Pulse seeds as promising and sustainable source of ingredients with naturally bio-encapsulated nutrients: Literature review and outlook

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Abstract

Pulse seeds are nutritious and sustainable matrices with a high level of intrinsic microstructural complexity. They contain high quality plant-based protein and substantial amounts of slowly digestible starch and dietary fiber. Starch and protein in pulses are located inside cotyledon cells that survive cooking and subsequent mechanical disintegration, hence preserving natural nutrient bio-encapsulation. In this context, several authors have explored a number of techniques to isolate individual cotyledon cells from these seeds, aiming to unveil their digestive and physicochemical properties. In recent years, isolated pulse cotyledon cells are also being highlighted as promising novel ingredients that could improve the nutritional properties of traditionally consumed food products. Even more, they could enable to implement an strategy for increasing pulse intake in populations where these seeds have not been traditionally consumed.

This review mainly focuses on the reported digestive, physicochemical and techno-functional properties of pulse cotyledon cells isolated through different techniques, preceded by a descriptive summary of the nutritional properties, structural organization and traditional process chain of pulse seeds. It also offers an outlook of research directions to take, based on the identified research gaps. All in all, it is clear that isolation of pulse cotyledon cells using diverse techniques constitutes a promising strategy for the development of pulse-based ingredients where natural bio-encapsulation of macronutrients is preserved. However, much more research is needed at the level of ingredient characterization to better understand the effect of starting pulse seed material, isolation technique, and isolation conditions on the nutritional and functional properties of the finished product(s) where the isolated cells are (to be) used.

1. Introduction

The relationship between dietary choices, environmental sustainability and human health is an area of increasing interest around the world. Diets with an excessive intake of refined carbohydrates, added sugars, red meats and edible oils, and a low consumption of legumes, vegetables and fruits, are significantly contributing to the upsurge in prevalence of metabolic disorders like obesity, type II diabetes, and coronary heart disease (Popkin, 2015; Tilman & Clark, 2014). They are moreover imposing a great burden on the planet, as current food production practices are responsible for 19 to 29% of global greenhouse gas emissions (Vermeulen, Campbell, & Ingram, 2012). Therefore, food matrices exerting a beneficial effect on both human and planet health are being strongly recommended as staple foods for current and future human diets. Pulses (common beans, chickpeas, lentils, etc.) are particularly relevant macronutrient delivery matrices of the so called 'win-win diet' (healthy and environmentally sustainable) (Tilman & Clark, 2014; Willett et al., 2019). These sustainably grown and nutrient-rich seeds contain substantial amounts of carbohydrates (composed of starch, non-starch polysaccharides and particular oligosaccharides) and protein (Bravo, Siddhuraju, & Saura-Calixto, 1998; De Almeida Costa, Da Silva Queiroz-Monici, Pissini Machado Reis, & De Oliveira, 2006; Reyes-Moreno, Paredes-López, & Gonzalez, 1993), as well as a diversity of micronutrients and non-nutrient bioactive compounds (which can be regarded as positive, negative or both (Champ, 2002)).

Carbohydrates, proteins, and the other constituent of pulses are located in a native microstructural network of high complexity. The main nutrient storage centers of these seeds are the cotyledon cells, in which starch granules are embedded in a protein matrix surrounded by

63 strong primary cell walls that are glued together by the middle lamella (Berrios, Swanson, &
64 Cheong, 2006; Garcia, Lajolo, & Swanson, 1993; Hughes & Swanson, 1985; Marconi, Ruggeri,
65 Cappelloni, Leonardi, & Carnovale, 2000). The complex native microstructural arrangement of
66 pulse seeds plays a highly significant role during processing and subsequent human digestion
67 (Verkempinck, Pallares Pallares, Hendrickx, & Grauwet, 2020).

68 Unlike other plant-based foods, pulses cannot be consumed without having been subjected to
69 some sort of processing. In most cases, treatments to induce softening and particle size reduction
70 are applied. A characteristic feature of pulse seeds is that they retain their microstructural
71 assembling (*i.e.* cotyledon cells prevail) after being thermally processed up to palatable
72 conditions and subsequently subjected to *in vitro* or *in vivo* mechanical disintegration (Pallares
73 Pallares, Loosveldt, Karimi, Hendrickx, & Grauwet, 2019; Tovar, De Francisco, Bjorck, & Asp, 1991;
74 Würsch, Del Vedovo, & Koellreutter, 1986). Preservation of microstructural assembly has been
75 observed even after human digestion (Noah et al., 1998).

76 The microstructural arrangement of pulses after processing and during passage through the
77 gastro-intestinal tract makes them interesting candidates to deliver specific nutritional
78 functionalities, such as (s)low starch digestibility and significant amounts of resistant starch
79 (Granfeldt, Björck, Drews, & Tovar, 1992; Noah et al., 1998). Nevertheless, consumption of pulse
80 seeds is not extensively widespread given the long cooking times usually required and/or the lack
81 of diet diversification in populations where they have not been traditionally consumed. In this
82 context, incorporation of pulse-based ingredients preserving natural nutrient bio-encapsulation
83 to already existing food products is being put forward as a feasible alternative to increase

consumption of pulses while improving the nutritional profile of the finished product (Delamare et al., 2020; Edwards et al., 2020).

Pulse samples with promising potential as novel ingredients, consisting exclusively of individual cotyledon cells, have been generated by distinct authors in recent years using diverse isolation techniques (Bhattarai, Dhital, Wu, Chen, & Gidley, 2017; Dhital, Bhattarai, Gorham, & Gidley, 2016; Do, Singh, Oey, & Singh, 2019; Edwards et al., 2020; Gwala, Pallares Pallares, Pälchen, Hendrickx, & Grauwet, 2020; H. Li, Gidley, & Dhital, 2019; P. Li et al., 2020; P. Li, Zhang, & Dhital, 2019; Pallares Pallares, Alvarez Miranda, et al., 2018; Rovalino-Córdova, Fogliano, & Capuano, 2019, 2018; Xiong, Zhang, Dhital, Huang, & Fu, 2019; Xiong et al., 2018). As a result of these studies, a better understanding of the effect of isolation conditions on digestive, physicochemical and/or techno-functional properties of pulse cotyledon cells has been obtained. This review hence aims to provide an overview of the research progress on isolation and characterization of pulse cotyledon cells, as well as an outlook on future studies based on the identified research gaps. In order to gain a basic understanding of the starting pulse seed material, this review begins with a descriptive summary of the nutritional properties, structural organization and traditional methods employed to process pulse seeds.

2. Pulses: sustainable and nutrient-rich foods with a complex structural organization

According to the Food and Agricultural Organization of the United Nations (FAO), pulses are annual leguminous crops harvested solely for use as dry grains. This term excludes vegetable crops harvested for consumption in their green form (*e.g.* green beans and green peas), as well

as those used for sowing (clovers, alfalfa) and oil extraction (soybeans, peanuts). In short, all pulses are legumes, but not all legumes can be classified as pulses. The FAO recognizes 11 pulse types, *i.e.* dry beans, dry broad beans, dry peas, chickpeas, , pigeon peas, lentils, Bambara groundnuts, vetches, lupines and minor pulses such as winged and velvet beans (FAO, 1994). Around 96 million tons of pulses were produced in the world in 2017 (FAO, 2019). Approximately 68% of the total was generated in 10 countries. India continues to be (by far) the major producer of pulses in the world, followed by Canada, Myanmar, China, Russia, Australia, Nigeria, Brazil, US, and Ethiopia. It is clear from this list that production of pulses is nowadays occurring in both developing and developed countries. In terms of consumption, it has been reported that the total human intake of pulses has increased over the last 40 years. Nevertheless, it is believed that such rise is a consequence of population growth rather than an increase in pulse consumption per capita (Watts, 2011). The most important grain legume for direct human consumption worldwide is *Phaseolus vulgaris*, ordinarily known as common bean (Broughton et al., 2003).

2.1. Sustainability aspects

Pulses are sustainable crops. Compared to animal-based and other plant-based food products, legumes stand out as the most nutrient- and protein-rich foods, while exhibiting the lowest greenhouse gas emissions (van Dooren, Douma, Aiking, & Vellinga, 2017). It has been reported that the greenhouse gas emissions per gram protein of legumes and pulses can be, respectively, 250 and 150 times lower than those of ruminant meats (Nijdam, Rood, & Westhoek, 2012; Tilman & Clark, 2014). Pulses also require a significantly lower land use per kilogram protein than beef and lamb, although they have similar requirements as poultry and seafood from aquaculture (Nijdam et al., 2012). Additionally, legume crops are unique on their capacity to fix nitrogen from

the air thanks to the symbiotic relationship they have with *Rhizobium* bacteria located in their root nodules (Martinez, Pardo, Palacios, & Miguel, 1985; Nair, Patel, Singh, & Kaushik, 1979; Yadav, 1981). The fixed nitrogen can be used by the plant itself or by the soil (Das & Ghosh, 2012), reducing the need for fertilizers and favoring sustainable agriculture. Apart from providing nitrogen to the soil, the role of pulse crops on improving soil quality extends to the enhancement of other features such as aggregate stability, low compaction degree, internal nutrient cycling and biological activity (Ganeshamurthy, 2009).

2.2. Biochemical composition and nutritional quality

Pulses are also nutrient-rich foods. Different macro- and micro-nutrients are found in these seeds, in addition to distinct non-nutrient bioactive substances. Biochemical composition and nutritional quality of pulses will be discussed in this section.

2.2.1. Macronutrients

Carbohydrates and protein predominate in pulses, as can be observed in **Table 1**. On the contrary, their lipid content is rather low, though of high quality. In common beans, for example, essential unsaturated fatty acids constitute 65 to 87% of the total lipid content (Hayat, Ahmad, Masud, Ahmed, & Bashir, 2014; Reyes-Moreno et al., 1993). **Table 1** shows the typical composition of four pulse types consumed as staple foods in different regions of the world (Asian, Latin American and African countries (De Almeida Costa et al., 2006)). These four pulse types are also frequently used in studies where cotyledon cells are isolated by different techniques (*cfr.* **Table 2**). Given that biochemical composition depends on pulse variety, as well as on growing and harvesting conditions, further variation will be found within each pulse type if different varieties and/or (pre)harvesting circumstances are considered. In this regard, differences in chemical composition

at the level of both seed coat and cotyledons were found between desi and kabuli chickpeas and within desi genotypes (Wood, Knights, Campbell, & Choct, 2014).

The carbohydrate fraction of pulses is composed of starch and non-starch polysaccharides, as well as some oligosaccharides (Bravo et al., 1998). Of all carbohydrates, starch is the one present in highest amount (Hayat et al., 2014). In common beans, starch contents between 35 and 60% (dry basis) have been reported (Reyes-Moreno et al., 1993), whereas values ranging from 43 to 46% and from 30 to 34% have been stated for chickpeas and wrinkled peas, respectively (Klamczynska, Czuchajowska, & Baik, 2001). This glucose polymer, synthesized by the plant as carbon storage structure, constitutes the main energy source for human nutrition.

Starch is formed by amylose and amylopectin. These are polymers characterized by abundant presence of linear glucose chains joined by α (1,4) glycosidic bonds. In amylopectin, α (1,6) links among glucose units are also found, resulting in a branched molecular structure. In the small intestine, most relevant phase for carbohydrate digestion, endogenous intestinal enzymes (*i.e.* pancreatic α -amylase) cleave the α (1,4) linkages of starch polymers and release small oligosaccharides (maltose, maltotriose, α -limit dextrin), which are then converted to glucose in the brush border membrane of the small intestine by additional intestinal enzymes. Glucose released in the upper gastro-intestinal tract is absorbed and subsequently utilized during ATP production. The remaining undigested starch, known as resistant starch (RS), is transported to the large intestine where it is fermented by saccharolytic bacteria to form short chain fatty acids that contribute to colon health (McCrory, Hamaker, Lovejoy, & Eichelsdoerfer, 2010). Pulses are considered an important source of RS type I (enclosed within intact cells). Depending on their

170 processing and/or storage history, RS type II (native, non-gelatinized starch) and/or RS type III
171 (retrograded starch) can be also found.

172 The non-starch polysaccharide fraction of pulses includes dietary fibers. According to the CODEX
173 Alimentarius Commission, dietary fiber is the term used to define those carbohydrate polymers
174 that are not digested by endogenous enzymes in the human small intestine (Jones, 2014). Plant
175 cell wall polysaccharides like (hemi)cellulose, pectin and lignin are therefore classified as dietary
176 fiber, and so are indigestible carbohydrates (*e.g.* resistant starch) and oligosaccharides (*e.g.*
177 raffinose, branched α -limit dextrin). Pulses are an excellent source of dietary fiber. The total
178 dietary fiber content of dry peas ranges from 14 to 26%, while in common beans it varies between
179 23 and 32% and in chickpeas and lentils between 18 and 22% (Tosh & Yada, 2010). Given their
180 significant dietary fiber content (undigested starch and non-starch carbohydrate fraction), the
181 potential of pulses as delivery systems of 'feed' for the microbiota has been recognized in recent
182 years (Ercolini & Fogliano, 2018; Williams, Grant, Gidley, & Mikkelsen, 2017).

183 Proteins in pulses are mostly found in the form of salt-soluble globulins and water-soluble
184 albumins (Adebowale, Adeyemi, Oshodi, & Niranjana, 2007; Boye, Zare, & Pletch, 2010). The major
185 storage proteins in pulse seeds are the globulins legumin (hexameric quaternary structure) and
186 vicilin (trimeric structure) (Boye et al., 2010). Pulse proteins have been reported to contain
187 substantial levels of essential amino acids, demonstrating their potentially high nutritional quality
188 upon digestion. In common bean globulins, high percentages of aspartic acid, glutamic acid,
189 lysine and arginine are present, whereas sulfur containing amino acids (methionine and cysteine)
190 and tryptophan are rather scarce (Carbonaro, 2006). Fortunately, cereals (with low lysine
191 contents but a good source of methionine) are a perfect complement to pulses when it comes to

achieving an adequate intake of all essential amino acids. Thus, the incorporation of cereal- and pulse-based ingredients in a given formulation could eventually lead to a finished food product containing a complete profile of all essential amino acids.

2.2.2. Micronutrients and non-nutrient bioactive substances

Several minerals (calcium, iron, copper, zinc) and B-vitamins (folic acid, vitamin B₆, niacin, riboflavin, thiamine) are found in pulses (Hayat et al., 2014; Reyes-Moreno et al., 1993). Although minerals and vitamins can be present in significant concentration levels in raw seeds, their bioaccessibility and bioavailability could still be low after processing and consumption. For instance, it has been reported that the *in vitro* mineral bioaccessibility of Bambara groundnuts is low after processing despite their significant level of minerals on a dry weight basis (Gwala, Kyomugasho, et al., 2020). According to the same authors, populations relying on the consumption of Bambara groundnuts could suffer mineral deficiencies as a serving of this pulse seed hardly meets the daily recommended nutrient intake requirements.

Non-nutrient bioactive substances such as phytates, phenolic compounds, and enzyme inhibitors are also present in pulse seeds (Campos-Vega, Loarca-Piña, & Oomah, 2010; Champ, 2002). The metabolic effects of these bioactive substances may be regarded as positive, negative, or both (Champ, 2002). Enzyme inhibitors negatively affect protein digestibility, although they rarely survive thermal processing of pulses. Phytic acid and some phenolic compounds might reduce mineral bioavailability, but they are also believed to exert positive effects on human health. As reviewed by some authors, phytic acid can potentially protect against some cancers, while phenolic compounds have important antioxidant properties with positive effects on preventing

oxidative stress-related chronic diseases and age-related disorders (Campos-Vega et al., 2010; Rousseau, Kyomugasho, Celus, Hendrickx, & Grauwet, 2020).

2.3. Health-related effects

Multiple health benefits have been associated with the regular incorporation of pulses into the human diet. In their publication based on different research articles and meta-analyses, Hutchins, Winham, & Thompson (2012) offered an overview on the impact of common beans and other dry grain pulses on glycemic response and chronic disease risk in human subjects. According to the authors, extensive published evidence supports that consumption of pulses (100-160 grams of cooked seeds per day) results in reduced postprandial glucose levels as compared to most starchy foods. In this regard, a significant blood pressure lowering effect of dietary pulses has been described in human subjects with and without hypertension, based on a meta-analysis of eight isocaloric pulse intervention trials including 554 subjects (Jayalath et al., 2014). Additionally, the effect of pulse consumption on improving markers of longer-term glycemic control in diabetic and non-diabetic individuals was demonstrated in another meta-analysis of 41 randomized controlled experimental trials. Pulses alone, as part of low-GI diets, or as part of high-fiber diets were considered by the researchers (Sievenpiper et al., 2009). Finally, in an observational trial where consumption of beans was correlated with particular health parameters, Papanikolaou & Fulgoni (2008) found that bean consumers exhibited higher overall nutrient intake levels, lower body weights and waist circumferences, lower systolic blood pressure, and had lower risk of being obese as compared to non-consumers.

In relation to the impact of pulses on chronic disease risk, a significant inverse relationship between pulse consumption and risk of coronary heart disease and cardiovascular disease was

found for US men and women in an epidemiologic follow-up study (Bazzano et al., 2001). This could be associated with the known effect of pulses on decreasing LDL-cholesterol, one of the risk factors for cardiovascular disease (Anderson & Major, 2002).

Different mechanisms have been proposed to explain the reducing effect on blood sugar levels produced by pulses. The nutritional composition of these food matrices is one of them. More specifically, it has been stated that the high fiber and protein content of pulses, as well as the presence of bioactive compounds, are possibly playing a role (Hutchins et al., 2012). Furthermore, the high amylose content of starch in pulses could partially explain their (s)low digestibility, given the lower susceptibility of this molecule to digestion as compared to amylopectin. Besides nutritional composition, the structural organization of pulses is another key aspect that could help understand why these food matrices lower the glycemic response. In recent years, the fact that food nutritional functionality might be substantially affected by the way nutrients are organized within the food matrix is being acknowledged and explored. In other words, the importance of food structure on nutrient behavior during processing and digestion is now widely accepted. Therefore, the structural organization of pulses will be described in the following section.

2.4. Structural organization

Pulse seeds exhibit differences in terms of physical appearance; distinct sizes, shapes and colors can be distinguished among types (*e.g.* spherical chickpeas vs round, flat lentils) and varieties (*e.g.* red beans vs white beans). Despite these variations, their structural characteristics are similar. Pulses are dicots (seeds can be separated in two halves) consisting of three parts at the macrostructural level: seed coat, cotyledon, and embryo. They constitute, respectively, 8-16%,

80-90%, and 1-3% of the total seed based on weight percentage (Dueñas, Hernández, & Estrella, 2002; Shiga & Lajolo, 2006; Shiga, Lajolo, & Filisetti, 2003). The two major constituent parts, seed coat and cotyledon, will be discussed in more detail in the next paragraphs.

2.4.1. Seed coat

The seed coat, cover of the pulse seed, acts as a protective barrier against damage of the internal structure (Reyes-Moreno et al., 1993). In most grain legumes, the seed coat structure has a rather similar blueprint (Lush & Evans, 1980; Smýkal, Vernoud, Blair, Soukup, & Thompson, 2014). When observed in the cross section, the seed coat of pulses shows three characteristic and distinctive cell layers, from its outer to its inner portion: palisade, sub-epidermal, and parenchyma cell layers (Berrios et al., 2006; Garcia et al., 1993; Hughes & Swanson, 1985, 1986; Lush & Evans, 1980; Smýkal et al., 2014; Wood, Knights, & Choct, 2011). It has been reported that the parenchyma cell layer of the seed coat displays thickness changes along the seed growth process, which might indicate that this layer supplies nutrients to the growing cotyledons (Hughes & Swanson, 1985). The seed coat parenchyma cell layer of common beans exhibits maximum thickness ($\approx 200 \mu\text{m}$) during the period of greatest seed growth, whereas a total seed coat thickness of around $90 \mu\text{m}$ is reached in fully mature beans (Hughes & Swanson, 1985). For chickpeas, total seed coat thicknesses between 251 and $423 \mu\text{m}$ have been described (Wood et al., 2011).

In terms of composition, the cell walls surrounding seed coat cells in pulses are predominantly composed of cellulose, although appreciable amounts of hemicellulose and pectin have been also reported (Gdala, 1998; Shiga & Lajolo, 2006; Zhong et al., 2018). It has been stated that the higher cellulose (56-65%) and lignin (1.4-1.9%) contents of seed coat cell walls as compared to cotyledon cell walls confer the outer part of pulses rigidity and a substantial resistance to water

(Reyes-Moreno et al., 1993; Srisuma, Ruengsakulrach, Uebersax, Bennink, & Hammerschmidt, 1991). Hemicelluloses can also contribute to the lack of water solubility exhibited by seed coats from pulse seeds.

Besides non-starch carbohydrates, seed coats of pigmented pulses also contain major amounts of polyphenols (as condensed tannins); it has been reported that removal of seed coats in common beans reduces their tannin content by 68-95% (Deshpande, Sathe, Salunkhe, & Cornforth, 1982). Furthermore, calcium is quite abundant in the seed coats of common beans and Bambara groundnuts (Chigwedere, Nkonkola, et al., 2019; Gwala, Kyomugasho, et al., 2020).

2.4.2. Cotyledon

Cotyledons constitute the major part of pulses, on mass as well as on volume basis. Therefore, it is not surprising that pulse seed cotyledons are the main nutrient storage center of these matrices. Carbohydrates (starch and non-starch polysaccharides) and proteins are primarily located in this part of the seeds, together with phytates (the main phosphorous reservoir of plants), iron, zinc, and magnesium (Chigwedere, Nkonkola, et al., 2019; Deshpande et al., 1982; Gwala, Kyomugasho, et al., 2020). Calcium is also present, although in less proportion as compared to the seed coat (Chigwedere, Nkonkola, et al., 2019; Gwala, Kyomugasho, et al., 2020). **Figure 1** shows the microstructural organization of the cotyledon in raw common beans (Marconi et al., 2000) as a representative case of pulse cotyledons.

As can be observed on the left micrograph, pulse cotyledons are formed by parenchyma cells. In the same way as other plant matrices, these cells are fixed to each other by the middle lamella, an outer envelope in direct contact with the primary cell wall and shared by adjacent cells. It is clear from the figure that starch granules are found inside the cells. In pulses, starch granules are

301 embedded in a protein matrix consisting of a dense material and small individual protein bodies.

302 Starch granules can be elliptic and/or spherical, with a size range of 10-50 μm , while protein

303 bodies have spherical to oval shapes, with sizes between 1-10 μm (Berrios et al., 2006; Garcia et

304 al., 1993; Hughes & Swanson, 1985; Marconi et al., 2000). The surrounding protein matrix can

305 also be identified in **Figure 1**. According to Hughes & Swanson (1985), starch granules are present

306 throughout the entire seed growing period (increasing in size and number as maturation time

307 increases), whereas protein bodies are only observed in later stages.

308 Starch and protein in the parenchyma cells are bio-encapsulated by primary cell walls, which are

309 surrounded by the middle lamella. Pectin is an important constituent of the middle lamella as

310 well as of the primary cell wall. In the latter, hemicellulose, cellulose and lignin are also present.

311 The cell walls in the cotyledon cells of pulses are mainly composed of pectic substances (29-41%

312 in total, 19-33% hot water soluble polysaccharides) and hemicellulose (16-24%). They have lower

313 cellulose (26-31%) and lignin (0.4-0.6%) contents than seed coat cell walls, which confers a much

314 more flexible structure to the cotyledon cell walls (Brillouet & Carré, 1983; Reyes-Moreno et al.,

315 1993; Srisuma et al., 1991; Zhong et al., 2018).

316 Cell walls of pulse cotyledon cells are rich in pectin released by strong alkali solution, on top of

317 their considerable amounts of loosely bound pectin polymers solubilized by water (Shiga &

318 Lajolo, 2006). According to Chigwedere, Nkonkola, et al. (2019) and Yi et al. (2016), the water

319 soluble and Na_2CO_3 (alkali) extractable fractions of pectin in common bean cotyledons are

320 between 45-50% and 30-40%, respectively. These findings, related to the pectin fractions present

321 on pulse cotyledon cell walls, have been the basis of the isolation techniques recently used by

322 different authors to obtain individual cotyledon cells from several pulse seeds.

323 Pectin encompasses a very complex family of plant cell wall polysaccharides, rich in covalently
324 linked galacturonic acid units (Mohnen, 2008; Willats, McCartney, Mackie, & Knox, 2001). Of the
325 several domains it includes, homogalacturonan (HG), rhamnogalacturonan I (RG I) and
326 rhamnogalacturonan II (RG II) are the most abundant ones. HG is a linear homopolymer formed
327 by α (1,4) linked galacturonic acids, which can be methyl-esterified at the C-6 carboxyl group
328 and/or acetylated at the O-2 or O-3 position (Mohnen, 2008). HG can also be substituted by β -D-
329 xylose residues at the O-3 position, thus forming the substituted galacturonan known as
330 xylogalacturonan (XGA) (Caffall & Mohnen, 2009; Vincken et al., 2003). RG I is a branched pectin
331 domain with repeated units of a disaccharide formed by α -D-galacturonic acid and α -L-rhamnose
332 as backbone. Common side chains in RG I are different neutral and acidic oligosaccharides such
333 as arabinan and galactan, substituting some of the rhamnose residues of the backbone (Van
334 Buggenhout, Sila, Duvetter, Van Loey, & Hendrickx, 2009). Finally, RG II is a branched domain of
335 pectin with a backbone similar to that of HG, constituted by around 9 galacturonic acid residues
336 joined by α (1,4) bonds. Side chains of this pectin building block consist of 4 heteropolymeric
337 chains of known and consistent length (Willats et al., 2001). RG I and II are known as the 'hairy'
338 regions of pectin. In pulse seeds, the pectin-rich cotyledon cell walls are structurally characterized
339 by the presence of xylogalacturonan and arabinose-rich rhamnogalacturonan rather than
340 homogalacturonan (Brillouet & Carré, 1983; Gdala, 1998; Shiga & Lajolo, 2006).

341 As a graphical synopsis of the structural organization of pulse seeds, **Figure 2** schematically
342 depicts the different structural levels of pulses discussed before, including some non-discussed
343 features of the starch polysaccharide fraction (*i.e.* granular structural architecture).

It is known that the strength of plant tissues is controlled by the mechanical properties of the cell walls, as well as by cell-cell adhesion (*i.e.* middle lamella status) and internal cell pressure (Van Buggenhout et al., 2009). In turn, internal cell pressure is influenced by the properties of the bio-encapsulated nutrients, which are starch and protein in the specific case of pulse seeds. All these structural components can be modified during processing. As a matter of fact, starch gelatinization, protein denaturation and pectin solubilization will occur upon application of thermal treatments under high moisture conditions. Process-induced biochemical modifications will undoubtedly play a role on determining parameters such as texture, particle size, and/or tissue failure mode during *in vitro* (*e.g.* milling) or *in vivo* (*i.e.* human mastication) mechanical disintegration. Hence, next part of this review will be dedicated to describe the traditional processing techniques applied to pulses, with special attention to their effect on biochemical and structural attributes of these food matrices.

3. Processing of pulses: effect on biochemical and structural attributes

Pulses, as many other foods, are made up of a range of compounds whose assembly and interactions determine the structural organization of the food matrix. This, in turn, affects the sensorial properties perceived (*e.g.* hardness) and the bioavailability of nutrients achieved upon consumption (Singh & Gallier, 2014). In the case of foods that can be consumed raw, such as fruits and vegetables, the intrinsic structural organization is the major factor controlling their nutritional functionality. However, this is not the scenario for food matrices such as pulses. In these seeds, processing is a requisite to guarantee adequate conditions for consumption and enhance susceptibility to digestion. Since the natural structural organization of pulses will be (partially) destroyed and/or transformed as a result of processing, the events observed in a

subsequent digestion process will be due to a combined effect of intrinsic and process-induced structural attributes. Furthermore, the effect of processing on food structure will depend on the conditions applied. Understanding the influence of processing (conditions) on structure is therefore highly important. The three most traditional processing steps applied to pulse seeds, namely soaking, thermal processing, and mechanical disintegration, will be discussed in the following sections.

3.1. Soaking

This process is done with the purpose of facilitating hydration of dry pulse grains. By enabling contact between the seeds and soaking media in excess, water uptake takes place and the cooking quality is improved, *i.e.* the time required for achieving adequate texture during subsequent thermal processing is reduced (Martínez-Manrique et al., 2011). Different soaking media can be used, as well as distinct temperatures and times. The most commonly used solution for soaking pulses is water, either alone or in combination with cations and/or anions from dissociated salts. Temperature-time combinations for soaking range from several hours at ambient temperature (*e.g.* 16 hours) to less than an hour at elevated ones (up to 100°C) (Matella, Mishra, & Dolan, 2012).

It has been reported that soaking common beans in Na₂CO₃ or NaHCO₃ induces faster reduction of hardness during subsequent thermal treatment (Chigwedere et al., 2018; Njoroge et al., 2016). Similar results were reported for cowpeas soaked in water containing NaHCO₃, which showed a decreased cooking time (Ávila et al., 2015). This is likely to be due to the displacement of divalent cations like calcium, interacting with the pectin of the middle lamella, by the monovalent cations (Na⁺) present in the soaking media. On the contrary, incorporation of divalent cations during

soaking of common beans hinders softening during cooking (Njoroge et al., 2016). Additionally, it is believed that soaking common beans in distilled water increases the activity of cell wall enzymes such as rhamnogalacturonase, galactanase, and polygalacturonase. As a result, the degree of polymerization of RG I is reduced while the solubility of polygalacturonan and galactan increases. Such changes in the chemical composition of pectic polysaccharides improve their thermosolubility (Martínez-Manrique et al., 2011). This is likely to be the case for other pulse types as well. A more detailed description of the phenomena happening during this processing step can be found in a recent publication made by Chigwedere, Njoroge, Van Loey, & Hendrickx (2019).

3.2. Thermal processing

Application of hydrothermal treatments is probably the most commonly used technique at both household and industrial levels to improve safety and palatability of foods. Several studies where this type of treatments are used to process pulse seeds have been published in recent years, as summarized in **Table 2**. Although obtaining microbiologically safe foods is the main goal of thermal treatments, in this section the discussion will be focused on the changes it induces on structural and biochemical attributes of pulses. These attributes, significantly affected by process-induced *in planta* interactions, are highly important during ingredient selection and food formulation (Dhital, Brennan, & Gidley, 2019). First, the effect of this processing technique on cell wall properties will be described. Afterwards, thermally induced starch and protein modifications will be explained.

3.2.1. Cell wall changes

Thermal processing enhances tissue softening. Hardness reduction, a macroscopically detectable event, is closely related to alterations occurring at the microstructural level. In plant-based foods, the cell wall is the structure playing the most influential role during softening. More specifically, high temperature is responsible for solubilization of pectic polysaccharides present in the middle lamella and primary cell wall, therefore increasing ease of cell separation (Waldron, Smith, Parr, Ng, & Parker, 1997). It has been reported for common beans that the rate-determining mechanism for softening is related to pectin thermosolubilization in the middle lamella and/or cell wall, whereas the role of starch gelatinization is rather insignificant (Chigwedere et al., 2018). Prevalence of cell walls enclosing starch and protein after thermal processing and *in vitro* mechanical degradation of different pulses has been observed by distinct authors (Berg, Singh, Hardacre, & Boland, 2012; Dhital et al., 2016; Gwala et al., 2019; Noah et al., 1998; Pallares Pallares, Rousseau, et al., 2018; Rovalino-Córdova et al., 2018; Tovar et al., 1991; Würsch et al., 1986). A number of studies have also evaluated the effect of thermal processing intensity (*i.e.* time) on the sensitivity of (post-harvest stored) pulses towards cell breakage/separation upon application of thermal processes followed by *in vitro* mechanical disintegration (Chigwedere et al., 2018; Gwala et al., 2019; Pallares Pallares, Rousseau, et al., 2018).

3.2.2. Starch changes

Another phenomenon induced by thermal processing is starch gelatinization. During this process, starch loses its pseudo-crystalline structure. When free starch granules are heated in presence of excess water, they start to hydrate and swell. At a certain temperature range, the rates of hydration and swelling increase considerably and a small amount of starch solubilizes. Further

temperature increase results in extensive leaching and collapse of the granular structure due to melting of crystallites, unwinding of double helices and breaking of hydrogen bonds (Ratnayake & Jackson, 2008; Wang, Li, Copeland, Niu, & Wang, 2015). The events happening during starch gelatinization might however get disturbed in food structures where starch is bio-encapsulated. According to Chigwedere et al. (2018), presence of cell walls in common bean cotyledon cells probably restricts the unwinding of double helices and subsequent pasting during starch gelatinization. However, it seems that swelling and initial disruption of the native molecular order of starch granules are not completely hindered. It was suggested in another study where different pulse types were considered that the cell wall limits the swelling of bio-encapsulated starch granules and hinders the melting of starch ordered structures (Dhital et al., 2016). The peak temperature of starch gelatinization in pulses is higher than the peak gelatinization temperature of most cereals (Edwards et al., 2015). In general, starch gelatinization is associated with improved starch digestibility.

3.2.3. Protein changes

A third event happening during high temperature processing of pulses is protein denaturation, related to the disruption of the protein native structure. Denaturation involves protein unfolding, which in turn favors the occurrence of intra- and inter-molecular interactions such as disulfide bridges (Drulyte & Orlie, 2019). These interactions sometimes give place to aggregation of the unfolded proteins, thus resulting in a loss of solubility. In this context, a decrease in protein solubility was reported in faba beans, lentils, chickpeas, and dry beans after autoclaving, compared to the raw seeds (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997; Carbonaro, Vecchini, & Carnovale, 1993). In relation to the effect of structural organization, it has

been stated that encapsulated proteins might exhibit an enhanced thermal stability compared to proteins in solution, as a result of macromolecular crowding and/or molecular confinement (Eggers & Valentine, 2001). On the whole, protein digestibility is positively impacted by denaturation whereas it is diminished when aggregation occurs. Cross-linked, aggregated proteins are less accessible to digestive enzymes (Drulyte & Orlie, 2019).

3.3. Mechanical disintegration

Wood & Malcolmson (2011) have defined three types of particle size reduction processes relevant for pulses, namely dehulling, splitting, and grinding. The phenolic content of pulses is significantly reduced when their seed coat is removed, as demonstrated by the loss of flavonoid compounds after dehulling of common beans, lentils, and dry peas described in literature (Deshpande et al., 1982; Dueñas, Estrella, & Hernández, 2004; Dueñas et al., 2002). Furthermore, dietary fiber and some minerals are partially removed as a result of this process. Regarding water uptake during soaking and cooking of common beans, faster rates have been found in cotyledons alone as compared to whole seeds (Chigwedere et al., 2018; Kinyanjui et al., 2015). In some occasions, dehulling is followed by seed splitting to produce *dhal*, or two separated cotyledons; pulses in countries like India are mostly consumed in this form (Wood & Malcolmson, 2011).

Dehulling and splitting are not likely to induce substantial modifications on the microstructural organization of pulses. In contrast, grinding or milling is a mechanical disintegration technique that affects microstructure while reducing particle size. Two characteristic failure modes, cell separation and cell breakage, are common to edible plant tissues when they are ground. Of course, a combination of both modes can also be observed. Predominance of cell breakage over cell separation is a result of stronger cell-to-cell adhesion forces as compared to the cell walls

themselves, and *vice versa* (Van Buggenhout et al., 2009; Waldron, Parker, & Smith, 2003). In this regard, it is important to mention that the sequence of processing techniques determines the predominant tissue failure mode, which subsequently can have consequences for later functionalities such as nutrient digestion. On the one hand, milling of raw pulses results in disruption of parenchyma cells and release of intracellular material. Upon pectin solubilization as a result of thermal processing, on the other hand, an evolution towards cell separation is observed, thus assuring significant levels of nutrient bio-encapsulation (Chigwedere et al., 2018; Gwala et al., 2019; Pallares Pallares, Rousseau, et al., 2018). In general, preservation of intact cell walls limits and/or delays digestion of the bio-encapsulated macronutrients. Yet, the barrier role of cell walls depends on intrinsic factors like plant species (having distinct cell wall composition and/or thickness) and extrinsic factors such as the level of cellular integrity maintained during processing, mastication and transit through the gastrointestinal tract (Grundy et al., 2016). Particle size reduction of pulses can be performed *in vivo*, as it is the case during mastication of whole cooked seeds, to reach adequate dimensions for gastrointestinal digestion. Furthermore, this mechanical disintegration process can also occur at different stages of the food process chain (*i.e.* raw or thermally treated pulses can be used). *In vitro* techniques of particle size reduction are normally used for the generation of pulse-based ingredients.

3.3.1. *In vivo* process

When extensive mechanical disintegration of pulses takes place during the first step of human digestion, mastication becomes a relevant operation for particle size reduction and microstructural modification of these seeds. Granfeldt, Björck, Drews, & Tovar (1992) performed an investigation where different starch-rich foods, including pulse seeds, were subjected to

human mastication. Nonetheless, the focus of such research did not cover the effect of mastication behavior or processing conditions on the microstructural properties of the generated boluses. To the best of our knowledge, only one study evaluating the role of human mastication on the (micro)structural properties of boluses from palatable, thermally treated pulse seeds is currently available (Pallares Pallares et al., 2019). This study was performed using common beans as a representative case of the group of pulses. The findings obtained, however, might also be expected in other pulse types.

Pallares Pallares et al. (2019) evaluated the influence of distinct process-induced hardness levels on the (micro)structural properties of *in vivo* generated boluses. To this end, a mastication study where 16 participants were asked to chew common beans after application of thermal treatments (95°C) of varying duration was executed, and the particle size distribution (PSD) of the *in vivo* generated boluses was determined. Five distinct cooking times, resulting in 5 different but palatable hardness levels, were considered. As shown in **Figure 3** (peak around 100 µm in left panel), the most characteristic fraction in all boluses was made up of individual cotyledon cells, regardless of process-induced hardness level or mastication behavior. Nonetheless, this characteristic fraction increased as process-induced hardness level decreased, linked to the anticipated higher pectin solubilization in samples with longer exposure times to high temperature. Partial least square regression analysis of the PSD data (right panel in **Figure 3**), in combination with repeated measures ANOVA, revealed that process-induced hardness had a statistically significant effect on the particle size distribution of oral boluses, while individual mastication behavior did not. Thus, the potential of thermal processing conditions as a tool to affect the (micro)structural properties of pulse seeds upon consumption was demonstrated.

Furthermore, it was also proved that process-induced changes to the starch-surrounding barriers (cell wall and/or protein matrix) were the main factors responsible for the patterns of *in vitro* starch digestion kinetics of oral boluses, given the predominant presence of individual cotyledon cells (Pallares Pallares et al., 2019).

3.3.2. *In vitro* techniques

Milling of raw seeds is a traditionally used step during generation of pulse flours, which can then be used as food ingredients in different preparations at household or industrial level. Nevertheless, several aspects required to properly control the milling process of pulses and obtain consistent high-quality ingredients are still unknown. The effect of seed coat content, as well as of seed size, hardness, and composition (*e.g.* cell wall content and thickness) on milling behavior are some of these unknown aspects (Thakur, Scanlon, Tyler, Milani, & Paliwal, 2019). Yet, pulse flours obtained from raw seeds have been already incorporated in distinct food products, mostly as partial or total replacers of wheat flour (Fujiwara, Hall, & Jenkins, 2017; Giuberti, Gallo, Fiorentini, Fortunati, & Masoero, 2016; Laleg, Cassan, Barron, Prabhasankar, & Micard, 2016; Zucco, Borsuk, & Arntfield, 2011).

Several sensorial and techno-functional aspects of the products developed using pulse flours have been evaluated. In relation to sensorial aspects, some authors state that consumers cannot detect differences between control samples and those containing pulse flours (Fujiwara et al., 2017), while others have reported that consumers have a major preference for food products without pulse addition (Zhao, Manthey, Chang, Hou, & Yuan, 2006). Regarding techno-functional properties, pastas formulated (partially) using pulse flours as ingredient have been described to be less cohesive and springy than cereal gluten-free pastas (Laleg et al., 2016), as well as to exhibit

540 lower overall quality and increasing firmness, pulse flavor, and color intensity when higher
541 percentages of pulse flour are used (Zhao et al., 2006). In the case of pulse-enriched cookies,
542 varying effects were identified depending on the particle size of the pulse flour used as
543 ingredient. Fine flours significantly increased product hardness and thickness whereas coarse
544 flours did not (Zucco et al., 2011). Although nutritional functionality was not evaluated by these
545 authors, coarse pulse flours might have offered an additional benefit in this regard, specifically a
546 lower glycemic index (GI). This hypothesis is supported by the expected presence of intact cells
547 and/or cell clusters in coarse pulse flours, based on the reported mean particle size for this
548 ingredient (150-190 μm (Zucco et al., 2011)). Fine pulse flours, on the contrary, seemed to be
549 mostly composed by free starch granules and other free cellular material (17-23 μm (Zucco et al.,
550 2011)).

551 Flours from distinct pulse types have been used as wheat replacers in several food products,
552 which has made evident that different outcomes in sensorial and techno-functional properties
553 are possible depending on the raw seed selected to generate the pulse flour ingredient (Zhao et
554 al., 2006; Zucco et al., 2011). All in all, it can be stated that a careful selection of pulse type, flour
555 particle size, and flour amount should be made depending on the type of product to be
556 formulated using pulse flours as (replacing) ingredients. Even though a number of sensorial and
557 techno-functional properties of pulse flours and the products developed with them need to be
558 improved, it has to be highlighted that products where pulse flours have been incorporated show
559 additional benefits from a nutritional point of view (lower GI, higher protein and fiber content)
560 when compared against their 100% cereal-based counterparts; besides, they offer an interesting

substitute for consumers with certain allergies such as gluten intolerance (Fujiwara et al., 2017; Giuberti et al., 2016; Laleg et al., 2016; Zucco et al., 2011).

According to Zhao et al. (2006), utilization of preheated pulse flours of diverse particle sizes could be a strategy to improve the overall quality of pulse-enriched products. In effect, raw pulse seeds are not the only material from which pulse-based ingredients can be obtained. A treatment to prepare pulse powders where nutrient bio-encapsulation is preserved was suggested very recently (Edwards et al., 2020). This unconventional treatment relies on the generation of powders from thermally processed pulse seeds which, after being cooked at 100°C, are mechanically disintegrated, sieved, and dried (Edwards et al., 2020). The potential of thermal processing followed by wet sieving as a strategy to isolate individual cells from pulse cotyledons had been previously highlighted in literature by different authors (Berg et al., 2012; Dhital et al., 2016; Pallares Pallares, Alvarez Miranda, et al., 2018; Tovar, Bjoerck, & Asp, 1990). This technique, on the contrary, is not effective to isolate endosperm cells from cereals like wheat and sorghum, as it results in major damage of cellular integrity (Bhattarai, Dhital, Mense, Gidley, & Shi, 2018). Differences in composition and/or thickness between cereal endosperm cell walls and pulse cotyledon cell walls could explain the higher fragility of cereal cells (Bhattarai et al., 2018) resulting in the lack of nutrient bio-encapsulation by cells wall after thermal treatment of the latter systems.

The prevalence of the cellular structure in pulse-based ingredients is becoming an important target parameter for food technologists working on the development of innovative food products. Likewise, the most characteristic microstructural fraction of palatable pulses after *in vivo* mechanical disintegration is constituted by individual cotyledon cells. As mentioned before,

modulation of nutrient release during human digestion could be achieved by preserving their bio-encapsulation after processing and mechanical disintegration of (pulse-based) foods. In other words, individual pulse cotyledon cells represent a highly relevant fraction in the context of controlled nutrition either when consuming whole pulse seeds or when these are used to obtain novel functional ingredients. Different authors recognize this premise nowadays and have been working on different ways to isolate cotyledon cells from pulses, aiming to achieve interesting digestive and/or techno-functional properties. Therefore, next part of this review will focus on these aspects.

4. Cotyledon cells from pulse seeds: digestive, physicochemical and techno-functional properties as influenced by isolation conditions

The first reports on prevalence of microscopically intact cotyledon cells after cooking of pulses trace back to the publications made by Würsch et al. (1986) and Tovar et al. (1990). An inactive period of around 20 years took place after these publications, until the studies of Berg et al. (2012) and Dhital et al. (2016) sparked renewed scientific interest in this research topic. As from 2016 (declared by the General Assembly of the United Nations as the International Year of Pulses), the number of scientific publications related to cotyledon cells isolated from pulse seeds has considerably increased. Thereby, major attention will be given in this part of the review to studies published throughout the last five years. In more detail, an integrated analysis of the processing conditions used for isolation and their influence on digestive, physicochemical and techno-functional properties of the generated samples will be provided. With regard to isolation aspects, three approaches have been used, namely thermal treatment above starch

gelatinization temperature (cooking), thermal treatment below starch gelatinization temperature, and acid/alkali treatment at room temperature. These will be widely discussed in the following sections. Furthermore, information on secondary processing of the isolated cells alone (to evaluate specific sample properties) or incorporated as novel ingredients in finished food products (to assess their effect on product properties) will be presented when available. **Figure 4** shows a graphic summary of the structural properties of cells isolated using the abovementioned techniques, whereas **Table 2** contains more detailed information on the processing conditions used for isolation and further processing of pulse cotyledon cells as based on the literature screened.

4.1. Isolation of pulse cotyledon cells via thermal treatment above gelatinization temperature

As mentioned before, thermal treatments above starch gelatinization temperature are usually applied to pulse seeds in order to improve their textural and digestive properties. The major changes occurring to the cell wall, starch, and protein components of pulses during this type of treatment were described in general terms earlier in this review. In this section, specific studies where thermal treatments reproducing household cooking conditions (90-100°C) have been used to isolate pulse cotyledon cells will be reviewed in more depth.

4.1.1. Digestive properties

Most studies where cotyledon cells have been isolated after cooking of pulses focus on *in vitro* starch digestion. Usually, the main interest is to understand the role of physical starch-surrounding barriers (cell wall and protein matrix) on the breakdown of the carbohydrate fraction at the small intestinal level. Different approaches have been followed to this end, including

626 evaluation of intact isolated cells versus isolated cells subjected to mechanical disintegration
627 and/or enzymatic cell wall degradation (Bhattarai et al., 2017; Dhital et al., 2016; Rovalino-
628 Córdova et al., 2018). Moreover, cooking time has been evaluated as a processing condition with
629 potential to modulate the starch digestive behavior of isolated pulse cells (Pallares Pallares,
630 Alvarez Miranda, et al., 2018). Very recently, the effect of hardness as a suitable food design
631 attribute to control starch and protein digestibility of pulse cotyledon cells was also highlighted
632 (Gwala, Pallares Pallares, et al., 2020). To this end, seeds within a specific processing time were
633 sorted based on hardness prior to the steps related to cell isolation. Cotyledon cells isolated from
634 various pulse types have been evaluated in some studies (Bhattarai et al., 2017; Dhital et al.,
635 2016; Gwala, Pallares Pallares, et al., 2020; Xiong et al., 2018), whereas other authors have
636 focused on cells isolated from cooked common beans only due to the importance of this specific
637 pulse in terms of production and consumption worldwide (Pallares Pallares, Alvarez Miranda, et
638 al., 2018; Rovalino-Córdova et al., 2018, 2019).

639 In general, two overall mechanisms for limitation of amylase action on starch are acknowledged,
640 namely presence of physical barriers encapsulating the substrate and starch structural features
641 in the absence of additional physical barriers (Dhital, Warren, Butterworth, Ellis, & Gidley, 2017).
642 Xiong et al. (2018) characterized the *in vitro* starch digestion kinetics of intact cotyledon cells
643 isolated from four pulse types, as well as some structural features (crystallinity, thermal
644 parameters) of the bio-encapsulated starch. No significant correlation was found between the
645 starch structural features and the kinetic parameters of *in vitro* starch digestion, leading to the
646 conclusion that starch digestibility in pulse cotyledon cells is purely controlled by the access
647 and/or binding of amylases to the starch substrate, which is in turn determined by process-

648 induced cell wall and/or protein matrix characteristics. This conclusion is in agreement to earlier
649 reports from other studies where cooking was used to isolate pulse cotyledon cells (Dhital et al.,
650 2016; Pallares Pallares, Alvarez Miranda, et al., 2018). In relation to the digestive behavior of the
651 four pulse types evaluated, variations in the rate and extent of starch digestion were identified
652 (Xiong et al., 2018). These variations could be the result of (i) intrinsic properties of each pulse
653 type, such as cell wall composition and structure or starch to protein ratio, and/or (ii) distinct
654 process-induced properties (*e.g.* different hardness values, possibly reflecting differences at the
655 microstructural level as well) resulting from the application of a same processing time to pulse
656 seeds with distinct initial features like size or initial hardness.

657 Differences among cotyledon cells from distinct pulse types in terms of starch digestion kinetic
658 parameters were observed as well by Bhattarai et al. (2017) and Dhital et al. (2016). Yet, the
659 hindering role of physical starch-surrounding barriers on starch digestion kinetics was
660 demonstrated by these authors regardless of pulse type after mechanical disintegration of the
661 isolated cells, which gave place to a significant increase in both digestion rate and extent. A
662 similar conclusion was reached by Rovalino-Córdova et al. (2018) when comparing intact
663 common bean cotyledon cells against those to which an enzymatic treatment with cell wall
664 degrading enzymes was applied before simulated digestion. Specifically, the starch digestion rate
665 of isolated cells was lower than that of enzymatically treated samples, which in turn exhibited a
666 lower digestion rate than mechanically disintegrated cells. This could be explained by the fact
667 that enzymatically treated cells retained structural properties of the cytoplasmic matrix (*i.e.*
668 cellular contents remained together after the treatment), whereas in mechanically treated cells
669 both the cytoplasmic matrix and the cell wall were completely disrupted. Finally, and not

670 surprisingly, free starch isolated from pulse seeds (*i.e.* a system where almost no cell wall or
671 protein material was present) exhibited the highest rate of carbohydrate breakdown (Rovalino-
672 Córdova et al., 2018; Xiong et al., 2018). Starch hydrolysis of this starch-rich system was however
673 reduced upon addition of cell wall components, which is attributable to the non-catalytic binding
674 of amylase to cell wall material (Bhattarai et al., 2017). This implies that the cell wall is not only
675 a physical barrier for diffusion of amylases but can also restrict starch digestion in pulse cells by
676 interacting with starch-degrading enzymes.

677 Even after diffusing through the cell wall, amylases will still encounter an additional barrier
678 preventing the immediate encounter with their substrate. In this regard, a recent study evaluated
679 the role of the intracellular protein matrix on *in vitro* starch digestion kinetics of common bean
680 cotyledon cells by systematically adding specific digestive enzymes during simulated digestion
681 experiments (Rovalino-Córdova et al., 2019). It was found that the rate and/or extent of starch
682 digestion were significantly reduced when one or more proteases were excluded from the
683 digestion protocol, hence demonstrating the barrier role of the starch-surrounding protein
684 matrix. The reduction effect was more pronounced in absence of small intestinal proteases
685 (trypsin and chymotrypsin) than in absence of pepsin (gastric protease). Expectedly, the lowest
686 amounts of starch digestion products were quantified in absence of all proteases. This last finding
687 also revealed that some of the starch bio-encapsulated in isolated pulse cotyledon cells is readily
688 available to amylases even when the surrounding cell wall and protein matrix seem to be intact
689 (Rovalino-Córdova et al., 2019). As a matter of fact, intactness of the physical barriers present in
690 isolated pulse cotyledon cells is normally evaluated using microscopic techniques, which does
691 not rule out potential damages at smaller length scales.

All of the abovementioned studies were performed using cotyledon cells isolated after cooking pulses for a fixed time (usually 60 min, see **Table 2**). Nevertheless, household cooking of pulses can have distinct intensities depending on consumer preferences in relation to textural properties. These, in turn, could have an influence on the digestive behavior of cooked pulse seeds. Hence, the impact of cooking time on the digestive properties of cotyledon cells isolated from common beans was evaluated for the first time by Pallares Pallares, Alvarez Miranda, et al. (2018). Despite looking highly similar from a microscopic and particle size point of view, the isolated cells showed variations in terms of digestive potential. It was demonstrated that modulation of *in vitro* starch digestion kinetics in common bean cotyledon cells is possible by varying the intensity of the thermal process (95°C) applied before cell isolation. More specifically, process-induced modifications of the starch-surrounding barriers governed the kinetics observed under simulated digestion. In detail, the rate-controlling mechanism for *in vitro* starch digestion of common bean cotyledon cells isolated after shorter processing times (*e.g.* 30 min) seemed to be the diffusion of pancreatic α -amylase through the cell wall, while the compactness of the cytoplasmic matrix where protein and starch co-exist appeared to be more important at longer processing times (Pallares Pallares, 2019).

The effect of processing time on digestive properties was also evaluated in a recent study where *in vitro* starch and protein digestion kinetics of cotyledon cells isolated from Bambara groundnuts was determined (Gwala, Pallares Pallares, et al., 2020). In a similar way as observed for common bean cells, the rate constant of *in vitro* starch digestion was higher for cells isolated after a longer cooking process. Nevertheless, no differences were observed at the level of the lag phase at early digestion times, which led to the conclusion that similar diffusion of pancreatic α -amylase

714 through the cell wall occurred regardless of cooking time. Hence, the observed differences in
715 starch digestion kinetics of Bambara groundnuts cotyledon cells were mainly attributed to the
716 starch-surrounding protein matrix (Gwala, Pallares Pallares, et al., 2020). In the same study, seeds
717 from a specific cooking time were sorted based on hardness aiming to understand the effect of
718 hardness categories from the same cooked population on starch and protein digestibility.
719 Expectedly, cotyledon cells isolated from low-hardness seeds of a specific cooking time exhibited
720 higher rates and/or extents of starch digestion than their high-hardness counterparts.
721 Interestingly, cells isolated from seeds of equivalent hardness but generated by application of
722 distinct cooking times showed the same starch digestion behavior, thus demonstrating the
723 valuable role of hardness as food design parameter to modulate digestive responses of pulse
724 cotyledon cells (Gwala, Pallares Pallares, et al., 2020). In this regard, it would be interesting to
725 study whether cotyledon cells obtained from distinct pulse types processed up to equivalent
726 hardness values have similar digestion patterns.

727 All in all, it is known up to date that both the cell wall and protein matrix surrounding starch in
728 cotyledon cells isolated from cooked pulses play an important role on *in vitro* starch digestion
729 kinetics. Nonetheless, an ultimate demonstration of the individual role of each barrier has not
730 yet been provided in open scientific literature, mainly due to the difficulties associated with
731 complete elimination of one barrier or the other. It is however fully clear that starch digestion on
732 these systems occurs inside the cellular space, as can be observed in **Figure 5** for the specific case
733 of common bean cotyledon cells stained with an iodine solution to visualize starch. Substrate
734 disappearance became evident in the remaining digested pellet as digestion time progressed,
735 while cell shape and size seemed stable. In relation to this, it was recently demonstrated through

real-time microscopic observations that navy bean cotyledon cells maintain cell shape and circularity when undergoing cooking (90°C) and simulated gastro-intestinal digestion (Do et al., 2020). **Figure 5** also shows the effect of processing time on starch digestion of common bean cotyledon cells, namely faster emptying and discoloration in cells isolated after longer cooking times.

The protein matrix present in pulse cotyledon cells is undoubtedly a physical barrier for *in vitro* starch digestion, as described before. Yet, these proteins could also be seen as a macronutrient playing a very significant role in (future) diets where alternative protein sources are to be included to maintain human and planet health. A few authors have lately evaluated the *in vitro* protein digestion of cotyledon cells isolated from cooked pulses (Bhattarai et al., 2017; Gwala, Pallares Pallares, et al., 2020; Rovalino-Córdova et al., 2019), in parallel to the main goal of understanding starch digestion. Similarly to starch, proteins are bio-encapsulated in pulse cotyledon cells, but only by the cell wall.

According to Bhattarai et al. (2017), the kinetic parameters of protein digestion were significantly higher in mechanically broken cellular systems as compared to intact cells isolated from distinct pulse types. As expected, the cell wall also constituted a physical barrier for protein breakdown. Furthermore, the authors confirmed that pancreatic proteases have a lower affinity for non-catalytic binding to cell wall components than pancreatic amylase. In other words, presence of cell wall material also decreased protein hydrolysis, but this reduction occurred to a much lesser extent than for starch hydrolysis. In relation to the role of the cytoplasmic matrix on protein digestion, it has been stated that the presence of undigested starch granules does not affect amino acid release in the small intestinal phase, contrary to the hindering effect of undigested

758 proteins on carbohydrate breakdown (Rovalino-Córdova et al., 2019). This seems to indicate that
759 (strong) starch-protein interactions are not present in pulse cotyledon cells, and that the protein
760 matrix mostly acts as a physical barrier protecting starch from enzymatic attack in these cellular
761 systems.

762 Although protein digestion of pulse cotyledon cells can take place in the gastric as well as in the
763 small intestinal compartment, intestinal proteases (trypsin and chymotrypsin) are much more
764 effective on digesting this macronutrient than the gastric protease pepsin. Rovalino-Córdova et
765 al. (2019) reported a 50% protein digestion after 360 min of a simulated gastro-intestinal
766 digestion experiment where pepsin was not included, whereas only 5% digestion was measured
767 upon addition of pepsin but not of trypsin and chymotrypsin. These results could be due to
768 differences in terms of enzyme specificity, mode of action, and/or conformational state of
769 proteins. In fact, most of the amino acids found in common beans are a preferred substrate for
770 trypsin (Rovalino-Córdova et al., 2019). Yet, it could still be the case that pepsin breaks down the
771 enclosed proteins to some extent during the gastric phase, although such action does not result
772 in a significant amount of small, soluble digestion products.

773 Gwala, Pallares Pallares, et al. (2020) have recently suggested a more complete analytical
774 approach to evaluate protein digestion in pulse cotyledon cells. Specifically, they have
775 established that a better understanding of the barrier role of the protein matrix for starch
776 digestion can be obtained when the amount of digested soluble protein is determined next to
777 the normally reported values of bioaccessible protein. According to these authors, quantification
778 of the digested soluble protein fraction in the supernatant allows to gather information on all
779 soluble protein fragments resulting from the action of digestive proteases, instead of amino acids

and small peptides only (as traditionally determined when measuring the bioaccessible protein fraction). Following this approach, around 29% of digested soluble protein was reported for Bambara groundnut cotyledon cells at the beginning of the small intestinal phase, hence demonstrating that a quite significant removal of starch-surrounding protein takes place during the simulated gastric phase (Gwala, Pallares Pallares, et al., 2020).

Protein digestion in pulse cotyledon cells can also be qualitatively evaluated by microscopic analysis. For instance, we detected at our research unit when working with common bean cotyledon cells that intrinsic protein fluorescence allowed to assess its *in vitro* digestion from a substrate disappearance point of view. The results of these microscopic observations, published for the first time in this review paper, are shown in **Figure 6**. The effect of cooking time was included as processing variable, rather than mechanical or enzymatic modifications. In a similar way as reported by several authors in the context of starch digestion in pulse cotyledon cells, protein disappearance was also evident from the cellular emptying that occurred at all processing times and progressed as digestion time increased. Emptying was accompanied by a reduction on the intrinsic fluorescence of proteins in the system, demonstrating their hydrolysis as a result of the action of protein-degrading enzymes. Cellular emptying and decrease of fluorescence intensity happened faster and to a greater extent in cotyledon cells isolated after longer cooking times of common beans, in agreement with the behavior observed in terms of starch digestion (**Figure 4**). Again, the importance of removing the protein matrix in pulse cotyledon cells in order to enhance starch breakdown by amylases was demonstrated.

It is noticeable in **Figures 4** and **5** that *in vitro* digestion of pulse cotyledon cells is not an homogeneous process. Whereas in some cells both starch and protein are significantly reduced

as digestion time increases, others appear to be (more) resistant to substrate disappearance (Pallares Pallares, Alvarez Miranda, et al., 2018). In a similar way, a study in which microscopic observation of pulse cotyledon cells during simulated digestion was made on a single-cell basis revealed that different rates of amylolysis can be observed in distinct cells of the same population possibly due to microstructural and compositional variability (Do et al., 2020). The substantial presence of undigested material in pulse cotyledon cells after simulated small intestinal conditions has started to trigger research studies addressing their fermentation potential. In two very recent publications, colonic fermentation experiments have been performed on intact, mechanically disintegrated and enzymatically modified common bean cotyledon cells (red kidney and pinto varieties) (Guan et al., 2020; Rovalino-Córdova, Fogliano, & Capuano, 2020). Both studies reported that the colonic fermentation rate is controlled by cell wall integrity, which also seems to influence the fermentation patterns observed. It has to be mentioned that these authors used non-digested pulse cotyledon cells, which might imply that their findings are not a complete representation of actual *in vivo* colonic fermentation potential.

4.1.2. Physicochemical properties

High temperature treatments are known to affect several food physicochemical properties. In pulse cotyledon cells, where the cell wall plays a fundamental role on modulation of digestive properties, cell wall porosity and/or permeability to specific enzymes is an important characteristic to evaluate. In some cases, fluorescently labelled dextran are used to determine the cell wall porosity of plant cells. Following this approach, H. Li et al. (2019) reported that cotyledon cells isolated from cooked (90 °C, 1 h) red kidney beans are permeable to 70 kDa but not to 150 kDa dextran. By quantitatively determining cellular diffusion rates, these authors were

824 able to conclude that cells isolated from cooked beans exhibited an intermediate porosity
825 between acid/alkali isolated cells (highest porosity) and those isolated from raw, immature pods.
826 Furthermore, it was also suggested that the densely packed intracellular environment possess an
827 additional constraint to the diffusion of representative probes (H. Li et al., 2019).
828 Whereas linear dextran molecules constitute a valuable probe for evaluation of cell wall
829 porosity/permeability, they do not fully represent what happens upon contact of digestive
830 enzymes with plant cells. For example, dextran probes fail to simulate the potential interactions
831 (non-catalytic binding) between cell wall polymers and digestive enzymes. Hence, some authors
832 have used fluorescently labelled pancreatic α -amylase to get qualitative information about the
833 cell wall porosity/permeability of cotyledon cells isolated from pulse seeds (Dhital et al., 2016;
834 Pallares Pallares, Alvarez Miranda, et al., 2018). According to Dhital et al. (2016), pancreatic α -
835 amylase cannot diffuse through the cell wall of cotyledon cells isolated from different cooked (95
836 °C, 1 h) pulse seeds. Instead, the enzyme accumulates at the cell wall. Experiments at a different
837 research unit also demonstrated accumulation of pancreatic α -amylase at the outer border of
838 cotyledon cells isolated from common beans cooked (95 °C) during 30 min and 60 min (Pallares
839 Pallares, Alvarez Miranda, et al., 2018). Nevertheless, the latter authors also observed enzymatic
840 diffusion into the cellular space despite the outer accumulation. The discrepancy among the two
841 reported studies could be due to the different enzyme amounts used during simulated digestion
842 experiments, as well as to distinct intrinsic cell wall properties of the pulse seeds used to isolate
843 the cotyledon cells. All in all, currently available quantitative and qualitative data on *in vitro*
844 starch digestion of cells isolated from cooked pulses seem to support the idea that particular
845 amounts of pancreatic α -amylase can diffuse towards the intracellular space (regardless of the

846 cooking time used to isolate the individual cells), where it hydrolyzes starch into soluble products
847 that diffuse out to the digestive supernatant.

848 Beyond the cell wall, the macronutrients inside the cellular space also undergo transformations
849 during isolation of pulse cotyledon cells via cooking. Starch changes have been quantified by
850 measuring residual gelatinization enthalpy and crystallinity. Very low residual gelatinization
851 enthalpies have been reported after differential scanning calorimetry (DSC) analysis of cotyledon
852 cells isolated from cooked pulse seeds (Dhital et al., 2016; Pallares Pallares, Alvarez Miranda, et
853 al., 2018; Xiong et al., 2018), indicating a significant loss of molecular order due to the thermal
854 treatment. Yet, some authors have stated as well that birefringence under polarized light remains
855 in cotyledon cells isolated from cooked pulses, despite the negligible residual gelatinization
856 values (Dhital et al., 2016; Pallares Pallares, Rousseau, et al., 2018). Hence, the presence of some
857 crystalline starch structure after thermal treatment of pulse seeds cannot be ruled out. In fact,
858 Xiong et al. (2018) found that the relative crystallinity of starch bio-encapsulated by cell walls of
859 pinto bean cotyledon cells was significantly higher than that of the cooked free starch, which
860 supports the idea that cell wall confinement hinders complete melting of starch ordered
861 structure during cooking due to space and water limitations. This was also recently observed in
862 a study where a real-time microscopic approach was used to evaluate the behavior of navy bean
863 cotyledon cells during cooking (24°C to 90°C, around 6.2°C/min) (Do et al., 2020). Rapid swelling
864 of bio-encapsulated starch granules was detected at temperatures higher than 80°C and it
865 continued until the available intracellular space was fully occupied. Entire occupation was
866 reached after 10 min of thermal processing, at a temperature of 86°C. No major changes on
867 swelling were identified from this moment onwards, even though the system was kept at a

868 temperature of 90°C for 10 min. The shape of the starch granules was greatly deformed, but they
869 did preserve their granular form and remained separated from each other within the cellular
870 space (Do et al., 2020).

871 Protein changes have also been described after isolation of cotyledon cells from cooked pulses,
872 although these modifications have not been as explored as the ones happening to the starch
873 macronutrient. Using Fourier transform infrared spectroscopy (FTIR), Rovalino-Córdova et al.
874 (2019) detected a β -I band in cotyledon cells isolated from cooked common beans instead of the
875 characteristic β -sheet peak of raw legume proteins. The β -I configuration seems to exhibit a
876 higher resistance to protein hydrolysis than the β -sheet conformation. It was hypothesized in the
877 same study that, in a similar way as observed for gelatinization of bio-encapsulated starch,
878 proteins in the intracellular space of pulse cotyledon cells might not be completely denatured
879 during cooking due to limited water uptake and unfolding (Rovalino-Córdova et al., 2019). In
880 relation to this, it was found in another study that the intensity of protein intrinsic fluorescence
881 was higher in common bean cotyledon cells isolated after longer thermal treatments (Pallares
882 Pallares, 2019). Such differences might be related to process-induced changes of the
883 conformational state of proteins in the system. According to Deshpande & Damodaran (1989),
884 thermal denaturation of phaseolin (major storage protein in common beans) gives place to
885 exposure of tyrosine, phenylalanine and tryptophan residues to the aqueous environment. These
886 amino acids have been reported as intrinsic fluorescent probes (Beechem & Brand, 1985; García-
887 Plazaola et al., 2015). Unfolding and subsequent exposure of hydrophobic aromatic amino acid
888 residues as a result of thermal denaturation might be related to dissociation of the phaseolin
889 subunit structure (Deshpande & Damodaran, 1989). Hence, higher intrinsic fluorescence appears

to indicate that an increased exposure of hydrophobic residues is present in samples isolated after longer processing times, probably due to a more extensive unfolding as a result of denaturation.

4.1.3. Techno-functional properties

Very limited information can be found in the open scientific literature about techno-functional properties of cotyledon cells isolated from pulse seeds, despite the significant influence they would have on the texture and other quality attributes of the food products where they might be incorporated as ingredients. Edwards et al. (2020) assessed the water holding capacity and swelling potential of pulse powders made up of individual cotyledon cells isolated after cooking, wet sieving, and drying distinct pulse types. They found that the water holding capacity of pulse powders was approximately double than that of pulse flours (made from raw seeds, hence not maintaining cellular integrity) generated from the same botanical source. With regard to the swelling capacity of pulse powders, it remained fairly constant at all incubation temperatures (37 to 95°C), opposite to the behavior of pulse flours for which the swelling capacity was affected by temperature.

4.1.4. Application potential

Isolation of pulse cotyledon cells via thermal treatments above starch gelatinization temperature constitutes the most reported technique up to date in scientific literature. This is probably due to the fact that cooking is the most traditionally applied process to achieve palatable conditions of pulse seeds. Additionally, these hydrothermal treatments can be applied under food grade conditions and have a high yield of individual cotyledon cells. These factors could also be the reason why a couple of research studies on isolated pulse cells have already evaluated their

912 potential as novel, functional ingredients. Although promising digestive and techno-functional
913 properties have been observed, other factors such as sensorial characteristics as perceived and
914 accepted by potential consumers are still to be determined. Likewise, exploration of additional
915 product types and formulations should be made.

916 The pulse cellular powders described in previous section have been reported to display highly
917 interesting characteristics from a nutritional point of view, since they are a source of RS type I
918 and exhibit low to medium glycemic index (Edwards et al., 2020). Because of this, they have a
919 lower starch digestibility (both rate and extent) than pulse flours prepared from raw seeds and
920 reduce the starch hydrolysis index of savory biscuits where they are incorporated to (partially)
921 replace wheat flour (Delamare et al., 2020; Edwards et al., 2020). The savory biscuits where a
922 chickpea pulse powder was used to replace 50% of wheat flour retained the microstructural
923 properties of the pulse-based ingredient, demonstrating that the biscuit making process did not
924 affect cellular integrity. Presence of intact cells in the formulation was also hypothesized to avoid
925 extensive gelatinization of wheat flour during biscuit baking (a limited water environment), which
926 was postulated to be the result of a higher water holding capacity of the pulse powder due to its
927 significant content of hydrophilic components (Delamare et al., 2020).

928 In another application, a pulse-based ingredient containing individual cells (although not all of
929 them intact) was used for the generation of gluten-free bread (Boukid et al., 2019). The authors
930 reported a product with decreased crumb hardness and increased crust yellowness when
931 replacing 20% of rice flour by cannellini bean flour rich in intact cells.

4.2. Isolation of pulse cotyledon cells via thermal treatment below gelatinization temperature

Cooking (90-100°C) is indisputably the most commonly used technique to isolate pulse cotyledon cells. Some recent scientific studies are however exploring the application of thermal treatments below starch gelatinization temperature to pulse seeds as a technique to obtain individual cotyledon cells. The purpose of this isolation technique is to preserve as much as possible the natural state of bio-encapsulated starch while enabling pectin solubilization in the middle lamella to achieve cell separation. Further processing of the isolated cells allows to gain insight into physicochemical changes of the starch macronutrient and its *in vitro* digestibility as affected by processing variables such as temperature and/or moisture content. Only two studies were found in open scientific literature using this isolation technique. In both cases, soaked pulse seeds were subjected to a 1 hour thermal treatment at 60°C before isolation of individual cells. The findings reported are discussed with more detail in the following sections.

4.2.1. Digestive properties

P. Li et al. (2020) subjected common bean and chickpea cotyledon cells to hydrothermal treatments in excess water at distinct incubation temperatures (70°C to 100°C) aiming to induce different degrees of starch gelatinization and, in turn, distinct responses to *in vitro* digestion. Expectedly, the increase on incubation temperature was reflected in a lower resistance to amylolysis as demonstrated by higher apparent digestion rate coefficients and (in some cases) digestion extents. Two main causes were identified for the observed trends, namely the physical barrier role of the cell wall on the one hand, and the lower effect exerted by enzyme inhibitors after treatments at high temperatures on the other hand. Starch structural features

(gelatinization degree, crystallinity) could have played a role as well on the starch digestibility of these samples. The latter result is opposite to what was described for cotyledon cells isolated via cooking of pulse seeds, for which starch structural features did not significantly influence starch digestion (Xiong et al., 2018). Finally, although data on cell wall permeability was not reported by P. Li et al. (2020), this could have also been a parameter affected by the secondary processing applied to the isolated cells and hence an additional one playing a role in subsequent digestion. In a similar way as described for cotyledon cells obtained after cooking distinct pulse types, the effect of botanical source has been observed as well in cells isolated using temperatures below starch gelatinization and further processed after isolation. In the abovementioned study, the *in vitro* starch digestion kinetics of common bean cells were more temperature-dependent than that of chickpea cotyledon cells (P. Li et al., 2020). According to the authors, this could be explained taking into account the differences in process-induced starch structural features among the two pulse types evaluated. Once more, cell wall properties and other intrinsic factors not evaluated in the study could have been also involved. Xiong et al. (2019) determined the effect of heat-moisture treatment (a process involving low moisture levels and high temperature) on the starch digestibility of common bean cells isolated using a thermal treatment at 60°C. Samples were initially equilibrated at 4°C during 24h at three different moisture levels, after which they were heated at 100°C for 8 h. A direct relationship was identified between moisture content of the heat-moisture treatment and starch digestion rate and extent. As a matter of fact, a higher moisture content could have enabled a more pronounced loss of starch crystalline structure and changes in cell wall properties (such as permeability) during exposure to high temperature. A further increase on starch digestion kinetic parameters

was identified upon cooking of the isolated cells subjected to heat-moisture treatment, which could once more be explained by additional cooking-induced changes on starch and cell wall characteristics.

4.2.2. Physicochemical and techno-functional properties

Information regarding cell wall permeability of cotyledon cells isolated at temperatures below starch gelatinization temperature was provided in the study made by Xiong et al. (2019). These authors concluded that cell walls of common bean cells isolated after a thermal treatment of 1 hour at 60°C were not permeable to a 20kDa fluorescently labelled dextran, whereas those subjected to heat-moisture treatment did allow passage of the probe through the cell wall barrier regardless of the moisture content used during the treatment. However, the fact that heat-moisture treatment could have also induced protein denaturation (and thus unfolding and exposure on intrinsically fluorescent amino acids) does not seem to have been tested by the authors although it could have influenced the conclusions made.

In terms of starch structural features, heat-moisture treatments at 100°C gave place to different effects depending on the moisture level of the process. At 15 and 25% moisture, an increase on relative crystallinity and gelatinization enthalpy was observed with respect to the control sample (not subjected to heat-moisture treatment), but this was not the case at 35% moisture where fairly low relative crystallinity and gelatinization enthalpy were determined (Xiong et al., 2019). The increased values were reported to be due to the simultaneous disruption and rearrangement of bio-encapsulated starch granules during heat-moisture, hence resulting in an even more stable crystalline structure than in the non-treated sample. In the case of cells processed at 35% moisture, the higher water availability probably enabled a more substantial thermal damage of

starch. In this context, microscopic observations made evident pronounced swelling of starch in the sample treated at 35% moisture, whereas not striking differences were detected between the non-treated sample and those processed at 15 and 25% moisture (Xiong et al., 2019).

When common bean and chickpea cotyledon cells isolated via thermal treatments below gelatinization temperature were subjected to hydrothermal treatments in excess water at different temperatures, the observed response depended on pulse type (P. Li et al., 2020). While birefringence of common bean cells did not seem to be substantially influenced by temperatures of up to 80°C, it decreased for chickpea cells in the same temperature range. Thermal treatment of the isolated cells using higher temperatures (up to 100°C), did cause a reduction on the birefringence of both pulse types although some residual Maltese crosses were still evident. Likewise, some crystallinity was retained even after the thermal treatment at 100°C, as observed by X-ray diffraction. According to the authors, these observations indicated incomplete starch gelatinization in the isolated and subsequently thermally processed pulse cotyledon cells. Nevertheless, it must be emphasized that minor gelatinization enthalpies (< 1 J/g) were quantified by DSC for chickpea and common bean cells treated at 90 and 100°C for 30 min, which is similar to what has been reported for pulse cotyledon cells isolated via cooking. There was a direct relationship between gelatinization temperatures and the temperature of hydrothermal treatment applied, which could be explained by the presence of residual crystallites of higher thermal stability remaining after hydrothermal treatments at higher temperatures. In general, temperatures associated to starch gelatinization were higher for common bean than for chickpea cells (P. Li et al., 2020).

No reported information on protein-related nor on techno-functional properties of pulse cotyledon cells isolated at a temperature below starch gelatinization temperature was found. This characterization is nevertheless of high importance if a thorough evaluation of the application potential of these pulse-based ingredients on finished food products would be pursued.

4.3. Isolation of pulse cotyledon cells using techniques at room temperature

A third approach for isolation of pulse cotyledon cells is the acid/alkaline process. Changes on the native status of the bio-encapsulated macronutrients are minimized by avoiding the use of elevated temperatures, whereas the middle lamella and cell walls do suffer alterations (*e.g.* acid hydrolysis of pectin) due to the severe pH changes achieved by use of HCl and NaOH solutions. These alterations enable cell separation by gentle mechanical disintegration after the acid/alkaline treatment (Do et al., 2019; Sila et al., 2009). As has been the case for the isolation method described in previous section, only limited studies (two) have been published up to date on this isolation technique and will be discussed below.

4.3.1. Digestive properties

Do et al. (2019) isolated cotyledon cells after subjecting diverse pulse seeds to an acid/alkali treatment. Before performing simulated digestion experiments, a cooking step was applied to the isolated cells. Not surprisingly, the results obtained in the context of starch digestion are similar to those described before for isolated cells using thermal treatments below and above starch gelatinization temperature. Individual cells obtained after the acid/alkali treatment had slower rates and lower extents of starch digestion as compared to isolated starches (Do et al., 2019). Differences on extent of starch digestion were identified as well among the distinct pulse

types, making evident once more the importance of intrinsic pulse properties like morphology and composition on the digestive behavior of pulse cotyledon cells. Of course, the cell wall and intracellular matrix remain the most important physical barriers for enzymatic diffusion and substrate access regardless of pulse type or isolation technique.

When acid/alkali isolated chickpea cotyledon cells were subjected to *in vitro* digestion without any additional treatment, they displayed the lowest digestion rate and extent in a study where cells isolated via thermal treatments above starch gelatinization temperature (70 – 100°C) were also evaluated (P. Li et al., 2019). A significant increase on the kinetic parameters of starch digestion was observed upon subsequent application of thermal treatments to the acid/alkaline isolated cells. Thermal treatments at 70 and 80°C resulted in similar increases, whereas the one at 100°C resulted in even higher kinetic parameters than the lower temperatures.

4.3.2. Physicochemical and techno-functional properties

It was reported, in terms of cell wall properties, that cotyledon cells isolated from common beans using the alkali/acid method imposed a lower degree of hindrance to 20 and 70 kDa fluorescently labelled dextran probes than those isolated via cooking. The acid/alkali isolated cell walls appeared to be permeable to a 150 kDa dextran as well, although the probe did not seem to homogeneously distribute inside the cellular space due to constraints inflicted by the intracellular contents (H. Li et al., 2019). On the contrary, another study where fluorescently labelled dextran probes of 70 and 150 kDa were used to obtain information about cell wall permeability of chickpea cotyledon cells showed that alkali/acid isolated cells were less permeable than cells obtained after a thermal treatment at 80°C, which had in turn a lower permeability of chickpea cells isolated using a pressure-heating method (P. Li et al., 2019).

1063 With regard to starch characteristics, broader gelatinization peaks and higher gelatinization
1064 transition temperatures were identified in acid/alkali isolated pulse cotyledon cells than in
1065 isolated starches from the same botanical sources (Do et al., 2019). Nevertheless, no significant
1066 differences were present at the level of gelatinization enthalpy between free and bio-
1067 encapsulated starch. In a publication where distinct isolation techniques were applied, it was
1068 found that chickpea cotyledon cells isolated via the acid/alkali process had higher gelatinization
1069 enthalpy and starch crystallinity than cells isolated using thermal treatments (70 – 100°C) or a
1070 pressure-heating treatment (P. Li et al., 2019). When the acid/alkali isolated cells were subjected
1071 to subsequent heating steps, decreasing gelatinization enthalpies and higher gelatinization
1072 temperatures were determined until, after a thermal treatment during 30 min at 100°C, a
1073 negligible gelatinization endotherm (< 1 J/g) was detected. This finding is similar to what was
1074 observed for common bean and chickpea cells isolated by thermal treatments below starch
1075 gelatinization temperature and subjected afterwards to an additional heating step (90 – 100°C),
1076 as well as to the reported results of starch gelatinization in pulse cotyledon cells isolated via
1077 cooking. It seems plausible that in all cases DSC results reflected melting of crystallites without
1078 necessarily indicating a complete transition from non-gelatinized granules to molecularly
1079 dispersed starch (Zhang & Hamaker, 1998).

1080 The swelling capacity of acid/alkali isolated cells was also lower than that of free starches isolated
1081 from the same pulse type (Do et al., 2019), although the values reported (5 g wet pellet / g dry
1082 sample) are similar to those found for pulse powders generated after cooking pulse seeds, also
1083 mostly containing individual cotyledon cells (Edwards et al., 2020). Finally, it has been reported
1084 that starch bio-encapsulated in acid/alkali isolated cells from different pulse types is more

thermally stable and exhibits higher resistance to swelling and shear-induced rupture than free starch, which is reflected in a lack of viscosity development during pasting experiments (Do et al., 2019).

Characterization of protein conformational status and other protein-related properties is missing in the available studies reporting on acid/alkali isolated pulse cotyledon cells. As mentioned before, these evaluations should be done in order to achieve a complete picture of the functionality and application potential of these pulse-based ingredients on finished food products.

Conclusion

Current knowledge on isolation conditions and characteristics of pulse cotyledon cells makes clear that it is possible to use specific processing conditions as engineering tool to generate novel pulse-based ingredients. Isolation of pulse cotyledon cells using diverse techniques (thermal treatment above or below starch gelatinization temperature, acid/alkali treatment at room temperature) constitutes a promising strategy for the development of pulse-based ingredients where natural bio-encapsulation of macronutrients is preserved. However, a comparison of isolation techniques in terms of parameters such as yield of individual cells achieved or assurance of food grade process conditions should still be made in order to completely assess their application potential for the development of food products with defined and desired (digestive) functionalities.

Based on the findings reported by different authors, there is no more doubt about the fundamental role exerted by the strong pulse cotyledon cell wall on modulating nutrient digestion in these systems. Yet, different isolation techniques might differently affect cell wall

1107 porosity and/or permeability (and thus digestion behavior of bio-encapsulated nutrients). Cell
1108 wall parameters will in turn be affected by the intrinsic properties of pulse seeds, which could
1109 make specific pulse types or varieties exploitable for targeted applications. Therefore,
1110 quantitative and qualitative evaluation of cell wall properties should not be forgotten in future
1111 investigations. At the qualitative level, attempts should be made towards the use of labelled
1112 digested enzymes on top of the traditionally used labelled dextran probes. At the quantitative
1113 level, cell wall composition as affected by isolation technique(s) could be determined.

1114 The effect of the densely packed cytoplasmic matrix as additional barrier for digestion has also
1115 been emphasized regardless of isolation technique, whereas enzyme (protein) inhibitors could
1116 play a role on those cells isolated by use of mild temperature conditions. Further research of
1117 these factors should also be conducted, especially in the context of protein digestion. While this
1118 is a nutrient of uttermost relevance in pulse cotyledon cells, its digestion patterns in these
1119 systems are not as well understood as those of starch.

1120 Furthermore, in light of current knowledge about the significant dietary fiber content and
1121 remaining undigested material after simulated digestion of pulse cotyledon cells, the
1122 fermentation functionality of pulse-based ingredients and (later on) of the products where they
1123 are incorporated should become a subject extensively addressed in future investigations.

1124 Additional studies are also required in relation to physicochemical and techno-functional
1125 properties of isolated pulse cotyledon cells, not only at the ingredient level but at the finished
1126 product level as well. Hence, studies directed to understand the effect of pulse type, isolation
1127 technique, and isolation conditions on nutritional and functional properties of the finished
1128 product(s) where the isolated cells are (to be) used should be performed. In this way,

optimization of product properties could be targeted by considering the digestive, technological and sensorial properties obtained after product (re)formulation using these novel and promising pulse-based ingredients.

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Author Contributions

A. Pallares Pallares conceptualized and drafted the manuscript, which was then critically revised and approved for submission by all other authors.

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List of Figures

Figure 1. Cross sectional view of the cotyledon parenchyma cells of raw common beans (left) and detail of a common bean cotyledon cell showing starch granules (right). Micrographs were obtained by Scanning Electron Microscopy (SEM). Scale bar = 20 μm . Adapted with permission from Marconi, E., Ruggeri, S., Cappelloni, M., Leonardi, D., & Carnovale, E. (2000). Physicochemical, nutritional, and microstructural characteristics of chickpeas (*Cicer arietinum* L.) and common beans (*Phaseolus vulgaris* L.) following microwave cooking. *Journal of Agricultural and Food Chemistry*, 48(12), 5986 – 5994. Copyright (2020) American Chemical Society.

Figure 2. Schematic representation of the different structural levels found in pulse seeds (macro to nanoscale), with emphasis on the carbohydrate (starch and cell wall polysaccharides) fraction.

Figure 3. *Left:* particle size distribution (PSD, as obtained by laser diffraction) of *in vivo* generated oral boluses from cooked (95°C) common beans using different cooking times (*i.e.*, 5 process-induced hardness levels). Error bars represent the standard error (n = 16 subjects in each level). *Right:* partial least square biplot describing the change in PSD as a function of process-induced hardness. The percentages of X- and Y-variance explained by the first two latent variables (LV) of the model are indicated on the respective axes. Particle size volumetric fractions are represented by open circles, while hardness levels are shown as different symbols. Adapted with permission from Pallares Pallares, A., Loosveldt, B., Karimi, S. N., Hendrickx, M., & Grauwet, T. (2019). Effect of process-induced common bean hardness on structural properties of *in vivo* generated boluses and consequences for *in vitro* starch digestion kinetics. *British Journal of Nutrition*, 122(4), 388 – 399.

1578

1579 **Figure 4.** Representative scheme showing the structural properties of pulse cotyledon cells

1580 isolated using different techniques. Further processing steps applied to the isolated cells are also

1581 depicted, with their respective impact on starch and (when information was available) cell wall

1582 fraction(s).

1583

Figure 5. Normal light microscopic pictures of cotyledon cells isolated from cooked common beans using different processing times (30 min, 90 min, and 180 min), taken at different digestion times of the small intestinal phase (0 min, 60 min, 120 min, and 180 min). Starch was labelled with an iodine solution, thus color changes give qualitative information about starch disappearance. Scale bar = 100 μ m. Reproduced from Pallares Pallares, A., Alvarez Miranda, B., Truong, N. Q. A., Kyomugasho, C., Chigwedere, C., Hendrickx, M., & Grauwet, T. (2018). Process-induced cell wall permeability modulates the *in vitro* starch digestion kinetics of common bean cotyledon cells. *Food & Function*, 9, 6545 – 6555, with permission from the Royal Society of Chemistry.

Figure 6. Normal light (odd rows) and epi-fluorescent (even rows) microscopic pictures of cotyledon cells isolated from cooked common beans using different processing times (30 min, 90 min, and 180 min), taken at different digestion times of the small intestinal phase (0 min, 60 min, 120 min, and 180 min). Intrinsic fluorescence changes give qualitative information about protein disappearance. Micrographs were taken on the same sample section using the two illumination sources. Scale bar = 500 μ m. Reproduced from Pallares Pallares, A. (2019). *Influence of distinct*

- 1602 *process-induced (micro)structures on the in vitro starch digestibility of common beans: A kinetic*
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Table 1. Chemical composition of different pulse types (g/100 g raw material), consumed as staple foods in Asian, Latin American and African countries. Adapted from De Almeida Costa, Da Silva Queiroz-Monici, Pissini Machado Reis, & De Oliveira (2006).

Pulse type	Carbohydrates	Protein	Lipids	Crude fiber	Ash	Moisture
Common bean	54.3 ± 2.9	20.9 ± 1.5	2.5 ± 0.2	8.6 ± 3.3	3.8 ± 0.3	9.9 ± 0.4
Chickpea	54.0 ± 3.3	18.5 ± 1.7	6.7 ± 0.6	9.9 ± 2.1	3.2 ± 0.2	7.8 ± 0.9
Lentil	56.4 ± 4.1	20.6 ± 0.4	2.2 ± 0.1	6.8 ± 2.4	2.8 ± 0.2	11.2 ± 0.3
Pea	52.5 ± 0.0	21.9 ± 1.5	2.3 ± 0.0	10.4 ± 2.3	3.0 ± 0.0	9.9 ± 0.8

1611 **Table 2.** Processing conditions used for isolation and further processing of pulse cotyledon cells
1612 (continues in next page).

Reference	Pulse type	Isolation technique(s)	Further processing of isolated cells
Dhital et al. (2016)	common beans, chickpeas, peas, mung beans	Soaking in water Dehulling Thermal treatment (95°C, 60 min) Mashing (mortar and pestle) Wet sieving (53 – 150 µm fraction) Refrigeration in a sodium azide solution	Mechanical disintegration of (a part of) the isolated cells using a shear mixing force (1 500 rpm)
Bhattarai et al. (2017)	common beans, chickpeas, peas, mung beans	Soaking in water Dehulling Thermal treatment (95°C, 60 min) Mashing (mortar and pestle) Wet sieving (53 – 150 µm fraction) Refrigeration in a sodium azide solution	Mechanical disintegration of (a part of) the isolated cells using a shear mixing force (1 400 rpm)
Pallares Pallares, Alvarez Miranda, et al. (2018)	common beans	Soaking in water Thermal treatment (95°C, f(t)) Dehulling Mashing (mortar and pestle) Wet sieving (40 – 125 µm fraction) Instant freezing (liquid N ₂)	
Roalino-Córdova et al. (2018, 2019)	common beans	Soaking in water Dehulling Thermal treatment (95°C, 60min) Mashing (mortar and pestle) Wet sieving (fraction retained at 90 µm) Storage in a sodium azide solution	Mechanical disintegration of (a part of) the isolated cells using a shear mixing force (1 500 rpm) Enzymatic degradation of (a part of) the isolated cells by using a commercial cocktail of cell wall degrading enzymes
Xiong et al. (2018)	common beans, chickpeas, green-split pea, black-eyed pea	Soaking in water Dehulling Thermal treatment (95°C, 60 min) Mashing (mortar and pestle) Wet sieving (50 – 150 µm fraction) Freeze drying	
Edwards et al. (2020)^a	common beans, butter beans, green lentils, red lentils, green-split pea, yellow-split pea, chickpeas	Soaking Thermal treatment (100°C, 15, 25, 30, 50, or 90 min depending on pulse type) Wet sieving (cells < 150 µm) Air drying (< 6 % final moisture)	
Gwala, Pallares Pallares, et al. (2020)	Bambara groundnuts	Soaking in water Thermal treatment (95°C, 40 and 120 min) Dehulling Hardness evaluation of each cooked seed Sorting based on hardness Mashing (mortar and pestle) Wet sieving (80 – 125 µm fraction) Instant freezing (liquid N ₂)	

^a Patented work, thus not all details of the isolation procedure are provided in the scientific publication.