Old and new technological strategies to improve Jack beans quality and promote its utilization

Fiametta Ayu Purwandari
Propositions

1. Structural changes play a more important role than the cell wall permeability in limiting protein digestibility.
   (this thesis)

2. Image quantification is a connecting bridge between chemical and microstructural properties.
   (this thesis)

3. If people are more aware of a balanced diet, they will purchase less street food.

4. Flipped learning makes it easier for university students to understand advanced topics.

5. International laws and conventions are ineffective in regulating and limiting the conduct of war.

6. The process is more important than the result.

Propositions belonging to the thesis, entitled

Old and new technological strategies to improve Jack beans quality and promote its utilization

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(Biobased, Biomolecular, Chemical, Food and Nutrition Sciences)
Old and new technological strategies to improve Jack beans quality and promote its utilization

Fiametta Ayu Purwandari
To my husband, Fahmi and my daughter, Atiqah.
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Chapter 1
General introduction
1.1 Underutilized legumes as a sustainable source of protein in human diets

Sustainable food systems are of vital importance in ensuring food security and promoting healthy nutrition for all individuals, while maintaining social, environmental, and economic sustainability for future generations [1]. The sustainability of agricultural and food systems is threatened as a result of various global concerns, with the inevitable growth of the world population being the most important among them. Given the current rate of global population growth, the increase in food demand was estimated to range from 35% to 56% between 2010 and 2050 [2], demanding a huge expansion in agricultural and food production. This development can cause significant environmental implications, such as an increase in greenhouse gas emissions, deforestation, and water use, contributing to ecological inadequacy and climate change [3].

In response to mounting concerns regarding sustainability, a transition towards more sustainable food sources is necessary. Animal food sources are accountable for the majority of negative impacts on land use [4], water use [5], and greenhouse gas emissions [6] in global food systems [7]. Currently, there has been a growing demand for alternatives to animal protein sources [8]. As a result, alternative protein options have gained significant attention, and among them, legumes have emerged as an economically viable and nutrient-rich source [9]. The EAT-Lancet recommends diets richer in legumes [10]. Legumes are considered a feasible solution for future security because they pose fewer technological challenges to production and innovation [11]. While there are many legumes available for consumption, only a few are commonly consumed. According to several studies, the most commonly consumed legumes include soybeans, common beans, peanuts, chickpeas, green peas, kidney beans, common peas, cowpeas, fava beans, and lentils [12,13]. On the other hand, there are many underutilized legumes, that have the potential to become a major source of high-quality protein in the human diet. Promoting the utilization of these legumes may provide a more biodiverse agricultural and food systems, providing healthy and nutritious food while at the same time ensuring sustainability.

Underutilized legumes refer to leguminous plants that are marginalized and disregarded by researchers, breeders, and policy makers [14]. Criteria used to classify a legume as underutilized include the local importance in production and consumption systems, the ability to adapt to specific agroecological niches, the representation by ecotypes/landraces, the cultivation and
utilization based only on indigenous knowledge, the limited representation in ex-situ collections, and the lack coordinated attention from national agricultural and biodiversity conservation policies, research and development, and the exclusion from the mainstream market system [15]. Many underutilized legumes are of local importance in developing countries for local communities, but their economic significance on the global market is rather low [16]. Based on these criteria, several species were included as underutilized legumes including winged bean (Psophocarpus tetragonolobus (L.) DC.), pigeon pea (Cajan cajan (L.) Huth), lablab (Lablab purpureus (L.)), lima bean (Phaseolus lunatus (L.)), Jack bean (Canavalia ensiformis (L.) DC.), sword bean (Canavalia gladiata (Jacq.) DC.), mung bean (Vigna mungo (L.) Hepper), Bambara groundnut (Vigna subterranea (L.) Verdc.), marama bean (Tylosema esculentum (Burch.) A. Schreib), kersting’s groundnut (Macrotyloma geocarpum (Harms) Maréchal & Baudet), African yam bean (Sphenostylis stenocarpa (Hochst. ex A. Rich.) Harms), rice bean (Vigna umbellata (Thunb.) Ohwi & H. Ohashi), adzuki bean (Vigna angularis (Willd.) Ohwi & H. Ohashi), cluster bean (Cyamopsis tetragonoloba (L.) Taub.), dolichos bean (Lablab purpureus (L.)), horsegram (Macrotyloma uniflorum (Lam.) Verdc.), red clover (Trifolium pratense L.), tepary bean (Phaseolus acutifolius A. Gray), common vetch (Vicia sativa L.), white lupin (Lupinus albus L.), grass pea (Lathyrus sativus L.), velvet bean (Mucuna pruriens (L.) DC.) and faba bean (Vicia faba L.) [15,17]. The nutritional composition of some of these beans is summarized in Table 1.1.
Table 1.1 Nutritional composition of several underutilized legumes

<table>
<thead>
<tr>
<th>Common name</th>
<th>Crude Protein (%db)</th>
<th>Carbohydrate (%db)</th>
<th>Starch (%db)</th>
<th>Crude Fiber (%db)</th>
<th>Crude lipid (%db)</th>
<th>Ash (%db)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adzuki bean</td>
<td>27.0</td>
<td>67.8</td>
<td>50.0</td>
<td>17.8</td>
<td>0.8</td>
<td>4.4</td>
<td>[18]</td>
</tr>
<tr>
<td>African yam bean</td>
<td>21.3</td>
<td>55.3</td>
<td>50.1</td>
<td>5.2</td>
<td>5.8</td>
<td>3.1</td>
<td>[19]</td>
</tr>
<tr>
<td>Bambara groundnut*</td>
<td>24.7</td>
<td>59.8</td>
<td>56.2</td>
<td>3.6</td>
<td>6.0</td>
<td>3.7</td>
<td>[20]</td>
</tr>
<tr>
<td>Jack bean</td>
<td>31.9</td>
<td>50.6</td>
<td>41.6</td>
<td>9</td>
<td>4</td>
<td>4.5</td>
<td>[21]</td>
</tr>
<tr>
<td>Kersting’s groundnut*</td>
<td>21.3</td>
<td>57.6</td>
<td>51.9</td>
<td>5.7</td>
<td>1.1</td>
<td>3.5</td>
<td>[22]</td>
</tr>
<tr>
<td>Lima bean*</td>
<td>19.4</td>
<td>75.1</td>
<td>54.3</td>
<td>20.8</td>
<td>1.4</td>
<td>4.0</td>
<td>[18]</td>
</tr>
<tr>
<td>Marama bean*</td>
<td>32.8</td>
<td>26.1</td>
<td>2.7</td>
<td>23.4</td>
<td>38.1</td>
<td>3.0</td>
<td>[23]</td>
</tr>
<tr>
<td>Mung bean</td>
<td>25.4</td>
<td>69.8</td>
<td>46.6</td>
<td>23.2</td>
<td>1.1</td>
<td>3.6</td>
<td>[18]</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>19.9</td>
<td>71.6</td>
<td>68.8</td>
<td>2.8</td>
<td>1.2</td>
<td>4.6</td>
<td>[24]</td>
</tr>
<tr>
<td>Rice bean</td>
<td>22.6</td>
<td>71.8</td>
<td>51.8</td>
<td>20.0</td>
<td>1.0</td>
<td>4.6</td>
<td>[18]</td>
</tr>
<tr>
<td>Sword bean</td>
<td>25.0</td>
<td>59.9</td>
<td>50.9</td>
<td>9.0</td>
<td>2.6</td>
<td>3.5</td>
<td>[25]</td>
</tr>
<tr>
<td>Velvet bean</td>
<td>31.4</td>
<td>52.6</td>
<td>47.4</td>
<td>5.2</td>
<td>6.7</td>
<td>4.1</td>
<td>[26]</td>
</tr>
<tr>
<td>Winged bean</td>
<td>31.0</td>
<td>32.2</td>
<td>19.4</td>
<td>12.8</td>
<td>20.2</td>
<td>3.9</td>
<td>[27]</td>
</tr>
</tbody>
</table>

*Average from different cultivars, accessions, or collections. Starch is estimated by subtracting fiber from carbohydrate content. db: dry basis

Table 1.1. shows the nutritional composition of several underutilized legumes and highlights the potential of legumes as a protein source. As one of the largest groups of plants, legumes offer a wide range of options for meeting the nutritional and dietary needs of various populations. The geographical distribution of the legumes is reported in Figure 1.1. The figures show that some of the legumes are distributed throughout the world, suggesting the potential for global consumption. On the other hand, several studies have shown the potential of underutilized legumes regarding their physiological and genetic adaptation to harsh environments, including extreme temperatures, soil pH, and salinity [28–30].
Legumes are indicated by yellow dots on the map. Jack beans occurrence map is presented in Figure 1.2.

**Figure 1.1** Occurrence of several underutilized legumes [31]
1.2 The potential and challenges of Jack bean

Jack bean, originally from South America, is primarily grown in tropical and subtropical regions [32]. Jack bean is widely distributed in Africa, Asia, and America, with large-scale cultivation reported in Congo and Angola [15]. The distribution of Jack beans around the world is illustrated in Figure 1.2.

![Figure 1.2 Jack beans occurrence around the world [31]](image)

Jack beans are indicated as yellow dots

**Figure 1.2** Jack beans occurrence around the world [31]

Jack bean can be cultivated in marginal land with either monoculture or intercropping systems [33]. Young pods of Jack beans are used as a vegetable. The seeds are sometimes eaten after boiling [34]. Jack bean is used as cover crops [35] and is mostly destined for livestock feeding [36]. The nutritional composition presented in Table 1.1 shows that Jack bean is high in proteins thus it has potential as a protein source. Moreover, the bean is a rich source of starch and fiber and poor in lipids. Notwithstanding its nutritional quality, Jack bean contains antinutrients such as concanavalin A, canavanine, canaline, canatoxin, protease inhibitors, phytates, cyanide, saponins, urease, and L-DOPA [37]. The concentration of antinutrients varies according to the Jack bean collection and most of them can be eliminated easily by processing the bean. Concanavalin A (Con A) was reported as the most important antinutrient in Jack bean seeds. It can agglutinate the erythrocytes of numerous animal species, clump certain bacteria, and precipitate glycogen and starch from solutions [38]. Con A negatively affected nutrient digestion by inhibiting digestive enzymes [39]. It has been reported to be highly resistant to heat treatments and to proteolytic digestion in the gastrointestinal tract [38]. The antinutrients and toxic compounds may impair the widespread use of Jack
beans as well as the long cooking time required to prepare the beans which is referred to as the hard-to-cook (HTC) phenomenon [40].

1.3 Hard-to-cook phenomenon

Beans are typically cultivated in tropical or temperate climates and stored for later consumption. The extended storage of beans under conditions with elevated temperature (>25°C) and high relative humidity (>65%) results in the development of a cooking issue known as the hard-to-cook (HTC) phenomenon [41]. HTC beans require a longer cooking time to soften the cotyledon to reach textural palatability. Several factors may determine the extent of the HTC development such as the length of the storage under adverse conditions [42], growing conditions in warm climates [43], and genetic differences [44] related to seed coat and cotyledon cell wall thickness [45].

HTC phenomenon has been observed for several legumes, such as Pinto bean [46], Jack bean [47], Bambara groundnut [48], chickpea [49], and kidney bean [50]. HTC phenomenon is mainly associated with structural and compositional changes within cotyledons such as the formation of tricellular junctions and changes in middle lamella [51]. Several hypotheses have been proposed to explain the mechanism of the HTC phenomenon such as the pectin-cation-phytate hypothesis, a mechanism involving phenolic compounds and the lignification-like theory, and changes involving starch, protein, and lipid.

1.3.1 Pectin cation phytate hypothesis

The pectin cation phytate hypothesis involved a dual enzyme mechanism i.e. phytase and pectin methyl esterase (PME). Phytase breaks down phytic acid (PA) reducing its chelation potential of calcium and magnesium ions. PME converts middle lamella pectins into calcium and magnesium pectates making the beans hard [52]. The resulting calcium and magnesium pectates are not readily soluble when the beans are boiled, which reduces cellular separation and consequently produces HTC beans. Research showed that PME activity remained constant during bean storage, implying that changes in pectin methyl esterification (DM) did not contribute significantly to HTC [42,53]. Instead, during the development of HTC, water-extractable pectin decreased while sodium carbonate-extractable pectin increased, indicating the transformation of loosely bound pectin into covalently bonded non-starch polysaccharides (NSP). The chelator extractable pectin fraction shows minor changes. Recent studies suggest that phytate breakdown and cation migration, rather than PME activity, primarily explain the pectin-cation-
phytate hypothesis in HTC bean formation. Additionally, microstructural changes involving calcium and magnesium pectates were linked to HTC [54]. Several recent studies have indicated hydrolysis of inositol phosphate and subsequent migration of Mg and Ca cations to be the most prominent explanation of the phytate-cation hypothesis [55,56].

1.3.2 Deposition of phenolic compounds and the lignification-like theory

Total phenolic content differs between easy-to-cook (ETC) and HTC beans with a reduction of the total phenolic content of the seed coat during HTC development [57]. Alteration of cell wall components reduced the release of phenolic compounds in HTC cotyledon, likely due to oxidative degradation [58]. Tannins, a group of high-molecular-weight compounds that contain multiple phenolic hydroxyl groups, can react with proteins to form persistent cross-links through peroxidase and free radicals produced by the breakdown of cell membranes. This reaction decreased the extractability of proteins [59]. It is proposed that storage under adverse conditions could induce the migration of tannins and other phenolics from seed coat to middle lamella and cotyledon where they could crosslink with pectin and proteins [60].

The lignification hypothesis suggests that lignin-like compounds deposit in cell walls and middle lamella of cotyledons, possibly due to reactions involving aromatic amino acids and polyphenolic compounds. Seed coat darkening was observed in chickpea [49]. Histological evidence supports this mechanism, revealing lignin deposition in stored beans [61]. However, true lignin formation remains debatable. Hydroxycinnamic acid was increased with ageing, especially ferulic acid, but no storage-induced lignin formation occurred [62].

1.3.3 Changes involving starch, protein, and lipid

Beans contain lipids, primarily phospholipids and triacylglycerols with minor components like sterol esters and other lipid components [63]. Beans contain essential polyunsaturated fatty acids, with linoleic acid being prominent [64]. The chemistry of fatty acids changes significantly during storage, especially at high temperatures, moisture content, and relative humidity, leading to lipid oxidation [65,66]. Lipid oxidation and polymerization, with an increase in saturation, are linked to alteration of membrane permeability which may affect the seed hardness [67].
Hydrolysis of lipids can lower bean pH during storage, resulting in protein insolubilization, reversible denaturation, and reduced extractability [68,69]. Furthermore, intracellular protein network formation during cooking was suggested to influence starch behavior, retaining the hardness in cooked cowpeas thus implying the involvement of the starch in HTC development during cooking [69]. Pseudo-retrogradation reactions were proposed as contributors to HTC in another study [67], contrasting with another report [70]. A previous study showed increased swelling and water absorption in starch isolated from HTC beans [71]. However, this seems not to influence the gelatinization behavior of Bambara groundnuts. In addition, the rate-limiting factor for bean softening in HTC beans was pectin solubilization, not gelatinization or protein denaturation [72].

HTC development can affect the nutritional quality of beans, affecting the bioaccessibility of minerals and protein and starch digestibility. A summary of nutritional implications related to the proposed hypotheses can be seen in Table 1.2.

**Table 1.2** HTC proposed mechanisms and their potential nutritional implications [73]

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Mechanism (summary)</th>
<th>Nutritional quality implication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin-cation-phytate theory</td>
<td>Redistribution of cations driving them into the cell walls and middle lamella.</td>
<td>Increased mineral chelation and a decrease in mineral bioaccessibility.</td>
</tr>
<tr>
<td>Lignification-like theory</td>
<td>Crosslinking of polyphenols with cell wall polymers or proteins.</td>
<td>Decreased protein digestibility.</td>
</tr>
<tr>
<td>Protein-starch hypothesis</td>
<td>A more robust protein network formation around starch granules. Reduced starch swelling.</td>
<td>A decrease in both protein and starch digestibility.</td>
</tr>
<tr>
<td>Lipid oxidation theory</td>
<td>A decrease in plasmalemma integrity.</td>
<td>Increased mineral loss during soaking and cooking. Synergistic effect with pectin-cation-phytate theory—decreased mineral bioaccessibility</td>
</tr>
</tbody>
</table>

General introduction
1.4 Starch and protein digestion of cooked beans in the upper gastrointestinal tract

Starch are digested by several enzymes in the gastrointestinal tract [74]. These include salivary and pancreatic α-amylase, isomaltase, and glucoamylase. Salivary amylase initiates starch digestion, continuing for up to half an hour in the stomach until the gastric acidity inactivates the enzyme. When the foods reach the small intestine, pancreatic α-amylase is released and starch digestion continues yielding maltose, maltodextrins and α-limit dextrins. Intestinal isomaltase, which is an α-1,6 glycosidase, cleaves the α-1,6 linkages in branched poly- and oligosaccharides, while glucoamylase digests small unbranched oligosaccharides.

Proteins undergo digestion in both the stomach and small intestine. In the stomach, pepsinogen is released and activated by gastric juice to form pepsin. Pepsin primarily cleaves peptide bonds containing aromatic amino acids [74]. In the small intestine, pancreatic juice contains three endopeptidases: trypsin, chymotrypsin, and elastase. Trypsin prefers peptide bonds involving basic amino acids, chymotrypsin favors bonds with aromatic amino acids, while elastase degrades elastin. Additionally, pancreatic juice contains two carboxypeptidases (A and B), with carboxypeptidase A showing a high specificity for bonds with basic amino acids like lysine or arginine. Brush border enzymes, particularly in the jejunum, break down products of proteolytic digestion into tripeptides, dipeptides, and amino acids, which are further degraded into amino acids by cytosolic tripeptidases and dipeptidases in the endothelial cells. A schematic illustration of starch and protein digestion along the gastrointestinal tract is depicted in Figure 1.3.
Starch and protein digestibility refers to the proportion of ingested nutrients that are hydrolyzed in the gastrointestinal tract into glucose (for starches) or amino acids (for proteins) made available for absorption. The protein digestibility of Jack bean was lower than Canavalia cathartica and different cultivars of *Phaseolus vulgaris* [75]. Jack bean was also reported to have low starch digestibility [75].

Beans require processing before consumption in order to enhance their nutritional value, and sensory attributes such as taste, aroma, and texture [76,77]. The most traditional process steps are soaking and boiling. Soaking is an important step to hydrate the seeds thereby shortening the required cooking time. During thermal processing, protein denatures and starch gelatinizes, causing an increase in their digestibility.
1.4.1 Starch digestion as affected by thermal processes

Cells are surrounded by a cell wall in all plants. In legumes, the cell wall is thick and can encapsulate intracellular nutrients if the cell integrity is retained at the point of swallowing, significantly hindering amylolysis during the *in vitro* digestion of pulses [78–82]. Amylolysis in cooked chickpea flours (with residual cellular intactness) is restricted due to low permeability of the cell wall to α-amylase, contrary to more permeable wheat endosperm cell walls [83]. Damage of the cell wall or hydrolysis of its constituents, caused by enzymatic or thermal processes, can affect starch digestion rate and extent [78,80,82]. The intact cell wall can also impede starch gelatinization during thermal processing due to cytoplasmic confinement [79]. Moreover, cell wall materials can delay starch digestion by binding α-amylase [79,84]. The protein matrix, which surrounds starch can limit α-amylase diffusion and forms a second barrier to amylolysis [85]. Starch digestibility depends on the gradual enzymatic degradation of the protein matrix during digestion [85,86], particularly during the small intestine phase. However, starch digestion does not seem to influence protein digestion [85].

Thermal treatment led to increased cell wall porosity, which enhanced starch hydrolysis rates, (i.e. shorter lag phases and higher reaction rate constants of *in vitro* starch hydrolysis) [87]. For Bambara groundnuts, a longer heating time did not significantly reduce the lag phase for starch digestion, possibly due to less cell wall pectin solubilization [86]. Differences in cell wall properties between different pulses may significantly influence the extent and the way in which cell walls are affected by processing, thereby altering macronutrient digestion [86]. Thus, for the whole pulses, thermal processing time can affect cell wall integrity and consequently the starch digestion rate through the porosity of the cell wall [87] and the degree of cell separation upon mechanical disintegration that leads to intact cells [88].

1.4.2 Protein digestion as affected by thermal processes

The effect of thermal treatment on protein digestion kinetics depends on the pulse type and processing method. Protein hydrolysis during the small intestinal phase is significantly higher for cells isolated from Bambara groundnuts with longer cooking times [86]. While autoclaving generally improved protein digestibility for several pulse types, boiling did not yield the same results, and no increase in protein digestion was observed with increasing thermal treatment time for *Canavalia* spp. [89]. Longer treatment times increase cell wall permeability [87], making protease diffusion more accessible. Thermal treatment can also reduce antinutrient factors, like trypsin...
inhibitors, and cause protein denaturation, making them more susceptible to digestive enzymes [90]. Moreover, protein aggregation can occur due to thermal treatment [91,92], impacting enzymatic hydrolysis. It has been proposed that protein aggregation occurs via Maillard-type reactions, protein oxidation reactions, interactions between amino acids, and interactions between protein and phenolics [93,94].

1.5 Fermentation of undigested fractions of beans in the lower gastrointestinal tract

Carbohydrates and proteins consumed in the human diet can reach the colon upon either escaping the upper gastrointestinal digestion once the amount consumed exceeds the rate of digestion or resisting the upper gastrointestinal digestion [95]. Several factors can influence digestive efficiency which in turn modulates the substrates available to the gut microbiota for consumption. The nondigested carbohydrates and proteins are fermented by several groups of bacteria including Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria and yield a wide range of microbial metabolites.

Non-digestible carbohydrates are fermented by the microbiota and result in short chain fatty acids (SCFAs) and gas (CO₂, CH₄, and H₂). The predominant SCFAs are acetate, propionate, and butyrate, which are absorbed by epithelial cells and metabolized by the host [96]. More recently, butyrate production has drawn much attention due to its health-related properties where the growth inhibition of colon-rectal cancer cells could be the most relevant [97]. Specific substrates, such as resistant starch have been found to stimulate the growth of certain bacterial strains capable of producing higher amounts of butyrate [97], hence its utilization in the design of functional foods has increased. The extent of fermentation of non-digestible carbohydrates by gut microbiota depends on several factors, including the type and amount of dietary carbohydrates, and the composition of the gut microbiota [96,98–100].

Proteins, peptides, and amino acids that escape digestion and absorption in the small intestine and reach the large intestine are also metabolized by the microbiota. First, they undergo proteolysis by microbial proteases and peptidases. In addition, residual proteases from the small intestine might contribute to proteolysis in the gut. The amino acids and peptides can be then taken up by the bacteria cells which they can be used for protein biosynthesis for bacterial cells or catabolized into metabolites such as SCFAs, branched short chain fatty acids (BCFAs), ammonia, hydrogen sulfide, carbon dioxide, phenols, and organic acids via deamination, decarboxylation, and
transamination [101]. Microbial fermentation of proteins depends on the proportion of available carbohydrates present concurrently with proteins [102]. Beyond the type of substrate present in the diet, transit time, and resulting pH condition due to substrate fermentation might influence the gut microbiota [101]. Different catabolic pathways and bacterial species are used for different amino acid substrates [103]. Some harmful metabolites compounds can be produced such as branched short-chain fatty acids (BCFAs). These BCFAs include isobutyrate, isovalerate, and 2-methylbutyrate which are fermented products of valine, leucine, and isoleucine, respectively [104]. Fermentation of sulfur-containing amino acids, such as methionine, cysteine, cystin, and taurine can produce H₂S [105]. Furthermore, degradation of undigested dietary protein by gut microbiota enzymes can release tryptophan and glutamate from the food matrices. The gut microbiota has a fundamental role in the transformation of Trp into indole and its derivatives [106]. Indolic compounds play a significant role in host immune homeostasis and barrier physiology as dietary or endogenous ligands of the aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR) [107–109]. In addition, γ-aminobutyric acid (GABA), an important inhibitory neurotransmitter in the nervous system for anxiety regulation [110], can be produced by gut bacteria through decarboxylation of glutamate likely mediated through the gut-brain axis [111].

1.6 Processing and cooking of legume seeds

Various processing methods have been employed to improve the consumption of beans. Soaking followed by boiling, herein referred to as cooking, is the most widely applied method to process beans [112]. Soaking usually plays an integral part in processing methods such as cooking, germination, and fermentation. The soaking profile of beans, including common beans [113], soybeans [114], and cowpeas [115], typically exhibits a high initial rate of water intake. The rapid water uptake is attributed to capillary filling on the seed coats and at the hilum, along with water absorption of intracellular matrix [76,116]. This phase is followed by a slower water uptake until equilibrium is reached, indicating the maximum water capacity of legume seeds. The decline in soaking rate is influenced by increased extraction rates of soluble materials and the filling of free capillary and intercellular spaces with water [117].

Water absorption during soaking depends on both soaking time and temperature, with higher temperatures increasing the hydration rate constant and decreasing soaking to achieve equilibrium [118]. A lag phase in water uptake is attributed to the seed coat in common beans [119], adzuki beans [120], and cowpeas [121]. Soluble compounds in beans, such as soluble sugar,
minerals, and some essential amino acids, may leach into the soaking water [122]. Soaking beans has been reported to reduce the content of antinutrients like phytate [123].

Cooking is applied to enhance palatability. The hydration kinetics observed during cooking are similar to those described in the soaking step. In the initial phase, water enters the intracellular space while starch gelatinization, protein denaturation, and dissolution of pectin in the middle lamella occur [72]. Pectin solubilization emerges as the rate-limiting step during bean cooking [72,124]. Pectin alterations in cell walls and middle lamellae influence texture decay during the cooking process. Cooking is important for effectively removing antinutrients such as tannin, phytate, lectin, and trypsin inhibitor [125–127], improving nutrients digestibility. Adequate cooking is crucial to inactivate phytohemagglutinin in beans, preventing potential toxicity [128].

Fermentation is a biotechnology process that makes use of the growth of microorganisms and their metabolic activities to extend food shelf-life. It results in chemical changes in the food matrix, improving its flavor and nutritional value [129]. Several microorganisms, such as bacteria, yeast, and fungi, and different fermentation types and conditions are used to ferment the beans. Fermentation can improve the nutritional quality of food such as increasing nutrient bioaccessibility [130], decreasing antinutritional components [131], improving nutrients digestibility [132], and generating bioactive compounds [133]. The fermentations resulted in modifications of chemical compositions as presented in Table 1.3.

**Table 1.3 Modification of chemical composition of fermented legumes**

<table>
<thead>
<tr>
<th>Bean type</th>
<th>Fermentation type</th>
<th>Fermentation form/conditions</th>
<th>Modifications in chemical composition</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>African yam bean</td>
<td>SSF</td>
<td>Controlled using <em>S. cerevisiae</em> 24h at 45°C</td>
<td>Increase in crude protein, ash, minerals, some AAs and IVPD. Decrease in fat content, fiber, CHO, PA and tannin.</td>
<td>[19]</td>
</tr>
<tr>
<td>African yam bean</td>
<td>SmF</td>
<td>Spontaneous 24h at 45°C</td>
<td>Increase in crude protein, CHO, and fat. Decrease in ash and fiber.</td>
<td>[24]</td>
</tr>
<tr>
<td>Bambara groundnut</td>
<td>SmF</td>
<td>Controlled using spore suspension of <em>R. oligosporus</em></td>
<td>Decrease in tannin, phytate, and trypsin inhibitor</td>
<td>[134]</td>
</tr>
<tr>
<td>Legume</td>
<td>Fermentation</td>
<td>Conditions</td>
<td>Changes</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>--------------</td>
<td>----------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Chickpea</td>
<td>SSF</td>
<td>0-72h (12h interval at 32°C) using <em>R. oligosporus</em> 51.3h at 34.9°C</td>
<td>Increase in crude protein, true protein, available lysine and IVPD. Decrease in lipid, ash, CHO, PA, and tannins.</td>
<td></td>
</tr>
<tr>
<td>Common bean</td>
<td>SmF</td>
<td>Controlled using <em>L. fermentum</em> 72h at 37°C</td>
<td>Increase in protein, ash, soluble fiber, soluble nitrogen, starch, and some AAs. Decrease in CHO, crude fiber, fatty acids, vitamins, soluble sugar and some minerals</td>
<td></td>
</tr>
<tr>
<td>Cowpea</td>
<td>SSF</td>
<td>Controlled using <em>R. oligosporus</em> 0-24h (3h interval) at RT</td>
<td>Increase in protein, fat, ash, and CHO.</td>
<td></td>
</tr>
<tr>
<td>Jack bean</td>
<td>SSF</td>
<td>Spontaneous 72 h, RT</td>
<td>Increase in crude fat, crude protein. Decrease in ash, crude fiber, CHO, trypsin inhibitor, PA, lectin, and tannin.</td>
<td></td>
</tr>
<tr>
<td>Lupin</td>
<td>SSF</td>
<td>Controlled using <em>A. sojae</em>, <em>A. ficuum</em> and their co-cultures 7 days at 30°C</td>
<td>Increase in fat, ash, crude fiber fractions, protein, starch, calcium and phosphorus. Decrease in IVPD and PA. Decrease and increase in soluble CHO</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>SSF</td>
<td>Controlled using <em>L. plantarum</em> Lp6 72h at 37 °C</td>
<td>Increase in IVPD and nitrogen solubility</td>
<td></td>
</tr>
</tbody>
</table>


Solid-state fermentation technique is predominantly applied on legume seeds rather than submerged fermentation techniques. The use of *Rhizopus* spp. represents a longstanding cultural practice, tempeh fermentation, that has been adopted and is available in the global market, suggesting consumer acceptance that may benefit a wider utilization of Jack bean. *Rhizopus* are known to secrete various enzymes, including α-amylase, esterase, cellulase, protease, and β-glucosidase [140]. This capability suggests their potential to
modify the chemical and structural properties of Jack beans, thus may enhance Jack beans nutritional quality.

1.7 Knowledge gap

When stored in adverse conditions, Jack bean can develop a hard-to-cook phenomenon, which often leads to a longer optimal cooking time of up to several hours and impaired nutritional quality. A growing body of literature has explored various mechanisms to explain the HTC phenomenon, with a strong emphasis on its impact on the structural organization of cotyledon’s middle lamella. Recent studies highlighted the significance of pectin fractions on the mechanism of HTC [54], as textural softening depends on cell separation due to pectin solubilization during the cooking process [72,124]. Nonetheless, each legume possesses different structural properties reflected as unique cell wall compositions, which may influence the pectin extractability affected by the HTC issue and no such information has been reported on the HTC Jack bean. Furthermore, several studies have successfully described the effectiveness of chemical cooking aids to tackle HTC [141–143]. However, they might have some drawbacks related to sensory properties [144], suggesting the need for alternative methods.

In the context of nutrients digestibility, there is a need to explore the nutritional quality of Jack bean as it is still underexplored. Additionally, the impact of the cooking process may differ depending on the type of legume, thus exploring new types of legumes can add more insight on the effect of cooking process in modulating the nutrients digestibility in legumes. In general, legumes showed limited nutrient digestibility when consumed as whole beans because a substantial fraction of cells remain intact at the point of swallowing. Fermentation has been identified as a method that can modify the chemical composition of legumes, potentially affecting nutrients digestibility. Comprehensive exploration of the digestibility and fermentability of fungal fermented legumes is still limited.

1.8 Aim and outline of the thesis

This thesis investigated the possible explanations of different optimal cooking times in Jack bean (Chapter 2), freezing as an alternative method to reduce cooking times (Chapter 3), and the nutritional implication on several Jack bean collections (Chapter 4) as well as the effect of applying fungal fermentation to enhance its nutritional quality (Chapter 5) (Figure 1.4).
In Chapter 2, we characterized the cell wall pectin of cotyledon powder from three Jack bean collections differing in the optimal cooking times as an indicator of HTC level. Quantitative microscopy was used to assess the influence of the optimal cooking times on bean microstructure. In Chapter 3, we selected one collection and applied freezing pretreatment as a strategy to reduce optimal cooking time. Different freezing conditions were tested and the microstructure of the treated samples was characterized. We further selected the freezing condition that proved the most effective in reducing cooking time and tested the in vitro protein and starch digestibility. This was compared to the in vitro starch and protein digestibility of beans prepared by soaking in alkaline solutions, a well-known strategy to reduce optimal cooking time. In Chapter 4, we evaluated the microstructure and in vitro protein and starch digestion of seven Jack bean collections differing in optimal cooking times. We investigated the correlations of nutrients digestibility with optimal cooking times of these collections. Furthermore, we evaluated the effect of different cooking times in one collection and evaluated protein and starch digestibility. We found that the digestibility of Jack bean proteins was lower than kidney bean. To improve protein digestibility, in Chapter 5, we performed fermentation using Rhizopus oligosporus to produce Jack bean tempeh. We compared the protein and starch digestibility of cooked Jack bean and cooked Jack bean tempeh. Furthermore, we characterized the fermentability of the
indigested fraction from cooked beans and cooked bean tempeh. We assessed the SCFAs, tryptophan metabolites, and GABA formation using an in vitro batch fermentation approach. Chapter 6 summarizes the findings and discusses the implications of the above-mentioned studies. The limitations and future directions are also discussed.
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Chapter 2

Chemical and microstructural characterization of easy- and hard-to-cook Jack bean (Canavalia ensiformis (L.) DC.) collections

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Abstract

Differences in the microstructure and the chemical composition of pectic polymers were investigated from three Jack bean collections with different hard-to-cook (HTC) levels, as confirmed by the optimal cooking time analysis. These collections showed different cell and starch granule sizes, where the hardest-to-cook bean (Jombang collection) had the smallest maximum cell perimeter (285 μm), the smallest starch granule perimeter (98 μm), and a highest frequency of small intercellular space indicating more linkage in the middle lamella. No significant difference was observed in the frequency of small intercellular spaces between Malang and Cilacap despite having different optimal cooking times. Jack bean cotyledon cell walls were mainly composed of insoluble polysaccharide rich in arabinose and galactose. The beans contained high covalently bound pectin fractions (NEF) ranging from 78%-83%. Higher bound phenolics (ferulic acid) were found with the increase in the optimal cooking times of the collections. Overall, the findings suggest the occurrence of cell wall linkage and highlight the importance of ferulic acid pectin crosslink in the HTC Jack bean. This study could help develop strategies to reduce cooking time to promote the utilization of Jack bean.

Keywords

Jack bean, pectin, hard-to-cook, microstructure, cell wall polysaccharide
2.1 Introduction

Due to sustainability and health issues, there is an increasing demand for alternatives of animal protein (Sá et al., 2020). Among plants, legumes represent an economic yet rich source of proteins (Friedman, 1996), and legumes are sustainable with lower technological challenges required for innovation (van der Weele et al., 2019). Legumes contribute substantially to the protein intake of a significant proportion of the world population, especially in developing countries, and are gaining more interest in developed countries. Whereas replacing animal proteins with plant proteins seems necessary for future food security, more alternatives to existing plant-based protein are needed, including promoting underutilized legumes such as Jack bean.

Jack bean (*Canavalia ensiformis* (L.) DC.) is one of the two utilized species from the genus *Canavalia* which counts approximately 60 other species (Schrire, 2005) that grow in the tropical and subtropical area (Ekanayake et al., 2000). They are grown in West Africa, Asia, South America, and the South Pacific (Sharasia et al., 2017). The plants are fast-growing and tolerant to drought, acidic, and nutrient-depleted soils (Sharasia et al., 2017; Udedibie & Carlini, 1998). Therefore, Jack bean has a good potential to tackle food security challenges in the future with its relatively high yield (2400-3400 kg/ha) compared to other legumes (Bressani et al., 1987). Jack bean is a cheap source of protein (content between 22-35 g/100g), carbohydrate (45-65 g/100g), and dietary fiber (4-17 g/100g) (Sridhar & Seena, 2006). Jack bean proteins contain high amounts of essential amino acids such as leucine, isoleucine, and threonine and are considered a good source of lysine (Bressani et al., 1987). It is rich in minerals and essential fatty acids such as linoleic and linolenic acid (Siddhuraju & Becker, 2001). Despite the nutritional benefits, the utilization of Jack bean is limited for human consumption and mostly destined for livestock feeding (Nwokolo, 1996). In addition, Jack beans are commonly hard-to-cook, which can be an obstacle for their utilization (Graham & Vance, 2003).

The hard-to-cook phenomenon (HTC) is a condition that does not allow beans to soften sufficiently after a reasonable soaking and cooking time. HTC phenomenon has been reported for several legumes, such as Pinto bean (*Phaseolus vulgaris* L.) (Njoroge et al., 2014), Jack bean (*Canavalia ensiformis* (L.) DC.) (Akpapunam & Sefa-Dedeh, 1997), Bambara groundnut (*Vigna subterranea* (L.) Verdc.) (Mubaiwa et al., 2017), chickpea (*Cicer arietinum* L.) (Reyes-Moreno et al., 2000), and kidney bean (*Phaseolus vulgaris* L.) (Parmar et al., 2017). HTC is often the result of storage under both high temperature...
(≥25 °C) and high relative humidity (≥65%) (Chigwedere, Njoroge, et al., 2019). Due to the HTC problem, cooking legumes become time-consuming, and less attractive due to its negative environmental impact since it requires more energy to process the legumes to reach acceptable palatability.

To explain the modifications of bean texture during cooking, several hypotheses have been put forward. The first suggests potential softening in the pectin-cation-phytate, implying linkage loss between demethylesterified pectin and cations (Reyes-Moreno et al., 1993). The second hypothesis involves phenolic compounds such as the ferulic acid linkage with arabinan and galactan residues of pectin (Fry, 1983) and the production of insoluble lignin as a result of oxidation and polymerization of polyphenols (Liu, 1995). The third hypothesis suggests the changes of starch and protein, which affect protein denaturation and starch gelatinization (Liu, 1995). Recent studies showed that changes in the cell wall, mainly pectin, may be the main contributor or the rate-limiting factor for tissue softening during cooking (Chigwedere, Nkonkola, et al., 2019; Shiga & Lajolo, 2006; Siqueira et al., 2018). Tissue softening is a result of cell separation, which is facilitated by the solubilization of pectin in the middle lamella (Chigwedere et al., 2018). During relatively high temperature and high humidity storage, new interactions between cell wall polymers could occur and make cell separation more difficult.

To date, there is still limited information on the HTC problem of underutilized legumes, and none specifically on Jack bean. Moreover, understanding the molecular basis of HTC is essential to develop a processing strategy to overcome the HTC problem. Therefore, in this research, we investigated the cotyledon cellular morphology, the microstructure of the cell wall and the chemical composition in pectic polymers and related these observations to the severity of the HTC phenomenon of three collections of Jack bean.

2.2 Materials and Methods

2.2.1 Materials

Jack bean (Canavalia ensiformis (L.) DC.) was harvested from three different regions (Malang, Cilacap, and Jombang) in Indonesia and stored in the farmers’/collectors’ warehouse at ambient temperature and moisture for 3, 2, and 14 months for Malang, Cilacap, and Jombang, respectively, before being shipped to the Netherlands. The storage in tropical conditions was known to trigger HTC development and represented the real condition we found in the
Indonesian market. The cooking profile of each collection was determined upon their arrival at the laboratory, and the remaining beans were stored at -80 °C until further use. All chemicals used were of analytical grade unless otherwise specified.

2.2.2 Determination of Jack bean cooking profile

The cooking profile was determined using the finger-pressing method as described in our previous study (Purwandari et al., 2023). In brief, one hundred Jack bean whole seeds were soaked in demineralized water (3 mL/g) at 25 °C for 16 h. Subsequently, the soaked seeds were boiled in demineralized water (2 mL/g). During the cooking process, ten beans were taken out every 30 minutes and their texture was assessed by pressing between the thumb and forefinger. A bean was considered as cooked once it could be easily disintegrated. The cooking profile was shown as the percentage of cooked beans at different cooking times. The optimal cooking time was the time when 9 out of 10 beans could be crushed.

2.2.3 Preparation of Jack bean cotyledon powder

A preliminary study was conducted on another collection of Jack bean which showed that the presence of seed coat does not change the cooking time (data not shown). Therefore, only the cotyledon was used in the present study. To prepare the cotyledon powder, raw Jack beans were manually dehulled using a knife and the seed coat was removed. The cotyledon was freeze-dried to further lower the moisture content and ground using a Ball mill (MM 400, Retsh, Germany) at frequency 1/30 s for 60 s. Cotyledon powder that passed the 425 µm sieve was collected and stored in a desiccator until further use.

2.2.4 Proximate analysis

Nitrogen was measured using the Dumas combustion method using a Flash EA 1112 NC analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the crude protein content was calculated using 5.4 as conversion factor (Mariotti et al., 2008). Crude fat content was determined gravimetrically by Soxhlet extraction using petroleum ether as a solvent, and moisture content was determined by drying the sample at 105 °C overnight until constant weight. Ash content was determined according to AOAC 923.03 in a muffle furnace at 550 °C overnight (AOAC, 2002). Carbohydrate content was calculated by difference. Phytic acid content was determined using the phytic acid assay kit (K-PHYT) from Megazyme Inc. (Bray, Ireland).
2.2.5 Microstructural analysis

Raw Jack beans were used for microstructural analysis. The samples for microstructural observation were prepared according to a previous report (Siqueira et al., 2018). A transverse midplane slice (thickness: 0.5-0.7 cm) was cut from Jack bean cotyledon before fixation in Karnovsky solution for 24 h (with a final concentration of 40 g/L paraformaldehyde, 20 mL/L glutaraldehyde, 0.1 mL/L Nonidet P-40 and 1 mmol/L 1,4-Dithiothreitol (DTT) prepared in 0.05 mol/L piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) biological buffer (pH 6.9)). The combination of both aldehydes in the fixative preserves cellular morphology by crosslinking and minimizes shrinkage. The bean was rinsed with the buffer and distilled water, dehydrated in increasing ethanol and water mixtures (1:9, 3:7, 1:1, 7:3, 9:1, 96:4, 1:0 for 2 h, respectively) containing 1 mM DTT to minimize oxidation and continued with absolute ethanol overnight. The bean samples were infiltrated with mixtures of absolute ethanol and rising methacrylate solution (100 mL Technovit 7100, 1 g hardener I and 2 mL PEG 400) (3:1, 1:1, and 1:3 for 3 h, respectively), followed by pure methacrylate solution overnight. Samples were polymerized in a mixture of T7100 methacrylate with freshly added Hardener II (in ratio 15:1) and incubated for 1 h at 37 °C. The polymerized block was glued with Technovit 3040 to the sample holder before cutting with a microtome to produce sections of 5 µm. Sections were stretched on water surface, lifted on an object slide, and dried for minimal 5 minutes at 80 °C hot plate to improve sticking prior to staining. Staining was performed with histological dyes that differentiate the composition of the cellular domain. Toluidine Blue (1 g/L) was applied for the overall staining of cell wall, cytoplasm, and nuclei and observed with bright field (Eclipse 80i, Nikon) with objective 20x/N.A. 0.50. For cell walls, Calcofluor White M2R (0.1 mL/L) was used as fluorescent dye and visualized with a confocal laser scanning microscope (LSM) (Stellaris 5 Confocal LSM, Leica, Germany) with argon laser excitation at 405 nm and objective 20x/N.A. 0.5. Images were acquired using LAS X software v.4.5.0 (Leica, Wetzlar, Germany) and were analyzed with ImageJ (Fiji) v.1.52 (NIH, Maryland, USA). To ensure comparability between collections, all images were obtained on the mature cells located approximately 5000 µm from the epidermis.

The area of intercellular space, as well as the size of cells and starch granules, was measured on confocal LSM images. The area of intercellular space, i.e. the void space between cells, was used to assess the cell wall connections. The images were turned to 8 bit and inverted, segmented with the “Find maxima” and “Segmented particles” functions. The void areas were selected using the wand tool and measured. We calculated the total intercellular space per image.
plane by summing up the intercellular space of one image plane. In addition, we examined the frequency distribution of each calculated area of intercellular spaces. Two image planes were used for the quantification of intercellular space. The number of measurements ranged from 160-186 measurements/image plane. For measuring the cell size, the maximum perimeter of the cells, refers to the outer part of the cell walls, was selected on the segmented images. For measuring the starch granule size, the 8-bit images were adjusted for the threshold to identify the starch granules. The perimeter of the starch granules was selected and measured. Similar to the area of intercellular space, the cell and starch granule sizes were calculated on two different image planes for each collection. A total of 100 measurements were taken to analyze the cell and the starch granules size.

2.2.6 Isolation of water-soluble and water-insoluble polysaccharides

Jack bean cotyledon powders were used to generate water soluble polysaccharides (WSP) and water insoluble polysaccharides (WIP) (Njoroge et al., 2014; Rovalino-Córdova et al., 2018). An enzymatic hydrolysis was performed to remove starch and protein using heat stable α-amylase (3000 U/mL), protease (350 Tyrosine U/mL), and amyloglucosidase (3300 U/mL) from Total Dietary Fiber kit that was purchased from Megazyme Inc. (Bray, Ireland). According to the protocol, 1 g of raw Jack bean cotyledon powder was mixed with 40 mL 0.05 mol/L 2(N-morpholino) ethanesulfonic acid (MES)/tris(hydroxymethyl) aminomethane (TRIS) buffer pH 8.2, added with 50 µL heat-stable α-amylase and incubated in a shaking water bath (70 rpm) for 30 min at 98-100 °C to hydrolyze starch. After cooling to room temperature, 100 µL of protease was added and incubated in a shaking water bath for another 30 min at 60 °C. Subsequently, the pH of samples was adjusted to 4.8 by adding HCl and 200 µL amyloglucosidase was added, followed by incubation at 60 °C for 30 min. Suspension was centrifuged for 15 min at 4700g.

The supernatant was brought to ethanol and water mixture (80:20), followed by precipitation at -20 °C for 24 h. The precipitates were carefully collected and washed with ice-cold ethanol (ethanol and water mixture (80:20)) three times, suspended in deionized water and freeze-dried. This fraction is referred as water-soluble polysaccharide fraction (WSP). The pellet from the centrifugation was collected and homogenized using an ultraturax homogenizer (3 x 6s) first in 64 mL, then 32 mL ethanol and water mixture (96:4), and finally in 32 mL ice-cooled acetone. After each homogenization step, samples were filtered under vacuum through 595½ filter paper (90mm,
Whatman). The residue, representing the water-insoluble fraction (WIP), was dried at 40 °C for 16 h and ground with mortar and pestle. WSP and WIP were stored in a desiccator until further use.

2.2.7 Fractionation of WIP

WIP from raw Jack bean cotyledon powder was fractionated into hot water extractable fractions (WEF), chelator extractable fractions (CEF), and Na$_2$CO$_3$ extractable fractions (NEF) (Chigwedere, Nkonkola, et al., 2019). Briefly, 0.5 g of WIP was suspended in 90 mL boiling demineralized water, followed by boiling the suspension for 5 min on a hot plate with stirring. The sample was cooled in cold water and vacuum filtered, as described in section 2.6. The obtained filtrate (Filtrate I) was adjusted to pH 5, topped up to 100 mL followed by dialysis against demineralized water at 4 °C for 72 h (with changes every 12 h). The residue was suspended in 90 mL 0.1 mol/L potassium acetate solution (pH 5) containing 0.05 mol/L cyclohexane-1,2-diaminetetraacetic acid (CDTA), incubated at 28 °C for 6 h on a plate stirrer and vacuum filtered. The filtrate obtained after CDTA addition (Filtrate II) was adjusted to pH 5, and topped up to 100 mL, dialyzed against 0.1 mol/L sodium chloride at 4 °C, for 36 h (with changes every 12 h) and then with demineralized water at 4 °C for 36 h (with changes every 12 h). The residue was mixed with 90 mL of 0.05 mol/L sodium carbonate solution containing 0.02 mol/L sodium borohydride, incubated at 4 °C for 16 h and 28 °C for 6 h and vacuum filtered. The filtrate obtained after sodium carbonate addition (Filtrate III) was adjusted to pH 5, topped up to 100 mL, and dialyzed against demineralized water at 4 °C for 72 h (with changes every 12 h). After dialysis, Filtrate I, II, and III were freeze dried and labelled as WEF, CEF, and NEF, respectively. The flow diagram of cell wall isolation and fractionation is provided in Figure 2.1.
Figure 2.1 Flow diagram of cell wall polysaccharide isolation and fractionation of Jack bean cotyledon powder. Water soluble polysaccharide (WSP), water insoluble polysaccharide (WIP), hot water extractable polysaccharide (WEF), chelator extractable polysaccharide (CEF), and Na$_2$CO$_3$ extractable polysaccharide (NEF) in WIP. CDTA: Cyclohexane-1,2-diaminetetraacetic acid.

2.2.8 Neutral sugar and galacturonic acid composition of WSP and WIP fractions

Neutral sugar and galacturonic acid composition of WSP, WIP, WEF, CEF, and NEF were determined by high-performance anion-exchange chromatography (HPAEC), using ICS-3000 ion chromatography high-performance liquid chromatography system equipped with a CarboPac PA-1 column (2 x 250 mm) in combination with a CarboPac PA guard column (2 x 25 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex, Sunnyvale, USA). One hundred milligrams of WSP, WIP, WEF, CEF, and NEF were weighed and hydrolyzed in sulphuric acid to generate monomeric sugars (Saeman et al., 1954). The hydrolysis was carried out in 5 mL 12 mol/L H$_2$SO$_4$ at 35 °C for 1 h. After diluting with distilled water to a final concentration of 2 mol/L H$_2$SO$_4$, the mixture was incubated for 1 h at 100 °C, then cooled on ice. A portion of hydrolysate was diluted with Milli-Q water until 1 mL volume was reached, and 2.5 μL 1 g/L bromophenol blue in ethanol was added. The pH was adjusted with barium carbonate until a clear blue colour was obtained (pH > 4.6). The remaining solution was filtrated using a 0.45 μm PTFE filter. The
amount of monomeric sugars was measured according to a previous report with modifications (Gilbert-López et al., 2015). A flow rate of 0.3 mL/min was used, and 0.1 mL/min for additional postcolumn (500 mol/L NaOH), column temperature was maintained at 17 °C. Elution was performed as follows: 0-63 min Milli-Q, 63-63.1 min 150 mmol/L NaOH, 63.1-83 min 500 mmol/L NaOH, 83-100 min Milli-Q. Monomeric sugars eluted from the CarboPac PA guard column were detected after adding 0.5 mol/L sodium hydroxide (0.15 mL/min). The monomeric sugars were quantified using a calibration curve generated with standards in the range of 0 – 0.1 mg/mL. Pectin content was estimated by the sum of polymeric Gal A and pectin-related neutral sugars (fucose, arabinose, rhamnose, galactose, xylose) from four fractions (WSP, WEF, CEF, and NEF). In this study, Jack bean contains 14.7-18.8 mg pectin/g dry cotyledon powder (Table S2.1).

**2.2.9 Quantification of bound phenolics in WIP**

Bound phenolics were extracted in duplicate using microscale alkaline treatment (Zavala-López & García-Lara, 2017). Firstly, 0.1 g of cotyledon powder was added with methanol and water mixture (80:20) to remove the soluble phenolics. The residue after centrifugation was added with NaOH and digested at 90 °C for 30 minutes under the nitrogen gas flush, followed by mixing for 5 minutes at 2500 rpm on Heidolph shaker. Samples were added with 2 mol/L HCl until the pH was 2, added with n-hexane to remove the fat layer. The bound phenolics were recovered by adding 1.5 mL ethyl acetate, mixed for 5 min at 2500 rpm, incubated at 25 °C for 10 minutes at 500 rpm and then centrifuged for 10 minutes at 2500 rpm. The ethyl acetate layer was removed carefully into a clean tube, and the extraction process was repeated twice. The extract was pooled and flushed with nitrogen until dry. The dry extract was resuspended with 1 mL methanol and water mixture (80:20) and stored at -20 °C until further analysis.

Total bound phenolics were analyzed using a colorimetric assay with Folin-Ciocalteau reagent, and gallic acid was used for generating a calibration curve (Urias-Lugo et al., 2015). The amount of bound phenolics was expressed as the relative amount of gallic acid equivalent to WIP by incorporating conversion factors from the yield of WIP/cotyledon powder in the calculation.

Ferulic acid was analyzed using a Dionex Ultimate 3000 apparatus (Thermo Fisher Scientific, Bleiswijk, The Netherlands). Prior to analysis, bound phenolic extracts were filtered through a 0.2 μm syringe filter. 10 μL extract was injected into the HPLC system with 1 mL/min flow rate. The separation was
achieved on a RP-Polaris C18-A (46 x 150 mm) equipped with a Polar RP security guard cartridge (4 x 3mm). The column temperature was set at 30 °C. A multistep gradient eluent was applied using water pH 2.5 (acidified with trifluoroacetic acid (TFA)) (A) and acetonitrile (B) for a total run time 35 min. The gradient program was 0 min 100:0 (A:B), 20-25 min 58:42 (A:B), 26-35 min 100:0 (A:B). Detection was performed at a wavelength of 290 nm. Ferulic acid standard was used to create calibration curve ranging from 0-0.1 mg/mL. Data processing was performed with Chromeleon v. 7.2 (Thermo Scientific, MA, USA).

2.2.10 Quantification of Boron and Calcium in WIP

Boron and calcium were measured on WIP of cotyledon powder. Sample (0.1 g) was digested using concentrated nitric acid, hydrochloric acid, and hydrogen-peroxide in a closed system microwave (MarsXpress, CEM corporation) (Novozamsky et al., 1996). The supernatant was injected to inductively coupled plasma optical emission spectroscopy (ICP-OES) system (Thermo iCAP-6500 DV, Thermo Fisher Scientific), and the result was expressed as the relative amount of boron and calcium to WIP.

2.2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. A one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons was used to test the difference among the three seed collections on the quantitative data except for intercellular spaces. The frequency distributions of intercellular space were compared by applying Chi square test. Significant differences were defined at p < 0.05.

2.3 Results and Discussion

2.3.1 Cooking profile of Jack bean from different collections

The cooking profile of the three collections of Jack beans selected in this study is presented in Figure 2.2 The three collections of Jack bean showed different optimal cooking times, i.e. cooking times to achieve a 90% cooked stage. Malang collections showed the shortest optimal cooking time (120 min), followed by Cilacap (195 min) and Jombang (300 min). No bean was cooked after boiling for 30 minutes for any of the three Jack bean collections. Interestingly, Cilacap showed a higher % cooked beans compared to the Malang collections at 60 and 90 minutes of cooking. However, the situation was reversed after 90 minutes of cooking. In fact, after 120 minutes the percentage of cooked beans was almost 100% in Malang, but Cilacap took an
additional 90 minutes to reach the same percentage of cooked beans as Malang. Jombang was the slowest to soften among the three collections, with 90% cooked beans after 300 minutes.

![Figure 2.2 Cooking profile of three Jack beans collections harvested and dried at different locations (N=2, error bars indicate standard deviation of the mean)](image)

A previous study classified beans that need 2 h or less to achieve 80% cooked beans as easy-to-cook (ETC), and else was considered as hard-to-cook (HTC) (Kinyanjui et al., 2015). Based on this classification, both Cilacap and Jombang should be grouped as HTC. Elevated storage temperature and relative humidity as well as prolonged storage time were reported as the main factors for the development of HTC phenomenon (Njoroge et al., 2015; Siqueira & Pereira, 2014). In this study, the Jombang collection, which was stored with the longest time (14 months), showed the longest optimal cooking times. However, Cilacap showed a longer optimal cooking time compared to Malang despite being stored for a shorter time. Beside the storage factors, the growing region can contribute to HTC. Growing regions with longer warm climates were reported to impact cell wall properties and produce a harder-to-cook Navy bean (Chu et al., 2020). In this study, all the three collections were grown at different regions (Table S2.2). Malang was grown in a mountainous area (elevation: 444 m) with lower temperature (<25 °C) compared to Cilacap and Jombang. This may partly explain the different longer cooking time of Cilacap (stored for 2 months) compared to Malang (stored for 3 months).
2.3.2 Proximate composition and phytic acid content of the three Jack bean collections

The proximate and phytic acid content of the three Jack bean collections are shown in Table 1. Proximate composition varied between collections except for ash content. Malang showed the highest carbohydrate content, followed by Jombang and Cilacap. Similar crude protein and crude fat content was found in Malang and Jombang collections, while Cilacap showed the highest crude protein and the lowest crude fat content among all collections.

Table 2.1 Proximate composition and phytic acid content of three different Jack bean collections (N=2, Mean ± SD)

<table>
<thead>
<tr>
<th>Collections</th>
<th>Carbohydrate (g/100g db)</th>
<th>Protein (g/100g db)</th>
<th>Fat (g/100g db)</th>
<th>Ash (g/100g db)</th>
<th>Phytic acid (g/100g db)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malang</td>
<td>67.2 ± 1.0a</td>
<td>26.2 ± 0.97a</td>
<td>3.4 ± 0.1a</td>
<td>3.2 ± 0.1a</td>
<td>1.6 ± 0.0a</td>
</tr>
<tr>
<td>Cilacap</td>
<td>61.8 ± 0.3b</td>
<td>32.0 ± 0.4b</td>
<td>2.9 ± 0.1b</td>
<td>3.3 ± 0.1a</td>
<td>2.2 ± 0.1b</td>
</tr>
<tr>
<td>Jombang</td>
<td>64.4 ± 0.1c</td>
<td>28.9 ± 0.3a</td>
<td>3.6 ± 0.1a</td>
<td>3.1 ± 0.0a</td>
<td>1.1 ± 0.0c</td>
</tr>
</tbody>
</table>

Different letters in the same column indicate significant differences (p<0.05). db: dry weight basis

The highest phytic acid content was found in Cilacap, followed by Malang and Jombang. The pectin-phytic acid-pectate theory states that storage in elevated temperature and relative humidity is favorable for activating endogenous phytase, causing dephosphorylation of phytic acid and releasing divalent minerals. Divalent minerals would migrate from parenchyma cells and concentrate around the middle lamella of the cell wall, possibly binding to the free carboxyl groups of pectin to form insoluble pectate (Kruger et al., 2015). Here, we observed a lower phytic acid content in Jombang but a higher phytic acid content in Cilacap compared to Malang. It is important to note that the development of HTC is related to the decrease of phytic acid content according to pectin-phytic acid-pectate theory. Therefore, the phytic acid content alone is not predictive of the optimal cooking time in Jack beans.

2.3.3 Microstructural characterization of the three Jack bean collections

Figure 2.3 gives an overview of Jack bean cotyledon cells cross planes. Cotyledon cells of Jack beans had a regular oval shape where Jombang had the lowest maximum perimeter (285 µm) compared to Malang (318 µm) and Cilacap (323 µm) (Table S2.3). Spherical protein bodies could be observed in all three collections. Large starch granules embedded in a protein matrix could be observed in all the collections with the HTC ones (Cilacap and Jombang),
showing smaller starch granule sizes (average: 100 and 98 μm) compared to Malang (106 μm) (Table S2.3). To our knowledge, there are no reported data on the size of the starch granules in beans with different HTC levels. The degree of starch crystallinity was reported to increase during high temperature and high relative humidity storage, increasing the gelatinization temperature (Yousif et al., 2007). However, starch was reported to be completely gelatinized after 30 minutes of cooking of beans, irrespective of its HTC level, which suggests that starch properties play a minor role in HTC (Chigwedere et al., 2018).

Figure 2.3 Overview of light microscope images of cotyledons of Malang (panel Malang), Cilacap (panel Cilacap), and Jombang (panel Jombang) stained with toluidine blue. s: starch granule, p: protein bodies, cw: cell wall, pm: plasmamembrane, ies: intercellular space.

An overview of the cell wall of the three Jack bean collections can be seen in Figure 2.4 Cell integrity seems preserved in all Jack bean collections, i.e. we did not observe any cell wall fracture in any of the three collections. However, in Carioca beans the HTC bean showed evidence of cell wall rupture compared to the ETC bean (Siqueira et al., 2018). We observed cell contacts, i.e. the cell wall area where two adjacent cells were connected (as indicated by the yellow arrow) (Figure 2.4) in all three collections. We examined the area of the intercellular space between cells as a measure of cell wall connections. It was reported that HTC bean showed less intercellular space compared to fresh bean due to the linkage in the middle lamella forming tricellular junctions (Perera et al., 2023). There was no significant difference in the total intercellular space areas of the three collections (Table S2.3). However, the
frequency distribution of Jombang collection was significantly higher than Cilacap and Malang (Figure 2.5) indicating more cell wall connections. However, the lack of a significant difference in the frequency distribution of the intercellular spaces between Malang and Cilacap demonstrates that the number of cell wall connections is not always correlated to optimal cooking times.

**Figure 2.4** Overview of confocal microscopy images of cotyledon of Malang, Cilacap, and Jombang collections. Cell wall (green) are stained with calcofluor white. Yellow arrow indicates the cell wall at the cell contacts.
Figure 2.5 Frequency distribution of the intercellular spaces of three Jack bean collections. Significant differences are indicated by an asterisk symbol (p<0.05).

2.3.4 Chemical characterization of Jack bean cell wall polysaccharides

The WSP and WIP yields obtained from the three collections of Jack bean are shown in Table 2.2. Representative chromatograms of sugar of the collections can be seen in Figure S2.1. There was a difference (p<0.05) between the amount of WSP extracted from the three collections where Jombang showed the least amount of WSP followed by Cilacap and Malang. This is in line with previous studies that reported a negative correlation between WSP and cooking time in common beans and Carioca beans (Chigwedere, Nkonkola, et al., 2019; Shiga et al., 2004). Unlike WSP, WIP yield was not significantly different among the three Jack bean collections and ranged from 135 to 151 mg/g dry cotyledon.

Sugar composition of WSP and WIP are presented in Table 2.2. The cell wall of Jack bean cotyledon was composed of mainly insoluble fractions. Among the three collections, Malang had a significantly higher total sugar content in the WSP fraction, while no significant difference was observed between Cilacap and Jombang. Arabinose and galactose were the main pectin-related neutral sugar in both polysaccharide extracts, probably derived from arabinans or arabinogalactan. High arabinose content is also reported in other legume cotyledon such as lentil, kidney bean, and Carioca bean (Bhatty, 1990;
In this study, a lower uronic acid content was observed compared to other varieties of Jack beans, as previously reported (Benítez et al., 2013). The difference is possibly due to the use of different part of the seeds. It is not clear whether only the cotyledon or the whole seed was used in the reported study, and GalA was mainly presented in the seed coat rather than in the cotyledon (Yi et al., 2016).

2.3.5 Chemical characterization of the pectin fraction

WIP was fractionated into WEF, CEF, and NEF fractions in which relative content and composition in neutral sugar, galacturonic acid, and glucuronic acid are displayed in Figure 2.6. We found low GalA in all three fractions of Jack bean cotyledon, suggesting that Jack beans contain limited homogalacturonan, as can be seen in soybean (Voragen et al., 2009).

All in all, there were small differences in the distribution of WEF, CEF and NEF among the three collections. Jombang showed the least relative amount of WEF and the highest relative amount of NEF (Figure 2.6A). WEF fraction contains the loosely bound pectin that can easily be solubilized with hot water extraction. No differences were observed in the sugar composition of the WEF fractions from the three collections (Figure 2.6B). CEF represents the calcium crosslinked pectin fraction. We observed a higher content in arabinose in Malang compared to the other two collections (Figure 2.6C). Cell wall connection is widely attributed to the properties of pectin, typically through the calcium crosslinked homogalacturonan (Daher & Braybrook, 2015; Jarvis et al., 2003). NEF represents the ester-linked pectin fractions. The link occurs between arabinan and galactan residues in Rhamnogalacturonan I with phenolic acids (such as ferulic acid); leading to the formation of ferulate dimers catalyzed by peroxidase and consequently enhancing the cell wall strength (Fry, 1983; Shiga et al., 2004). In addition, apiofuranosyl residue of xylose-containing side chains in Rhamnogalacturonan II may ester link with borate (Ishii et al., 1999). Arabinose and galactose in NEF fraction were higher in Cilacap and Jombang compared to Malang, showing that these two sugars occur more in the ester-linked form in the first two collections than in the Malang collection (Figure 2.6D). Previously, it was postulated that some loosely bound pectin is converted into covalently bound pectin during development of HTC (Chigwedere, Nkonkola, et al., 2019). We must mention, however, that the differences in NEF content are rather small ranging from 78% to 83%.
### Table 2.2 Yield, sugars composition and total sugars of water-soluble (WSP) and water-insoluble polysaccharides (WIP) from the three Jack bean collections (mg/g dry cotyledon) (N=2, Mean ± SD)

<table>
<thead>
<tr>
<th>Collections</th>
<th>Yield</th>
<th>Sugars</th>
<th>Total sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fuc</td>
<td>Ara</td>
<td>Rha</td>
</tr>
<tr>
<td>Malang</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSP</td>
<td>141.7</td>
<td>0.3 ±</td>
<td>8.2 ±</td>
</tr>
<tr>
<td></td>
<td>0.1a</td>
<td>0.5a</td>
<td>0.1a</td>
</tr>
<tr>
<td>WIP</td>
<td>144.3</td>
<td>0.4 ±</td>
<td>38.7 ±</td>
</tr>
<tr>
<td></td>
<td>0.0A</td>
<td>1.1B</td>
<td>0.0A</td>
</tr>
<tr>
<td>Cilacap</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSP</td>
<td>129.0</td>
<td>0.2 ±</td>
<td>4.9 ±</td>
</tr>
<tr>
<td></td>
<td>0.0a</td>
<td>0.2b</td>
<td>0.0a</td>
</tr>
<tr>
<td>WIP</td>
<td>135.1</td>
<td>0.3 ±</td>
<td>37.0 ±</td>
</tr>
<tr>
<td></td>
<td>0.0A</td>
<td>1.9B</td>
<td>0.0A</td>
</tr>
<tr>
<td>Jombang</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSP</td>
<td>103.4</td>
<td>0.1 ±</td>
<td>5.3 ±</td>
</tr>
<tr>
<td></td>
<td>0.0a</td>
<td>0.3b</td>
<td>0.0a</td>
</tr>
<tr>
<td>WIP</td>
<td>151.1</td>
<td>0.4 ±</td>
<td>42.1 ±</td>
</tr>
<tr>
<td></td>
<td>0.0A</td>
<td>1.3A</td>
<td>0.0A</td>
</tr>
</tbody>
</table>

Different lowercase and capital superscript letters within a column indicate significant differences (p<0.05). Fuc: fucose, Ara: arabinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose, GalA: galacturonic acid, Glu: glucuronic acid.
Figure 2.6 Relative amount of hot water extractable polysaccharide (WEF), chelator extractable polysaccharide (CEF), and Na$_2$CO$_3$ extractable polysaccharide (NEF) in WIP fraction from the three Jack bean collections (panel A). Neutral sugar, GalA and Glu composition of WEF (panel B), CEF (panel C) and NEF (panel D) from three different Jack bean collections. Fuc: fucose, Ara: arabinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose, GalA: galacturonic acid, Glu: glucuronic acid. Significant differences are indicated by asterisk symbol (p<0.05). (N=2, error bars indicate standard deviation of the mean).

In WIP fractions, we found a similar amount of calcium and boron content but higher bound phenolic/ferulic acid (p < 0.05) with increasing optimal cooking times (Table 2.3). This corresponds to the covalently bond pectin crosslink, possibly the ferulic acid pectin crosslink. Ferulic acid has been linked to the crosslink of pectic arabinans and galactans in several dicotyledons (Fry, 1983; Wefers et al., 2014). A recent study with immunolabelling microscopy of red Haricot bean showed evidence of higher feruloylated pectin crosslinking at intercellular junction zones of aged beans compared to non-aged beans (Chen et al., 2021). It is important to note that the amount of ferulic acid that has been measured may give an approximate indication of the actual level of
crosslinks in the Jack bean cell wall. The crosslink of ferulic acid and polysaccharides occurs via ferulic acid dimers and trimers (Waterstraat et al., 2016), and those are not quantified by our method. In sugarbeet and beetroot, the feruloylated moieties have an impact on the textural degradation during heat treatment (Waldron et al., 1997). Sugarbeet, where 20% of the feruloyl moieties are integrated into dimers, does not completely soften after several hours of heating at 100 °C, whereas beetroot, where 10% of the feruloyl moieties are integrated into dimers, softens in 20-30 minutes.

<table>
<thead>
<tr>
<th>Collections</th>
<th>Calcium (g/kg WIP)</th>
<th>Boron (mg/kg WIP)</th>
<th>Bound phenolic (µg GAE/g WIP)</th>
<th>Ferulic acid (µg/g WIP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malang</td>
<td>1.2 ± 0.0b</td>
<td>26.0 ± 0.0a</td>
<td>341.0 ± 26.8c</td>
<td>12.0±1.3c</td>
</tr>
<tr>
<td>Cilacap</td>
<td>1.5 ± 0.1a</td>
<td>27.5 ± 2.5a</td>
<td>502.9 ± 25.1b</td>
<td>20.5±0.2b</td>
</tr>
<tr>
<td>Jombang</td>
<td>1.4 ± 0.0ab</td>
<td>22.5 ± 1.5a</td>
<td>636.8 ± 17.1a</td>
<td>29.5±1.9a</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences (p<0.05).

### 2.4 Conclusion

This paper provides, for the first time, a characterization of microstructure and composition in Jack bean collections with different levels of HTC induced by different storage times and growing regions. We found modest differences in the microstructure of these three Jack bean collections. Our finding, however, reveal that the hardest-to-cook Jack bean collection (Jombang) has more cell wall connections, as showed by a higher frequency number of the fractions of small intercellular space, compared to the other two collections. The proximate and phytic acid content differs among collections, with no apparent correlation to the optimal cooking time. The water-soluble polysaccharides (WSP) and the covalently bound pectin fractions (NEF) are associated to the increasing optimal cooking times in Jack bean. Whether this difference in the cooking behavior of Jack bean can be linked to molecular modifications remains to be studied. Another possible tool, such as FTIR, can be utilized to evaluate the chemical bond caused by HTC on the cell wall fractions. To date, there is still limited study on the ferulic dimers or oligomers on legume cell wall related to the development of HTC. Furthermore, it is necessary to investigate the role of ferulic-pectin crosslinking on the development of textural softening upon cooking to gain a mechanistic understanding of the HTC Jack bean. This can be done, for instance, by quantifying the ferulic dimer or oligomer in the pectin fractions during different cooking times and
eventually finding collections or providing solutions to reduce optimal cooking times.

**CRediT authorship contribution statement**

**Fiametta Ayu Purwandari**: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Norbert C. A. de Ruijter**: Methodology, Investigation, Data curation, Writing – review & editing. **Vincenzo Fogliano**: Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration. **Edoardo Capuano**: Conceptualization, Methodology, Visualization, Supervision, Writing – review & editing, Project administration, Resources.

**Declaration of competing interest**

The authors declare no conflict of interests that may influence the work reported in this paper.

**Acknowledgements**

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References


Chemical and microstructural characterization of easy- and hard-to-cook Jack beans

genotypes. *LWT - Food Science and Technology, 97*, 117–123. https://doi.org/10.1016/j.lwt.2018.06.048


Supporting information

Table S2.1 Mean and standard deviation of pectin content of three Jack bean collections

<table>
<thead>
<tr>
<th>Collections</th>
<th>Pectin content* (mg/g cotyledon db)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malang</td>
<td>18.8 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cilacap</td>
<td>14.7 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jombang</td>
<td>15.8 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Pectin content has been estimated from the sugar data. Different letters indicate significant differences (p<0.05).

Table S2.2 Geographical growing conditions of three Jack bean collections

<table>
<thead>
<tr>
<th>Location</th>
<th>Malang</th>
<th>Cilacap</th>
<th>Jombang</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dampit, Malang, Indonesia</td>
<td>Kawunganten, Cilacap, Indonesia</td>
<td>Marmoyo, Kabuh, Jombang, Indonesia</td>
</tr>
<tr>
<td>Latitude</td>
<td>8.24 South</td>
<td>7.59 South</td>
<td>7.4 South</td>
</tr>
<tr>
<td>Longitude</td>
<td>112.74 East</td>
<td>108.9 East</td>
<td>112.15 East</td>
</tr>
<tr>
<td>Altitude</td>
<td>444.00m/1456.69ft</td>
<td>4.00m/13.12ft</td>
<td>112.00m/367.45ft</td>
</tr>
<tr>
<td>Average temperature</td>
<td>75 °F</td>
<td>81 °F</td>
<td>81 °F</td>
</tr>
</tbody>
</table>

<sup>1</sup>elevationmap.net  
<sup>2</sup>weatherspark.com

Table S2.3 Mean and standard deviation of total intercellular spaces, maximum cell perimeter, and starch granule perimeter of three Jack bean collections

<table>
<thead>
<tr>
<th>Collections</th>
<th>Total intercellular spaces (µm²/plane)</th>
<th>Maximum cell perimeter (µm)</th>
<th>Starch granule perimeter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malang</td>
<td>108390 ± 6824&lt;sup&gt;a&lt;/sup&gt;</td>
<td>318 ± 64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>106 ± 14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cilacap</td>
<td>107603 ± 6267&lt;sup&gt;a&lt;/sup&gt;</td>
<td>323 ± 61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jombang</td>
<td>116463 ± 10772&lt;sup&gt;a&lt;/sup&gt;</td>
<td>285 ± 57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences (p<0.05).
Chapter 3

Freeze-thaw procedure as an alternative method to speed up the cooking time of Jack bean (*Canavalia ensiformis* (L.) DC)

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Manuscript under preparation
Abstract

This study aimed to investigate the effect of freeze-thaw procedures on the Jack bean's cell wall, cooking times, and nutritional quality of the frozen-thawed cooked beans, including mineral content, hemagglutination activity, and in vitro protein and starch digestibility.

Confocal laser microscopy was used to examine cell wall changes. Optimal cooking times, hemagglutination activity, and mineral content were evaluated on beans subjected to freeze-thaw procedures and those cooked with sodium bicarbonate (AC) and demineralized water (DC), respectively. The freeze-thaw procedure that yielded a significant reduction in cooking times, AC, and DC samples were used to generate intact cells for in vitro protein and starch digestion.

Freeze-thaw procedure at -20 °C for 24 h resulted in the biggest cell separations and reduction effect in cooking time but was less effective compared to alkaline salt cooking. No hemagglutination activity was observed in all cooked beans and mineral loss was correlated with the duration of cooking times. Reduction of cooking times showed by freeze-thaw cooked beans increased protein digestibility compared to control beans cooked in demineralized water, with no effect on starch digestibility. The finding implies that freezing-induced microstructural alterations can be used to reduce cooking times and increase protein digestibility, making it a viable method for Jack bean preparation. Additionally, the absence of hemagglutination activity and the observed mineral loss emphasize the importance of efficient cooking methods in preserving nutritional properties.

Keywords:
Freezing, microstructure, beans, cooking times, protein digestion, starch digestion
3.1 Introduction

One of the global challenges established by FAO [1] is to find a more sustainable source of protein, and increasing the consumption of legumes plays a crucial role in addressing this issue [2]. Legumes are renowned for being rich in protein, high in fiber and minerals, and they require less land and water, and produce fewer greenhouse gas emissions compared to animal husbandry and other crops [3]. Among legumes, there are underutilized legumes that are not consumed on a global scale. One of them is Jack bean, which protein content ranges from 23% to 35% while having low lipid levels and high fiber content [4]. Jack bean contains the antinutrient lectin concanavalin A that has hemagglutination activity and thus can be inactivated by the thermal processes [5]. Despite its nutritional potential, Jack bean utilization is limited by the so-called hard-to-cook (HTC) phenomenon [6].

HTC Jack bean is associated with more cell connections and more covalently bound pectin within the cell walls [7]. These factors may result in a more pronounced difficulty in separating the cells, which is a crucial microstructural feature linked to textural degradation. Specifically, the textural alterations during cooking are driven by the solubilization of pectin in the middle lamella, which in turn, leads to cell separation upon mechanical disintegration [8]. Various approaches have been explored to reduce cooking times for beans such as soaking and dehulling [9]. Nevertheless, the role of seed coat in softening during cooking was minor [8]. Methods such as micronization, gamma irradiation, pressure cooking, and germination were reported to decrease cooking durations [10–15]. Alkaline solutions were used to reduce cooking times either as a soaking or cooking medium [8,16,17]. This technique offers a simple way to shorten cooking times, suitable for resource-constrained regions. However, the use of alkaline solutions may darken the beans, impacting sensory qualities and overall acceptability [16,18].

Freezing is a commonly used process for preserving the quality and extending the shelf life of fruits and vegetables. Freeze-thaw process is usually deleterious food quality due to the cellular damage and textural loss after thawing, especially in plant tissue with high water content [19]. Freezing involves the crystallization of water in the cellular structure, forming ice crystals. Fast freezing formed small intracellular ice crystals, while slower rates caused intracellular water to leave the cell and form larger extracellular ice crystals [20,21], creating more cellular damage. Slow freezing rates at -20 °C resulted in significant damage to the cellular structure of mango cell walls, whereas fast freezing rates at -80 °C yielded cells similar to those found in
fresh tissues [22]. In the case of HTC Jack beans, our hypothesis is that a freeze-thaw treatment may damage the beans' structure or facilitate cell separation, thus potentially leading to a reduction in cooking time.

To the best of our knowledge, no literature has reported the use of freeze-thaw treatments to reduce cooking time in beans. This study aimed to investigate the influence of freeze-thaw treatments on the Jack bean cell wall, cooking times, and nutritional quality such as mineral content, hemagglutination activity, and in vitro protein and starch digestibility.

3.2 Materials and Methods

3.2.1 Materials

Jack bean was grown and harvested in Malang (Indonesia). The bean was stored in the warehouse at ambient temperature before being shipped to the laboratory in the Netherlands. All other chemicals were of analytical grade unless stated otherwise. The flow diagram of the experiments can be found in Fig. 3.1.

![Figure 3.1 Schematic illustration of experimental design](image-url)
3.2.2 Determination of water absorption kinetic of Jack bean

Fully soaked stage is important for the effectiveness of freeze-thaw procedure on the reduction of the cooking time. In our preliminary study, we observed a statistically significant difference (p<0.05) in the cooking time of beans treated with 50% hydration and those treated with 100% hydration, both of which were subsequently frozen for 24 hours, on another batch of Malang collection (data not shown). In this study, we evaluated the kinetics of water absorption during soaking to determine the time to achieve the fully soaked stage according to a previous report [9]. In brief, the whole beans (10 g) were added to demineralized water at the ratio bean to water 1:10 (w/v). Soaking was performed in a water bath at 25 °C with a total soaking time of 40 h. The weight of the soaked beans was monitored every 30 min during the 0-8, 14-28, and 36-40 h and the beans were returned to the soaking water. Excess surface water was removed from the beans with paper towels. The soaking tests were carried out in duplicate. The results were expressed as g water/g dry matter, representing % moisture content for the Peleg model below.

The dry matter content of the whole beans was determined by weighing a known amount of beans powders and stored in an incubator oven at 105 °C overnight. The dried powders were weighed until constant weight.

Data on the water absorption were modelled by using a Peleg model (Equation 3.1) [23] from the duplicate data.

\[ M(t) = M_0 + \frac{t}{(k_1 + k_2t)} \]  

\( M(t) \) (%db) is the moisture content at time \( t \), \( M_0 \) (%db) is initial moisture content, \( t \) (h) is soaking time, \( k_1 \) (h.%-1) is the Peleg rate constant, and \( k_2 \) (%-1) is the Peleg capacity constant. The parameters were estimated by minimizing the residual sum of squares (RSS) using Solver. The goodness of fit between the experimental data and the model was examined using the mean relative percentage deviation modulus \((E)\) as described in Equation 3.2.

\[ E = \frac{1}{N} \sum_{i=1}^{n} \left| \frac{M_{\text{exp}} - M_{\text{pred}}}{M_{\text{exp}}} \right| \times 100\% \]  

\( E \) (Equation 3.2)
where $M_{\text{exp}}$ is the experimental data, $M_{\text{pred}}$ is the predicted value, $N$ is the number of experimental data. It is widely adopted that $E$ value below 10% indicates a good fit for practical purposes [24]. The moisture equilibrium ($M_E$), when $t \to \infty$, was estimated using the following equation (Equation 3.3) [23].

$$M_E = M_0 + \frac{1}{k_2}$$  \hspace{1cm} (Equation 3.3)

### 3.2.3 Freezing treatment

Jack beans (500 g) were soaked in demineralized water (1:10 w/v) for 24 h to ensure optimum water absorptions were obtained. This soaking time was based on the experimental results determined by the method described in section 3.2.2. The soaked beans were divided into four groups and subjected to different freezing treatments: slow freezing at -20 °C for 1 h (SF1) and 24 h (SF24), and fast freezing at -80 °C for 1 h (FF1) and 24 h (FF24). The frozen bean samples were thawed in an incubator at 25 °C for 2 h. The thawed beans were further used for analysis.

### 3.2.4 Microstructural analysis

Microstructural analysis was performed on thawed beans from various freezing conditions (SF1, SF24, FF1, FF24) to evaluate the impact of freezing on the microstructure of the Jack bean. A control (NF) was prepared from beans that had been soaked in demineralized water (1:3 w/v) for 24 h. Cotyledon from each treatment and control was thinly sliced (0.5-0.7 cm) to generate two slices per bean cotyledon. The sliced samples were stained with 0.1% calcofluor white to examine the cell wall and left overnight to ensure complete dye diffusion and homogeneity [25]. Cell microstructure was observed with a confocal laser microscope Zeiss 510 (Carl Zeiss microscopy, Oberkochen, Germany), with an argon laser set at 405 nm. Images were taken at objective 20x Plan-Neofluar (0.30) and processed with Zen blue software v.2.3 (Carl Zeiss, Oberkochen, Germany). At least 10 images were randomly captured from different areas for each sample treatment. To assess cell separation, image quantification was carried out using Image J 1.52 (Fiji) software (NIH, Maryland, USA). The software was employed to measure the shortest perpendicular distance between two adjacent cells. A total of 100 measurements were analyzed for each sample group.
3.2.5 Cooking time determination

Finger pressed method
Cooking times were determined on the thawed beans according to the previous study [26]. In brief, the thawed Jack beans were cooked in boiling demineralized water (1:2 w/v) at 97 °C. Cooking times were also determined on the unfrozen beans i.e. soaked in demineralized water and cooked in alkaline solution (NaHCO₃, 5 g/L) (AC) and soaked and cooked in demineralized water (DC), representing positive and negative control, respectively. During the cooking process, ten beans were removed every 30 min and classified as cooked when they easily disintegrated upon pressing between thumb and forefinger. The results were expressed as a percentage of the cooked beans (%) and modelled using a logistic model as described in the following equation (Equation 3.4):

\[ y = \frac{a}{1 + e^{b-kt}} \]

(Equation 3.4)

where \( y \) (%) is the percentage of cooked beans, \( t \) (min) is the cooking time, and \( a, b, \) and \( k \) are the kinetic parameters. The parameters were estimated by minimizing the residual sum of squares (RSS) using Solver. The model was used to determine the cooking time, defined as the time at which 9 out of 10 beans were cooked (i.e. \( y=90\% \), CT₉₀). The cooking time was determined in duplicate.

Texture analysis
An objective method for evaluating the hardness of the beans during cooking was performed on the same set sample as described in section Finger pressed method. During the cooking process, ten beans were withdrawn every 30 min and the hardness was measured on the dehulled seed using a texture analyzer (TA-X2i, Stable Micro Systems, England). Therefore, each seed was considered a replicate. The test was done using 50 kgf cell load equipped with a cylindrical probe with a diameter of 30 mm. The pre-test, test, and post-test speeds of the probe were 2, 1, and 2 m/s, respectively. The hardness was measured as the peak of maximum force (N) required to compress a cotyledon to 75% strain. The obtained data were modelled using a fractional model (Equation 3.5).

\[ F_t = F_{\infty} + (F_0 - F_{\infty}) \times e^{-kt} \]

(Equation 3.5)

where \( F_t \) (N) is the hardness at time \( t \), \( F_{\infty} \) (N) is the hardness at equilibrium or the nonzero maximum retainable hardness, \( F_0 \) (N) is the initial hardness at time
0, \( k \) (min\(^{-1}\)) is the rate constant, and \( t \) (min) is the cooking time. The model was used to determine cooking time, the time at which the beans reached a textural hardness of 42N [27].

### 3.2.6 Preparation of Jack bean flours and intact cells

Jack bean was frozen using the condition as described in section 3.2.3. The thawed beans were cooked in demineralized water at 97 °C as previously mentioned. The beans were cooked at different times based on the freezing treatments, which were 213, 166, 214, and 193 min for SF1, SF24, FF1, and FF24, respectively. Two additional samples were prepared from AC and DC with cooking times of 125 and 216 min, respectively. These cooking times were the optimal cooking times previously determined in Section 3.2.3. Finger pressed method. The cotyledons and seed coat were separated and freeze-dried. To generate flours, the dry samples were milled using a ball mill MM 400 (Retsch, Haan, Germany) at 30 Hz for 60 s followed by sieving through a 425 μm filter. The flours that passed through the filter were collected and stored at room temperature until further use for hemagglutination and mineral analysis. In addition, flours were prepared from the cotyledon of raw jack beans (dry beans) (as a control sample for hemagglutination analysis) and cotyledon and seed coat of raw beans (soaked beans) (as a control sample for mineral analysis).

Intact cells were isolated from cooked cotyledon samples SF24, AC, and DC. Briefly, the cooked cotyledons were mashed with mortar and pestle and wet-sieved. Particles between 71 and 125 μm filters were collected and flushed with demineralized water three times to remove free starch granules. This range was used to collect only individual cells without the presence of cluster cells. Intact cells were collected, added with sodium azide (0.02% in the final mixture), and stored at 5 °C until further use.

### 3.2.7 Hemagglutination activity

Hemagglutination activity was determined on the protein extract of raw and cooked jack bean flours (SF1, SF24, FF1, FF24, AC, DC). Phosphate saline buffer (PBS) (0.2M, pH 7.4) was used to suspend bean flours (0.2 g/mL). The mixtures were stirred (450 rpm) for 2 h at 4 °C followed by centrifugation of 4500g for 15 min. The supernatant was dialyzed against demineralized water at 4 °C for 20 h using dialysis tubes (MWCO 12-14 kDa). The extracted protein was stored at -20 °C until further use. Protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay kit (Thermo Fisher Scientific, Waltham, USA). Hemagglutination activity was performed according to a previous study.
[28]. In brief, erythrocyte 2% was prepared from erythrocyte stock (10%) (MyBioSource Inc., San Diego, USA) by diluting with PBS. Protein extracts were prepared in serial dilutions (2-fold). In duplicates, 25 μL of diluted protein extracts were placed in a 96-well round-bottom microplate, followed by the addition of 25 μL erythrocyte (2%) and incubation for 2h at room temperature. One hemagglutination unit (HU) is the minimal concentration of protein expressed in μg/mL that could agglutinate $10^6$ erythrocyte cells.

### 3.2.8 Mineral content

Mineral content was analyzed on the cotyledon and seed coat flours from cooked beans (SF1, SF24, FF1, FF24, AC, DC) and raw soaked beans. The ash determination method according to AOAC 923.03 [29] was used to determine the mineral content by using a muffle furnace at 550 °C overnight. Dry matter content was analyzed by drying a known amount of bean flour followed by oven incubation at 105 °C overnight. Mineral data were expressed as % dry weight.

### 3.2.9 Protein and starch digestibility

Static *in vitro* intestinal digestion was performed on intact cells of SF24, AC, and DC based on INFOGEST protocol as previously described [30]. For the oral phase, bean samples (5 g) were mixed with simulated salivary fluid (SSF) (with no salivary α-amylase), CaCl$_2$ and Milli-Q water to produce a final volume of 10 mL. Subsequently, the bolus was mixed with simulated gastric fluid (SGF) and the pH was adjusted to 3 with HCl. CaCl$_2$, Milli-Q water, and finally porcine pepsin enzymes were added to the mixture (enzyme concentration in the final mixture was 2000 U/mL) before incubation for 2 h at 37 °C. To stop the enzymatic reaction in the gastric phase, the pH was adjusted to 7 with NaOH. Gastric chyme was combined with simulated intestinal fluid (SIF), bile salt solution, CaCl$_2$, porcine trypsin, and pancreatic enzyme (100 U/ml trypsin activity and 200 U/mL amylase activity in the final mixture). The tubes were incubated for 2 h at 37 °C. Blank digestion was prepared by replacing the sample with Milli-Q water. All samples were run in triplicate. During the digestion phase, aliquots were collected at different times (time 0 and 120 min in the gastric phase and time 15, 30, 60, and 120 min in the intestinal phase). Absolute ethanol and Pefabloc were used to stop the amylase and protease activity, respectively, and the samples were stored at -20 °C until further analysis. The concentration of free amino groups was determined by ω-phthaldialdehyde (OPA) on the pefabloc-added samples as described in a previous study [31]. The degree of protein hydrolysis (DH%) was calculated based on the level of free amino groups in digested samples, undigested
samples, and completely hydrolyzed samples in 6 M HCl at 110 °C for 24 h [32]. To the ethanol-added supernatants, amylglucosidase treatment was applied to convert the amylase hydrolysis products into glucose [25] followed by D-glucose assay using GOPOD protocol (Megazyme Inc., Bray, Ireland). Starch hydrolysis was expressed as grams of hydrolyzed starch/100 g of dry starch.

### 3.2.10 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics v.26 (IBM, New York, USA). One-way analysis of variance (ANOVA) was used to analyze the effect of different freezing temperatures and freezing times on the cooking time as well as the mineral data. Mood’s median test was used to analyze the effect of different freeze-thaw conditions on the length of cell separation. A significance level was determined at the p <0.05.

### 3.3 Results

#### 3.3.1 Water absorption kinetics

![Figure 3.2 Water absorption profile of Jack beans during soaking](image)

Figure 3.2 shows the water absorption profile during the soaking of whole Jack beans and reflects the increase of moisture content during soaking times. The Jack bean initial moisture content was 14.4 (% dry weight). The seed absorbed a larger quantity of water during the initial stage of soaking, and the rate of water absorption decreased as the soaking time increased. The Peleg model was used to estimate the parameter kinetics, the \( k_1 \) and \( k_2 \) values were \( 0.57 \times 10^{-2} \) h.%\(^{-1}\) and \( 3.71 \times 10^{-2} \) %\(^{-1}\), respectively. The \( E \) value and \( M_E \) were 3.7%
and 190.2%, respectively. An $E$ value less than 10% implied a good fit of the Peleg model to model water absorption [24].

### 3.3.2 Freeze-thaw procedures change beans microstructure

Figure 3.3 displays representative microstructure images of Jack beans and the measured cell separation after freeze-thaw procedures compared to soaked beans as a control. Jack bean cells had an ovoid shape and no occurrence of cell wall damage was apparent in any of the images after freeze-thaw procedures. Changes in the microstructure after freeze-thaw were observed as cell separation indicating enlargement of intercellular spaces. SF showed the biggest changes in cell separation compared to other procedures. Meanwhile, FF procedures were likely to maintain the bean microstructure where small changes in the cell separation could only be detected after 24 h.

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Cells separation (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF1</td>
<td>3.6 ± 1.2^a</td>
</tr>
<tr>
<td>SF24</td>
<td>4.7 ± 2.3^a</td>
</tr>
<tr>
<td>FF1</td>
<td>0 ± 0^b</td>
</tr>
<tr>
<td>FF24</td>
<td>1.6 ± 0.8^b</td>
</tr>
<tr>
<td>NF</td>
<td>0 ± 0^c</td>
</tr>
</tbody>
</table>

**Figure 3.3** Representative micrographs and the extent of cell separation of Jack bean after freeze-thaw procedures (SF1, SF24, FF1, FF24) compared to soaked bean (NF). White and yellow arrows indicate intercellular spaces and cell contacts, respectively. SF1: slow freezing at -20 °C for 1 h, SF24: slow freezing at -20 °C for 24 h, FF1: fast freezing at -80 °C for 1 h, FF24: fast freezing at -80 °C 24 h. Cell separation presented as median and 95% Confidence Interval (N=100).
3.3.3 Freeze-thaw procedures reduce cooking time of the beans

Cooking times of Jack beans after different freeze-thaw procedures and soaked beans cooked with two different mediums are summarized in Table 3.1. Two different approaches of hardness evaluation were used (i.e. finger press method and texture analyzer): they provided comparable results. The cooking times of Jack beans after the freeze-thaw procedures were not significantly different compared to DC (p>0.05). Based on our observations, SF24 showed the biggest reduction effect on bean cooking time (by 23% and 17% measured by finger press and texture analyzer, respectively). The result indicates that the freeze-thaw procedure could affect the cooking times of Jack beans (p = 0.07 (Finger press) and p = 0.12 (Texture analyzer)). The alkaline cooking procedure remained the most effective in reducing cooking time, a golden standard to speed up the optimal cooking times in beans.

Table 3.1 Optimal cooking times of Jack bean after frozen and cooked in demineralized water (SF1, SF24, FF1, FF24), soaked and cooked in demineralized water (DC), and soaked and cooked in alkaline solution (AC) extrapolated from cooking profiles determined by finger press (CT90) and texture analyzer (CT42N)

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Finger Press (CT90)</th>
<th>Texture Analyzer (CT42N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF1</td>
<td>212.6 ± 6.5a</td>
<td>185.0 ± 22.6ab</td>
</tr>
<tr>
<td>SF24</td>
<td>166.1 ± 16.5ab</td>
<td>159.2 ± 35.1bc</td>
</tr>
<tr>
<td>FF1</td>
<td>214.2 ± 3.9a</td>
<td>198.8 ± 19.2a</td>
</tr>
<tr>
<td>FF24</td>
<td>192.8 ± 10.2a</td>
<td>187.5 ± 42.9ab</td>
</tr>
<tr>
<td>DC</td>
<td>215.9 ± 0.0a</td>
<td>191.8 ± 28.0ab</td>
</tr>
<tr>
<td>AC</td>
<td>124.2 ± 11.2b</td>
<td>129.7 ± 11.8c</td>
</tr>
</tbody>
</table>

SF1: slow freezing at -20 °C for 1 h, SF24: slow freezing at -20 °C for 24 h, FF1: fast freezing at -80 °C for 1 h, FF24: fast freezing at -80 °C 24 h. CT90: cooking time at which 9 out of 10 beans could be easily disintegrated; CT42N: cooking time at which the hardness level of cooked beans is 42N. Different letters indicate significant differences at p<0.05.

3.3.4 Hemagglutination activity and mineral content of frozen-thawed and cooked Jack beans

The levels of hemagglutination activity of the cotyledon and mineral content of cotyledon and seed coats of cooked Jack beans with and without freeze-thaw treatments are reported in Table 3.2. Hemagglutination activity was only present in raw cotyledon (0.0128 HU) and no hemagglutination activity was detected in all cooked beans. The mineral contents did not show a significant difference for both cotyledon and seed coat for all Jack beans cooked in demineralized water with/without freezing treatments (SF1, SF24, FF1, FF24).
Higher mineral content in cotyledon was found in alkaline cooked beans (AC) as compared to DC. All cooked Jack beans showed lower mineral contents for both cotyledon and seed coat in comparison to raw beans (3.1% for cotyledon and 5.4% for seed coat, respectively). No significant variation in the mineral content of all treatments compared to the raw bean, except for AC, was observed.

**Table 3.2** Hemagglutination activity and mineral content of Jack beans after freezing and cooking in demineralized water (SF1, SF24, FF1, FF24), soaking and cooking in demineralized water (DC), and soaking and freezing in alkaline solution (AC) as compared to raw bean (Mean and standard deviations, N=2)

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Hemagglutination activity (HU)</th>
<th>Mineral content (% dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cotyledon</td>
</tr>
<tr>
<td>SF1</td>
<td>N.H.</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>SF24</td>
<td>N.H.</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>FF1</td>
<td>N.H.</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>FF24</td>
<td>N.H.</td>
<td>1.9 ± 0.0</td>
</tr>
<tr>
<td>DC</td>
<td>N.H.</td>
<td>1.8 ± 0.0</td>
</tr>
<tr>
<td>AC</td>
<td></td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Raw bean</td>
<td>0.0128</td>
<td>3.1 ± 0.0</td>
</tr>
</tbody>
</table>

SF1: slow freezing at -20 °C for 1 h, SF24: slow freezing at -20 °C for 24 h, FF1: fast freezing at -80 °C for 1 h, FF24: fast freezing at -80 °C 24 h. HU: hemagglutination unit. N.H.: no hemagglutination was detected. Different letters indicate significant differences at p<0.05.

**3.3.5 In vitro protein and starch digestibility of frozen-thawed and cooked Jack beans**

Figure 3.4 displayed the protein digestibility of intact cotyledon cells of Jack beans after different treatments (SF24, AC, and DC). In general, reducing cooking times increases protein digestibility. We observed that despite different cooking times, SF24 and DC showed similar starch digestibility. In addition, AC showed a lower starch digestibility compared to SF24 and DC.
3.4 Discussion

Freeze-thawing has been known to alter the microstructure of plant cells [19,21,22], offering a potential solution to the extended cooking time in HTC beans. This is the first study that investigated the influence of freeze-thaw procedures on the microstructure of the Jack bean in relation to its cooking durations, as well as the implications of the procedures on the nutritional quality of the cooked beans. Since the outcome depends on the formation of ice crystals, it is essential to assess the kinetics of water absorption to achieve optimal water uptake prior to the freezing stage.
Jack beans exhibited a rapid initial water absorption with no appearance of a lag phase at the beginning of the soaking process (Figure 3.2). The initial rapid water absorption could be attributed to the filling up of capillaries located on the surface of the seed coat and the hilum [33]. As the soaking continued, the rate of water absorption slowed down, which was due to the increased extraction of soluble materials as well as the filling up of the free capillary and other intercellular spaces [34]. Compared to unsoaked beans, soaked beans provided adequate moisture to facilitate rapid starch gelatinization and protein denaturation, and pectin solubilization was the rate-limiting factor influencing cooking times [8]. For Jack bean, the water absorption rate began to decrease at time 15 h of soaking. To optimize water absorption while preventing fermentation [34] and avoiding extensive solid loss, we chose a soaking time of 24 h at the equilibrium phase to prepare optimum hydrated beans before freeze-thaw treatments. This hydration duration corresponds to 136.9 g water/100 g dry matter.

We observed that the freeze-thaw procedure increased cell separation, i.e. enlargement of intercellular spaces. We did not find any changes related to the cell shape nor damage in the cell wall on frozen-thawed Jack beans. Our finding is in line with a previous report on soybeans where no cell rupture was visible in cotyledon cells after freezing at -5°C for 4 days [35]. Cell separation can be a result of the formation of extracellular ice crystals that may separate the cells and solubilization of pectin induced by freezing. The formation of extracellular ice crystals could cause the expansion of intercellular spaces, weakening of intercellular adhesion [36].

Freezing conditions, such as the rates and durations, affected the severity of microstructure changes. In apple tissue, larger intercellular spaces were found after freezing at -20 °C while no difference in intercellular space was found after freezing at -80 °C compared to fresh samples [37]. This may explain the fact that the microstructure of the beans after freezing at -80 °C looks similar to that of the control beans. During prolonged freezing times, recrystallization tends to occur naturally when ice crystals change shape, increase in size, and decrease in number, due to the distribution of water from small to large ice crystals [20]. In the present study, increasing trends of cell separation were found with the longer freezing procedures, suggesting that the freezing duration may induce larger ice recrystallization in frozen-thawed beans.

Slow freezing for 24 hours (SF24) on soaked beans yielded the highest reduction in the optimal cooking times compared to other freeze-thaw procedures. Slow freezing in mango increased the level of water-soluble pectin.
fractions [22]. Moreover, electron micrographs showed that the slow freezing process in carrots caused a loosening of cellulose microfibrils [38]. During the hydrothermal process, pectin in the middle lamella will solubilize causing the cotyledon cells to separate upon mechanical disintegration [8]. It is possible that freezing may induce modifications in cell wall pectin in Jack beans allowing faster pectin solubilization and reducing cooking times. In the present study, beans cooked in alkaline solution (AC) have the shortest cooking time, indicating a fast pectin solubilization rate. It can be assumed that a combination of freeze-thaw and alkaline cooking may further enhance the rate of pectin solubilization during cooking. Cooking with sodium bicarbonate was reported to promote the rearrangement of side chains of rhamnogalacturonan-I and β-elimination reactions that degrade the backbone of the chelated pectin chains, and lead to degradation into short-chain molecules [39].

We compared the use of a finger press and texture analyzer to determine cooking times. At 42N hardness, the cooking times between these two methods follow the same trend. The results showed that both methods gave similar trends of cooking times, suggesting that the finger press method is reliable and that the two methods can be used interchangeably. A previous report used cutting force 11.16 N to discriminate between cooked and uncooked beans and plot the hardness level into %cooked beans. The converted profiles showed similar categorization as when the finger press method was used [9].

Reduction of cooking time is advantageous, yet it may have consequences on other nutritional (i.e. mineral loss and nutrients digestibility) or toxicological aspects (i.e. the inactivation of hemagglutination activity). Therefore, we assessed whether the strategies would not compromise Jack bean utilizations or may even enhance their nutritional qualities. We found that raw bean showed hemagglutination activity and no hemagglutination activity was observed in all treated samples, regardless of the different pretreatments and cooking methods employed (Table 3.2). This finding suggests that shorter cooking times used in this study can effectively eliminate the hemagglutination activity, improving the safety for consumption.

Table 3.2 shows that a significant mineral loss occurred in the cotyledon part where it is significantly correlated with cooking times (Table S3.2). We found no changes in the mineral content in the seed coat part after freezing and cooking treatments as compared to raw beans. This result was in agreement with a previous study showing that the changes in mineral content occurred in
the cotyledon and not the seed coat [40]. During cooking, minerals will diffuse from the cotyledon cell through the cell walls causing mineral leaching. The minerals can form chelates with compounds such as phytic acid and dietary fiber such as pectin [41]. The seed coat contains more galacturonic acid and free carboxyl groups than the cotyledon, whereas the cotyledon contains more phytate than the seed coat [42]. Seed coat pectin, is less sensitive to β-elimination, due to its lower degree of methoxylation compared to cotyledons, and that might explain the retained mineral content in the seed coat, i.e. more pectin is left in the seed coat that can chelate the minerals diffusing out of the cotyledon cells. In addition, cooking in alkaline solutions (AC) resulted in an increase in the mineral content in the seed coat compared to the raw beans. The use of bicarbonate solution may provide an additional source of minerals. The mineral binding capacity of phytic acid and pectin can be changed with the pH. It is possible that the pH of AC induce more dissociation of PA and pectin, causing mineral binding despite the leaching and solubilization of these molecules. Moreover, the effect of mineral binding is more pronounced in the seed coat, having more pectin with less susceptible to β-elimination, resulting in an increase of mineral content in AC in the present study.

We evaluated the protein and starch digestibility of three cooked Jack beans (SF24, AC, and DC) as depicted in Figure 3.4. There was a trend of reduction of optimal cooking times yielded a higher protein digestibility. At optimal cooking times, we could expect a similar degree of pectin solubilization and cell wall permeability [43]. More protein aggregation possibly occurred when the beans were cooked for prolonged times, causing a decrease in protein digestibility [44–46]. Hence, the observed decrease in protein digestibility with optimal cooking times may not depend on differences in cell wall permeability to proteases but on protein aggregation. It is likely that similar cell wall permeability may cause a similar diffusion rate of amylase and thus can be observed as a similar starch digestibility (SF24 and DC). In the case of AC, alkaline cooking conditions can induce pH changes in intracellular regions. The higher pH within the isolated cells may not be effectively neutralized after adjustment of pH in the in vitro digestion simulation. This higher pH “as sensed by amylase” might potentially affect its activity during the in vitro digestion.

In this study, we demonstrated that freeze-thaw procedures can reduce Jack bean cooking times. To the best of our knowledge, this is the first investigation on the use of freeze-thaw methods to shorten bean cooking durations. Freezing induces cell separation without any signs of cell rupture. These changes in microstructure appear to be the primary factors responsible for the reduction in Jack bean cooking times. It is important to note that the rates and
duration of freezing play crucial roles in altering the microstructure of Jack beans. However, it is not clear whether the microstructural changes can be directly linked to the changes in the pectin fractions. Further exploration may be performed as well on the effect of freeze-thaw treatments in the protein or starch structure in the cytoplasmic matrix. In summary, freeze-thawing can be used as a simple, practical alternative method to reduce Jack bean cooking time, which in turn may prevent loss of minerals without impacts on protein and starch digestibility but its application at industrial or domestic scale may depend on resource availability as well as sustainability considerations.

**CRediT authorship contribution statement**

FIametta Ayu Purwandari: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. Raehana Saria Gahari: Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. Vincenzo Fogliano: Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration. Edoardo Capuano: Conceptualization, Methodology, Visualization, Supervision, Writing – review & editing, Project administration, Resources.

**Declaration of competing interest**

The authors declare that they have no competing interests or personal relationships that could have appeared to influence the work reported in this study.

**Acknowledgement**

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References


Supporting information

<table>
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<tr>
<th>Procedures</th>
<th>Estimated parameters</th>
<th>RSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooking 1</td>
<td>k: 0.04 b: 6.63 a: 105.47</td>
<td>242.07</td>
</tr>
<tr>
<td>Cooking 2</td>
<td>k: 0.05 b: 8.70 a: 103.77</td>
<td>115.63</td>
</tr>
<tr>
<td>SF24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooking 1</td>
<td>k: 0.04 b: 5.56 a: 97.89</td>
<td>252.40</td>
</tr>
<tr>
<td>Cooking 2</td>
<td>k: 0.10 b: 13.27 a: 100.06</td>
<td>3.93</td>
</tr>
<tr>
<td>FF1</td>
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<td>Cooking 1</td>
<td>k: 0.07 b: 13.44 a: 100.33</td>
<td>127.33</td>
</tr>
<tr>
<td>Cooking 2</td>
<td>k: 0.05 b: 8.14 a: 104.52</td>
<td>498.34</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cooking 1</td>
<td>k: 0.07 b: 9.55 a: 98.83</td>
<td>143.01</td>
</tr>
<tr>
<td>Cooking 2</td>
<td>k: 0.06 b: 9.03 a: 99.09</td>
<td>139.82</td>
</tr>
<tr>
<td>DC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooking 1</td>
<td>k: 0.05 b: 8.57 a: 103.39</td>
<td>156.09</td>
</tr>
<tr>
<td>Cooking 2</td>
<td>k: 0.05 b: 8.57 a: 103.39</td>
<td>156.09</td>
</tr>
<tr>
<td>AC</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>245.44</td>
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<td>Cooking 2</td>
<td>k: 0.08 b: 8.41 a: 102.05</td>
<td>14.79</td>
</tr>
</tbody>
</table>

**Figure S3.1** Kinetic parameters and representative data and the corresponding fitted model of Jack bean (SF1) cooking profile estimated by finger press. Cooking experiments were done in duplicate for each procedure (Cooking 1 and Cooking 2).

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Estimated parameters</th>
<th>RSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF1</td>
<td>k: 0.010 b: 271.05 c: 0.00</td>
<td>916.7</td>
</tr>
<tr>
<td>SF24</td>
<td>0.012 b: 245.39 c: 8.26</td>
<td>1128.1</td>
</tr>
<tr>
<td>FF1</td>
<td>0.011 b: 276.03 c: 14.40</td>
<td>180.8</td>
</tr>
<tr>
<td>FF24</td>
<td>0.012 b: 282.85 c: 12.01</td>
<td>1226.6</td>
</tr>
<tr>
<td>DC</td>
<td>0.011 b: 256.38 c: 12.03</td>
<td>578.4</td>
</tr>
<tr>
<td>AC</td>
<td>0.014 b: 258.79 c: 0.00</td>
<td>1215.6</td>
</tr>
</tbody>
</table>

**Figure S3.2** Kinetic parameters and representative data (dot) and the corresponding fitted model (line) of Jack bean (SF1) cooking profile estimated by texture analyzer.
### Table S3.1 Mean and standard deviations of soluble protein concentration

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Protein concentration (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF1</td>
<td>135.3 ± 2.4</td>
</tr>
<tr>
<td>SF24</td>
<td>196.6 ± 4.8</td>
</tr>
<tr>
<td>FF1</td>
<td>169.3 ± 4.1</td>
</tr>
<tr>
<td>FF24</td>
<td>170.6 ± 11.7</td>
</tr>
<tr>
<td>DC</td>
<td>153.9 ± 0.0</td>
</tr>
<tr>
<td>AC</td>
<td>131.2 ± 5.7</td>
</tr>
<tr>
<td>Raw bean</td>
<td>837.5 ± 15.3</td>
</tr>
</tbody>
</table>

### Table S3.2 Pearson correlation of cooking times and mineral content

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cooking time determined by finger press</th>
<th>Cooking time determined by texture analyzer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral content (cotyledon)</td>
<td>-0.935*</td>
<td>-0.960*</td>
</tr>
</tbody>
</table>

*Asterisk symbol indicates a significant correlation at the 0.05 level (2-tailed)*
Freeze-thaw procedure as a method to speed up the cooking time of Jack bean
Chapter 4

Proximate composition, microstructure, and protein and starch digestibility of seven collections of Jack bean (Canavalia ensiformis) with different optimal cooking times

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Abstract

Because of its high protein content, Jack bean (*Canavalia ensiformis*) is a promising alternative protein source. However, the utilization of Jack bean is limited due to the long cooking time to achieve palatable softness. We hypothesize that the cooking time may influence protein and starch digestibility. In this study, we characterized seven Jack bean collections with different optimal cooking times in terms of their proximate composition, microstructure and protein and starch digestibility. Kidney bean was included as a reference for microstructure and protein and starch digestibility. Proximate composition showed that Jack bean collections have a protein content ranging from 28.8 to 39.3%, a starch content ranging from 31 to 41%, a fiber content from 15.4 to 24.6%, and a concanavalin A content in the range 35-51 mg/g dry cotyledon. Particles of size ranging between 125 and 250 µm were chosen as a representative sample of the whole bean to characterize microstructure and digestibility of the seven collections. Confocal laser microscopy (CLSM) revealed that Jack bean cells have an oval shape and contain starch granules embedded in a protein matrix similar to kidney bean cells. The diameter of Jack bean cells was measured by image analysis of CLSM micrographs and ranged from 103 to 123 µm, while the diameter of starch granules was 31-38 µm, comparatively larger than that of the kidney bean starch granules. Isolated intact cells were used to determine the starch and protein digestibility in the Jack beans collections. The digestion kinetics of starch followed a logistic model, whereas the digestion kinetics of protein followed a fractional conversion model. We found no correlation between optimal cooking time and kinetic parameters of protein and starch digestibility, implying that optimal cooking time is not predictive of protein and starch digestibility. In addition, we tested the effect of reduced cooking times on protein and starch digestibility on one Jack bean collection. The result showed that reducing cooking time significantly reduces starch digestibility, but not protein digestibility. The present study contributes to our understanding of the effect of food processing on protein and starch digestibility in legumes.

Keywords:

Intact cells, protein digestion, starch digestion, effect of food processing, optimal cooking times, cell wall barrier
4.1 Introduction

Legumes are considered sustainable staple foods since they generate less greenhouse gases than food from animal sources (Poore & Nemecek, 2018). Underutilized legumes, i.e. legumes that have not been used as a feeding crop on a global scale, are one of the primary strategies for improving global food security. This is because the legumes can be used not only as a support for food security challenges in developing countries but also as a sustainable protein source in the protein transition towards plant-based proteins (Padulosi et al., 2013). One such underutilized legume is Jack bean (*Canavalia ensiformis*). Jack bean is grown in tropical and subtropical regions in South America, West Africa, Asia and the South Pacific (Sharasia et al., 2017). The legume has a protein content of 23-35%, a carbohydrate content of 46-65%, and a fiber content of 5-11% (Sridhar & Seena, 2006). The main concern related to Jack bean human consumption is its content of a lectin named concanavalin A. Lectins have several physiological effects, such as agglutination of erythrocyte, binding of glycoprotein in the small intestine, and inhibition of digestive enzymes (Lajolo & Genovese, 2002). Concanavalin A can be denatured by heat treatment, and soaking before heating has been reported to improve its inactivation (Carlini & Udedibie, 1997).

During cooking of legumes, solubilization of pectin in the middle lamella occurs, to an extent depending on the cooking intensity, together with starch gelatinization and protein denaturation. This solubilization causes cell separation upon mechanical disintegration and is most likely to be the rate limiting factor for textural alteration rather than the gelatinization and protein denaturation (Chigwedere et al., 2018). The solubilization of pectin can be delayed due to the complex linkages between pectin molecules and other cell wall components in the middle lamella.

Furthermore, changes in the cell wall may have nutritional significance because the cell wall is made up of indigestible carbohydrates. In intact cells, the cell wall can reduce nutrient digestibility by acting as a barrier to enzyme diffusion. It has been reported that the cell wall delays the access of the enzyme to the substrate, thus influencing protein and starch digestibility (Dhital et al., 2016; Rovalino-Córdova et al., 2018). Different intensities of cooking influence cell wall permeability. In case of Canadian wonder beans and Bambara groundnuts, different levels of cell wall permeability alter the rate of enzyme diffusion and influence starch digestibility (Gwala et al., 2020; Pallares Pallares et al., 2018). In soybean, cooking process intensities significantly affect protein digestibility, but the magnitude of the effect is modest (Zahir et
It is also important to mention that, in legume cotyledon cells, starch granules are embedded in the protein matrix in the densely packed cytoplasm, which may further limit α-amylase diffusion to starch (Rovalino-Córdova et al., 2019). This additional barrier effect exerted by the protein matrix can be modulated by cooking time through the extent of protein denaturation and protein conformation (Carbonaro et al., 2012; Rovalino-Córdova et al., 2019).

To the best of our knowledge, whether differences in cooking times may affect the digestibility of starch and protein has not been studied for Jack beans. In this study, we, therefore, aimed to investigate the influence of optimal cooking time on in vitro protein and starch digestibility using seven Jack bean collections. We collected Jack bean samples with a wide range of cooking times from different collections. We characterized the proximate composition, starch, fiber, and concanavalin A of the raw material and observed the corresponding microstructure at optimal cooking time. Furthermore, different cooking intensities were applied to one of the collections to investigate further the effect of heat treatment on protein and starch digestibility.

### 4.2 Materials and Methods

#### 4.2.1 Materials

Jack bean (*Canavalia ensiformis*) collections were harvested from seven locations in Indonesia: Banyuwangi, Kebumen, Wonogiri, Malang, Yogyakarta, Lampung, and Temanggung. Kidney bean was purchased from a local supermarket (Jumbo, the Netherlands). On arrival at the laboratory, Jack beans were sorted to remove any damaged seeds. Some of the beans were immediately analyzed for their cooking time (section 2.4), and the rest were stored at -80 °C until further use. Pepsin from porcine gastric mucosa (4205 U/mg), trypsin from porcine pancreas (309 U/mg), pancreatin from porcine pancreas (2.5 U/mg trypsin activity; 41 U/mg amylase activity) were purchased from Sigma-Aldrich (Missouri, USA) and amyloglucosidase (3300 U/mL) was supplied by Megazyme Inc. (Bray, Ireland). All other chemicals were analytical grade unless stated otherwise.

#### 4.2.2 Production of Jack bean cotyledon powder

Raw Jack beans were manually dehulled, freeze-dried, and ground using a ball mill (MM 400, Retsch) for 60 s at a frequency 1/30 s. The powder was sieved using 425 µm to remove big particles. The big particles were milled again at the same condition as previously described. The powder that passed through the sieve was collected and stored at room temperature until further analysis.
4.2.3 Proximate composition, starch, fiber, and concanavalin A

Proximate composition was analyzed on cotyledon powder. Nitrogen was quantified with the Dumas combustion method (FlashEA 1112NC, Thermo Scientific). The protein content was calculated by multiplying the nitrogen value by the conversion factor 6.25. We selected this conversion factor to be able to compare with other studies. Different conversion factors have been reported for legumes (Mariotti et al., 2008). Our data is likely an overestimation of the protein content, which would require a specific factor for Jack bean to calculate an accurate value. Lipid content was determined with the Soxhlet method by using petroleum ether as a solvent. Ash content was analyzed by incinerating the sample at 550 °C for 16 h following an AOAC 923.03 method. Carbohydrate content was calculated by difference, i.e. the residual weight after subtracting the amount of protein, fat, and ash. Moisture content was determined after drying a known amount of samples overnight in an oven at 105 °C to a constant weight. Starch was determined on cotyledon powder according to the protocol of the Total Starch Assay (amyloglucosidase/α-amylase method) (Megazyme Inc., Bray, Ireland). Fiber is calculated by subtracting starch from carbohydrate content. All measurements were performed in duplicate and presented on dry weight.

Concanavalin A content was analyzed on the protein extract of the cotyledon powder by using an ELISA kit from CloudClone (SEC179Ge, Texas, USA). The protein was extracted by using the manufacturer’s standard protocol with modification. Cotyledon powder was mixed with 0.02 M phosphate buffer saline (pH 7.3) (1:8 w/v) under shaking condition (1000 rpm at 25 °C) for 1.5 h. The mixture was centrifuged at 10500g for 10 min, and the supernatant was collected and stored at -20 °C. Additionally, cooked Jack bean powder from the Malang collection was extracted for protein using the same method previously described. The bean was cooked following the method in section 4.2.5. After dehulling, the bean was freeze-dried, milled, and sieved as described in section 4.2.2. The assay was performed according to the protocol in triplicate.

4.2.4 Determination of the optimal cooking time

Finger pressing was used to measure cooking time where the cooking degree is presented as % cooked bean seeds, following the protocol described in a previous study (Kinyanjui et al., 2015; Wahome et al., 2023). One hundred Jack bean seeds were soaked in demineralized water (1:3 w/v) for 16 h at 25 °C and subjected to a thermal treatment at 97 °C. During this process, 10 beans were taken out every 30 minutes and were categorized as “cooked” once they easily disintegrated when pinched between a thumb and forefinger. The cooked
degree was calculated as the ratio of the number of easily pressed beans with
the number of beans taken in each sampling. The cooking profile was
expressed as the percentage of cooked beans at different cooking times and
was modelled by using a logistic model with three kinetic parameters
(Equation 4.1).

\[ y = \frac{a}{1 + e^{(b-kt)}} \]

(Equation 4.1)

\( y \) is the value of cooked beans (%), \( t \) is the cooking time (min), and \( a, b \) and \( k \)
are the kinetic parameters. Minimization of residual sum of squares (RSS) was
used for the estimation of the parameters. The model was used to extrapolate
the optimal cooking time, defined as the time at which 90% beans were cooked
(CT\(_{90}\)). The determination of the optimal cooking time was performed in
duplicate for each of the collections.

### 4.2.5 Isolation of cotyledon cells

Isolation of intact cotyledon cells was carried out as described in a previous
study (Rovalino-Côrdova et al., 2018) with modifications. In brief, 30 g beans
were soaked and cooked under the same condition as previously described in
section 2.4. The shortest cooking time that gave 100% of cooked beans (CT\(_{100}\))
was used instead of CT\(_{90}\) to ensure cell separation and prevent the collection
of residual cell clusters or the rupture of the cells during mechanical grinding.
Banyuwangi, Kebumen, and Wonogiri collections were cooked for 180
minutes, Malang, Yogyakarta, and Lampung for 210 minutes, and Temanggung
for 270 minutes. We included kidney bean as a reference bean for
microstructure and digestibility analysis. Kidney beans were boiled for 45
minutes; this cooking time was previously evaluated using the same cooking
time determination method as Jack bean (data not shown). All cooked beans
were dehulled, and the seed coats were discarded. The cotyledons were
mashed with mortar and pestle and then wet-sieved and the particles with size
ranging between 125-250 µm were selected for further characterization. This
range was selected because it gave the highest yield while mostly containing
individual cells, therefore representing an homogeneous sample of the whole
beans. Particles that remained at 125 µm were flushed with demineralized
water three times to remove free starch granules. The cotyledon cells were
collected and stored at 5 °C after adding sodium azide with a concentration of
0.02% in the final mixture.
We randomly selected one Jack bean collection to eliminate the compositional differences caused by different collections. Additional cotyledon cells were isolated from the Malang collection after processing the collection at two different cooking times (90 and 135 minutes). Therefore, isolated cells from the Malang collection were generated from three different cooking times. The selection of cooking times represents different % cooked beans (see Figure 4.1). The higher the % cooked beans, the lower the hardness of the cooked beans (Wainaina et al., 2021). This means that samples at different cooking times and % cooked beans would have different hardness levels. As a result, we could compare, after in vitro digestion, the digestion kinetics of the same collection at three different hardness levels.

![Figure 4.1](image)

**Figure 4.1** Cooking profile (panel a) and CT$_{90}$ (panel b) of the seven Jack bean collections. Data expressed as mean ± standard deviation from two replicates. CT$_{90}$: estimated time to achieve 90% cooked beans.

### 4.2.6 In vitro digestion

*In vitro* digestion was performed in duplicate according to the harmonized INFOGEST protocol with modifications (Brodkorb et al., 2019; Zahir et al., 2018). In the oral phase, 5 g cotyledon cells of Jack beans and kidney bean were added with simulated salivary fluid (SSF) without salivary α-amylase. In the gastric phase, simulated gastric fluid (SGF) was added to the previous mixture and adjusted to pH 3 with HCl. CaCl$_2$ was added to the mixture, and finally porcine pepsin (2000 U/mL enzyme concentration in the final mixture). The tubes were incubated at 37 °C for 2 h. Aliquots were taken at digestion times (t) 0 and 120 minutes during the gastric phase. Absolute ethanol and Pefabloc (0.1 M) were added immediately into the aliquots to stop the activity of amylase and proteases, respectively. The ratio to sample was 4:1 and 1:20 for absolute ethanol and Pefabloc. At the end of the gastric phase, the pH was
adjusted to pH 7 with NaOH to stop the enzymatic reaction. For the intestinal phase, gastric chyme was combined with simulated intestinal fluids (SIF), bile solution, CaCl₂, porcine trypsin, and pancreatic enzyme (100 U/mL of trypsin activity and 200 U/mL of amylase activity in the final mixture). The tubes were incubated at 37 °C for 2 h. Aliquots were taken at 15, 30, 60, 120 minutes during intestinal phase. The t=120 minutes for the gastric phase was used as t=0 for the intestinal phase. Absolute ethanol and Pefabloc were added, as above mentioned. Incubation in all of the phases were performed under mixing conditions using rotatory equipment at 20 rpm. A blank digestion was prepared by using Milli-Q water instead of cotyledon cells and was run under the same digestion condition. All aliquots were centrifuged at 20000g for 10 minutes, and the ethanolic and pefabloc-added supernatants were collected and stored at -20 °C until use for the digestible starch and digestible protein analysis.

**Determination of in vitro protein digestibility**

Free amino groups released after *in vitro* digestion were measured using a spectrophotometric assay as described in a previous study (Zahir et al., 2018). The degree of protein hydrolysis (DH%) was calculated using Equation 4.2 as follows:

\[
DH\% = \frac{NH_{2final} - NH_{2initial}}{NH_{2acid} - NH_{2initial}} \times 100
\]  

*(Equation 4.2)*

where \(NH_{2final}\) is the free amino groups content in the hydrolysate of the digested sample, \(NH_{2initial}\) is the free amino groups content in the hydrolysate of the undigested sample (at time 0 of gastric phase), \(NH_{2acid}\) is the free amino groups content after hydrolysis of sample with 6N HCl at 110 °C for 24 h.

Data from the protein hydrolysis were modelled by using a fractional conversion model (Gwala et al., 2020) from duplicate data (Equation 4.3).

\[
Protein_t = Protein_f + (Protein_i - Protein_f) \times e^{-kt}
\]  

*(Equation 4.3)*

where \(Protein_t\) (%) is the DH% at time t of digestion, \(Protein_f\) (%) is the estimated plateau at extended digestion times, and \(Protein_i\) (%) is the value of DH% at the beginning of the small intestinal phase. \(k\) represents the reaction rate constant (min⁻¹). Residuals and RSS were used to assess the goodness-of-fit of the model.
**Determination of in vitro starch digestibility**

Ethanolic supernatants were subjected to amyloglucosidase treatment to convert products of amylase hydrolysis into glucose (Rovalino-Córdova et al., 2019). D-glucose assay procedure was performed to quantify the amount of glucose at digestion time (0 and 120 minutes during gastric phase, 15, 30, 60, and 120 minutes during intestinal phase) using a protocol of GOPOD FORMAT K-GLUC 09.14 (Megazyme Inc., Bray, Ireland). A conversion factor of 0.9 was used to convert glucose to starch. The starch hydrolysis was presented as grams of hydrolyzed starch/100 g of dry starch.

Data from the starch hydrolysis were modelled by using a logistic model (Equation 4.4) as reported in several studies (Gwala et al., 2020; Pallares Pallares et al., 2018) from duplicate data.

\[
\text{Starch}_t = \frac{\text{Starch}_f}{1 + e^{4 \times k_{max}(\lambda - t) + 2}}
\]  

(Equation 4.4)

where \(\text{Starch}_t\) (%) is the digested starch at time \(t\), \(\text{Starch}_f\) (%) is the plateau of the digested starch, \(k_{max}\) (%starch/min) is the rate constant, and \(\lambda\) (min) is lag phase time. Residuals and RSS were used to assess the goodness-of-fit of the model.

**4.2.7 Microscopy analysis**

Intact cells of seven Jack bean collections and kidney bean, as a reference, were visualized using confocal laser scanning microscope (LSM-510, Zeiss). Cell walls were stained with 0.1% calcofluor white, and protein bodies were visualized with 0.0001% rhodamine B. Staining was performed overnight to ensure the complete penetration of dyes on the cell wall and protein, respectively. Microscope setting with 405 nm blue/violet laser for calcofluor white and 543 nm HeNe laser for rhodamine B were applied. All images were taken at objective 20x EC Plan-Neofluar (0.50) and processed with Zen blue software v.2.3 edition (Carl Zeiss, Oberkochen, Germany).

Images were processed using ImageJ (Fiji) v.1.52 Fuji software. All images were converted into 8-bit binary before adjusting the threshold. The “invert” and “fill hole” tools were applied to the images to fill the area of starch granules and enabled the selection of cells along the cell’s perimeter. Additionally, the “watershed” tool was used if the cells were clustered. The cell area was selected using the wand tool and was measured. Cell diameter (\(D_{cell}\)) was calculated from the cell area, assuming the cells were round. For starch area
measurement, we eliminated the cell wall by inactivating the calcofluor white panel and used only the rhodamine B panel, which contained starch granules. The images were converted into 8-bit binary, and the threshold was adjusted. The watershed tool was then used to separate the overlap area of the starch granules. The area of the starch granule was selected and measured. Similar to cell diameter, starch granule diameter ($D_{\text{starch}}$) was calculated assuming the granule was round. When selecting the area of starch granule, the processed image was compared with the raw image to ensure that the selected area conforms to a single starch granule. For each Jack bean collection, 100 measurements of $D_{\text{cell}}$ and $D_{\text{starch}}$ were collected.

### 4.2.8 Particle size distribution of the isolated intact cells samples

Standard percentile ($D_{V10}, D_{V50}, D_{V90}$) and mean diameter of cells ($D_{3,2}, D_{4,3}$) were determined using a Mastersizer 3000 laser diffraction particle size analyzer with a hydro HV dispersion system (Malvern Panalytical Ltd., Malvern, UK). Intact cells were suspended in deionized water (1 g/ml). The particle refractive index was set at 1.46 for legume cells (Edwards et al., 2020). Dispersant refractive index and particle absorption index were set at 1.33, and 0.01, respectively. All measurements were performed within 12-14% obscuration range.

### 4.2.9 Statistical analysis

Kinetics parameters were estimated using the Solver add-in in Microsoft Excel by minimizing the residual sum of squares. Analysis of variance was used to compare the nutritional properties and kinetic parameters of protein and starch digestion across collections. Pearson correlations were used to examine the correlations between kinetic parameters of protein and starch digestion and cooking time ($CT_{90}$), geometrical properties ($D_{4,3}, D_{\text{cell}},$ and $D_{\text{starch}}$) and proximate composition as well as between protein and starch digestion. Both the variance and correlation analysis were run using GraphPad Prism 9. The level of significance was set at $p<0.05$.

### 4.3 Results

#### 4.3.1 Cooking profile, proximate composition, starch, fiber, and concanavalin A of seven Jack bean collections with different cooking times

Figure 4.1a shows the cooking profiles of the seven Jack bean collections. It can be seen that the kinetics of the cooking profile follow the logistic model
The Jack bean collections exhibited different optimal cooking times ranging from 137 to 254 minutes (Figure 4.1b).

Table 4.1 shows the proximate composition, starch, and concanavalin A of the seven Jack bean collections. Jack bean protein content ranged from 28.8 to 39.3% of the dry weight. The highest protein content was found in the Kebumen collection. Carbohydrates ranged from 53.8 to 63.1% of the dry weight, with a starch content ranging from 31 to 41% of the dry weight. The collections contain a significant amount of dietary fiber, with an average of 21.5%. The content of concanavalin A varied between 35 and 51 mg/g dry cotyledon.
<table>
<thead>
<tr>
<th>Collections</th>
<th>Protein (%db)</th>
<th>Fat (%db)</th>
<th>Ash (%db)</th>
<th>Carbohydrate (%db)</th>
<th>Starch (%db)</th>
<th>Fiber (%db)</th>
<th>Concanavalin A (mg/g dry cotyledon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banyuwangi</td>
<td>36.8±0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>56.1±0.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>32.0±0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.0±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.5±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kebumen</td>
<td>39.3±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53.8±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.0±1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.7±0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>43.0±4.6&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wonogiri</td>
<td>28.8±0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.9±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3±0.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>63.1±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.5±1.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>24.6±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.1±4.9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malang</td>
<td>36.5±0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.4±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0±0.1&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>56.1±0.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>39.3±1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.8±2.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>43.5±3.5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Yogyakarta</td>
<td>35.6±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.3±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.2±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.0±0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.2±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.9±4.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lampung</td>
<td>35.8±0.1&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>4.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>57.5±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.7±0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>22.8±0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>44.9±0.9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Temanggung</td>
<td>35.6±0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.4±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.3±0.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>40.9±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.4±1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.8±2.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscript letters within the same column indicate significant differences (p<0.05). dry basis (db). Data expressed as mean ± standard deviation from two replicates for protein, fat, ash, carbohydrate, starch, and fiber and three replicates for concanavalin A.
4.3.2 Microstructure characteristics of seven Jack beans collections

An overview of Jack bean and kidney bean cotyledon cells is shown in Figure 4.2. Jack bean intact cotyledon cells have an oval shape similar to kidney bean cells. Jack bean has big starch granules embedded in the protein matrix and encapsulated by the cell walls. Mostly single cells and a limited amount of small cluster cells with two or three cells (yellow arrow in Figure 4.2) were observed on samples obtained by mashing and sieving with 125-250 µm filter.

Figure 4.2 Representative confocal laser microscopy images of cooked Jack bean (1-7) and Kidney bean cells (8). Proteins are displayed in red, cell walls are displayed in light blue. Banyuwangi (1), Kebumen (2), Wonogiri (3), Malang (4), Yogyakarta (5), Lampung (6), Temanggung (7), Kidney bean (8). Yellow arrows indicated cluster of cells.
Table 4.2 shows the diameters of cell and starch granules of the particles of size ranging between 125 and 250 µm of the seven Jack bean collections measured by particle analyzer and image analysis. Jack bean collections show different cell diameters as measured by either a particle analyzer (D_{4.3}) or calculated from the images (D_{cell}). We observed a lower diameter value based on the microscopy (103-123 µm) compared to the particle analyzer (123-165 µm). For starch granules, similar starch granule diameters (D_{starch}) were observed in the seven Jack bean collections (31-38 µm). It is clear that Jack beans have a bigger starch granule diameter than kidney bean (29 µm).

**Table 4.2** Standard percentile (D_{V10}, D_{V50}, D_{V90}) and mean diameter of cell (D_{3.2}, D_{4.3}, D_{cell}) and starch granule (D_{starch}) of seven Jack bean collections and one kidney bean collection

<table>
<thead>
<tr>
<th>Collections</th>
<th>Particle size analyzer</th>
<th></th>
<th>Image analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DV_{10} (µm)</td>
<td>DV_{50} (µm)</td>
<td>DV_{90} (µm)</td>
</tr>
<tr>
<td>Banyuwangi</td>
<td>92^d</td>
<td>138^d</td>
<td>209^d</td>
</tr>
<tr>
<td>Kebumen</td>
<td>79^f</td>
<td>119^f</td>
<td>177^f</td>
</tr>
<tr>
<td>Wonogiri</td>
<td>90^e</td>
<td>138^d</td>
<td>211^d</td>
</tr>
<tr>
<td>Malang</td>
<td>101^b</td>
<td>156^a</td>
<td>241^a</td>
</tr>
<tr>
<td>Yogyakarta</td>
<td>80^f</td>
<td>124^e</td>
<td>190^e</td>
</tr>
<tr>
<td>Lampung</td>
<td>102^ab</td>
<td>154^ab</td>
<td>234^b</td>
</tr>
<tr>
<td>Temanggung</td>
<td>103^a</td>
<td>153^b</td>
<td>231^b</td>
</tr>
<tr>
<td>Kidney bean</td>
<td>97^c</td>
<td>147^c</td>
<td>226^c</td>
</tr>
</tbody>
</table>

Values with different superscript letters in the same column are significantly different (p<0.05). \(D_{V10}:\) the size of particle below which 10% of the particles are found, \(D_{V50}:\) median of the volume distribution, \(D_{V90}:\) the size of particle below which 90% of the particles are found, \(D_{3.2}:\) the surface weighted mean, \(D_{4.3}:\) the volume weighted mean, \(D_{cell}:\) diameter of cell measured by image analysis, \(D_{starch}:\) diameter of starch granule measured by image analysis.

### 3.3 In vitro protein and starch digestion kinetics of Jack bean collections with different optimal cooking time

The kinetic parameters of starch and protein digestion are presented in Table 4.3. A logistic model was used to model the starch digestion in intact cotyledon cells as described in previous reports (Pallares Pallares et al., 2018; Rovalino-Córdova et al., 2019). The model has three kinetic parameters: a lag phase at the initial phase of digestion (\(\lambda\)), rate constant (\(k_{max}\)) and digested starch at the final intestinal phase (\(Starch_f\)). Jack bean starch was digested slowly at the initial phase of digestion, as shown by the \(\lambda\) values, ranging from 14.0 to 34.9 min. The lag phase was followed by a rapid increase in starch digestion and reached final values ranging from 38.1 to 63.9%. Compared to kidney bean, Jack bean collections showed higher starch digestion rate, which resulted in a
higher final starch digestion ($Starch_f$). For protein digestibility, we observed differences in initial protein digestion ($Protein_i$), rate constant ($k$), and final digested protein ($Protein_f$) during the intestinal phase among the seven Jack bean collections. We observed a low % digested protein at the beginning of the intestinal phase of digestion, showing a limited ability of pepsin to digest and generate free α-amino groups during the gastric phase. During intestinal digestion, the amount of digested protein increased, and Jack bean showed a % digested protein ranging from 21.6 to 30.8% at the end of the intestinal phase. In general, the final % digested protein in Jack beans was, on average (25.2%), slightly lower than kidney bean (31%).

Pearson correlation coefficients between kinetic parameters of starch and protein digestibility and CT$_{90}$ are summarized in Table 4.4. No significant correlation was found between CT$_{90}$ and any of the kinetic parameters of starch and protein digestibility, meaning that the effect of optimal cooking times on starch and protein digestion is limited. Moreover, we examined the correlation between kinetic parameters of protein and starch digestion and diameter of cells, diameter of starch granules, and proximate composition (Table 4.4). The results showed a limited effect of the cells and starch granules size on starch and protein digestibility, perhaps due to the narrow variability of the geometrical properties of the seven Jack bean collections used in this study. We did find a significant positive correlation between carbohydrate content and starch digestion rate constant. A negative correlation at $p<0.1$ was observed between protein content and starch digestion rate constant ($k_{max}$) ($p=0.057$) as well as final digested protein ($Protein_f$) ($p=0.062$).
Table 4.3 Kinetic parameters of *in vitro* protein and starch digestion of cells isolated from seven Jack bean collections and one kidney bean collection

<table>
<thead>
<tr>
<th>Collections</th>
<th>Protein digestibility</th>
<th>Starch digestibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein&lt;sub&gt;i&lt;/sub&gt; (%)</td>
<td>k (protein%/min)</td>
</tr>
<tr>
<td>Banyuwangi</td>
<td>2.1±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.036±0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kebumen</td>
<td>1.8±1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.044±0.009&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wonogiri</td>
<td>2.3±0.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.022±0.004&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malang</td>
<td>0.8±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.017±0.002&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Yogyakarta</td>
<td>1.2±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.020±0.002&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lampung</td>
<td>1.1±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.015±0.003&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Temanggung</td>
<td>2.5±0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.035±0.004&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney bean</td>
<td>1.0±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.015±0.000&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Protein and starch digestibility from two replicates data are fitted to fractional conversion model and logistic model, respectively. Protein<sub>i</sub>: digested protein at the beginning of the intestinal phase; k: protein digestion rate constant; Protein<sub>f</sub>: digested protein at end of the intestinal phase; λ: digested starch at beginning of the intestinal phase; k<sub>max</sub>: starch digestion rate constant; Starch<sub>f</sub>: digested starch at the end of the intestinal phase. Values with different superscript letters in the same column indicate significant differences (p<0.05).
Table 4.4 Correlation matrix of starch/protein digestibility and cooking time (CT90), geometrical properties (D4,3, DCell, Dstarch) protein, fat, ash, carbohydrate, starch, fiber, and concanavalin A

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>CT90</th>
<th>D4,3</th>
<th>Dcell</th>
<th>Dstarch</th>
<th>Protein</th>
<th>Fat</th>
<th>Ash</th>
<th>Carbohydrate</th>
<th>Starch</th>
<th>Fiber</th>
<th>Concanavalin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ</td>
<td>0.61</td>
<td>0.67</td>
<td>0.44</td>
<td>0.37</td>
<td>0.22</td>
<td>-0.08</td>
<td>-0.16</td>
<td>-0.21</td>
<td>0.30</td>
<td>-0.47</td>
<td>-0.45</td>
</tr>
<tr>
<td>kmax</td>
<td>-0.28</td>
<td>0.25</td>
<td>-0.58</td>
<td>-0.21</td>
<td>-0.74</td>
<td>0.61</td>
<td>-0.35</td>
<td>0.78*</td>
<td>0.17</td>
<td>0.42</td>
<td>0.54</td>
</tr>
<tr>
<td>Starchf</td>
<td>0.45</td>
<td>0.30</td>
<td>-0.24</td>
<td>0.11</td>
<td>-0.52</td>
<td>0.07</td>
<td>0.51</td>
<td>0.49</td>
<td>0.61</td>
<td>-0.25</td>
<td>-0.18</td>
</tr>
<tr>
<td>Proteinf</td>
<td>0.05</td>
<td>-0.15</td>
<td>0.39</td>
<td>-0.26</td>
<td>-0.34</td>
<td>0.42</td>
<td>0.27</td>
<td>0.24</td>
<td>0.17</td>
<td>0.01</td>
<td>-0.28</td>
</tr>
<tr>
<td>k</td>
<td>-0.04</td>
<td>-0.49</td>
<td>0.59</td>
<td>-0.14</td>
<td>0.44</td>
<td>-0.15</td>
<td>0.21</td>
<td>-0.51</td>
<td>-0.35</td>
<td>-0.04</td>
<td>-0.18</td>
</tr>
<tr>
<td>Proteinf</td>
<td>-0.26</td>
<td>0.23</td>
<td>-0.38</td>
<td>0.21</td>
<td>-0.73</td>
<td>0.76*</td>
<td>-0.04</td>
<td>0.68</td>
<td>0.59</td>
<td>-0.09</td>
<td>-0.17</td>
</tr>
</tbody>
</table>

Asterisk symbol indicates a significant correlation p<0.05 (2-tailed).
4.3.3 Effect of different cooking time on protein and starch digestion in one Jack bean collection

Starch and protein digestibility of the Malang collection cooked at different times are presented in Figure 4.3. The processing intensity clearly showed an influence on starch digestion. We found a significant increase in starch digestibility by prolonging the cooking time (Table S4.1). On the contrary, a limited effect of cooking time was observed on protein digestibility. We can observe that increasing processing intensity reduced the initial protein digestion from 1.35 to 0.78%, with no significant effect on the rate constant and the final digested protein (Table S4.1).

![Figure 4.3 Protein and starch digestibility of isolated intact cells of Jack bean cooked at different times. Malang collection was used to generate intact cells.](image-url)
4.4 Discussion

This study reports the variability of nutritional parameters of seven Jack bean collections having different optimal cooking times (Figure 4.1). The Jack bean collections have a protein content ranging from 28.8 to 39.3% (Table 4.1), showing the nutritional potential of Jack bean as a source of proteins. In other studies, the protein content in whole Jack bean was reported to be 26.8% (Agbede & Aletor, 2005) and 31.9% (Vadivel & Janardhanan, 2001). The difference in the protein content is probably due to the beans analyzed here were dehulled prior to analysis. The Jack bean hull contains three times less protein than in the cotyledon (Oliveira et al., 1999). Concanavalin A content in our collections ranged from 35 to 51 mg/g dry cotyledon. This value is higher than previously reported (15-35 mg/g dry weight) (Sridhar & Seena, 2006). Once cooked, the coagulation activity can be partially lost due to denaturation (Udedibie & Carlini, 1998). We found a lower amount of concanavalin A in optimally cooked Malang collection (9.5 mg concanavalin A/g dry cotyledon) than raw bean (43.5 mg concanavalin A/g dry cotyledon), which suggests that the lectin was modified during the heat treatment process. Longer cooking times may be beneficial for removing all hemagglutination activity, and the activity of this residual concanavalin A should be considered when strategies to reduce the cooking time of Jack beans are implemented.

We show that image processing can be useful as an alternative method to measure some microstructure characteristics of plant material, especially when comparing samples. This is the first time that this approach is used and the first time that cell size of Jack beans is determined. However, we found that the cell size measured by image analysis is lower than the cell size measured by particle analyzer (either D₄,₃ and D₃,₂ values) (Table 4.2; Figure S4.2). This can be explained by (1) cell clusters that may have been measured by particle analyzer and not by image analysis; (2) the fact that with the 2D images, we cannot always measure the diameter of the cells in correspondence to the center of the cells. Compared to the particle analyzer, image analysis offers the flexibility to select and analyze more than one parameter of interest in one-time preparation while at the same time being able to differentiate single cells over cell clusters. This approach should be further optimized, for instance, by evaluating the sensitivity of other microstructure parameters (such as Feret’s diameter or perimeter) or using 3D scanning images to locate and measure only the center of the cells.

The starch granule size of kidney beans determined by image analysis (Table 2) falls in the range of the starch granule size determined by laser diffraction
in another research (Romero & Zhang, 2019). The authors reported that the size distribution and mean diameter (D₄,₃) of kidney bean starch granules measured by particle analyzer are 10-55 µm and 27 µm, respectively. In our study, the mean diameter of kidney bean was 29 µm. The cell size of kidney bean in this study (112 µm) is slightly bigger than the value tentatively estimated in a previous study (100 µm) (Rovalino-Córdova et al., 2018), which can be due to the different sieve range used in our study.

To study the in vitro starch and protein digestibility, we used isolated intact cells for three reasons: (1) to appreciate the barrier effect of the cell wall that would have been lost if flour was used, (2) to uniform the legume particle size since it has been shown to affect macronutrients digestibility in intact plant materials (Zahir et al., 2018), and (3) intact cell is the most important microstructural fractions to represent the digestive behavior of the whole seeds cooked at optimal cooking times (Duijssens et al., 2023). Overall protein digestibility varied across collections, as seen from all kinetic parameters (Table 4.3). The DH% at the beginning of the intestinal phase (Proteinᵢ) suggests that the pepsin can penetrate the isolated cells of Jack beans and kidney bean during the gastric phase. At the end of the intestinal phase, the digested protein (Proteinᵢ) ranged from 21.6-30.8% showing that Jack bean collections had lower protein digestibility than kidney bean, which may affect the overall quality of Jack bean compared to kidney bean. We found no correlation between optimal cooking times and all protein digestibility kinetic parameters (Table 4.4). This implies that optimal cooking times from the seven Jack bean collections are not predictive of in vitro protein digestibility. Cooking time is mainly correlated with the progressive solubilization of pectin in the middle lamella (Chigwedere et al., 2018). When legumes are cooked at the optimal cooking time, the level of pectin solubilization is perhaps comparable, as well as the barrier effect exerted by the cell wall, and the trypsin can easily diffuse into the cells (Zahir et al., 2020). It has been reported that processing may induce protein aggregation, thus reducing protein digestibility (Miraji et al., 2021). Therefore, it is likely that different levels of aggregation may also contribute to the differences in protein digestibility across collections.

Starch digestibility of Jack bean collections followed a logistic model, likely due to the accumulation of amylase in the cells as mechanistically modelled in kidney bean (Rovalino-Córdova et al., 2021). In general, all the kinetic parameters of starch digestion varied between Jack bean collections (Table 4.3). The slow progression of starch digestion at the initial phase (λ) reflects barriers to enzyme diffusivity due to cell wall integrity which were shown by
previous reports (Pallares Pallares et al., 2018; Rovalino-Córdova et al., 2021). Jack bean collections (except Kebumen) demonstrated a higher rate constant ($k_{max}$) than kidney bean, resulting in a greater extent of starch digestion ($Starch$). We found no correlation between cooking time and any of the kinetic parameters of starch digestion (Table 4.4). Similar to protein digestion, optimal cooking time is not predictive of in vitro starch digestion in Jack beans. The differences in starch digestibility suggest that cell wall permeability is not the only factor hindering amylase diffusion. Other mechanisms may influence amylase diffusivity, such as the role of cellulose in the cell wall in binding amylase (Dhital et al., 2015) and the role of densely packed protein matrix in the cell cytoplasm (Dhital et al., 2016; Rovalino-Córdova et al., 2019).

It was reported that the protein matrix can act as an additional barrier to amylase diffusion to starch (Do et al., 2023; Rovalino-Córdova et al., 2019). We did not find any correlation between kinetic parameters of protein digestibility with starch digestibility in Jack beans (Table S4.2), but we observed a negative correlation between protein content and starch digestion rate constant ($p=0.057$) (Table 4.4). At the optimal cooking time, when the cell wall permeability is likely similar, the protein matrix surrounding the starch granules may have a major role in starch digestion, i.e. the higher the protein content, the slower starch digestion.

The protein/starch digestibility has no correlation with the diameter of cells and the diameter of starch granules. In this study, we isolated the cells within a narrow range (125-250 µm) that produced a small cell size variability, with a ratio between the largest and the smallest cell of around 1.2 (Table 4.2). Despite this difference is magnified when the whole surface area is considered, this is still too small to produce differences in enzyme diffusion within the cells and this possibly explains the limited correlation we found in this study. An influence of particle size is reported within fractions with a larger size range (71-125 µm to 1000-2000 µm), i.e. an inverse relationship between particle size and protein digestibility (Zahir et al., 2018).

Our findings suggested that protein and starch digestibility is not correlated to optimal cooking time when different collections were used, possibly due to the variability in macronutrients that may have larger impact than cooking times on the digestibility. In order to limit the variability, we selected one collection to confirm the effect of cooking times on protein/starch digestibility in Jack bean. Using only one collection, we can expect different cell wall permeability due to a different degree of pectin solubilization. We selected Malang collection for this investigation. The results showed that increasing
cooking time in one collection would increase starch digestibility but not protein digestibility (Figure 4.3; Table S4.1). It can be assumed that increasing cooking time would increase the permeability of cell walls to digestive enzymes as reported in previous studies (Bhattarai et al., 2018; Pallares Pallares et al., 2018; Zahir et al., 2018). The effect of the increased cell wall permeability may be more pronounced for amylase (51-54 kDa) than trypsin (23.4 kDa). The digestive enzyme with a smaller size can diffuse more quickly than the bigger ones. In soybean cells, trypsin has been shown to easily diffuse inside the cells (Zahir et al., 2020). Another study mentioned a higher affinity of amylase towards non-specific cell wall binding than proteases (Bhattarai et al., 2017). The difference in size or relative characteristics of digestive enzymes possibly explains the effect on starch digestion ($Starch_f$) and the existence of a lag phase ($\lambda$) for starch digestion that is not evident for protein digestion (Figure 4.3; Table S4.1). It has been reported that starch gelatinization and protein denaturation occurred during 30 minutes of boiling treatment in short and longer-to-cook beans (Chigwedere et al., 2018). Another study mentioned that a complete gelatinization had occurred on Canadian wonder beans ($Phaseolus vulgaris$) cooked at 95 °C for 30 minutes, showing no residual gelatinization enthalpy compared to a longer processing time (180 minutes) (Pallares Pallares et al., 2018). Therefore, in the present study, we can assume that the starch would be fully gelatinized even after the shortest cooking time (90 minutes). The increase in starch digestibility with cooking time is in agreement with a previous study for Bambara groundnut (Gwala et al., 2020). In contrast, prolonged cooking times have no effect on the final starch digestion for lentil (Duijsens et al., 2023) and black bean (Pälchen et al., 2022). In this study, the lack of effect on protein digestibility was found, similar to earlier report on other Canavalia species (Torres et al., 2016). Longer cooking times may enable more protein unfolding, making the protein more prone to enzymatic attack. On the other hand, prolonged cooking times may produce protein aggregation, which is known to decrease protein digestibility (Zahir et al., 2021). It could be possible that the heating time utilized in our study (90 to 210 minutes) generated more protein aggregation, therefore counteracting the effect of an increase cell wall permeability to trypsin (Gwala et al., 2020).

4.5 Conclusion

In this study, we have characterized the proximate composition of seven Jack bean collections. The collections can be a potential source of protein with a valuable amount of fiber. We found that optimal cooking time cannot be used as an the only factor to predict starch and protein digestibility in Jack beans.
However, increasing cooking time for a single collection would increase starch digestion but not protein digestibility. Furthermore, the current study demonstrated for the first-time quantification of cells and starch granules size in Jack bean using image analysis. Image analysis has the advantage of excluding cell clusters, thus improving the accuracy of cell size quantification. Reliable results can be obtained for starch granules without the need for starch isolation as compared to the particle distribution analyzer. The findings of this study suggest that strategies for reducing cooking time in Jack bean will be advantageous for lowering starch digestibility without impairing protein digestibility.

**CRediT authorship contribution statement**

Fiametta Ayu Purwandari: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. Christien Westerbos: Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. Keumwoo Lee: Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. Vincenzo Fogliano: Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration. Edoardo Capuano: Conceptualization, Methodology, Visualization, Supervision, Writing – review & editing, Project administration, Resources.

**Declaration of Competing Interest**

The authors declare no conflict of interests that may influence the work reported in this paper.

**Acknowledgements**

We thank Erik Meulenbroeks for the help with confocal laser microscopy analysis. This work was supported by Indonesia Endowment Fund for Education (LPDP) (S-682/LPDP.4/2019).
References


Supporting information

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<th>Collections</th>
<th>a</th>
<th>b</th>
<th>k</th>
<th>RSS</th>
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<tbody>
<tr>
<td>Banyuwangi</td>
<td>101.25</td>
<td>3.47</td>
<td>0.04</td>
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<td>53.37</td>
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<tr>
<td>Kebumen</td>
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<td>94.79</td>
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<td></td>
<td>100.73</td>
<td>4.85</td>
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<td>260.53</td>
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**Figure S4.1** Kinetic parameters of Jack beans cooking profile and representative data (dot) and the corresponding fitted model (line) of Jack bean (Banyuwangi2) cooking profile.

**Figure S4.2** Histograms of cell and starch granule diameter of seven Jack bean collections and one kidney bean collection.
### Table S4.1 Kinetic parameters of in vitro starch and protein digestion of Malang collection with different cooking time

<table>
<thead>
<tr>
<th>Cooking time</th>
<th>Protein digestibility</th>
<th>Starch digestibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{Protein}_i$ (%)</td>
<td>$k$ (protein%/min)</td>
</tr>
<tr>
<td>90 min</td>
<td>1.35±0.64a</td>
<td>0.020±0.002a</td>
</tr>
<tr>
<td>135 min</td>
<td>0.79±0.43a</td>
<td>0.020±0.001a</td>
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<tr>
<td>210 min</td>
<td>0.78±0.62a</td>
<td>0.017±0.002a</td>
</tr>
</tbody>
</table>

### Table S4.2 Correlation matrix between protein and starch digestion of seven Jack bean collections

<table>
<thead>
<tr>
<th></th>
<th>$\lambda$</th>
<th>$k_{\text{max}}$</th>
<th>$\text{Starch}_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein i</td>
<td>-0.29</td>
<td>0.13</td>
<td>-0.24</td>
</tr>
<tr>
<td>Kprot</td>
<td>-0.35</td>
<td>-0.50</td>
<td>-0.70</td>
</tr>
<tr>
<td>Protein f</td>
<td>-0.43</td>
<td>0.39</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Asterisk symbol indicates a significant correlation $p<0.05$ (2-tailed). Red to blue color code indicates $r = -1$ to $r = 1$. 
Chapter 5

Tempeh fermentation improves nutritional and functional characteristics of Jack bean (*Canavalia ensiformis* (L.) DC)

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Abstract

The effect of two processing methods of Jack beans (i.e. cooked beans (CB) and cooked tempeh (CT)) on \textit{in vitro} digestibility of protein and starch, as well as the production of short-chain fatty acids (SCFAs), \(\gamma\)-aminobutyric acid (GABA), and Tryptophan (Trp) metabolites after \textit{in vitro} colonic fermentation was investigated. CT was obtained by fungal fermentation after cooking in acidic conditions. CT had significantly higher protein, lower digestible starch, lower total fiber, higher free phenolic, and higher ash content compared to CB. Bean fermentation caused a better \textit{in vitro} protein digestibility than CB and less glucose released than CB. A comparable concentration of total SCFAs and GABA was produced after \textit{in vitro} fermentation of CB and CT, but CB produced more indole than CT, resulting in more total Trp metabolites. To summarize, our findings show tempeh fermentation improves the nutritional quality of Jack bean and describes the impact of the fermentation on the digestibility of nutrients and the formation of metabolites during colonic fermentation.

Keywords:

Fungal fermentation, gastrointestinal digestion, colonic fermentation, SCFA, Trp metabolites.
5.1 Introduction

Underutilized pulses refer to pulses that are not globally consumed. Promoting the consumption of underutilized pulses can play a significant role in combating hunger while balancing the economy [1]. Jack bean (*Canavalia ensiformis*), an underutilized pulse, is high in protein (23-35%) and complex carbohydrates (46-65%, including fiber), minerals, and vitamins, while being low in fat (2-12%) [2]. This favorable nutritional profile in Jack bean, and pulse in general, is restricted by the lower protein digestibility compared to other protein-rich sources. In the pulse matrix, cell walls can physically hinder proteases diffusion to proteins lowering their digestibility [3]. Moreover, the presence of antinutritional factors such as protease inhibitors can also impair protein digestion [4].

Solid-state fermentation (SSF) with fungal biomass has long been employed for the production of fermented foods. An attractive example of SSF product is tempeh, a fermented product, originated from Indonesia, that is made from soybeans. Tempeh processing involves the employment of *Rhizopus* sp. fungi, mainly *R. oligosporus* or *R. oryzae*, which results in the formation of a dense white mycelium that binds the soybeans together. *Rhizopus* mycelia grow on cooked beans and secrete a wide range of enzymes including α-amylase, esterase, cellulase, protease, and β-glucosidase [5]. These enzymes produce chemical and structural changes in the bean, resulting in the production of bioactive peptides [6], improvement of flavor [7], decrease of antinutritional factors [8] and modification of cell wall observed as cell walls damage [9]. Cell wall modification in kidney beans after tempeh fermentation and subsequent mastication has been reported to produce a significant increase in protein digestibility [9].

There has been a growing interest in fungal fermentation in the past decades. Many studies have reported the use of fungal fermentation on pulses other than soybean, such as chickpea (*Cicer arietinum*), pigeon pea (*Cajanus cajan*), red kidney bean (*Phaseolus vulgaris*), Jack bean (*Canavalia ensiformis*), faba bean (*Vicia faba*), and lentils (*Lens culinaris*) [9–12]. The main focus of these studies was on nutritional quality and production of microbial metabolites during tempeh fermentation. Few studies investigate the behavior of tempeh products in the gastrointestinal tract and none in the human colonic environment.

This study aimed to compare Jack bean cooked bean (CB) and cooked tempeh (CT) in terms of *in vitro* digestibility of protein and starch during small
intestinal digestion of Jack bean, as well as the production of metabolites derived from colonic fermentation of non-digestible carbohydrates (i.e. SCFAs) and amino acids (i.e. Trp metabolites and GABA).

5.2 Material and Methods

5.2.1 Material

Jack bean was harvested and purchased from local Indonesian farmer. The bean was grown in Malang, East Java, Indonesia and was stored in a vacuum plastic bag at room temperature upon delivery to the laboratory. All chemicals and enzymes were analytical grade unless otherwise stated.

5.2.2 Production of cooked bean and cooked tempeh

Whole Jack bean seeds (1000 g) were washed and soaked in demineralized water overnight (16h, 25 °C) with a ratio of bean to soaking water 1:3 (w:v). Half of the soaked beans were cooked in boiling demineralized water (97 °C) for 270 min with a ratio of bean to cooking water 1:2 (w:v). The cooking time was previously determined using the finger press method as described in our previous study [13]. The beans were dehulled, and the cotyledons were freeze-dried before being processed in a ball mill (MM400 Retsch, Germany) at a frequency 1/30 s for 60 s. The flour was sieved to pass through 425 μm filter and was labelled as CB. The other half of the soaked beans were dehulled and cooked in 1% lactic acid solution with a ratio of bean to cooking solution 1:2 (w:v) for 30 min at 97 °C. Acidification is one critical step that mimics natural fermentation thus preventing the growth of spoilage bacteria and creating a favorable environment for the fungal growth (pH 5.5). After cooking, the cotyledons were split and sliced into 4 pieces slab-shaped cotyledon for each cooked seed. The sliced seeds were cooked for 30 min in boiling demineralized water at a bean-to-water ratio of 1:2 (w:v). They were drained and cooled to room temperature before inoculation with Rhizopus oligosporus starter (Raprima, Bandung) (0.2%). To create regulated aeration, the inoculated beans were placed in a perforated polypropylene plastic bag (uniformly perforated with a distance of 1.5 cm between holes) and finally incubated in a ventilated incubator oven (BF 260 Binder, Germany) at 30 °C for 48 h. The tempeh was cut into cube size and steamed for 10 min. It was freeze-dried, milled, and sieved to pass through 425 μm filter. The resulting sample was labelled as CT sample. Both CB and CT were stored in a tight plastic container at room temperature until use within 8 months.
5.2.3 Compositional analysis

We dried a known amount of samples at 105 °C until constant weight for dry matter analysis, measured the crude lipid using Soxhlet extraction and nitrogen with Dumas method. The crude protein was calculated by using the factors of 5.4 [14]. Fibers (soluble and insoluble) and starches (available and resistant starch) were analyzed according to the protocol from Megazyme (K-TDFR) based on AOAC 991.43 [15] and Megazyme (K-RSTAR) based on AOAC 2002.02 [16], respectively. Free and bound phenolics were extracted [17] and determined by using Folin-Ciocalteau method [18]. The amino acid composition was measured on raw, CB, and CT according to a previous report [19], except for tryptophan [20]. Individual essential amino acid score was calculated by comparing the amino acid values with amino acid requirement values for humans within the age group of older child, adolescence, and adult [21]. All compositional analyses, except total amino acid, were performed in triplicate and expressed on a dry weight basis.

5.2.4 Particle size distribution (PSD)

PSD was determined on the sample flours by laser diffraction using a Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, UK). A pressure of 400 kPa was used and the volume-based particle size distribution was calculated using the Fraunhofer theory.

5.2.5 In vitro gastrointestinal digestion

In vitro gastrointestinal digestions were carried out on CB and CT flours according to our previous study [13]. In brief, 2 g of CB and CT flour were added with 3 mL Milli-Q water to generate a paste-like mixture. The oral phase involved mixing 5 g of CB and CT mixture with simulated salivary fluid (SSF) without salivary α-amylase and CaCl₂. The gastric phase included the addition of simulated gastric fluid (SGF) adjusted to pH 3 with HCl, along with CaCl₂ and porcine pepsin. The mixture was incubated at 37°C for 2 h. For the intestinal phase, the pH was adjusted to 7 with NaOH. The gastric chyme was combined with simulated intestinal fluids (SIF), bile salt solution, CaCl₂, porcine trypsin, and porcine pancreatic enzyme. These two enzymes are required to obtain 100 U/mL of trypsin activity and 200 U/mL of amylase activity in the final mixture. The mixture was incubated for another 2h at 37 °C. Three independent tubes were taken at 6 time points at t=0, 120 min in the gastric phase and t=15, 30, 60, and 120 min in the intestinal phase. The tubes were immediately placed in a boiling water bath for 10 min to stop the enzymatic reaction. An aliquot (1 mL) of supernatant was withdrawn from each tube and stored at -20 °C until
further use for starch and protein digestion analysis. Blank digestion was prepared by replacing the same amount of samples with Milli-Q water. Digestion experiments were run in three independent replicates for each time point.

**Determination of protein and starch digestion**

Protein digestibility was examined by assessing the free amino acids in digested supernatant using o-phthaldialdehyde (OPA) spectrophotometric assay in microplates after trichloroacetic acid (TCA) addition [22]. L-serine standard was used to obtain a calibration curve. The calculation of protein hydrolysis degree is based on a previous report [23]. The starch digestion was assessed by adding an aliquot of digested supernatant (0.1 mL) with ethanol, and quantifying D-glucose concentration after amyloglucosidase incubation as mentioned in the earlier study [3].

5.2.6 *In vitro* batch colonic fermentation

For *in vitro* batch fermentation sample preparation, at the end of the intestinal phase (section 5.2.5), a centrifugation technique (at 4500 g, 4 °C, for 20 min) was used instead of thermal shock to stop the enzymatic reaction. The pellet was separated from the supernatant and stored at -20 °C until further use. Soluble fiber was recovered from the supernatant using alcohol precipitation and alcohol washing (adapting K-TDFR protocol by Megazyme). The fiber obtained was carefully added back to the pellet maintaining the same ratio of the pooled pellet and the pooled fiber. Therefore, 10 mL of sample for colonic fermentation was prepared by dissolving 1.5 g digested pellet CB + 0.03 g fiber CB or 1.5 g digested pellet CT + 0.04g fiber CT in sterile Milli-Q water. The sample (10 mL) was combined with 21.5 mL of sterile buffered colon medium, containing 5.22 g/L K2HPO4, 16.32 g/L KH2PO4, 2.0 g/L NaHCO3, 2.0 g/L yeast extract, 2.0 g/L special peptone, 1.0 g/L mucin, 0.5 g/L L-cysteine HCl, and 2.0 mL/L Tween 80, in 120 mL sterile glass penicillin bottles. The bottles were then covered with a rubber cap, sealed with an aluminum ring, and flushed with nitrogen for 30 min to create an anaerobic environment. A 3.5 mL of human fecal inoculum was then injected into each penicillium bottle, and the fermentation was carried out for 24 h at 37 °C, with constant shaking of 100 rpm. The human fecal inoculum was prepared by combining 40 g of fresh feces with 200 mL phosphate buffer (8.8 g/L K2HPO4, 6.8 g/L of KH2PO4, and 0.1 g/L of sodium thioglycolate). The mixture was then homogenized into a stomacher for 10 min at speed 300 and centrifuged for 2 min at 500g. The feces were obtained from 3 healthy donors (aged 25-35 years old) who had not been treated with antibiotics for the previous 6 months, stored in anaerobiosis and
used within 4 h after collection. This research did not need ethics approval according to the guidelines of the Medical Ethical Advisory Committee of Wageningen University (METC-WU). In parallel, two controls were prepared: 1. A sample control without the fecal supernatant was included to determine the release of metabolites from the food matrix. 2. A fecal control without sample addition was used to normalize the metabolite production from the fecal inoculum. All fermentations were repeated in duplicate. Sample tubes were taken at 0, 8, and 24 h after inoculation. The samples were immediately centrifuged at 4500g, 4 °C, for 20 min, and the supernatants and pellets were separated. An aliquot of supernatant (5 mL) was then further centrifuged at 14,000 g for 5 min (4 °C) and filtered with 0.2 μm regenerated cellulose filter (Phenomenex, Torrance, USA). The filtrate was divided into small tubes and stored at -20 °C for further analysis (i.e. SCFA, Trp metabolites, and GABA). Gas production and pH were recorded as quality control parameters using manometers and pH meter, respectively.

**Determination of SCFA**

SCFAs were determined using a gas chromatography system equipped with a flame ionization detector (GC-2014, Shimadzu, Hertogenbosch NL) as reported in the previous study [24]. Acetic, propionic, butyric, valeric, iso-butyric, and iso-valeric acids were used to obtain calibration standards. Internal standard (2-ethylbutyric acid) was prepared in 0.3 M HCl and 0.09 M oxalic acid (1.6 mg/mL) and were added to samples/standards prior to injection. Concentrations of SCFA in colonic fermentation were expressed as μmol per g dry matter.

**Determination of Trp and Trp metabolites**

Tryptophan was extracted from CB and CT flours (~200 mg) using ice-cold 80% methanol at a ratio of 1:10 (w/v) according to a previous report [25]. Extracts were diluted 16-fold for CB and 80-fold for CT with Milli-Q water and filtered with 0.2 μm regenerated cellulose filter (Phenomenex, Torrance, USA). The supernatant of colonic fermentation and bean extracts were injected to a targeted Trp catabolite by Nexera XR LC-20 ADxr UPLC system coupled with a Shimadzu LCMS-8050 mass spectrometer (Shimadzu, Kyoto, Japan). Separation was done using Kinetex® 1.7 μm EVO C18 LC column (100 × 2.1 mm) (Phenomenex, Torrance, USA). The operation condition, calculation of recovery and matrix effect were carried out according to a previous method [26]. Data analysis was carried out on LabSolutions LCMS 5.6 (Shimadzu, Kyoto, Japan). Concentrations of Trp catabolites in colonic fermentation were corrected for recovery and matrix effect and normalized by the fecal control.
Determination of GABA
To the CB and CT flours, glutamate, and GABA were extracted using 70% ethanol as described elsewhere [27]. The dry extract was reconstituted using 2 mL ACN 50%. Meanwhile, ACN was added to the supernatant of batch fermentation (1:1) (v/v). 5 μL of bean extracts and colonic fermentation supernatants were injected to Nexera XR LC-20 ADxr UPLC system coupled with a Shimadzu LCMS-8050 mass spectrometer (Shimadzu, Kyoto, Japan). Separation was done using a SeQuant® 3.5 µm ZIC HILIC column (4.6 × 150 mm) (Merck, Darmstadt, Germany) equipped with a SeQuant® ZIC HILIC PEEK coated guard column (20 × 2.1 mm) (Merck, Darmstadt, Germany). The operation conditions were carried out with a flow rate of 0.7 mL/min and a column temperature at 40°C. The mobile phases consisted of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The elution program (t in min/%B) was 0.0/90; 4.0/70; 10.0/20; 13.0/20; 15.0/90 and 18.0/90. Data were processed with LabSolutions (Shimadzu corporation, Kyoto, Japan). Calculations of recovery and matrix effect were performed according to section Determination of Trp and Trp metabolites. Data analysis was performed on LabSolutions LCMS 5.6 (Shimadzu, Kyoto, Japan). Concentrations of GABA in colonic fermentation were corrected for recovery and matrix effect and normalized by the fecal control.

5.2.7 Statistical analysis
Significant differences in the compositional data, DH%, amount of digested starch/g dry matter and starch digested% were tested by t-Test (two-tailed). Significant differences in the concentration of colon fermentation metabolites among fermentation times and types of process (CB and CT) were tested by Repeated measure ANOVA (two-ways) followed by post-hoc analysis using Tukey for comparing more than two samples or paired test with Šidák correction for comparing the difference between two samples. Graph Pad Prism 9 software was used for the statistical analysis and a value of p<0.05 was considered significant.

5.3 Results
5.3.1 Compositional and structural properties of cooked Jack bean and cooked Jack bean tempeh
Table 5.1 shows the chemical composition of CB and CT. CT had a higher protein content (31.6%) and lower total starch (35.7%) than CB (26.6% protein; 41.3% starch). Lower total starch was found in CT compared to CB, and both flours exhibited similar resistant starch content (p>0.05). In terms of fiber
compositions, CT (16.6%) contained less insoluble fiber than CB (20.6%). The absence of soluble fiber in CT is perhaps related to the enzymatic activity from the *Rhizopus* fermentation process. Our finding indicates that CT has a lower carbohydrate content than CB. Furthermore, CT displayed a comparable fat content to CB and higher ash content compared to CB. In comparison to CB, CT had a 5 times higher content of free phenolic acids and an equivalent content of bound phenolic acids. The total amino acids profile of the two samples is reported in Table 5.2. We found a slight decrease of the amount of amino acids after cooking and after cooking+fermentation. However, this decrease does not impair the individual essential amino acid score. All of the essential amino acid scores exceed the body requirement [21], except for sulfur-containing amino acids.
Table 5.1 Compositional analysis of cooked Jack bean and cooked Jack bean tempeh (N=3, Mean ± SD)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (Nx5.4) (%db)</th>
<th>Non-resistant starch (%db)</th>
<th>Resistant Starch (%db)</th>
<th>Total Starch (%db)</th>
<th>Insoluble fiber (%db)</th>
<th>Soluble fiber (%db)</th>
<th>Total Fiber (%db)</th>
<th>Lipid (%db)</th>
<th>Ash (%db)</th>
<th>Free phenolic acid (mg GAE/g dry flour)</th>
<th>Bound phenolic acid (mg GAE/g dry flour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooked bean</td>
<td>26.6 ±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.3 ±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.7 ±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.9 ±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.8 ±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7 ±1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.6 ±1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8 ±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.65 ±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4 ±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05 ±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cooked tempeh</td>
<td>31.6 ±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.6 ±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.1 ±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.7 ±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.6 ±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.6 ±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.2 ±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.71 ±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.9 ±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06 ±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

%db: %dry weight basis. Different letters in the same column indicate significant differences (p<0.05)
<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Raw beans (g/kg protein)</th>
<th>Cooked beans (g/kg protein)</th>
<th>Cooked tempeh (g/kg protein)</th>
<th>Individual essential amino acid score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-essential amino acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>46.8</td>
<td>44.5</td>
<td>48.8</td>
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<tr>
<td>Arginine</td>
<td>56.1</td>
<td>52.4</td>
<td>44.8</td>
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<td>Aspartate</td>
<td>110.3</td>
<td>104.7</td>
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<td>Cysteine</td>
<td>9.7</td>
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<td>Glutamate</td>
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<td>114.8</td>
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<td>Glycine</td>
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<td>34.3</td>
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<td>Proline</td>
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<tr>
<td>Serine</td>
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<td>54.9</td>
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<td>1.0</td>
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<tr>
<td>Tyrosine</td>
<td>39.5</td>
<td>38.5</td>
<td>39.7</td>
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<td><strong>Essential amino acids</strong></td>
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<td>Histidine</td>
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<td>28.1</td>
<td>30.2</td>
<td>1.8</td>
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<tr>
<td>Isoleucine</td>
<td>43.4</td>
<td>43.9</td>
<td>40.9</td>
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<tr>
<td>Leucine</td>
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<td>83.1</td>
<td>71.1</td>
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<td>Lysine</td>
<td>59.3</td>
<td>55.5</td>
<td>51.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>12.5</td>
<td>12.2</td>
<td>11.7</td>
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<tr>
<td>Phenylalanine</td>
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<td>47.6</td>
<td>43.8</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>45.1</td>
<td>43.8</td>
<td>43.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Tryptophan</td>
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<td>11.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Valine</td>
<td>48.6</td>
<td>49.3</td>
<td>45.9</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Sulfur-containing amino acids (SAA)</strong></td>
<td>22.2</td>
<td>20.3</td>
<td>20.8</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Aromatic amino acids (AAA)</strong></td>
<td>96.7</td>
<td>97.4</td>
<td>94.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>

SAA includes methionine and cysteine, AAA includes phenylalanine, tyrosine and tryptophan. Individual amino acid scores were the ratio of amino acid of samples compared to the same amino acid of established amino acids requirement values for humans within the age group of older child, adolescence, and adult [21].

Tempeh fermentation improves nutritional and functional characteristics of Jack bean.
5.3.2 Protein and starch digestibility of cooked Jack bean and cooked Jack bean tempeh

Figure 5.1 displays the PSD of CB and CT flours. CT contained a larger number of smaller particles than CB, possibly deriving from the fungal mycelium. Figure 5.2 reports *in vitro* protein (panel A) and starch digestibility (panel B) of CB and CT, respectively. A higher protein hydrolysis was observed at time 0 of the intestinal phase. We also observed a higher free amino group in CT (1 mg/ml) compared to CB (0.1 mg/ml) at the beginning of the gastric phase (time 0) (data not shown). At the end of intestinal digestion, protein digestibility in CT is 4% higher than in CB (in absolute increase), even though the difference is not statistically significant.

Figure 5.2B shows that starch is rapidly hydrolyzed by amylase during the 15 min of intestinal digestion. A gradual increase of starch hydrolysis was observed in CB but not CT. In CT, the starch digestion remained stable after 15 min until the end of intestinal digestion. The starch digestion behavior suggests that the available starch in CT is completely digested.

![Figure 5.1 Particle size distribution of flours from cooked Jack bean (dash) and cooked Jack bean tempeh (line). An averaged curve is shown for each flour (N=3)](image)
5.3.3 Metabolites production during \textit{in vitro} colonic fermentation of cooked Jack bean and cooked Jack bean tempeh

\textbf{Short-chain fatty acids}

We examined the formation of six SCFAs i.e. acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate during fermentation at different times (Figure 5.3). Similar concentration was produced by CB and CT for almost all of the SCFAs, except propionate (panel A-F). Propionate was found in higher concentration as well as the percentage in CT (0.68 mmol/g DM; 31\% of the total SCFA) than in CB (0.55 mmol/g DM; 26\% of the total SCFA) (panel B, H). In comparison to the fermentation control, i.e. a fecal control without sample addition, the formation of valerate, isovalerate, and isobutyrate was suppressed in both CB and CT (panel D-F). There was no significant difference in the total SCFAs between CB and CT (panel G). In terms of individual SCFAs, acetate was the major SCFAs produced (58-60\%) (panel H) followed by propionate, butyrate, valerate, isovalerate and isobutyrate.
Figure 5.3 SCFA (panel A-F), total SCFAs (panel G), and relative contribution of individual SCFAs (panel H) of cooked Jack bean and cooked Jack bean tempeh during in vitro colon fermentation with human inoculums. Control samples consist of inoculums and a buffer without the addition of bean samples (panel A-G) (N=3, Mean values with error bars that indicate standard error of the mean). Different letters indicate a significant different between cooked bean, cooked tempeh, and control samples at the same colonic fermentation time point.

**Tryptophan metabolites**

Figure 5.4 shows the concentration of Trp catabolites during colonic fermentation normalized by the metabolites produced in the fecal control as explained in the methodology part. We found an increase of Trp in both CB and CT at t=8 h compared to t=0 h fermentation. The concentrations then decreased significantly at t=24 h fermentation. During fermentation, Trp metabolites are produced in both CB and CT with the highest Trp metabolites concentration obtained at the end of colonic fermentation. We observed that CB produced higher total Trp metabolites compared to CT at t=24 hours of colonic fermentation.
Figure 5.4 Tryptophan and total metabolites released during in vitro colonic fermentation of cooked Jack bean and cooked Jack bean tempeh. Different lowercase and uppercase letters indicate significant differences within the same sample for cooked Jack bean and cooked Jack bean tempeh, respectively. Asterisk symbol indicates a significant difference between cooked bean and cooked tempeh at the same fermentation time (N=3, Mean with error bars that indicate standard error of the mean).

Production of Trp metabolites such as Indole, IAA, ILA, IPA, I3A, TA, Kyn, and Oxi is displayed in Figure 5.5. Almost all Trp metabolites formed during fermentation and reached the highest concentration at the end of fermentation except ILA. ILA, as an intermediate compound, shows an increase and later decrease during colonic fermentation (p<0.05). Trp is mostly converted into Indole, accounting for approximately 95.7% and 94.6% of total metabolites from CB and CT, respectively (Table S5.4). Lower formation of Indole, IAA, and IPA were found in CT than CB at 24 h colonic fermentation (p<0.05).
Figure 5.5 Tryptophan metabolites (A–H) produced during *in vitro* colonic fermentation of cooked Jack bean and cooked Jack bean tempeh. Different lowercase and uppercase letters indicate significant differences within the same sample for cooked Jack bean and cooked Jack bean tempeh. Asterisk symbol indicates a significant difference between cooked bean and cooked tempeh within the same fermentation time. (N=3, Mean with error bars that indicate standard error of the mean). IAA: Indole-3-acetic acid, ILA: Indole-3-lactic acid, IPA: 3-indolepropionic acid, I3A: Indole-3-aldehyde, TA: tryptamine, Kyn: Kynurenine, Oxi: Oxindole.
Figure 5.6 describes the changes in glutamate and GABA during colonic fermentation of CB and CT after normalization with fecal control. The production of glutamate raised at the beginning of colonic fermentation and then declined at the end of colonic fermentation (Figure 5.6), similar to the trend observed previously for Trp (Figure 5.4). GABA, a decarboxylated product from glutamate, showed a significant increase during the colonic fermentation (p<0.05). No difference could be found in the GABA formation between CB and CT at any fermentation time point (p>0.05).

![Graphs showing glutamate and GABA production](image)

**Figure 5.6** Glutamate (A) and GABA (B) produced during *in vitro* colonic fermentation of cooked Jack bean and cooked Jack bean tempeh. Different lowercase and uppercase letters indicate significant differences within the same sample for cooked Jack bean and cooked Jack bean tempeh, respectively. No asterisk symbol means no significant difference between cooked bean and cooked tempeh within the same fermentation time (N=3, Mean with error bars that indicate standard error of the mean).

### 5.4 Discussion

*Rhizopus* fermentation has been reported to facilitate chemical and structural changes in the food being fermented which may further impact nutrient bioavailability as well as the production of microbial metabolites in the large intestine [5,28]. In the present study, we compared the nutritional quality as well as the digestibility and fermentability of Jack bean tempeh to that of...
cooked bean, as an example of commonly used domestic preparation. We observed several compositional differences between CB and CT (Table 5.1). In line with previous reports on chickpea, pigeon pea, and soybean [12], we found a higher concentration of protein in CT than in CB. This can be due to the development of the fungal biomass richer in proteins compared to the bean. Pure biomass of *Rhizopus oryzae* might contain protein as much as 50.6% per dry biomass [29]. It is predicted that 5.9% (dry weight basis) of tempeh consists of fungal biomass [30]. The growth of fungal biomass requires the utilization of starch as a carbon source, which may explain the lower total starch content in CT compared to CB. Lower starch content was also reported in fermented grains due to the activity of microbial enzymes [31]. In another study, a decrease in monosaccharides and disaccharides was also found after *Rhizopus* fermentation [5]. The total fiber was lower in CT than in CB. During fermentation, fungal enzymes (e.g. pectinase and xylanase) may have partly hydrolyzed insoluble fiber into soluble fiber, leading to their loss by leaching during the subsequent cooking process. Another possible explanation for the lower concentration of fiber in CT may be the utilization of part of the fibers for fungal growth. Fungal carbohydrazes will breakdown the polymer of fiber releasing sugar monomers. Glucose, fructose, galactose, maltose, and xylose have been reported to be excellent carbon sources for *Rhizopus oligosporus* growth [32]. We found similar lipid values but higher ash content in CT than CB. This showed tempeh processed material is able to retain more minerals than CB. The concentration of free phenolic acid is higher in CT than in CB. Phenolic compounds mostly occur in insoluble forms which are covalently bound to structural components of the cell wall [33]. Fungal cell wall degrading enzymes may not only release part of the insoluble fiber but also some phenolic acids in the fermented bean. In our study, however, tempeh processing retained the free phenolic acids better than the cooked bean. The duration of cooking time applied for preparing cooked beans may lead to the leaching of the part of free phenolics, producing larger losses in CB. In addition, CT showed a rather small change in the amino acid composition than CB. It is possible that tempeh fermentation did not cause changes in the amino acid composition but rather a release of free amino acids due to the proteolytic activity of the fungus.

A higher degree of protein digestion was observed in CT compared to CB at the start of the gastric phase, indicating that the protein had been pre-digested during tempeh fermentation, as well as at the start of the intestinal phase (Figure 5.2A). A previous study reported on the hydrolysis of legume proteins into small soluble proteins, peptides, or amino acids, confirmed by the disappearing of high molecular weight polymers, that correlated with the
protease activity during tempeh fermentation with *Rhizopus oligosporus* [5]. A recent microscopy study found the disappearing of protein, especially in the cells surrounded by mycelia [9]. In our study, a small increase in protein digestibility by 4% (in absolute increase) at the end of the intestinal phase was observed. A small increase in protein digestibility (~3% in absolute increase) was also observed in a previous study [34]. Furthermore, consumption of the same weight of the two samples, (i.e. based on the mg L-serine released/ml digestive supernatant), would result in more amino groups released from the matrix of CT than CB (Figure S5.1). All in all, the higher degree of protein digestion observed in CT than CB can possibly be attributed to several factors: a higher degree of cell wall permeability of the bean cells; a lower level of protein aggregation; more fraction of smaller particle size providing more surface area for enzymatic attack; and a lower barrier effect of fungal cell wall. During the fermentation process of tempeh, fungi secreted a mix of cell wall degrading enzymes which contribute to the disruption of cell wall integrity [9], facilitating protein digestion. Prolonged heat treatment (CB: 270 min; CT: 70 min) could promote protein aggregation, which hinders protease attack during intestinal digestion [4]. On the other hand, the fungal cell wall of *Fusarium venenatum* was reported to be a less effective barrier to proteases than the plant cell wall [35]. Finally, in CT, a fraction of small particles occurs that most likely correspond to mycelium particles (Figure 5.1) and those smaller particles may have offered less resistance to proteases penetration.

We observed a higher amount of digested starch/g dry matter at time 0 of the intestinal phase which indicates starch hydrolysis during tempeh fermentation (Figure 5.2B). No lag phase was observed at the beginning of the intestinal phase, with starch rapidly digested in both CB and CT flours. A previous study on Jack bean intact cells demonstrated that *in vitro* starch digestion followed a logistic model with a lag phase at the beginning of intestinal digestion [13]. This is most likely due to the use of flour which includes a certain fraction of free starch granules (i.e. not encapsulated into intact cells), allowing for a rapid starch digestion during the initial phase of starch digestion. We observed that the amount of starch digested was smaller at the end of the intestinal phase for CT compared to CB, meaning that the same amount of dry matter from the two prepared samples is expected to produce a lower post-prandial blood glucose peak from CT. CT’s starch has been digested completely at time 15 min intestinal digestion but the final starch digestion is lower compared to CB. The lower starch digestion in CT than CB may be due to the lower starch content. Moreover, if the %starch digested is calculated (i.e. the amount of digested starch is divided by the initial amount of starch, Table S5.1), CT showed lower
digestibility than CB at the end of intestinal digestion. This observation deserves further investigation on the structural properties of starch after fermentation. Taken all together, our data indicates that fungal fermentation can increase protein digestibility while reducing the glycemic load, thus would be particularly beneficial from the nutritional perspective. At the end of intestinal digestion, we found less pellet remained in CT compared to CB (data not shown), indicating a greater leaching of soluble components in CT.

Non-digestible carbohydrates that escape gastrointestinal digestion are mainly converted into SCFAs by gut microbiota. Dietary fibers have been reported to affect microbial diversity thus influence metabolite formation in the gut, including SCFAs [36]. The most abundant SCFA produced during colonic fermentation was acetate, followed by propionate and butyrate (Figure 5.3A-C), in line with a prior study in kidney beans [37]. Notably, a higher concentration of propionate was found after in vitro fermentation of digested CT compared to CB. One of the mechanisms for propionate production is via acrylate pathway, using lactate as a substrate [38]. Hence, one possible explanation may be due to the presence of residual lactic acid in the CT matrix, which was not metabolized by Rhizopus and remained after gastrointestinal digestion. As previously mentioned, lactic acid was used in one of the cooking steps during tempeh preparation. Another explanation may be due to the different types of fiber in CT that can modulate the microbial ecology (i.e. composition and activity) resulting in differences in the concentration of metabolites, including propionate [39]. At the end of colonic fermentation, a higher concentration of butyrate was formed compared to the control (Table S5.2). In contrast, the fermentation of CT produced less butyrate than the control, and no statistical difference was observed between CB and CT. The colonic fermentation contained a similar amount of fibers for CT and CB (CB: 0.12 g fiber, CT: 0.11 g fiber). Therefore, we can expect similar SCFAs production (Figure 5.3G).

When fermentable fibers are limited, gut microbiota uses less energy-efficient sources for growth, such as amino acids from dietary or endogenous proteins. Most of the ingested dietary proteins are digested and absorbed in the small intestine, however, a significant amount of proteins may still enter the colon and be metabolized by the gut microbiota [40]. In this study, a lower content of undigested protein was found in the pellet after digestion of CT (i.e. less proteins have been exposed to the gut microbiota, CB: 0.21 g protein; CT: 0.18 g protein). The lower protein digestion in CB in the small intestine suggests that it could be used as a more effective system to deliver undigested proteins in the gut than CT. We found that the starting material of CT contains higher
free amino acids (i.e. Trp, Glutamate, and GABA) than CB flour (Table S5.3), which is in line with the proteases activity of *Rhizopus*. A unique product of protein fermentation is branched-short chain fatty acids (BCFAs) such as isobutyrate and isovalerate, which are derived from branched-chain amino acids (valine, isoleucine, and leucine) [41]. The production of branched-chain amino acids was suppressed in our study by the addition of the digested pellet of Jack bean (Table S5.2). This may be due to the presence of extra fermentable carbohydrates from the digested bean that may have suppressed protein fermentation.

During colonic fermentation, Trp and Glutamate were released from undigested proteins and further metabolized by gut microbiota producing indole derivatives and GABA respectively, hence the bell-shaped curve observed in Figure 5.4 & 5.6. This is in line with another study in soybean cells [42]. Trp metabolites play a significant role as endogenous ligands for the aryl hydrocarbon receptor (AhR), impacting host immune homeostasis and barrier physiology [43,44]. In addition, IPA, particularly in the presence of Indole, has been identified as a ligand of pregnane X receptor (PXR), regulating intestinal barrier function [45]. On a different note, GABA serves as an important inhibitory neurotransmitter in the nervous system, primarily involved in modulating responses to anxiety [46]. It is likely that the modulation of microbiota-derived GABA is mediated through the gut-brain axis [47].

We found that CB produced more Trp metabolites than CT (Figure 5.4) with significant differences in the concentrations of Indole, IAA, and IPA (Figure 5.5). The most likely explanation may be the lower protein load in the CT pellets compared to CB in the fermentation system. However, even after normalization of the values per g protein (Figure S5.2 & S5.3), we could still observe lower Trp metabolites formation in CT samples, as indicated by indole, the most abundant Trp metabolites formed during colonic fermentation (Table 5.4). Other factors may also play an influence in the Trp metabolites such as the fiber content and the integrity of the plant matrix. A high-fiber diet promotes the production of certain Trp metabolites like IAA, ILA, IPA, I3A, IA, and 5-HT, while a high-protein diet favors the production of TA, Kyn, Ind, and Oxi [48]. However, since the two pellets contain a similar amount of fiber, it is unlikely that the concentration of fiber may influence the Trp metabolites. The integrity of the plant matrix, specifically of the cell wall, influences microbial catabolism by affecting the accessibility of Trp to the microbiota [42]. Nevertheless, it remains uncertain whether the integrity of the bean cell wall was altered during *Rhizopus* fermentation, and on the other hand the fungal cell wall may be more accessible to the microbiota enzymes. This aspect
requires further investigation. Unlike Indole, we found that GABA production was similar in CB and CT (Figure 5.6).

5.5 Conclusion

In conclusion, our study showed that fungal fermentation significantly affects the nutritional quality of Jack beans resulting in an improved protein digestibility compared to cooked beans while at the same time reducing the amount of digestible starch and limiting starch digestion as well as in a better preservation of phenolic compounds. Processing into tempeh did not affect concentrations of SCFA and GABA produced during in vitro fermentation but reduced the production of Trp metabolites. This work provides evidence that fungal fermentation is a useful strategy for individuals with certain dietary requirements since it improves protein quality, and potentially controls the blood sugar levels, without compromising the production of beneficial gut microbial metabolites such as SCFA and GABA.

CRediT authorship contribution statement

**Fiametta Ayu Purwandari**: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Vincenzo Fogliano**: Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration. **Edoardo Capuano**: Conceptualization, Methodology, Visualization, Supervision, Writing – review & editing, Project administration, Resources.

Declaration of competing interest

The authors declare that they have no competing interests that may affect the work reported in this study.

Acknowledgements

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References


Tempe fermentation improves nutritional and functional characteristics of Jack bean


Supporting information

Figure S5.1 Hydrolyzed protein expressed as L-serine in digestive supernatant

Figure S5.2 Tryptophan and total Tryptophan metabolites produced per g protein of cooked bean and cooked tempeh during colonic fermentation. An asterisk symbol indicates a significant difference
Figure S5.3 Tryptophan metabolites produced per g protein of cooked bean and cooked tempeh during colonic fermentation. An asterisk symbol indicates a significant difference. (N=3, Mean with error bars that indicate standard error of the mean) IAA: Indole-3-acetic acid, ILA: Indole-3-lactic acid, IPA: 3-indolepropionic acid, I3A: Indole-3-aldehyde, TA: tryptamine, Kyn: Kynurenine, Oxi: Oxindole.
Table S5.1 | Starch digestibility of cooked bean and cooked tempeh as expressed as g digested starch/100 g starch (N=3, Mean ± standard deviations)

<table>
<thead>
<tr>
<th>Intestinal phase (min)</th>
<th>g digested starch/100 g starch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cooked bean</td>
</tr>
<tr>
<td>0</td>
<td>0.0±0.1</td>
</tr>
<tr>
<td>15</td>
<td>32.2±1.0</td>
</tr>
<tr>
<td>30</td>
<td>35.4±1.5</td>
</tr>
<tr>
<td>60</td>
<td>46.2±3.0</td>
</tr>
<tr>
<td>120</td>
<td>48.9±1.7</td>
</tr>
</tbody>
</table>

Asterisk symbol indicates a significant difference between cooked bean and cooked tempeh.

Table S5.2 | SCFAs produced by cooked bean and cooked tempeh during colonic fermentation after normalization with blank samples (N=3, Mean ± standard error of the mean)

<table>
<thead>
<tr>
<th>SCFAs</th>
<th>Time (h)</th>
<th>Cooked bean (mmol/g DM)</th>
<th>Cooked tempeh (mmol/g DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>8</td>
<td>0.328±0.020</td>
<td>0.347±0.088</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.490±0.175</td>
<td>0.463±0.090</td>
</tr>
<tr>
<td>Propionate</td>
<td>8</td>
<td>0.148±0.028</td>
<td>0.211±0.023</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.216±0.037</td>
<td>0.342±0.052*</td>
</tr>
<tr>
<td>Butyrate</td>
<td>8</td>
<td>-0.005±0.004</td>
<td>-0.013±0.028</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.041±0.051</td>
<td>-0.006±0.039</td>
</tr>
<tr>
<td>Valerate</td>
<td>8</td>
<td>-0.019±0.010</td>
<td>-0.021±0.020</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-0.024±0.021</td>
<td>-0.046±0.030</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>8</td>
<td>0.000±0.000</td>
<td>0.000±0.001</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-0.037±0.013</td>
<td>-0.039±0.015</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>8</td>
<td>0.000±0.0001</td>
<td>-0.001±0.003</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-0.034±0.006</td>
<td>-0.039±0.013</td>
</tr>
<tr>
<td>Total SCFAs</td>
<td>8</td>
<td>0.451±0.049</td>
<td>0.524±0.119</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.650±0.251</td>
<td>0.676±0.153</td>
</tr>
</tbody>
</table>

Asterisk symbol indicates a significant difference between cooked bean and cooked tempeh.
### Table S5.3 Tryptophan, glutamate, and GABA of dry bean flour (N=3, Mean ± standard deviations)

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Cooked bean (mg/g dry flour)</th>
<th>Cooked tempeh (mg/g dry flour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>0.02 ± 0.00</td>
<td>8.12 ± 0.09</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.92 ± 0.03</td>
<td>18.56 ± 0.20</td>
</tr>
<tr>
<td>GABA</td>
<td>1.10 ± 0.06</td>
<td>8.06 ± 0.02</td>
</tr>
</tbody>
</table>

### Table S5.4 %abundance of Tryptophan metabolites

<table>
<thead>
<tr>
<th>Trp metabolites</th>
<th>%abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CB</td>
</tr>
<tr>
<td>Indole</td>
<td>95.7</td>
</tr>
<tr>
<td>IAA</td>
<td>0.3</td>
</tr>
<tr>
<td>ILA</td>
<td>0.2</td>
</tr>
<tr>
<td>IPA</td>
<td>0.9</td>
</tr>
<tr>
<td>I3A</td>
<td>0.2</td>
</tr>
<tr>
<td>TA</td>
<td>0.3</td>
</tr>
<tr>
<td>Kyn</td>
<td>0.1</td>
</tr>
<tr>
<td>Oxi</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Tempeh fermentation improves nutritional and functional characteristics of Jack bean.
Chapter 6
General discussion
To address the increasing global food demands, promoting the cultivation and consumption of underutilized crops is crucial. These crops are considered sustainable and can potentially enhance food and nutrition security, particularly in emerging countries. One promising underutilized legume is Jack bean. However, its utilization has been hindered by various factors, including the hard-to-cook phenomenon and concerns about its nutritional quality [1,2]. This thesis has been conducted to provide solutions for these issues. In Chapter 2, we characterized the cell wall properties and microstructure of Jack bean collections with different cooking behaviors. Then, we evaluated freezing treatments as alternative strategies to reduce cooking time (Chapter 3). Considering that hard-to-cook can be related to nutritional digestibility [3,4], we examined the effect of optimal cooking times on protein and starch digestibility in Chapter 4. We further explored the impact of the *Rhizopus* fermentation process on Jack bean on chemical composition, protein and starch digestion, and metabolite formation during colonic fermentation (Chapter 5). The main findings are summarized in Table 6.1 and are generally discussed in this section.
Table 6.1 Summary of the main findings in this thesis

<table>
<thead>
<tr>
<th>Objectives</th>
<th>Main findings</th>
</tr>
</thead>
</table>
| **Hard-to-cook in Jack beans** | **HTC Jack bean collections (Cilacap and Jombang) showed smaller starch granule sizes than the collection with the shortest cooking time (Malang).**  
**Jack bean collection with the longest cooking time (Jombang) has the highest fraction of small intercellular spaces, indicating more cell wall connections.**  
**Longer optimal cooking times in Jack beans are associated with lower concentration of water-soluble polysaccharide (WSP), higher percentage of covalently bound pectin (NEF), and higher concentration of bound ferulic acids.** |
| **To study the effect of freezing treatments on the microstructure of cell walls, duration of cooking times, and starch and protein digestibility in the small intestine (Chapter 3).** | **Slow freezing treatments (-20°C) demonstrated higher microstructure alteration compared to fast freezing treatments (-80°C), as showed by more cell separation.**  
**Bean stored at -20°C for 24 hours showed the shortest cooking time compared to other freezing treatments.** |
| **Starch and protein digestion** | **At optimal cooking times, frozen treated beans showed an increase in protein digestibility and similar starch digestibility compared to kidney beans.**  
**Alkaline-cooked beans showed higher protein digestibility and lower starch digestibility than kidney beans cooked in demineralized water.** |
| **To examine the effect of processing conditions on in vitro protein and starch digestibility (Chapter 3)** | **Protein and starch digestion of Jack beans did not correlate with optimal cooking times.**  
**Bean cooked for longer cooking time increased starch digestion, but not protein digestion, in one Jack bean collection.** |
| **Jack bean tempeh**          | **Cooked tempeh showed higher protein, ash, and free phenolic acids than cooked beans. Cooked tempeh showed lower non-resistant starch and total fiber than the cooked bean.**  
**The digestibility of protein in cooked tempeh shows an increase compared to cooked beans.**  
**The digestibility of starch in cooked tempeh is lower than in cooked beans.**  
**Tempeh produced similar concentrations of SCFA and GABA and lower concentrations of Tryptophan metabolites than cooked beans after intestinal digestion and colonic fermentation.** |
| **To investigate the nutritional quality of cooked beans and tempeh (Chapter 5).** | |
6.1 Towards understanding the HTC phenomenon in Jack bean and alternative strategies to reduce cooking time

The HTC phenomenon has been mainly attributed to the prolonged storage in high temperature and relative humidity conditions, particularly in tropical countries. In many studies, therefore, bean samples are prepared that are stored in such conditions to induce the HTC phenomenon and to evaluate either the microstructural or chemical changes [5,6]. However, in the market, beans are not only stored but also originated from various regions, reflecting different growing conditions. It has been reported that different growing conditions can produce beans with longer cooking times [7]. This leads to an additional question of how chemical and microstructural modifications can be observed in the collections that are reflective of the diverse conditions encountered in the market. To answer the question, we collected beans from three different regions (i.e. Malang, Cilacap, and Jombang), each with distinct optimal cooking times, and evaluated their microstructure and cell wall properties (Chapter 2). To our knowledge, this is the first time that these aspects of Jack beans are characterized.

Previous studies reported on the alteration of the pectin fractions in aged beans [5,8,9] and the rate of pectin solubilization was reported as the main determining factor for the textural degradation of beans during cooking [10]. Therefore, we conducted a comprehensive compositional analysis with a specific focus on the pectin fractions on three Jack bean collections. Results showed that the primary pectin fraction in Jack bean is the covalently linked pectin, accounting for more than 75% of the total pectin fractions. This was followed by water-soluble pectin and then chelator pectin fractions in descending order. There was a correlation between cooking times and the relative percentage of covalently linked and water-soluble pectin fractions. We observed lower water-soluble polysaccharides and higher covalently bound pectin and bound phenolics as the cooking time increased. One previous report highlighted the importance of covalently linked pectin fractions, rather than chelator pectin fractions, in the context of HTC beans [9]. Moreover, we observed a significant difference in the level of bound ferulic acids as the optimal cooking time increased. This suggests that ferulic oligomers may play a role in crosslinking pectin within the cell walls of HTC beans, influencing texture changes during cooking, as observed in other plant tissues like sugar beet [11]. A recent study documented the presence of ferulic acid monomer and dimers in the cell wall of HTC Red haricot beans, whereas none were found in the fresh beans [12]. As far as we are aware, there has been
limited research focused on the bound phenolic compounds that are linked to pectin fractions or other cell wall polysaccharides in the cotyledon of HTC beans.

We observed a shift towards a distribution pattern characterized by smaller intercellular space in the bean tissue as optimal cooking times increased. These intercellular spaces were associated with cell wall connections, an indicative feature of HTC beans [13]. Quantifying microstructural features using images obtained at an appropriate scale may open new opportunities for the design of products with desired properties. This approach enables the connection of microstructure, such as cell wall connections, to material properties (i.e. texture) and facilitates a deeper insight into the underlying physicochemical processes. Since we observed a slight increase in the proportion of covalently bound pectin fractions with the optimal cooking time, further work is necessary to confirm if there is a causal relationship between the intercellular spaces and the amount of bound phenolics in HTC beans.

Longer cooking time has an environmental impact since it requires additional energy. The utilization of alkaline salts, either through the soaking or cooking step, was reported to be able to reduce cooking time [10,14,15]. However, several findings reported adverse effects of the alkaline treatment, such as increased pH level and coloration in contrast to beans cooked in distilled water [16]. Another study reported a decrease in sensory acceptability in common beans prepared by soaking and cooking in alkaline solutions [17]. In Chapter 3, we explored the use of freezing methods to reduce cooking time. Our results demonstrated that subjecting the beans to slow freezing treatments at -20 °C for 24 hours prior to cooking leads to cell separation, suggesting that the formation of ice crystals may enlarge the intercellular spaces. Consequently, the pretreated bean showed shorter optimal cooking times than the untreated one (Chapter 3).

Our observations showed differences in the effects of alkaline and freezing techniques on reducing cooking times, emphasizing the effectiveness of chemical aid in pectin solubilization during cooking. Notably, the freezing method does not involve chemical additives that could alter pH levels and the appearance of beans. In addition, it is possible that the ice crystallization may not only affect the breakdown of the cell wall connections but also induce changes in the cytoplasmic matrix, depending on the freezing rate and time. An increase in intercellular spaces was reported on soybeans after freezing at -5 °C for 4 days compared to control beans [18].
Usually, the severity of the HTC phenomenon is connected to the storage time: the bean with the most extended storage duration exhibited the longest optimal cooking times (Jombang). However, the Cilacap collection showed a longer optimal cooking time than the Malang collection despite being stored for a shorter period. This may suggest that growing conditions may also play a role, as discussed in Chapter 2. Previous studies reported the influence of other parameters on the HTC phenomenon, i.e. the bean variety and the growing regions [7,19]. A summary of factors affecting cooking time is presented in Figure 6.1. Overall, our observations toward understanding the HTC phenomenon in Jack bean provide important implications for bean breeders, farmers, and food technologists. The goal is to prevent the production of HTC beans on farms. It is preferable to cultivate beans in controlled environmental conditions, such as cooler climes or mountainous areas. In addition, it is essential to investigate the genetic characteristics associated with the development of the HTC phenomenon. In regions where the implementation of controlled storage conditions is challenging, the freezing treatment can be used as a potential alternative method for reducing cooking times. This approach can be adapted at a domestic scale, especially where freezing technology is available. It is crucial to assess the overall sustainability of the freezing approach since freezing also requires energy despite resulting in shorter cooking times. We believe the implications extend to a broader range of bean types as cell wall connections as well as cell separations are common features of HTC legumes and optimally cooked
beans, respectively. Therefore, an approach toward mitigating HTC beans and inducing cell separation can benefit a broader spectrum of beans, promoting the utilization of legumes.

6.2 Nutritional composition of Jack beans and the effect of processing

Chapter 4 reported the nutritional composition of Jack beans from seven different collections. Jack beans have high protein content, ranging from 28.8 to 39.3%, low-fat content, and high amounts of fiber and starch up to 24.6% and 40.9%, respectively. These findings suggested that Jack bean may be a potential alternative source of proteins compared to other underutilized legumes such as adzuki bean (19.9%) [20], Bambara groundnut (20%) [21], and velvet bean (31.4%) [22].

Microstructure changes can occur during hydrothermal processing and mechanical disintegration of the HTC Jack bean, as illustrated in Figure 6.2. The microstructure of soaked cotyledon can be described as a compact assembly of ovoid-shaped cells connected to each other by the middle lamella of adjacent cells. During the hydrothermal process such as boiling, pectin that is located in the middle lamella of the cell wall undergoes solubilization. This solubilization improves cell wall permeability [23], making it more accessible to digestive enzymes. Pectin solubilization can also cause cell separation, resulting in a loosening of cells’ connection thus increasing intercellular spaces in cooked bean. The solubilization rate of pectin determined the speed of cooking time that related with textural properties [10]. Mechanical disintegration such as mastication or milling of optimally cooked beans mostly can result in individual intact cotyledon cells with retention of the cells shape (i.e. ovoid shape).
Processing of Jack beans may impact their nutritional components. For instance, tempeh fermentation can increase protein content and free phenolic acids but reduces available starch and total fiber (Chapter 5). Many studies have documented the influences of fermentation on the nutritional composition of different types of legume seeds. African yam bean showed an increase in crude protein, ash, minerals, and certain amino acids, and a decrease in fat, fiber, carbohydrate, and anti-nutritional factors such as phytic acids and tannin after Saccharomyces cerevisiae fermentation [24]. Meanwhile, an increase in iron, zinc, and in vitro bioavailability of minerals was reported on black-eyed peas due to Aspergillus oryzae fermentation [25].

In Jack beans, both cooking and fermentation slightly decrease amino acid compositions without compromising quality, as essential amino acid levels still surpass the recommended body requirement recommended by FAO [26], except for sulfur-containing amino acids (Chapter 5). This aligns with findings for other legumes such as chickpea and lentil, which also exhibit high essential amino acids but limited sulfur-containing amino acids such as methionine and cysteine [27]. Moreover, cooking reduces mineral contents in Jack bean cotyledons, while minimizing cooking time helps limit the mineral loss (Chapter 3). Cooking effectively inactivates hemagglutination activity caused by lectin, an antinutrient in Jack beans (Chapter 3). Notably, reduced cooking time retains this benefit, further enhancing the nutritional value of Jack beans. The cooking process can also reduce antinutrients such as phytates and
tannins [28] and inactivate trypsin inhibitors and lectin [1]. Therefore, a prolonged hydrothermal process, such as in the HTC bean, may not only influence its nutrient compositions but also digestibility as discussed in the next section.

6.3 Starch and protein digestibility and the effect of processing

We investigated the *in vitro* digestibility of protein and starch in seven Jack bean collections (Chapter 4). The collections represent a suitable range of optimal cooking times and are collected from the most populated areas in Indonesia. Protein and starch digestion of Jack bean collections showed a slightly lower protein, but higher starch digestibility as compared to kidney beans. The different protein and starch digestion of Jack beans compared to kidney beans can be attributed to variability in cooking time, macronutrient, and antinutritional compounds because different types of beans were used. Limited studies have investigated the digestibility of nutrients in Jack beans. To the best of our knowledge, existing literature reported *in vitro* protein digestibility in Jack bean flour using an *in vitro* digestion model [1] which differs from the method employed in this thesis. Furthermore, we observed that protein and starch digestibility were not significantly correlated with optimal cooking time due to variability across collections (Chapter 4).

To better understand the impact of cooking times on protein/starch digestion, we randomly selected one collection and subjected the beans to varying cooking durations (Chapter 4), corresponding to beans that are fully cooked, partly cooked, and uncooked. This allowed us to generate samples with different hardness levels corresponding to different cell wall permeability levels. Our findings indicate that longer cooking times increase starch digestibility but not protein digestibility. As previously mentioned, nutrient digestibility can be affected by cell wall permeability. When the cell walls are more permeable, digestive enzymes such as α-amylase can easily diffuse into the cytoplasmic matrix, facilitating starch digestion. Conversely, an increase in permeability has a limited effect on the diffusion of trypsin inside the cells, which may be because trypsin has a smaller size compared to amylase as discussed in Chapter 4. In addition, protein digestibility can also be affected by its structure, such as protein aggregation. Moreover, during thermal treatment, protein experiences denaturation where the globular structure unfolds and makes the proteins more easily attacked by proteases, resulting in increased protein digestibility. However, longer cooking time can further
aggregate the unfolded protein, thus reducing digestive enzyme accessibility and decreasing protein digestibility [21,22] partly counteracting the effect of an increased cell wall permeability.

In Chapter 3, slow freezing for 24 hours showed a reduction of optimal cooking time compared to untreated beans as a control. This treated bean showed an 8% absolute increase in protein digestibility than control beans, even though they were not statistically different (p = 0.09). Notably, starch digestion was similar in both beans (p = 0.62), likely due to similar cell wall permeability when the beans reached similar hardness after cooking [23]. Achieving shorter yet optimal cooking times tends to improve protein digestibility, likely due to the limited formation of aggregate. In addition, when beans were cooked in alkaline salts, shorter cooking times significantly improved protein digestibility but reduced starch digestibility compared to control beans. This effect may be attributed to a higher pH within the cells, which may still be higher than the pH of the digestive supernatant after its neutralization in the in vitro digestion simulation. α-amylase has a pH optimum of 6.9 to digest starch [29]. This higher pH, as sensed by amylase in the cells, might have an impact on the enzymatic activity. On the other hand, trypsin has a pH optimum at 8-8.2 [30], slightly higher than amylase, thus may favor the protein digestion on the sample after cooking with alkaline. These findings suggest that achieving the desired texture with reduced cooking times favors Jack bean nutrient digestibility.

Starch digestibility in intact cell cotyledon and how this impacted by cooking time can be significantly differed between legume types. The effect of cooking duration on starch digestibility in Jack beans is similar to Bambara groundnut [31] and Canadian wonder beans [23]. Cell wall permeability modification occurred as the cooking duration proceeded, induced by pectin solubilization [23]. In other legumes, such as lentils [32] and black beans [33], the starch digestibility can be more influenced by the permeation through the protein matrix rather than the cell wall permeability [32]. Thus, increasing cell wall permeability did not affect the starch digestibility in those legumes. The protein matrix could act as a barrier for starch digestibility, in addition to the cell wall, in plant intact cells [34–36]. This means starch digestion could increase when more protein is digested. In the case of Jack bean, we did not observe a positive correlation between the extent of protein and starch digestion, as discussed in Chapter 4. Increasing processing time did not affect protein digestibility in Canavalia brasiliensis [37]. Overall, the abovementioned findings suggest the need to investigate different legumes to gain more
insight into the mechanism of the plant matrix in modulating nutrient digestibility.

Overall, reducing cooking time can be used as a strategy to decrease starch digestion for certain legumes. A delay in the blood glucose rise after consumption can benefit both healthy and diabetic patients and improve glucose metabolism in the long run. More importantly, it is essential to consider a reduced optimal cooking time that reaches palatable hardness to be applicable as reported in Chapter 3, for instance, using alkaline cooking. The impact on the sensory properties needs to be considered. Investigation of different types of legumes and the processing conditions that may limit starch digestibility will allow the design of more beneficial diets in diabetes management and carbohydrate metabolism disorders.

Fermentation can be used as a strategy to improve the nutritional quality of food. Fermentation could enhance nutrient bioaccessibility [38], reduce antinutritional components [39], improve nutrient digestibility [40], produce bioactive compounds [41], and support gut health [42,43]. Implementing a fermentation process on beans, such as Rhizopus fermentation or tempeh process, may enhance the nutritional quality of plant-based foods, such as protein digestibility. On the other hand, applying the fermentation process can also contribute to a diversification of dietary patterns for many cultures worldwide.

The protein digestibility of the Jack bean collections is considered low (Chapter 4). Legumes had low protein digestibility due to their cellular integrity and the presence of antinutritional factors [44,45]. Fungal fermentation by Rhizopus oligosporus on Jack bean could increase protein digestion by 4% (absolute increase), which could be possibly due to compositional and structural modifications in the legume matrix (Chapter 5). An overview of possible modifications after tempeh fermentation in relation to protein digestibility is illustrated in Figure 6.3.
During fungal fermentation, the fungi grow and secrete enzymes that break down carbohydrates and proteins in the beans, releasing nutrients that the fungus can use for energy and biomass production. *Rhizopus* could attach to the cell surface and produce cell wall degrading enzymes, allowing modification of the cell wall [46]. The fungus also secretes proteases, allowing the digestion of bean protein [47]. We observed that cooked tempeh produced on average smaller particles when subjected to mechanical disintegration, possibly because of the contribution of fungal mycelium, than the cooked beans. The effect of small fragments produced by the fungus may allow more accessibility to proteases, thus improving protein digestion in cooked tempeh. A study demonstrated that the barrier to proteases of the fungal cell wall of *Fusarium venentatum* was not as effective as plant cell walls [48]. Conversely, the starch digestion is lower than in cooked beans, which requires further investigation of the structural properties of the starch after fermentation. Increased protein digestion and nitrogen solubility were reported on soybeans fermented by lactic acid bacteria [40,49,50]. It was reported that soybeans fermented by a combination of fungi and bacteria showed a higher degree of protein hydrolysis compared to a single starter [51]. However, it is important also to mention that the fermentation of several beans, such as Bambara groundnut [52] and black bean [53], resulted in the conversion of resistant starches into available starches, which possibly can lead to an increase in starch digestibility.
6.4 Behavior of Jack beans in the large intestine and the effect of processing

During colonic fermentation, the non-digestible carbohydrates are converted into short-chain fatty acids (SCFAs) by gut microbiota, with a high-fiber diet induces the production of SCFAs [54]. The total SCFAs production between cooked tempeh and cooked bean is similar, possibly due to a similar amount of fibers in the two pellets after digestion (Chapter 5). The highest SCFA produced was acetate, followed by propionate and butyrate. We found similar levels of acetate, lower levels of propionate, and similar levels of butyrate in cooked beans than in cooked tempeh (Chapter 5). Moreover, the production of branched short-chain fatty acids (BCFAs) such as isobutyrate and isovalerate were suppressed, which may be related to the limited protein fermentation.

Dietary proteins are digested and absorbed in the small intestine. However, a significant amount of proteins may still enter the colon and be metabolized by gut microbiota [55]. In Chapter 5, we evaluated the Tryptophan (Trp) metabolites and γ-Aminobutyric acid (GABA) production during colonic fermentation. Due to the enhanced protein digestion in cooked tempeh, amino acids become more available for absorption in the small intestine. Therefore, less protein goes to the colon when cooked tempeh is consumed. Consuming the same portion of cooked bean may produce higher levels of Trp metabolites than cooked tempeh (Chapter 5). After normalization by the same amount of proteins exposed to gut microbiota, cooked bean still gives higher Trp catabolites than cooked tempeh as indicated by indole formation (p<0.05). The formation of Trp metabolites can be influenced by the amount of fiber and the integrity of the cell wall in the plant matrix [56,57]. Since the amount of fiber is similar between the two pellets, one possible explanation may be due to the cell wall integrity modification after fermentation and/or the effect of fungal cell wall which remain to be investigated in future studies. On the other hand, we found a similar level of GABA produced at the end of colonic fermentation for cooked beans and cooked tempeh.

This study provides evidence that fungal fermentation on beans may be used as a promising method for modification of the compositional properties and enhancement of nutritional quality, including the influences on Trp metabolites and GABA formations. Metabolites derived from Trp play a crucial role as ligands for the aryl hydrocarbon receptor (AhR), influencing the immune homeostasis and barrier physiology of the host [58,59]. Additionally, Indole-3-propionic acid (IPA) has been recognized as a ligand for the pregnane
X receptor (PXR), regulating the intestinal barrier function [60]. GABA, possibly mediated via the gut-brain axis [61], plays a vital role as an inhibitory neurotransmitter in the nervous system, contributing to the modulation of anxiety responses [62].

Bacterial and fungal fermentation can be applied to legume seeds via both solid-state and sub-merged techniques [40,52,63,64]. Bacterial fermentation typically requires less time than fungal fermentation [65], and both fermentation processes can effectively reduce undesirable off-flavors, resulting in distinct fermented bean flavor and texture properties. For instance, Natto, a soy product fermented by *Bacillus subtilis* var. *natto*, has a sweet umami and slightly bitter taste with the texture of a viscous polymer [66]. *Bacillus* grows on the soybean surface and produces various viscous polymers such as poly-γ-glutamic acid and polyfructan [67,68]. For bean consumption, the selection of a fermentation method must consider these two critical factors: the substrate characteristic and the intended consumer application. Since beans are a solid food, the solid-state fermentation technique may be the most suitable. Among solid-state techniques, the tempeh process has widespread popularity, especially for Indonesian consumers.

**6.5 Methodological considerations**

**6.5.1 In vitro digestion models**

Simulating digestion in the gastrointestinal tract is necessary to understand the impact of food matrix and composition on macronutrient digestibility. *In vivo* (human or animal) intervention trials may be the best method, however, this method can be difficult to undertake and expensive or is not justifiable on ethical grounds [69]. Moreover, high variability due to physiological aspects makes the *in vivo* method unsuitable for sample comparison as we did in our studies. For these reasons, we opted for *in vitro* models to simulate digestion. In this thesis, we applied the INFOGEST method [69] to estimate the protein and starch digestion of Jack beans. This method is internationally recognized for standardizing static *in vitro* digestion protocols based on the human digestive process in adults. This model consists of a sequential series simulating physicochemical and enzymatic conditions of three different oro-gastrointestinal compartments. Static *in vitro* model offers simplicity, reproducibility, relatively low cost, and no ethical concerns. Sequential series allow an easy assessment of each of the digestion phases. Thus, the method can be suitable for mechanistic studies, hypothesis testing, and screening [69].
This means that the method can be relevant for studying the food matrix effect, comparing between samples, and dealing with many samples, which are the primary considerations for selecting the static in vitro digestion method in this thesis. However, this sequential approach omits the dynamic nature of digestive physiology, such as the gradual decreases of pH in the gastric phase, the enzyme release to reach the enzyme-substrate ratio, and gastric emptying [69,70]. Previous studies reported good correlations between the INFOGEST method versus in vivo models for protein [71–73]. However, contrasting evidence was also reported [74]. INFOGEST method was reported to give a rapid and accurate prediction of glycemic index [75], suggesting a good comparability with in vivo trials. For more accurate simulation models, dynamic in vitro models can be used [76]. Among dynamic models are the Human Gastric Simulator [77] and Dynamic Gastric Model [78], which mimic the peristaltic movements of the gastric compartment in computer-controlled mechanical simulators, or the TNO gastrointestinal Model [79]. The TNO model allows controlling many parameters in separate compartments, such as peristaltic movements, transit time, and flow rates.

We decided to use intact cotyledon cells to study nutrients digestibility in Chapter 4. Beans are typically consumed as a whole cotyledon, and intact cells are the main fractions generated after optimal cooking and mastication [80]. Thus, the use of isolated intact cells may well represent the form of bean bolus presented at the beginning of the gastric phase. However, isolating intact cells can be challenging for certain processed foods like those processed with Rhizopus fermentation. Considerable parts of filamentous fungi can be retained on the first screen, and only a small amount of mycelium can be isolated during the wet sieving process. This can result in isolated fractions which are not representative of tempeh. Therefore, we used flours instead of isolated intact cells in Chapter 5. In this way, we were able to collect samples representing tempeh composition. The particle size distribution showed that the majority of the fractions are bean cells within the range of Jack bean intact cells diameter (~100 μm). The milling condition used in this thesis was possibly mild and thus did not cause lots of small particles. The use of intact cells and flour may impact the digestion of the nutrients. One clear observation is that starch undergoes rapid digestion at the beginning of the intestinal phase. This rapid digestion prevents us from capturing the lag phase previously observed when only intact cells were utilized. Differences in cooking time due to the use of different batches in Chapter 4 and 5, may also influence the extent of digestibility. Consequently, meaningful comparisons can only be made in relation to the digestion profile and not the extent of digestibility.
6.5.2 In vitro colonic fermentation models

Undigested nutrients may escape from the upper gastrointestinal tract and be fermented by the gut, resulting in the production of metabolites. Investigation of the fate of undigested food components in the large intestine is crucial for gaining a comprehensive understanding of the nutrient fate in the gastrointestinal tract. In vitro fermentation models are the most frequently used to simulate the human gut. They are considered excellent tools for being simple, fast, and reproducible [81,82]. In this thesis, we selected the in vitro batch model to assess the impact of dietary compounds having different microstructures and compositions, such as cooked beans and cooked tempeh, on metabolite formation by gut microbiota. These in vitro batch models are particularly valuable for studying the microbial fermentation of specific dietary compounds and assessing the resulting metabolites [82]. However, in vitro batch models have one significant drawback i.e. their inability to simulate the functionality of different colon regions accurately. This model uses a single closed system chamber where substrates and microbiota are combined under anaerobic conditions at the start of fermentation, and they typically involve short incubation periods. This limitation arises because undigested fractions pass through the small intestine and are transferred to various segments in the colon. Different colon segments exhibit distinct pH levels, substrate availability, microbial populations, and densities [83], which cannot be replicated effectively using batch fermentation models. Furthermore, batch simulations may constrain microbiota growth due to substrate depletion, pH reduction, and metabolite accumulation. This shift from the initial microbial balance in batch models can impact their relevance to in vivo conditions, especially in longer simulations [84]. As a result, batch models are typically restricted to incubation times of 24 to 48 hours [85]. To address these limitations and better simulate the complexities of the human colon, more sophisticated continuous in vitro models have been developed to understand colonic fermentation processes and their relevance to human physiology. These continuous models involve one or several interconnected pH-controlled chemostats that are inoculated with fecal microbiota and aim to simulate different regions of the human colon. Examples of dynamic models include the Reading model, PolyFermS, SHIME, TIM-2 [81] and the scalable MiGut set up [86].

6.5.3 Cooking time determinations

Cooking time is crucial in achieving the desired texture and palatability for bean consumption. Cooking time is one important parameter to distinguish HTC and non-HTC beans [87]. Depending on the cooking time, beans can
undergo chemical and microstructural alterations impacting nutrient digestibility, such as starch gelatinization, protein denaturation, cell separation due to pectin solubilization [10,88], and cell wall permeability increase [23]. Therefore, determining optimal cooking time is necessary from the perspective of the HTC and the nutrient quality. In this thesis, the finger press method was employed to determine the optimal cooking time as an indicator for HTC level (Chapter 2), comparing the effectiveness of pretreatment and cooking process (Chapter 3), and preparing beans for digestibility study (Chapter 4 and 5). This cooking method offers multiple benefits due to its simplicity, cost-effectiveness and efficiency. As a subjective method, finger press relies on individual preferences to determine cookability and requires training to ensure repeatability and reproducibility [89]. Despite the limitations, this method is still valuable for comparing samples, as presented in this thesis. Several objective methods are available, such as a texture analyzer and a Mattson bean cooker equipped with pointed plungers placed on the surface of bean seeds. There is an automated version of the Mattson device which can automatically record the time when the plungers drop [90]. In Chapter 3, we compared the subjective finger press method and the objective compression test to determine the optimal cooking times of Jack beans. The results indicated that the finger press method is reliable for determining Jack bean cooking times, as also reported in another legume [87].

6.5.4 Image quantification

Image analysis has gained prominence in scientific research due to accessible computational power and advanced digital camera technology to collect images at high resolution. This method has been used for quantifying various parameters such as polymer dimensions, cell area, nuclei count, fibronectin/protein content and co-localization [91–93]. Image analysis has the advantage of providing detailed insights into cellular and molecular phenomena, revealing distribution patterns at the individual cell level rather than generalizing across cell populations [94]. It allows experiments on limited sample tissues as starting material. In this thesis, we applied image analysis to confirm changes in intercellular spaces related to the increase of optimal cooking time in HTC beans (Chapter 2). We quantified the distance between adjacent cells to assess the impact of freeze-thaw procedures on the microstructural changes in beans (Chapter 3). Additionally, we demonstrated that image analysis is a viable method for evaluating starch granule size, exhibiting good comparability with the existing particle size analyzer techniques (Chapter 4). Successful image analysis depends on proper microscopy instruments and adequate sample preparation to achieve
sufficient image quality. Plant cells can vary in structure, size, and shape, depending on tissue location. Thus, it is essential to locate tissues at a similar developmental stage for meaningful sample comparisons. Moreover, it can be challenging to determine an appropriate parameter for quantification that aligns with the specific research question.

### 6.6 Nutritional Implication

Protein bioavailability, particularly from intact plant cell sources, is strongly influenced by the food structure (i.e. cell wall integrity and particle size) [95]. The combination of pre-digestion, and possibly an increase in accessibility of the matrix to digestive enzymes provided by fungal biomass (Chapter 5), may increase the protein digestibility in Jack bean. Nevertheless, this fermentation process can limit the starch digestibility. On top of that, the chemical composition showed that tempeh gives a higher content of minerals than unfermented beans. These findings imply that tempeh can be used as a strategy to combat triple-burden malnutrition, which refers to the coexistence of undernutrition, overnutrition, and micronutrient deficiencies [96]. The low starch digestion in tempeh has potential benefits, for example, for obese or diabetic patients who benefit from low glycemic index for carbohydrate management. Tempeh fermentation offers an affordable and relatively low-cost process of nutritious food and thus can be used as a suitable choice for people in developing countries who often rely on plant-based food for consumption.

### 6.7 Societal Implication

Food sovereignty is a concept and a movement that emphasizes the right of people to healthy and culturally appropriate food produced through ecologically sound and sustainable methods and their right to define their own food and agriculture systems [97]. The application of tempeh fermentation to Jack beans can be implemented to support this movement providing access to nutritious and sustainable protein sources for individuals and communities. On the other hand, tempeh fermentation approach can be extended as a model to other hard-to-cook legume varieties or any other legumes. Nowadays, tempeh processing is gaining popularity worldwide and is reported to be successfully applied to several bean varieties such as faba beans [98], kidney beans [46], chickpeas, and pigeon peas [99]. The use of local/diverse legumes reduces the reliance on specific food sources, which can be harmful to biodiversity, providing better food security. Thus, the fermentation technique demonstrates an ecologically sound and sustainable method by promoting
resource efficiency and supporting local communities. In addition, the application of tempeh processes can preserve cultural practices and knowledge related to food for Indonesian people.

Understanding the chemical and microstructural properties of Jack beans with different optimal cooking times is crucial to enable the development of new strategies, such as the freeze-thaw procedure, to reduce cooking times. The knowledge derived from these studies can have wider implications, especially in the context of resource optimization, particularly in developing countries. By identifying ways to reduce cooking times, researchers and communities can make more efficient use of available resources, which is particularly vital where there may be limitations in energy sources or time constraints. Moreover, the application of cooking time reduction strategies may not be limited to Jack bean alone and can be extrapolated to other legumes with long cooking time. This broader application enhances the generalization of the findings and makes them relevant to a wider range of agricultural and culinary practices. The wider adoption of Jack bean beyond Indonesia emphasizes the global potential of this research, as Jack bean is also available in other countries. If the benefits of reduced cooking times and optimized resource utilization can be extended to other regions, it could have a positive impact on combating food insecurity. Jack beans, or the strategies developed for them, could become part of a larger movement contributing to protein transitions, which is significant for addressing nutritional challenges on a global scale.

6.8 Future perspectives

Here, we proposed some possible future directions for further research based on the results of this thesis:

Chapter 2 showed that a significant portion of Jack bean pectins is covalently linked, indicating potential involvement from bound phenolic acids, including ferulic acid, in HTC bean. It is important to note that research on the role of phenolics bound to the cotyledon cell in HTC beans is still limited. Therefore, further investigations are required to study the role of phenolic crosslinks within the cell wall and their influence on textural changes. In Red haricot beans, it has been reported that several phenolic compounds, apart from ferulic acid, are involved in the HTC bean [12]. Future research should also aim to verify whether the phenolic complex exclusively binds to pectin fractions or extends to other polysaccharide fractions. Additionally, it is also crucial to understand how these linkages modulate structural changes during the cooking process.
Chapter 3 discussed how freezing treatment can induce cell separation, leading to a reduction in cooking time. However, we still do not know the effect of freezing treatment on the cytoplasmic matrix of HTC beans. Notably, the coalescence of protein and oil bodies has been reported in soybeans after freezing storage [18]. Investigation into the effect of freezing treatment on the cytoplasmic matrix of different types of legumes needs to be conducted, especially to link it with protein interactions during the hydrothermal process. Insights into protein interactions during the thermal process could help gain more understanding on protein digestibility. In addition, further research is essential to quantify the ecological impact of employing freeze-thaw techniques to reduce cooking times, such as the energy consumption and potential implications for water resources.

*Rhizopus* fermentation induced compositional changes in HTC Jack bean. However, it is still unclear how the microstructural changes due to this fermentation can affect protein digestion. Moreover, the effect of oral mastication on the particle size of fungal mycelium and such fermented products requires investigation. It is essential to consider and apply mechanical disintegration methods that mimic physiological oral mastication to simulate gastrointestinal tract digestibility better. Our results demonstrated that fermented beans exhibit lower starch digestion than unfermented cooked beans, suggesting further investigation on the structural modification of starch during fermentation. Nevertheless, an intervention study may be employed to confirm the physiological effects of consuming Jack bean tempeh in lowering glycemic index.
References


Summary

Sustainable food systems play a vital role in global food security, balancing social, economic, and environmental factors. The imminent rise in food demand due to the increasing world population raises concerns about heightened emissions and deforestation. Addressing these challenges requires a shift toward more sustainable food sources. The negative environmental impacts by animal protein source has driven the demand for alternatives, with legumes being nutrient-rich and recommended by the EAT-Lancet. While many legumes exist, only a few are commonly consumed. On the contrary, underutilized legume such as Jack bean has potential as a valuable protein source. The limited utilization of Jack beans is often due to the long cooking times and limited protein digestibility. This thesis aims to investigate the possible explanations of different optimal cooking times in Jack bean, explore freeze-thawing as an alternative method to reduce cooking times and assess the nutritional quality of several Jack bean collections and the effect of several processing techniques, including fungal fermentation.

In Chapter 2, we examined the pectin composition and the microstructural properties of three Jack bean collections with varying optimal cooking times. Jack bean with the longest cooking time (Jombang) exhibited a higher fraction of small intercellular spaces, indicating more cell wall connections. Longer optimal cooking times in Jack beans correlated with a lower concentration of water-soluble polysaccharide (WSP), a higher percentage of covalently bound pectin (NEF), and higher concentrations of bound ferulic acids.

Moving to Chapter 3, we selected one Jack bean collection and implemented freeze-thawing pretreatment as a strategy to reduce optimal cooking time. Various freezing conditions were tested, and the microstructure of the treated samples was examined. We found that slow freezing treatments at -20 °C produced a greater cell separation compared to fast freezing treatments at -80 °C. Beans stored at -20 °C for 24 hours demonstrated the shortest cooking time compared to other freezing treatments. We further selected this freezing condition and tested the in vitro protein and starch digestion. The shorter cooking times observed in freeze-thaw cooked beans increase protein digestibility compared to control beans, with no impact on starch digestibility. In addition, the shorter cooking time obtained by freeze-thaw treatments is sufficient to inactivate hemagglutination activity and limit mineral loss.
Starch and protein digestibility was further investigated in Chapter 4, where we included seven Jack beans collections with varying optimal cooking times and evaluated their microstructure and in vitro protein and starch digestion. We used kidney bean as a comparison. We found that digestibility of Jack beans proteins was lower than kidney bean and protein and starch digestion of Jack beans did not correlate with optimal cooking times. To isolate the effect of cooking from compositional differences among accession, we further selected one collection and evaluated the effect of different cooking times in protein and starch digestibility. Longer cooking times increase starch digestion, while protein digestion remained unaffected.

Chapter 5 focused on testing the effect of fermentation using Rhizopus oligosporus to produce Jack bean tempeh on nutritional composition and protein digestibility. The nutritional quality of jack bean tempeh was compared to cooked Jack bean. Additionally, we studied the behavior of the cooked bean and tempeh undigested fractions in an in vitro model of colon fermentation. We assessed the SCFAs, tryptophan metabolites, and GABA formation. Cooked tempeh showed higher protein content and lower starch content, while both fulfilling FAO's essential amino acids recommendations, except for sulfur-containing amino acids. An increase of protein but decrease in starch digestion was found in cooked tempeh compared to cooked beans. In the colon, cooked tempeh produced similar concentrations of SCFA and GABA and a lower concentration of Tryptophan metabolites than cooked beans.

Chapter 6 summarizes the findings and discusses their implication, along with limitations and future directions. This thesis shows that Jack beans protein digestibility can be improved by new approaches such as freeze-thaw before cooking or by using fermentation techniques such as in the production of tempeh. The undigested fractions of beans and tempeh showed similar potential for GABA and SCFAs production. Overall, the findings in this thesis help improving nutritional quality of Jack beans and promoting its consumption. This finding could aid in the development of strategies to enhance plant protein digestibility, particularly where there may be limitations in energy sources or time constraints such as on hard-to-cook beans and can guide further research on the effect of processing on nutritional quality of Jack beans and other underutilized legumes.
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About the author

Fiametta Ayu Purwandari was born on July 30, 1989 in Bantul, Indonesia. She obtained her BSc in Food and Agricultural Product Technology from Gadjah Mada University, Indonesia in 2010. She spent 6 months at Chalmers University of Technology, Sweden to conduct her BSc thesis on anaerobic digestion of oil palm empty fruit bunch. Fiametta pursued her MSc in Food Science and Technology at Gadjah Mada University with a fully funded scholarship from Indonesian Ministry of Culture and Education, completed in 2014. For her MSc thesis, she explored the impact of copigmentation and encapsulation on anthocyanin stabilization. During her studies, she worked as a lecturer assistant in the Microbiology lab and Food engineering lab.

She has been working as a lecturer in the Department of Food and Agricultural Product Technology, Gadjah Mada University in Food and Nutrition lab since July 2016. She has been involved in undergraduate courses such as nutrition science, quality control, sensory evaluation, product and process development, and packaging technology. Fiametta has received several research grants for the young lecturer competitive program, where she explored chemical characterization of Jack bean under different cooking methods and the optimization of tempeh process from local legumes. She was a member of Indonesian association of food technologist (PATPI).

In July 2019, she was awarded a scholarship and research funding from Indonesia Endowment Fund for Education (LPDP) to pursue a PhD degree in Food and Quality Design, Wageningen University and Research, the Netherlands. Her research project is entitled “Canavalia legumes as an indigenous resource to enhance food security in Indonesia”. The results of her research are presented in this thesis.

Fiametta enjoys spending time with her family and friends, as well as watching movies. She can be reached at fiametta@ugm.ac.id.
List of publications

List of Publications from this thesis:


3. **Purwandari, F.A.**, Gahari, R.S., Fogliano, V., Capuano, E., Freeze-thaw procedure as an alternative method to speed up the cooking time of Jack bean (*Canavalia ensiformis* (L.) DC) (Manuscript in preparation)

4. **Purwandari, F.A.**, Fogliano, V., Capuano, E., Tempeh fermentation improves nutritional and functional characteristics of Jack bean (*Canavalia ensiformis* (L.) DC) (Manuscript in preparation)

Other works:


# Overview of completed training activities

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<th>Discipline specific courses/conferences</th>
<th>Organizing Institute</th>
<th>Year</th>
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<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; NIZO Plant protein functionality conference</td>
<td>NIZO</td>
<td>2020</td>
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<td>35&lt;sup&gt;th&lt;/sup&gt; EFFoST conference&lt;sup&gt;a&lt;/sup&gt;</td>
<td>EFFoST</td>
<td>2021</td>
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<tr>
<td>Reaction kinetics in food science</td>
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<td>2021</td>
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<tr>
<td>Healthy food design</td>
<td>VLAG</td>
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<tr>
<td>Summer course: Tempe heritage food</td>
<td>IPB University</td>
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<td>Ecophysiology of food-associated microorganism and roles in health and disease</td>
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<tr>
<td>7&lt;sup&gt;th&lt;/sup&gt; International Conference on Food Digestion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>INFOGEST</td>
<td>2022</td>
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<td>Advanced food analysis</td>
<td>VLAG</td>
<td>2022</td>
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<th>General courses</th>
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<tr>
<td>PhD week</td>
<td>VLAG</td>
<td>2019</td>
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<td>Improve your English: self help guide</td>
<td>WGS</td>
<td>2019</td>
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<td>Project and time management</td>
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<td>Introduction to R</td>
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<td>Philosophy and ethics of food science and technology</td>
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<td>Brain friendly working and writing</td>
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<td>Applied Statistics</td>
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<td>Scientific writing</td>
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<td>Effective and efficient communication in academia and beyond</td>
<td>WGS</td>
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<th>Assisting in teaching and supervision activities</th>
<th>Organizing Institute</th>
<th>Year</th>
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<tr>
<td>FQD-21306 Cases of Food Packaging and Design</td>
<td>FQD</td>
<td>2020</td>
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<tr>
<td>FQD-24306 Case Studies on Product Quality</td>
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<td>2021</td>
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<tr>
<td>Thesis supervision (2 bachelor thesis, 4 master thesis)</td>
<td>FQD</td>
<td>2020</td>
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<tr>
<th>Other activities</th>
<th>Organizing Institute</th>
<th>Year</th>
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<tr>
<td>Preparation of research proposal</td>
<td>FQD</td>
<td>2019</td>
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<tr>
<td>Internal scientific meetings, seminars, colloquia</td>
<td>FQD</td>
<td>2019</td>
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<td>Organizing committee PhD trip 2020 (Cancelled due to COVID-19)</td>
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<td>Management committee</td>
<td>Indonesian PhD-Postdoc community</td>
<td>2021</td>
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<sup>a</sup>poster presentation; <sup>b</sup>oral presentation; VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences; WGS: Wageningen Graduate School; FQD: Food Quality and Design
Colophon

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