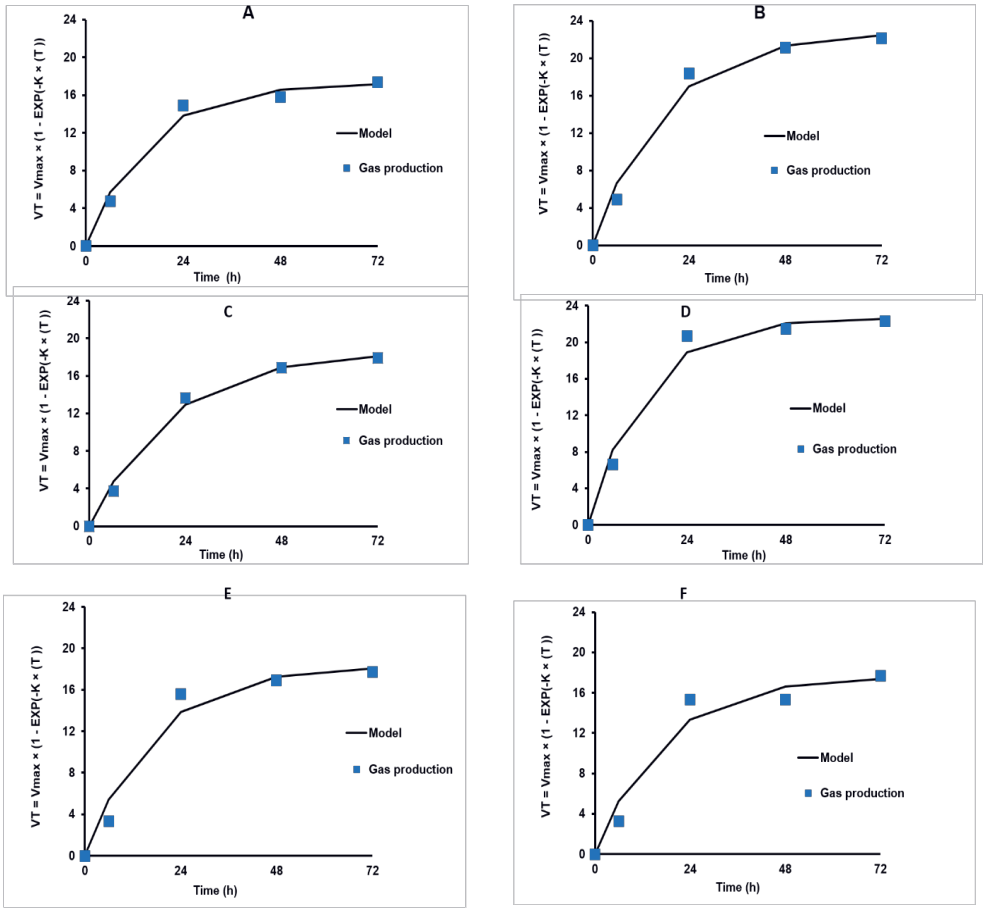
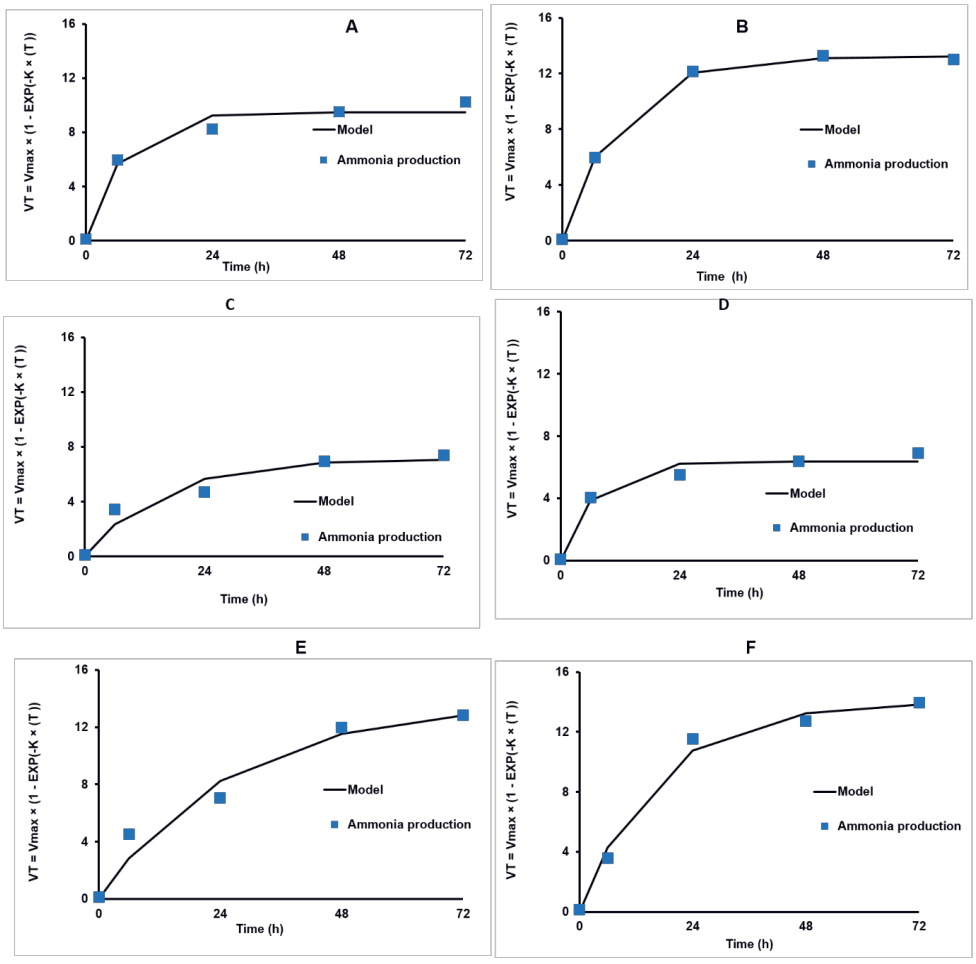
Supporting information



S.I- Fig.5.1. Fitting the time course of gas production during in vitro fermentation of different soybean tissues to a simple exponential model, A= undigested intact cells (IC), B = undigested broken cells (BC), C = predigested intact cells (DIC), D = predigested broken cells (DBC), E= raw concentrated protein (RCP), and F = heated concentrated protein (HCP).



S.I- Fig.5.2. Fitting the time course of ammonia production during in vitro fermentation of different soybean tissues to a simple exponential model, A= undigested intact cells (IC), B = undigested broken cells (BC), C = predigested intact cells (DIC), D = predigested broken cells (DBC), E= raw concentrated protein (RCP), and F = heated concentrated protein (HCP).

Chapter 5

Chapter 6

General discussion

6.1. Introduction

The digestibility and utilization of macronutrients, which play an important role in the human diet, are a concern to consumers, researchers, and food industry processors. In-plant foods, in which macronutrients are surrounded by cell walls, the digestibility of macronutrients deserves special attention, since the structural barrier may determine the bioavailability of nutrients during gastrointestinal digestion. Over the past years, studies have been focused on the starch and lipid digestion in cereals, legumes, and nuts using different levels of cellular integrity [1-4]. Plant protein digestion, as effected by cell wall integrity, has been overlooked in many of those studies. Moreover, very scarce literature is available regarding the relationship between food processing (intensity/condition) and cellular integrity to protein digestibility and colonic fermentability [2]. Therefore, the overall aim of this thesis was to understand the role played by the cellular integrity of legumes tissues in limiting protein digestion and fermentation. The potential role of food processing in modulating cell wall integrity and protein digestion was also considered. Soybean particle sizes and isolated intact cells were used as a food tissue model to monitor their cell wall integrity/ porosity and to assess their protein digestibility. The present chapter intends to discuss the main findings obtained in the research chapters (Table. 6-1) and to offer a broader perspective about the implications, the relevance to improving plant protein digestibility, and future perspectives in this field.

Table 6.1. Summary of the main results obtained in this thesis.

	objective	Main findings
Chapter 2	• To understand the behavior of cell separation during cooking.	• Only the severe thermal treatment gave satisfactory cell separation and facilitates cell isolation from soybean cotyledons.
	• To evaluate cell wall breakage upon milling/mashing.	• Cell breakage during the milling process is different according to whether they are milled/mashed in raw form or cooked form.
	• To assess the role of the particle size in modulating protein digestion.	• Particle size reduction improves protein digestibility
	• To evaluate the kinetics of protein digestion contained within soybean particles.	• The kinetics of protein hydrolysis in soybean particles can be described by double exponential kinetics.
	•To understand the role of the oil bodies in modulating protein digestion	• The oil bodies which form borders ringing the protein bodies act as a barrier affecting protein hydrolysis.
Chapter 3	• To highlight the role of pectin solubilization in modulating cell wall porosity.	• Pectin solubilization/degradation from cell walls increases the porosity of cell walls.
	• To address the role of fermentation and germination in modulating cell wall porosity and protein digestibility.	• Fermentation and germination enhance cell wall porosity and protein digestibility.
	• To understand the potential role played by the digestion process in modulating cell wall permeability.	• The digestion process, particularly the action of protease enzymes, may have a contributing role in modulating cell wall permeability.
	• To visualize the diffusion of trypsin into the cell space.	• Cell walls of cooked soybean are not an absolute barrier to pancreatic proteases, however, the diffusion of pancreatic proteases into cells within particles is limited by the particle size and thickness.
Chapter 4	• To understand the role of cell walls integrity of non-germinated and germinated soybean in attenuating the physicochemical changes during cooking.	• The cell wall integrity have a protective effect on the heat-induced protein physicochemical changes, and a significant effect was observed for the protein of non-germinated compared to germinated soybean
	• To link between protein conformational changes during cooking and its digestibility.	• The distinct changes in protein conformation which were induced by germination and/or boiling, translated into an increment in protein digestibility.
Chapter 5	• To understand the role of the cell wall in protein colonic fermentation.	• The cell wall of intact cells delays and limit colonic fermentation of protein.
	• To highlight the effect of thermal treatment in modulating protein colonic fermentation.	• Cooking has a limited role in modulating protein fermentation.

6.2. Role of the cell walls in modulating protein digestion and fermentation.

Accumulating evidence shows the natural structural barriers, particularly of the cell wall component play an important role in regulating the digestion of starch and lipids within an intact plant matrix [5-14]. However, the effect exerted by the plant cell wall on protein digestibility in legumes has not been conclusive. In this thesis, the levels of cellular integrity were shown to restrict the access of digestive enzymes and limit protein digestion. In **Chapter 2**, using soybean particles of different sizes as substrates for in vitro protein digestion, we have demonstrated that protein digestibility in soybean is limited by the size of the soybean particle and its cellular integrity. The microscopic examination of recovered particles after *in vitro* digestion (**Chapter 2**) showed that the encapsulated proteins within the intact cells remained undigested or were not fully digested. Whereas when the cellular structure was damaged, a greater proportion of the intracellular proteins were easily digested depending on the degree of damage. Microscopy observations of the particle also revealed that a greater proportion of cells within particle prepared from milled and then boiled cotyledons are damaged compared with the particle prepared from boiled and then milled cotyledons. Macroscopy evidence from an earlier study on almond showed that the intracellular nutrients (i.e., lipid, and protein) in structurally intact cells showed no signs of digestion. In contrast, those nutrients in fractured cells were fully digested [15].

By using a kinetic modeling approach to describe the data of *in vitro* protein digestibility of different particle sizes **(Chapter 2**)**,** we observed that the protein hydrolysis in soybean particles occurred with kinetics which might reflect differences in protein accessibility within the particles. Based on the microscopy observations, we observed that proteins were located in two distinct fractions of the particle; (i) the fraction of particle surface that comprised ruptured cells where proteins are released from the cells (ii) the fraction of particle core that comprised intact cells where the protein was enclosed inside cells. The proteins that were in ruptured cells can be digested at a faster rate while the proteins that were locked within intact cells of the particle can be digested at a slower rate. The model-computed digestibility curves of particle revealed two distinct linear phases, in which the slope of each distinct phase provides a rate constant. Thus, the kinetics of protein hydrolysis in soybean particles can be described by double exponential kinetics. A previous study had shown that starch hydrolysis in wheat and chickpea particles occurred by a two-phase process that reflected differences in starch accessibility within particles [16].

For a closer examination of the role of cell wall integrity in regulating protein digestion, intact cotyledon cells were isolated and subjected to *in vitro* digestion (**chapter 2)**. In such a way,

it was possible to ensure that the results obtained after *in vitro digestion* reflected homogeneous conditions in terms of cell wall integrity and intracellular protein accessibility. The data of *in vitro* protein digestibility of isolated intact cells together with the microscopy observations after *in vitro* digestion suggested that the digestion of proteins in intact cells is limited by the diffusion of digestive enzymes through cell walls. The *in vitro* trypsin diffusion observation using fluorescently labelled trypsin visualized by confocal microscopy was reported for the first time in this thesis (**chapter 3**). Results demonstrated that the intact cell wall is not an absolute barrier for trypsin diffusion into the cell space of single individual cells. Nevertheless, the digestibility of protein of intact cells (**chapter 3**) is limited, independently from the rate of fluorescently labelled trypsin diffusion. By monitoring the diffusion of trypsin into particle or cluster cells compared to single individual cells, we found the diffusion of trypsin into the particles is very limited. This is the most critical point in terms of protein accessibility and digestion as plant foods (e.g. legumes) are typically consumed as whole tissue and they are broken down into particles of different sizes rather than single individual cells during human mastication.

The barrier effect exerted by the plant cell wall on the bioavailability of protein contained within intact cells during small intestinal digestion is also likely to modulate microbiota accessibility to the protein of intact cells and thus modulate its colonic fermentation. However, studies assessing the effect of cellular entrapment on protein colonic fermentability, and microbiota composition, as a result of protein fermentation, are very rare [8, 17]. Most studies regarding dietary protein focus on the detection of altered fermentation products [17]. In some studies, a drop in the abundance of the end products of protein fermentation was observed following the consumption of fermentable carbohydrates such as resistant starch and fibers products [18, 19]. However, the effect exerted by fibers when they occur in their complex supramolecular assembly as cell walls on plant protein colonic fermentation is still not well understood. Therefore, in **Chapter 5**, the potential role of cell wall integrity in modulating protein colonic fermentability was studied by comparing two different structural matrices (intact cells, and broken cells). Confocal microscopic observations of intact cells made at different time points of *in vitro* fermentation provided a qualitative impression that the cellular structure of soybean cells is able to resist prolonged fermentation treatment. Moreover, intact cells were associated with fewer fermentation end-products (BCFAs and ammonium) compared to broken cells. On the contrary, the production of short-chain fatty acids (SCFAs) was higher for broken cell than what was found for intact cells. Therefore, we concluded that the ammonium and BCFAs production (the two main end-products of protein fermentation) could be limited by the presence of cellular integrity of plant cells during colonic fermentation. It is important to highlight that lower SCFA production and higher production of ammonia and BCFAs, as a result of protein fermentation, have been considered potentially harmful to host health and have been implicated in the pathogenesis of large intestinal diseases. In particular,

their roles in colorectal cancer, inflammatory bowel disease, and functional bowel disorders have been proposed [17, 20-22]. Thus reducing protein colonic fermentability is highly relevant to human health.

Apart from the effect exerted by the plant cell wall integrity on protein accessibility during digestion, the cell wall may also modulate protein physicochemical changes induced by thermal treatments such as protein denaturation [7, 23]. Denaturation of protein is a key factor in protein hydrolysis since it facilities the digestive enzymes reaching their internal sites of hydrolysis [24]. In **Chapter 4** we studied the differential response of soybean protein to heat-induced physicochemical changes when it is heated inside intact cells, as it is the case for cooked intact cotyledon, or in flour. The study results revealed that the presence of an intact cell wall during cooking limited protein physicochemical changes induced by thermal treatment. Differences were observed when comparing protein denaturation temperature, level of residual trypsin inhibitor activity, and the changes in protein surface hydrophobicity, and protein secondary structure between boiled whole cotyledon and boiled flour. Compared to soybean particles protein, soybean flour protein showed lower denaturation temperatures as determined by differential scanning calorimeter. The short boiling treatment (30 or 90 min) resulted in a lower level of residual trypsin inhibitor activity and more distinctive changes in protein surface hydrophobicity, and protein secondary structure for soybean flour compared to soybean cotyledon. These distinctive changes in protein physicochemical properties were translated into an increment in protein digestibility, as demonstrated through determining *in vitro* protein digestibility values for boiled flour and boiled cotyledon **Chapter 4**. Interestingly, a short boiling treatment was efficient to improve soybean protein digestibility when soybean flour devoid of cellular structures was cooked. Such a phenomenon was already observed for kidney bean protein when bean flour was boiled for 30 min [7].

In Chapter 4, we have also shown that germination before boiling was able to produce some distinctive changes in protein physicochemical properties and eventually limited the effects exerted by the plant cell wall on the protein physicochemical changes during cooking of non-germinated soybean. The occurrence of plant cell wall integrity during cooking limited the heat-induced protein conformational changes of the boiled whole soybean cotyledon. However, this effect was limited when a germination process was applied before boiling. It is possible germination may have changed the molecular structure of storage proteins due to metabolic reactions [25-27], thereby allowing heating to induce more distinctive changes in protein physicochemical properties. The overall picture indicated that the germination process could be used as a strategy to reduce the detrimental effects exerted by the plant

cell wall on heat-induced protein conformational changes and improve soybean protein digestibility.

6.3. Role of food processing in modulating cell walls porosity and permeability

At the moment, there is limited knowledge about the role of food processing in modulating the permeability of cell walls to protease enzymes. The current study explicitly demonstrates the alteration in cell wall porosity and permeability as a result of food processing. By comparing different cooking times that are expected to induce different degrees of softening of soybean texture (**chapter 2)**, it was possible to observe changes in the behavior of soybean cotyledon cells upon mechanical processes (milling and sieving). For soybean cotyledons that were boiled at 100 °C for 1 h, cotyledon cells showed resistance to separate upon mechanical processes. Conversely, when more severe thermal treatments (boiling at 100 °C for 3.5 h or autoclaving for 10 min at 121 °C) were applied, cotyledon cells showed a higher tendency to separate and the proportion of isolated cells increases as particle size decreases. Previous studies reported the degree of cell separation upon cooking depends on thermal pectin solubilization within the middle lamella that binds two adjacent cells [28-30]. The solubilization of pectin from middle lamella and primary cell wall may lead to different degrees of cell wall permeability, even when isolated cells preserved their physical intactness [28-30]. From the in vitro protein digestibility data presented in **chapter 2**, it was concluded that intracellular protein could be hydrolyzed despite being encapsulated within cell walls, indicating that the cell wall of isolated cells from cooked soybean cotyledon is not an absolute barrier to pancreatic proteases. Using different thermal process intensities for cooking common beans, Pallares, et al [5]. demonstrated that diffusion of fluorescently labelled pancreatic α-amylase inside the isolated cells of cooked common beans is enhanced by the increasing cooking time. The authors hypothesized that the increased cell wall permeability upon the cooking was attributable to the magnitude of pectin solubilization. The effect of pectin solubilization/degradation was assessed in this thesis after enzymatic treatment with pectinase aimed to remove more pectin from cell walls (**Chapter 3**). By measuring the permeability of cell walls base on the penetration of different dextran probes into soybean cells, we provided empirical evidence that pectin solubilization/degradation from cell wall alter its permeability.

Furthermore, in **Chapter 3**, we investigated the role of the food processes such as fermentation and germination in increasing the permeability of cooked soybean cells and found that they become more permeable to the dextran probes after the combined treatment of boiling and germination or fermentation. The exact mechanism of increasing cell wall

porosity and permeability by fermentation and germination process, in soybean cells, is far from being fully understood. Hypothetically, cell wall composition, and structural architecture have been modified during fermentation and germination. Previous researchers found that the solubilization of soybean cell wall polysaccharides (dietary fibre) is increased after either germination or fermentation and attributed that to cell wall polysaccharides mobilization or the degradation which may occur during germination or fermentation [31-37]. The changes in cell wall dietary fibre solubilization upon food processing may affect cell wall architecture and, in turn, increase the size of cell wall pores.

Besides, it is important to mention that the effect of germination and fermentation is not limited to cell wall porosity. Differences in packing levels of macronutrients inside cells were observed between isolated cells from soybean cotyledon that were boiled with or without previous germination or fermentation **(Chapter 3)**. Boiled cells were densely packed with intracellular macronutrients while cells that were first germinated or fermented were loosely packed with intracellular macronutrients. Comparing the diffusion of different FITC-dextran probes into the three different isolated cells we found that boiled cells are not permeable to 20 kDa dextran probe but germinated/ fermented treated cells showed an extensive permeability to different dextran probes sizes (20 kDa, 40 kDa, 70 kDa, and 150 kDa). This suggests that the voids which were observed for germinated/ fermented treated cells allow the mobility of probes molecules inside cells. A previous study showed extensive permeability of dextran probes of 150 kDa for the loosely packed cells of cooked potato. Starch inside the potato cells was completely gelatinized without any distinguishable granular structure after successive treatments with acid/alkali and cooking (70 °C, 20 min), This contrasts with legume cells that retained granular structure even after cooking for 1 h at 90 °C. [38]. All the above-mentioned structural changes are important to consider in the design of food processing for plant foods such as cereals and legumes.

6.4. Protein structural changes during food processing and relevance to its digestibility values.

It is important to consider that the plant protein digestibility is not only affected by cell wall integrity but also affected by the presence of trypsin inhibitor, as well as protein structural properties [39-42]. In this view, this thesis (**chapter 4**) addressed the effects of food processing (e.g., boiling and germination) on the inactivation of trypsin inhibitor, protein aggregation, surface hydrophobicity, and secondary structure as well as consequences of these changes for protein digestibility. Given the relevance of heat-induced reduction in trypsin inhibitor activity, the result showed that boiling treatment resulted in substantial inactivation of the trypsin inhibitors in soybean. Around 90 % of inactivation has been

achieved after 30 min of boiling. Increasing boiling time to 180 min reduced the level of trypsin inhibitor activity to 6.4 % for boiled whole cotyledon and 3.3 % for boiled flour of the total activity that was found in raw soybean. When investigating the role of the germination process in inactivating trypsin inhibitor, it was observed that 44% of inactivation was achieved after the germination process. Natural inactivation of trypsin inhibitors, as a result of the germination process, may have positive repercussions on protein digestibility compared to thermal inactivation which is well-known for its negative repercussions (e.g. protein aggregation or cross-linking), especially when severe heat treatments are used [43-45]. Indeed, as demonstrated through analysis of the SDS-PAGE protein profile in **Chapter 4**, the formation of protein aggregates upon long heating was more visible in non-germinated soybean compared to germinated soybean. We also have shown that germination produced distinctive changes in protein surface hydrophobicity, and protein secondary structure when compared to non-germinated soybean protein. This is not surprising as it has been already reported that the protein surface hydrophobicity increased proportionally to the growth of hypocotyls length and this attributable to changes in protein conformation during germination as a result of the action of endogenous proteases of the germination process [46]. Besides the distinct changes in physicochemical properties of soybean after germination, a natural phenomenon in seed regeneration, an improvement in protein digestibility was observed for soybean proteins after the combination of germination with heat treatment.

6.5. The alterations in cell integrity and matrix during digestion and its role in facilitating the digestion of macronutrients.

Mastication, which consists of breaking down the food ingested into small pieces, is the first physical transformation of food matrices during eating [47, 48]. From a food structure – digestion perspective, food breakdown, and size reduction during oral processing represent the most important processes in plant food digestion. This because the nutrient release from the food matrix in the subsequent digestion compartments is highly dependent on the disintegration of food structure and matrix during oral processing [49]. In **chapter 2**, we used the grinding process to simulate the mastication process and to study the digestive fate of protein of soybean particles with a wide range of sizes (< 70 to > 2000 µm). Apart from the effect of heating treatment on protein digestibility, the results indicated that the rate and extent of intestinal digestion of soybean proteins depend on the size of the particle and the proportion of ruptured cells within the particle. This can be explained by the fact that the relatively small-sized particle and structural damage of the cell wall within the particle would facilitate the exposure of the intracellular protein to pancreatic proteases.

In the stomach, the physiological conditions that include the peristaltic contractions, digestive juices, low acid environment, and digestive enzymes can alter plant cell structure [50].

Thanks to the action of acidic gastric juice, pectin hydrolysis during gastric digestion may occur to some extent [50]. An early study reported a 7–18% of pectin hydrolysis after simulated gastric digestion for four hours [51]. It could be stated that the pectin hydrolysis/ solubilization, if any, occurring during gastric processing, may modulate cell wall porosity [50, 52, 53]. Empirical evidence has demonstrated that the simulated gastric environment decreases plant cell adhesion, increases cell separation, and induces breakage and breach of cell walls [52, 54]. The biochemical digestion of protein and lipids could be also relevant in modulating cell wall porosity of plant cells, thus facilities the digestive enzymes diffusion and enhance intestinal digestion of nutrients in plant tissues. Rovalino-Córdova, et al [7]. investigated the role played by *in vitro* gastric protein digestion in modulating *in vitro* starch digestion in the isolated intact cell from kidney bean and concluded that the proportion of protein hydrolyzed at gastric phase, although small, produced a 20% increment in the amount of starch digested at the small intestine. Analogous information for the role played by gastric lipase is not available. This is due to the lack of *in vitro* lipolysis studies in the gastric compartment as a result of the unavailability of a relevant and convenient replacement for human gastric lipase [55]. In an attempt to describe the role of lipids hydrolysis during digestion in modulating protein digestion, *in vitro* protein digestion of intact cells was conducted with or without the simultaneous hydrolysis of lipids as described in **chapter 2.** In general, it was observed that protein digestion in soybean was improved by the simultaneous hydrolysis of lipids.

In the small intestine, the simultaneous biochemical digestion of nutrients by the action of pancreatic enzymes (amylases, lipases and, proteases) continues even further [56]. Again, the digestion of nutrients in the initial stage of intestinal digestion may play a relevant role in modifying cell wall porosity/permeability and facilitate the diffusion of digestive enzymes. In **chapter 3**, by conducting in vitro diffusion experiment using dextran probes of different sizes and without or with simultaneous protein digestion using trypsin and chymotrypsin and microscopy visualization, we have shown for the first time that cell walls of soybean cells become permeable to dextran probes during digestion. We, therefore, hypothesized that the access of dextran probes inside the cells might be facilitated by the digestion of intracellular proteins and /or cell wall protein by the action of proteases enzymes. When this happens, cell wall porosity and the packing levels of macronutrients inside cells are expected to be modulated. A recent study showed the amount of extracted protein from the hyphal structure of mycoprotein was increased after pancreatic enzyme treatment and suggested that digestive enzymes can diffuse through the cell walls and facilitate the release of protein [57]. Altogether, we argue that the food digestion process can also induce microstructural

changes in plant foods matrix and these changes should be considered as relevant factors in the rate and extent of plant nutrients digestion and also colonic fermentation.

6.6. Methodological aspects

6.6.1 *In vitro digestion model for estimating protein digestibility*

Scientists have developed *in vitro* digestion models aimed at simulating human gastrointestinal digestion. These models are relatively simple, inexpensive, and provide a useful tool for investigating the breakdown of foodstuff, and the digestibility of its components. Furthermore, *in vitro* digestion models have better reproducibility, less variability between replicates, and do not have ethical restrictions compared to *in vivo* digestion models [58-60]. *In vitro* digestion models mimicking the physiological conditions of the human gastrointestinal tract can be either dynamic or static. The most well-known sophisticated dynamic models are those developed by the Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO) [62], or by Institut National de la Recherche Agronomique (INRA) [61]. There is evidence that these models are suitable for simulating and representing the digestive processes (e.g. absorption, peristalsis, and the flow of food). However, they are relatively complex, expensive to set up and maintain, and therefore are less accessible than static models in which only the biochemical processes involved in digestion are reproduced [62, 63].

The work presented in this thesis used the standardized static in vitro digestion model developed by the COST Action INFOGEST [62, 63]. This static model addresses key biochemical components of *in vivo* digestion and simulates the main processes of the human digestive tract compartments (i.e. oral, gastric, and duodenal phases). The choice of this static model for studying protein digestion in the plant tissue matrix was driven by the fundamental nature of the study and the fact that we aimed at understanding the protein digestion kinetics and the mechanism of digestive enzymes diffusion. We are aware that the static *in vitro* digestion model may not perfectly predict the accurate digestibility of protein contained within an intact plant matrix due to the limitation of the simulation of realistic conditions and physical characteristics of *in vivo* digestion. However, this static model was an effective tool to describe the role played by the plant food structure (i.e. cell walls) during protein digestion which was the main objective of this thesis. More recently, the INFOGEST consortium has produced a standardized semi-dynamic *in vitro* model which representing the digestion process occurring in the upper gastrointestinal tract of an adult human. This model is easy to use in laboratories across the world and for a wide range of foods [64].

In this thesis, we were interested in the protein digestibility comparison between different samples that varied in their cellular integrity. The nutritional quality of the protein of the soybean tissues was not an objective in this thesis. Therefore, the degree of protein digestibility was determined based on the O-phthaldialdehyde (OPA) method. This method has become popular in recent years as a useful tool for predicting protein digestibility of different protein sources [65]. In a recent study [66] it was found that the digestibility values of different protein sources determined by OPA method after hydrolyzed using the INFOGEST static digestion model were in agreement with the protein digestibility of the same protein sources measured by protein digestibility-corrected amino acid scores (PDCAAS) and in vivo digestible indispensable amino acid score values (DIAAS), the more recent methods recommended by FAO/WHO committees for assessing the nutritional quality of dietary protein [67, 68]. Therefore, it has been suggested that more validation experiments on other protein sources need to confirm the utility of *in vitro* digestion for digestibility predictions. As a next step toward in vitro DIAAS values and to have a better prediction of the digestibility of proteins of different foods, analyzing the individual amino acids after *in vitro* digestion and comparing their values with the *in vivo* data were recommended [66].

6.6.2 In vitro fermentation models

The restriction to study colonic fermentation kinetics of a diet within *in vivo* has led the researcher to develop *in vitro* gut fermentation models [69]. The rationale of *in vitro* models is to represent the physiological conditions of *in vivo* fermentation. A wide range of techniques, from continuous or semi-continuous fermentation models to batch in vitro inoculated with feces (human and animal), have been developed [70-72]. These models are a useful tool to predict the impact of diet on the composition and functionality of the gut microbiota, and also capture the colonic fermentability variation between different foods substrates. Batch fermentation models are accepted as the simplest, most versatile, and accessible technique because it is characterized by a closed anaerobic environment and a short time simulation [69]. In **chapter 5** of this thesis, we used in vitro fermentation model to investigate the role of the cellular structure (e.g. cell walls) in modulating the microbial fermentation of protein in legumes. The use of batch *in vitro* fermentation models allows us to capture the colonic fermentability variation between studied samples as well as to define the role played by food structure and composition in modulating colonic fermentation of protein. However, it should be noted that *in vitro* fermentation methods cannot perfectly reproduce the conditions of *in vivo* fermentation. For example, during *in vivo* fermentation, short-chain fatty acids (SCFAs) are rapidly absorbed across the colon wall [73]. The accumulation of SCFAs in the closed environment of a batch *in vitro* system, where no removal mechanism operates, may change the microbial metabolism [74]. Thus, the

production and absorption of SCFA occur in the colon is not represented well in the batch method, and this one of the drawbacks with the batch method. This may explain why a false indication of the amount of SCFA produced in the colon is obtained when measured in feces [70, 75]. The same may be true for the accumulation of metabolites of protein fermentation (e.g. BCFAs, ammonia).

6.6.3 Microscopic observation of cellular structure.

The usefulness of including microscopic observation of the cellular microstructure to interpret digestibility data has become clear and popular in recent years. In view of this, we considered and observed the fate and characteristics of the cellular structure of soybean tissues after food processing and *in vitro* digestion. Confocal laser scanning microscopy (CLSM) was chosen over conventional wide-field microscopy to visualize the changes in soybean cellular integrity after food processing and digestion due to its ability to produce images with clear contrast, differentiating one food component (e.g. cell walls, fat, or protein) from the other. This is achieved using fluorescent dye specific to a food component. Examples of fluorescent dyes are calcoflour white, rhodamine B, and Nile red. In our study, samples were probed with calcoflour white. rhodamine B and Nile red to stain cell walls, proteins, and fat respectively. Labeling food components with fluorochromes would provide incredible information about their behavior upon food processing and food digestion.

Besides the macrostructure visualization, CLSM permitted us to have a closer examination of the porosity and permeability of cell walls and the potential diffusion of digestive enzymes through the cell walls. We have initially visualized the penetration of fluorescein isothiocyanate conjugate tagged dextran (FITC-dextran) into soybean cells to measure the changes in cell wall porosity upon food processing. However, the diffusion of a molecule through the cell wall may not only depend on the size of the cell wall pores. The confirmation and flexibility of cell wall pores as well as the level packing of intracellular macronutrients inside cells during the diffusion experiment may also play role in regulating the diffusion of molecular probes. Indeed, it is important to consider that such a diffusion experiment should be performed under an environment that reproduces the physiological conditions of digestion (e.g. mixing and the presence of digestive enzymes). **In chapter 3**, we performed the probes diffusion experiment under mixing conditions and showed a weak penetration of 20 kDa dextran isolated cells of the boiled soybean cotyledon soaked in a salt solution. Moreover, the presence of digestive enzymes (e.g. trypsin and chymotrypsin) to the aqueous medium during dextran probe diffusion was shown to induce cell permeability, thus extensively facilitate the diffusion of the molecular probes (20, 40, 70, and 150 kDa). When comparing the probes diffusion results obtained in this thesis to those obtained by other studies [38, 76,

77] where no mixing nor digestive enzymes addition were considered during the probes diffusion experiment, we can therefore conclude that the mixing regime and presence of digestive enzymes could potentially enhance the cell wall porosity and facilitate the probes diffusion into intact cells. Using the digestive enzymes-sized FITC dextran probe alone is not sufficient to predict the rate and extent of diffusion of the digestive enzymes into intact plant cells. Therefore further diffusion experiments were carried out using pancreatic trypsin that was labelled with Alexa Fluor® 488 reactive dye to monitor the diffusion. of trypsin into soybean cell space. This allowed us to have a better prediction of the accessibility of protease enzymes to plant foods proteins and provides further explanation of why the plant food proteins have limited digestibility. However, It is important to mention that even though the micrographs evidence provided in this thesis was useful to draw clear conclusions about the cell wall permeability of processed plant foods to digestive enzymes, this microscopy evidence is based on a qualitative approach. For this reason, we believe that quantitative image analysis or fluorescence recovery after photobleaching analyses, as described by other researchers [38], might be essential in these types of studies and it is indeed desirable and scientifically intriguing. Fluorescence recovery after photobleaching (FRAP) is widely used for measuring the transport of fluorescent molecules in small systems such as individual living cells [78]. FRAP can be used to indicate the degree of diffusion of fluorescently-labelled probes/enzymes into the individual cell [38].

6.7. Implications of findings

6.7.1 Applicability for modulating protein digestibility in cereals

In this thesis, we have demonstrated that the cell walls of soybean cells act as encapsulating material governing the passage of digestive enzymes into soybean cell space and consequently limit the digestibility of the intracellular protein. However, it was observed that the degree of protein digestibility has relied on the proportion of ruptured cells within the soybean particles, which in turn is determined by the degree of particle size reduction and the way soybeans are processed, e.g., milled before or after cooking. Microscopy observations of the particle preparations revealed that a greater proportion of cells of particles prepared from boiled soybean flour were found to be damaged compared with particles prepared from boiled whole cotyledons (**Chapter 2**). Since the cereal storage proteins are also located intracellularly, the integrity of cell walls of cereal cells would likely exert similar effects on protein digestibility. To test this hypothesis, an *in vitro* protein digestibility of particles of different sizes, prepared either from boiled maize flour or from boiled maize seeds, was measured and the results are shown in Fig. 6.1. In a similar fashion to the protein digestibility of soybean particles (**chapter 2**), an inverse relationship between maize protein digestibility and the degree of particle size reduction was found. Though we have not

performed a microscopic observation of the cellular microstructure of maize particle sizes after milling, the increase in protein digestibility with decreasing the particle sizes would indicate that there was a difference in protein bioaccessibility between different particle sizes. The accessibility of digestive enzymes to intracellular protein is likely to have been facilitated by the fraction of broken cells in the particle. The smaller particles are also expected to have a higher proportion of ruptured cells when comparing with bigger particles which in turn, facilitate the exposure of intracellular components to digestive fluids during digestion.

However, cereal cells of raw and boiled maize seeds may behave similarly when they are subjected to mechanical force after boiling treatment, and the proportion of ruptured cells are expected to be the same for particles of the same size of boiled flour (BF) and boiled seeds (BS). It is well-known that when mechanical stress is applied to boiled cereals, the individual cells tend to fracture rather than separate due to the absence of pectin in cereal cell walls. In legumes, pectin is the major component of cell walls and is well-known for its thermal solubilization property. Numerous studies including this thesis studies have stated that pectin thermal solubilization during boiling was the main mechanism responsible for the separation of individual cells of legume when a mechanical force is applied after boiling [6, 7, 11-13, 28-30].

In general, increase the proportion of ruptured cells of plant tissue increases protein digestibility, as the surface area for proteases contact will be increased. However, this is not the only factor affecting protein hydrolysis. Protein conformational changes during heating might also have a major effect on the rate and extent of protein digestion. Thus, the marked differences in protein digestibility between particles of maize BF and particles of BS of the same size and which might have similar levels of ruptured cells could indicate that the occurrence of cellular integrity during maize cooking can also limit protein digestibility. This is possibly due to the effects of cell wall integrity on the heat-induced protein conformational changes, as it has already shown for soybean (**chapter 4**). Overall, the processing procedure used in this thesis for soybean and maize, could be generalized for other legumes or cereals and open up new perspectives for plant food preparation aimed at the improvement of plant protein digestibility.

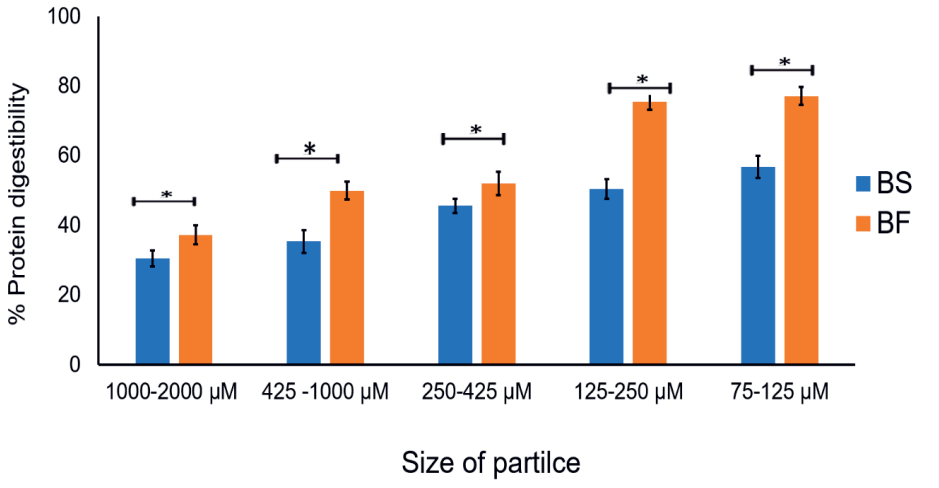


Fig.6.1. Degree of protein digestibility at the end of *in vitro* intestinal digestion of maize particles of different sizes, BS = particles prepared from milled maize that previously boiled as whole seeds; BF= particles prepared from maize flour that boiled as flour. Maize particle sizes were prepared following the same procedure described for soybean particles in **chapter 2.** Following the same procedures of **chapter 2,** the in vitro digestion experiments and protein digestibility quantification of maize particles were carried based on the harmonized INFOGEST protocol and the o-phthaldialdehyde method (OPA) respectively. Data are expressed as a mean of 3 replicates ± SEM, with *p < 0.05.

6.7.2. Information for food security.

It has been widely recognized that the majority of the people, particularly in developing countries, live on a diet based on plant foods with minimal or no foods of animal origin. Cereals and legumes are the most important plant groups in developing countries and account for a major portion of daily protein intake [79-81]. Higher daily consumption of cereals and legumes has been associated with protein malnutrition. One possible explanation for this nutritional phenomenon is the limited essential amino acid content and digestibility of cereal and legume proteins [82, 83]. As the digestibility of protein is a determinant of its biological utilization by the human body [39, 84], the diets with limited protein digestibility are not capable of ensuring nutritional security [85, 86]. According to the world food summit (FAO, 1996), food utilization is embedded as a key dimension of food security along with availability, access, and stability [87]. Food utilization refers to the process through which the body utilizes food nutrients. It also requires hygiene practices and proper food preparation [88]. The data brought together in this thesis shows that proper food preparation/processing enhances the protein digestibility of plant foods. In **chapter 2,** we showed that grinding seeds before boiling improve protein digestibility in soybean. Furthermore, **chapter 4** showed that cooking design

in which grinding seeds is applied before boiling indicated distinct conformational changes that are associated with an increment in protein digestibility. The improvement in protein digestibility was also observed in **chapter 3** when germination or fermentation was combined with boiling treatment. The knowledge on the relationship between plant foods preparation and structure and the resulting implications in protein digestibility can be useful in the development of strategies for food and nutrition security.

6.7.3. Information for nutrition interventions

The nutritional content of a food does not necessarily reflect its nutritional value because not all the nutrients contained within a portion of food are available for digestion and utilization by the human body [2]. However, the consumers and health care providers often rely on the nutrient composition of food to assess its nutritional value and its metabolizable energy, as the information and understanding of nutrient availability in most plant foods for human digestion and absorption are still limited. This can lead to misconceptions about the true nutritional value and the actual energy content of a food. In this view, knowledge of the relationship between food processing and structure to protein digestion, like those provided in this thesis, is quite important in human nutrition. For individual consumers, it could help the consumer make informed decisions on the feasible way to cook plant foods, and the amount of foods to consume to meet the recommended daily allowances of proteins. Recent studies have reviewed the role of the food matrix and digestion on the calculation of the actual energy content of food and elegantly showed that metabolizable energy is affected by food processing and structure properties. Moreover, these studies claimed that the Atwater factors normally employed for calculating the metabolizable energy may provide an overestimation of the energy content of plant foods [2, 89]. Therefore, it is of particular importance to consider these findings when designing the dietary intervention based on plant food origin for preventing and alleviating protein-energy malnutrition.

6.8. Further prospectives

This thesis brought a comprehensive description of the main factors affecting digestion and fermentation of protein contained within intact plant tissues and the potential to improve the plant protein digestibility using a feasible food processing approach. However, more research should be performed to bring more light into understanding the relationship between plant foods processing and structure to its protein digestion. Following the concept of the relevance of particle size, further work should focus on establishing the effect of particle size reduction during oral processing in modulating protein digestion. This is of importance as it could help

predict the amount of absorbed proteins from the particle size distribution of bolus obtained from the mastication of plant food which still needs to be addressed for protein digestion. For this, the novel approach of combining *in vivo* particle size reduction (mastication), and *in vitro* gastrointestinal digestion model to predict protein digestibility will need to be standardized.

Whilst this thesis provided insights on how the cellular structure and matrix of soybean, as well as its protein digestibility, are modulated by natural food processing such as fermentation and germination, the mechanisms of fermentation or germination in inducing plant cells structure alterations is not well understood. Further work could include the investigation of cell wall monosaccharide composition analysis. The measurement of monosaccharides of cell wall before and after these treatments could help to explain the difference in cell wall porosity and of fermented/germinated legumes and cereals.

Another aspect that might be worth exploring is to determine the amino acid composition in the digested protein fraction as opposed to the undigested protein fraction. This could help to predict the biological value of proteins and to validate *in vitro* models of digestion. Developed and validated in vitro protein digestion model can be justified as an alternative to PDCAAS and DIAAS which are typically determined through in *vivo* experiments and are not always desirable due to the ethical and economic considerations involved [65].

Concerning plant cell wall structure and protein colonic fermentation, this thesis showed that cell walls may limit protein colonic fermentation. However, further work is needed to provide more information about the cell wall structure, and composition changes during in vitro colonic fermentation. It is of interest to see the changes in the gut microbiota composition using whole food and diverse food substrates instead of purified ingredients. Moreover, the role of simultaneous colonic fermentation of protein alongside carbohydrates in microbial biomass production needs to be addressed [90].

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Summary

Summary

Plant foods are organized into hierarchical structures that range in scale from centimeter dimensions of plant tissue to the nanometre scale of intracellular macronutrients (starch, lipid, and protein) inside plant cells. This natural encapsulation system may restrict the access of digestive enzymes to macronutrients during gastrointestinal digestion, particularly when the integrity of plant cell walls is preserved after food processing. Thus, addressing the effect exerted by the physical integrity of plant tissues on the bioaccessibility of plant protein is highly relevant to human nutrition, and health, especially in populations where minimal or no animal proteins are consumed as part of the daily diet. Moreover, a better understanding of the triangular relationship between plant food structure, food processing, and food digestion is of great importance and it is essential for designing and developing the strategies that could help to improve plant protein digestibility and utilization by the human body. In this thesis, we have provided more insights into the effect of cell wall integrity on plant protein digestion and colonic fermentation. The role of food processing, (e.g., milling, boiling, germination, and fermentation), in inducing structural changes in cell wall integrity and intracellular protein and the implications of these changes for protein digestibility was investigated. Soybean particles and intact cells were used as plant food models.

Chapter 2 aimed to elucidate the impact of grinding alone or in combination with boiling on the fate of the cellular integrity of soybean tissues and its *in vitro* protein digestibility. Initially, different particle sizes of raw and boiled soybean (as whole cotyledon or flour) were prepared and used to compare the digestibility of protein between these different particles. The protein digestibility increases as the particle size decrease. This was also associated with the proportion of broken cells within the particle which in turn, was confirmed by the use of confocal microscopy to be determined by the degree of particle size reduction, and how cotyledons are treated, e.g. whether milled before or after boiling. The proportion of ruptured cells was greater for particle preparations of boiled flour compared to the particle preparations of boiled cotyledon or raw flour. To investigate the individual contribution of the cell wall in modulating protein digestibility, isolated intact cells and isolated protein were digested. It was observed that cell intactness limited protein digestibility, possibly via limiting the access of digestive enzymes to the protein within intact cells. Moreover, it was also found that the interaction of oil bodies and protein bodies inside soybean cells contributed to reducing protein digestion. These findings raise questions about the role of cell wall porosity and permeability of isolated cells from processed soybean cotyledons in modulating protein digestion.

In **Chapter 3**, we investigated the effect of food processing on cell wall permeability of soybean cotyledon cells and the accessibility of trypsin to intracellular protein. The penetration of fluorescein isothiocyanate dextrans (FITC-dextran) probes into isolated cells from boiled seeds combined with either germination or fermentation was initially visualized by using confocal microscopy to determine the cell walls porosity and permeability of processed soybean cells. The confocal observations showed that intact boiled cells are not permeable to the fluorescent probe (20 kDa) while germinated, and fermented treated cells showed an extensive permeability to different FITC-dextran sizes (20, 40, 70, and 150 kDa). The diffusion behavior of fluorescently labelled trypsin which was used to predict the access of protease enzymes into intact cell protein was different compared to the diffusion behavior of dextran probes. Fluorescently labelled trypsin was able to diffuse through the cell walls and accumulate in the cell space of different isolated cells, irrespective of the treatments (boiling, germination, and fermentation). This difference indicated that the action of trypsin might have modulated the cell porosity and permeability, hypothesizing that labelled trypsin can hydrolyse proteins and “eats its way” through the cell wall or, most likely, the intracellular space allows its accumulation inside the cells. Indeed, it was observed that boiled cells that are not permeable to dextran probes become more permeable to the dextran probes when trypsin and dextran probes were simultaneously added into the aqueous medium during dextran probe diffusion. The comparison of the in vitro protein digestibility of the differently pre-treated isolated cells showed that protein digestibility was increased when boiling was combined with fermentation or germination. This is probably due to the pre-digestion of storage proteins and inactivation of trypsin inhibitors which occur during the fermentation and germination processes.

In **Chapter 4**, the relationship between the protein physicochemical properties (e.g. trypsin inhibitors levels, surface hydrophobicity, secondary structure, and thermal denaturation and aggregation) and protein digestibility of boiled soybean was defined to understand the influence on digestibility played by proteins physicochemical changes during food processing. The physicochemical properties and protein digestibility of boiled whole cotyledons of germinated or non-germinated soybean were initially compared. The results showed that the boiled cotyledon germinated soybeans had distinct physicochemical properties which in turn translated into an increment in protein digestibility compared to boiled cotyledon of non-germinated soybeans. Besides, the physicochemical properties and protein digestibility of boiled cotyledon were compared to those of boiled flour to assess the individual contribution of cellular integrity during cooking in protein physicochemical changes. Results indicated that the preservation of an intact cell during cooking, as it is the case for boiled

cotyledon, contributed to reducing physicochemical changes during cooking and limiting soybean protein digestion. Nevertheless, the effect exerted by cellular integrity on physicochemical changes during cooking was limited when the germination process was applied before boiling. This is most likely due to the role of the germination process in changing the molecular structure of storage proteins due to metabolic reactions naturally occurring during germination. The role of cooking time was also investigated for both boiled germinated and non-germinated soybean either as whole cotyledon or flour. The results showed that the differences in cooking times either among cotyledon or flour samples of both germinated and non-germinated soybean resulted in slight changes in protein physicochemical properties that were accompanied by a limited improvement in the protein digestibility.

The data provided in chapters 2, 3, and 4 together demonstrated that the structural attributes of soybean cells limited protein digestion in the small intestine, regardless of food treatments or processing procedures. Therefore, in **Chapter 5** we have studied the role played by the soybean cell structure and composition in the regulation *in vitro* protein colonic fermentability using intact cells, broken cells, and isolated protein. Results indicated that the fermentability of protein within intact cells decreased by cellular integrity as evident from the difference in BCFAs and ammonia production between intact and broken cells. In a separate experiment, to simulate protein digestion and fermentation processes in humans, intact and broken cells were pre-digested using trypsin and chymotrypsin before incubated with a fecal human inoculum. The findings of this experiment revealed that following the *in vitro* gastrointestinal digestion, higher BCFAs, and ammonia was produced during the fermentation of intact cells compared to what was found for broken cells. At the same time, intact cells produced low gas, and SCFAs compared to broken cells. Apart from the differences in the structural integrity of intact and broken cells, the differences in food composition, as a result of differences in the amount of digested protein during the pre-digestion process, may explain why broken cells had lower BCFAs and ammonia and higher SCFAs compared to intact cells. To investigate the role of heat treatment in protein fermentability, raw, and heat-treated soybean protein were compared. The time course of BCFAs and ammonia production showed the degradation of raw soybean proteins was lower compared to its heat-treated protein counterpart.

In the general discussion (**Chapter 6**), the effect of plant-tissue structure in limiting protein digestion and the potential role of food processing in improving plant protein digestibility were discussed in a broader context. It was concluded that food processing is an indispensable tool to manage digestive barriers in plant-based foods and improve their protein digestibility.

This finding could aid in the development of strategies to enhance plant protein digestibility, particularly in populations where there is a need for improved protein nutrition. Furthermore, in **Chapter 6**, the methodological approach was evaluated and its limitations were defined. These limitations can be used as an indication for future research. This section ends with an overview of the perspectives for further studies on plant protein digestion using in vitro protein digestion models.

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About the author

About the author

Mostafa Zahir was born in Al Hudaydah, Yemen, on December 23th, 1980. He obtained his BSc degree in Food Science and technology from Sana'a University (Yemen) in 2004. The year after, Zahir followed MSc program in Food science at Sana'a university. During his MSc studies, he got an invitation from Prof Nasser Zawia (the farmer Dean of the Graduate School at the University of Rhode Island, USA) to college of Pharmacy where he conducted his MSc thesis research activities under Prof. Zawia's supervision. After 9 months, Zahir returned to Sana'a University where he defended his MSc thesis and got his MSc degree in Food science in 2008. He was awarded Al-Saeed Foundation for Science and Culture Prize for his MSc thesis research. In 2008, Zahir joined Yemen Customs Authority as head of the department of tariff nomenclature. Due to his passion for academia, he worked (part-time) as a lecture for food science and nutrition (Bachelor courses) at several universities, Yemen from 2008-2015. Zahir was awarded IDB Merit-PhD Scholarship for PhD program, and in February 2016 he started his PhD project at Food Quality and Design Group, Wageningen University under the supervision of Prof. Dr. Vincenzo Fogliano, and Dr. Edoardo Capuano. The results of his research are presented in this thesis.

List of publications

- **Zahir, M.,** Fogliano, V., & Capuano, E. (2018). Food matrix and processing modulate in vitro protein digestibility in soybeans. ***Food & function*, *9*(12), 6326-6336.**
- **Zahir, M.,** Fogliano, V., & Capuano, E. (2020). Effect of soybean processing on cell wall porosity and protein digestibility. **Food & Function, 11(1), 285-296.**
- **Zahir, M.,** Fogliano, V. and Capuano, E., 2020. Soybean germination limits the role of cell wall integrity in controlling protein physicochemical changes during cooking and improves protein digestibility. **Under revision on Food Research International**.
- **Zahir, M.,** Fogliano, V. and Capuano, E., The role of plant cell wall integrity and thermal treatment in modulating in vitro protein colonic fermentation (**Submitted for publication**)

Others

- Myriam M.-L. Grundy, Evan Abrahamse, Annette Almgren, Marie Alminger, Ana Andres, Renata M. C. Ariens, Shanna Bastian-Net, Claire Bourlieu, André Brodkorb, Maria R. Bronzei, Irene Comi, Leslie Couëdelo, Amélie Deglaire, Didier Dupont, Sedef N. El, Tara Grauwet, Christine Heerup, Ana Heredia, Marcos R. Infantes Garcia, Christian Jungnickel, Ilona E. Kłosowska-Chomiczewska, Marion Létisse, Adam Macierzanka, Alan R. Mackie, David J. McClements, Olivia Menard, Anne Meynier, Marie-Caroline Michalski, Ana-Isabel Mulet-Cabero, Anette Mullertz, Francina M. Payeras Perelló, Irene Peinado, Mélina Robert, Sébastien Secouard, Ana T. Serra, Sandra D. Silva, Gabriel Thomassen, Cecilia Tullberg, Ingrid Undeland, Claudia van den Braak, Carole Vaysse, Gerd E. Vegarud, Michelle Viau, **Mostafa Zahir**, Ruojie Zhang and Frédéric Carrière. INFOGEST inter-laboratory recommendations for assaying gastric and pancreatic lipases activities before in vitro digestion studies (**Submitted for publication**)

Overview of completed training activities

Discipline specific activities

Course

- Advanced Food Analysis (VLAG, Wageningen, NL,2017)
- Nutri-Science Global nutrition: from nutrients to whole diets (VLAG, Wageningen, NL,2017)
- Reaction kinetics in food science (VLAG, Wageningen, NL,2016)
- Post graduate course "Healthy Food Design (VLAG, Wageningen, NL,2018)
- Microscopy and Spectroscopy in Food and Plant Sciences (VLAG & EPS, Wageningen, NL,2018)
- Advanced Course Bioprocess Design (VLAG, Wageningen, NL,2019)
- Food proteins: functionality, modifications and analysis (VLAG, Wageningen, NL, 2019)
- The intestinal Microbiome and diet in Human and Animal Health VLAG, Wageningen, NL

Conferences and meetings

- 5th International Conference on Food Digestion (COST INFOGEST, Rennes, FR, 2017)
- Symposium 'Food, microbiome and immunity (FQD, Wageningen-NL, 2019)
- 6th International Conference on Food Digestion (COST INFOGEST, Granada, ES, 2018)
- 33rd EFFoST International Conference 2019 (ELSEVIER, , Rotterdam,-NL, 2019)

General Courses

- VLAG PhD week (Baarlo, NL,2016)
- Philosophy and Ethics of Food Science and Technology (VLAG, Wageningen, NL,2018)
- Applied Statistics, (VLAG, Wageningen, NL 2018)
- Career assessment (WGS, Wageningen, NL,2019)
- Career Perspectives (WGS, Wageningen, NL, 2020)

Optional activities

- Preparation of research proposal (Wageningen-NL, 2016)
- PhD study tour, (Italy, 2016)
- PhD study trip (Australia, 2018)
- Organizing PhD study tour (Australia, 2018)
- Meetings and colloquia (FDQ, Wageningen, NL, 2016-2020)

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences
WGS: Wageningen Graduate School
EPS: The Graduate School Experimental Plant Sciences
FQD: Food Quality and Design

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