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Process-induced cell wall permeability modulates the *in vitro* starch digestion kinetics of common bean cotyledon cells†

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The presence of cell walls entrapping starch granules in common bean cotyledons, prevailing after thermal processing and mechanical disintegration, has been identified as the main reason for their (s)low *in vitro* starch digestibility. Nevertheless, it is unknown if the role of cell walls on starch digestion changes as processing conditions (e.g. time) are modified. In this study, it was hypothesised that cell wall permeability would be differently affected depending on thermal process intensity, giving origin to distinct *in vitro* starch digestion kinetic profiles. Cotyledon cells were isolated from common beans by applying processing conditions normally found at the household level (95 °C and times between 30 and 180 min (palatable range)). Isolated cells were characterised and subsequently subjected to *in vitro* simulated digestion. Microstructural properties, the starch gelatinisation degree, and the total starch content were similar among samples. In contrast, a higher diffusion of fluorescently labelled pancreatic α -amylase inside the cells was evident as processing time increased. From the kinetic analysis of digestion products, it was determined that longer lag phases and slower reaction rate constants were present in samples with a lower degree of process-induced cell wall permeability. The qualitative analysis of the remaining pellets showed that cellular integrity was maintained throughout *in vitro* digestion. A mechanism for the *in vitro* starch digestion of isolated common bean cotyledon cells as well as an alternative kinetic model to describe this process were proposed. Overall, our work demonstrated that the *in vitro* starch digestion kinetics of common bean cotyledon cells can be modulated by influencing cell wall permeability through thermal processing time.

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1. Introduction

Lifestyle behaviours play a dominant role in human metabolism, as they may influence the long-term energy balance of individuals.^{1,2} Among these, certain dietary patterns (high intake of refined carbohydrates, added sweeteners, edible oils, and meats and low intake of legumes, vegetables, and fruits) are widely acknowledged as one of the major contributing factors to the increase in the prevalence of obesity, type II diabetes, and other metabolic disorders.^{3,4} Therefore, renewed interest has been directed to the intake and digestion of starch (the major source of energy in the human diet), given

the relevance of this macronutrient on various chronic non-communicable diseases. In this regard, special efforts are being made to understand the digestive behaviour of food matrices with slow starch digestion, given that postprandial blood glucose responses could be potentially controlled by choosing starchy foods with specific susceptibilities to amylolysis.⁵

During amylolysis, the starch polysaccharide is first hydrolysed by salivary and pancreatic α -amylase to small oligomers (mostly maltose, maltotriose and limit α -dextrins), which are then broken down to glucose units by brush border membrane enzymes such as maltase–glucoamylase and sucrose–isomaltase. In the human body, these enzyme-catalysed reactions occur in the mouth and the small intestine. Although characterisation of this process has been traditionally performed based on the classification proposed by Englyst *et al.* (1992),⁶ in the last couple of years starch digestion is being evaluated using *in vitro* kinetic approaches.^{7–14} The main reason for this is that, by identifying and understanding key factors influencing the rate and extent of starch digestion, changes could be

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made at different levels (food matrices, processing conditions, *etc.*) to manipulate the kinetic behaviour of starch hydrolysis in the gastrointestinal tract.⁵

Starch is naturally found in the form of semi-crystalline granules. The enzyme-catalysed hydrolysis of such granules implies three key steps: (i) enzyme diffusion to the surface of the granule, (ii) enzyme adsorption on the solid surface, and (iii) hydrolysis of the mobile starch chains.^{5,9} Based on these steps, it has been proposed that the rate of starch digestion can be restrained by delaying or avoiding either the enzyme–substrate encounter (by the presence of barriers such as cell walls, protein matrices, *etc.*) or the enzyme action (by inherent starch structural features, when access is not restricted).^{5,15} In solid matrices, where starch is most of the time a part of a complex structural system, the first mechanism is believed to play the major role in limiting the digestion of this macronutrient.

Common beans are among the starchy foods with potential to allow controlled starch digestion, due to their high structural complexity. In this matrix, starch granules are surrounded by a protein matrix and enclosed by strong cell walls.^{16–18} This structural arrangement prevails after the application of thermal processing and mechanical disintegration, and has been identified by different authors as the main responsible for the (s)low digestibility of starch in the gastrointestinal tract.^{12–14,19–22} Yet, as we have put forward in our previous publication,¹² some discrepancies regarding the exact role of cell walls in limiting starch digestion are still present in the available literature. It is stated in some studies (with lack of direct evidence) that cell walls of common beans are a permeable barrier to digestive enzymes, therefore implying that the rate of starch digestion is reduced due to the tightly packed cytoplasmic contents.^{12,19,21,22} In contrast, some authors have concluded that pancreatic α -amylase cannot penetrate common bean cell walls, *i.e.*, that this barrier completely prevents starch breakdown.^{13,14}

The above-mentioned divergences are likely to be a result of different methodological factors. For example, the *in vitro* digestion protocols used by different authors, and more specifically, the enzymatic activities considered (which are directly proportional to the amount of enzyme added), greatly vary from one study to another. This fact becomes especially important given the strong binding affinity of α -amylase to cell wall components,¹³ which will most certainly influence the results observed depending on the amounts used when performing experiments. Another aspect possibly influencing is the intensity (temperature–time combination) of the thermal process applied to isolate the individual cells. As already reported by some authors, thermal processing induces changes on certain cell wall properties,^{23–25} which in the case of common beans are mostly related to pectin solubilisation.^{12,26–28} The latter process is temperature- and time-dependent, reason for which it is pertinent to question the function of processing conditions on determining the role of cell walls as a barrier for starch digestion in common beans.

To the best of our knowledge, processing intensity (*i.e.*, time) has not yet been included as a variable in any of the available studies about starch digestion of cotyledon cells isolated from common beans. So far, studies have been focused on the starch digestion of intact cells as compared to mechanically broken^{13,14,19–21} and/or enzymatically damaged^{13,22} cells. Nevertheless, given that thermal treatments (95 °C) of different durations have been proved an effective tool for the generation of intact cells from common beans,¹² it becomes relevant to explore the digestive behaviour of samples with the same microscopic structure obtained by distinct processing times. In this regard, we suggested in our previous study that individual cells from thermally processed common beans had different *in vitro* starch digestion profiles due to dissimilarities in cell wall porosity/fragility induced by variations in process intensity.¹² In the present study, we have approached this in detail by doing a kinetic evaluation of the *in vitro* starch digestion of individual intact cells isolated from thermally processed common beans using different processing times. The hypothesis being put forward is that the barrier role of cell walls during digestion of enclosed starch granules in common beans changes as the processing intensity (*i.e.*, time) used to isolate individual cells is modified. Such change of the barrier role is hypothesised to be a result of different process-induced cell wall permeability degrees affecting enzyme accessibility to the substrate to different extents.

2. Materials and methods

The present study involved an experimental set-up divided in three parts. First, individual intact cells from cotyledons of thermally processed common beans were isolated and characterised (section 2.2). Next, the process-induced cell wall permeability of the isolated cells to pancreatic α -amylase was qualitatively assessed using a microscopic technique (section 2.3). Finally, the isolated cells were subjected to a static *in vitro* digestion procedure and the kinetics of starch digestion were assessed qualitatively and quantitatively (section 2.4).

2.1. Plant material

Canadian wonder beans (*Phaseolis vulgaris* L.), harvested and dried in Kenya during the harvesting season of April 2015, were obtained from the Kenya Agricultural and Livestock Research Organisation (KALRO), Thika Station, Kenya. The plant material was manually cleaned upon arrival to the laboratory in Belgium, after which it was stored at –40 °C until use.

2.2. Isolation and characterisation of common bean cotyledon cells

2.2.1. Isolation procedure. The isolation of individual cotyledon cells from common beans was achieved after sequential application of different processing techniques: soaking, thermal processing, dehulling, mechanical disinte-

gration, and wet sieving. The conditions of such techniques are described in the following paragraphs.

Soaking was done by putting common beans in contact with demineralised water at a ratio 1 to 5 for 16 hours at room temperature. Subsequently, the soaking water was discarded and the soaked beans were transferred (at a ratio 1 to 5) to a Duran glass bottle containing demineralised water at 95 °C. A thermal process was applied at this temperature for a specific period of time, using an oil bath (ONE 10, Memmert, Germany) as heating media. A non-tight closing lid was placed on top of the glass bottle to avoid significant evaporation of water and pressure build-up during heating. Five processing times were considered, namely 30, 60, 90, 120, and 180 min. These times result in high presence of individual intact cells upon mechanical disintegration, as described in the findings of our previous study.¹² In addition, they are within the palatable range of common beans, as determined in our research unit by human mastication tests.

Once the required processing time was completed, the thermal process was stopped by rapidly decreasing the temperature using an iced water bath. Following, the processing water was discarded and the seed cotyledons were isolated by manual removal of the seed coat. The isolated cotyledons were mechanically disintegrated using a mortar and pestle until a paste-like consistency was obtained.

Individual intact cells were isolated from mechanically disintegrated cotyledons using a vibratory sieve shaker (AS 200, Retsch, Germany) equipped with a universal wet sieving clamping device. Two runs were performed, each with a vibratory amplitude of 2.5 mm and a duration of 4 min. During the first run, demineralised water was supplied to the system by using a water spray located on the top of the sieve stack. Such wet sieving was done in order to facilitate the separation of particles into the respective size ranges. In the second run, the water supply was suspended aiming to eliminate residual water. The fraction containing individual cells (40–125 µm) was collected, frozen using liquid nitrogen, and kept at –40 °C until analysis.

2.2.2. Characterisation techniques

Microstructural characterisation. Two techniques were used in assessing the microstructural properties of the isolated samples: laser diffraction and microscopy. The first technique allowed to determine the particle size distribution of the fraction of individual cells, while the second made possible to observe their microstructure.

During laser diffraction analysis, a LS 13 320 particle size analyser (Beckman Coulter Inc., US) equipped with a Universal Liquid Module was used. Samples were added into a stirring tank filled with demineralised water and thereafter pumped into the measuring cell (pumping rate 30%). The laser light (wavelength main illumination source: 750 nm; wavelengths of halogen light for polarisation intensity differential scattering: 450 nm, 600 nm, and 900 nm) was scattered by the dispersed particles and the volumetric particle size distribution was calculated from the intensity profile of the scattered light according to the Fraunhofer optical model using the instru-

ment software. All measurements were carried out in duplicate.

For the microscopy analysis, samples were suspended in demineralised water, after which 10–20 µl of the suspension was taken and the microstructure visualised using an Olympus BX-51 light microscope (Olympus, Optical Co. Ltd, Japan) equipped with an Olympus XC-50 digital camera and a photo-analysing software (Cell^F). Observations were done in differential interference contrast (DIC) mode using the objective of 40× magnification. Representative images of each sample were taken.

Total starch content. A portion of each isolated sample was lyophilised (Alpha 1–4 LSCplus, Martin Christ, Germany) and pounded in a Ball mill (MM 400, Retsch, Germany) prior to the determination of its total starch content. These steps were done in order to align moisture contents and guarantee full exposure of starch granules to enzymatic hydrolysis during the procedure. The total starch content was determined in duplicate for each sample using the Megazyme Total Starch Assay Kit (AA/AMG), following the instructions given by the manufacturer.

Moisture content. This parameter was determined using a method based on weight loss on drying, by placing a known amount of wet sample in an oven (Jouan, France) and allowing it to dry until a constant weight (105 °C, 24 h) was achieved.

Residual starch gelatinisation enthalpy. Differential scanning calorimetry (DSC) was used to identify thermal transitions occurring in the isolated samples. The procedure suggested by Salgado-Cruz *et al.* (2017)²⁹ was adopted with the minor changes described by Pallares Pallares *et al.* (2018).¹² The thermograms generated by the machine were used to compare the residual gelatinisation enthalpy of the isolated common bean cotyledon cells from different thermal processing times.

2.3. Qualitative evaluation of process-induced cell wall permeability to pancreatic α -amylase

2.3.1. Enzyme labelling. Pancreatic α -amylase (A6255 Sigma, Sigma Aldrich, Belgium) was labelled using a protein labelling kit (Atto 488, 38371 Sigma, Sigma Aldrich, Belgium), following the instructions of the manufacturer. Briefly, the enzyme and the reactive dye were each dissolved in sodium bicarbonate buffer (pH = 9.5). Following, the two solutions were mixed (13.8 molar excess dye to protein) and incubated at room temperature for 2 h while gently stirring. The labelled enzyme was separated from the non-reacted dye using a gel filtration column (PD-10), pre-equilibrated with a phosphate buffer solution (pH = 7.5). Using this kit, pancreatic α -amylase was labelled *via* conjugation of the succinimidyl ester group of the dye to primary amine groups.

Following labelling, the purity of the resultant enzyme solution was confirmed by HPLC-DAD (data not shown). From this analysis, it was concluded that the fluorescently labelled enzyme was pure (*i.e.*, that there were no remaining amounts of free, non-reacted dye in solution). In addition, a solution containing non-labelled enzyme was also analysed to confirm that its labelled equivalent had the same size. Finally, the strong

emitted fluorescent signal of the labelled enzyme was verified by preliminary *epi*-fluorescent microscopy analysis. The labelled enzyme solution, with a concentration of 0.325 mg ml^{-1} , was divided in aliquots and stored at $-20 \text{ }^\circ\text{C}$ until use.

2.3.2. *epi*-Fluorescence microscopy. 20 mg of the isolated cotyledon cells from each processing time were put in contact with $2 \text{ } \mu\text{l}$ of calcofluor-white (1% v/v in Milli-Q water), $25 \text{ } \mu\text{l}$ of labelled pancreatic α -amylase and $133 \text{ } \mu\text{l}$ of demineralised water. The dispersion was continuously mixed for 1 min and then the solids were allowed to sediment. The supernatant was discarded and the remaining solids were washed twice and dispersed again in demineralised water. From the new suspension, exactly $20 \text{ } \mu\text{l}$ was taken and microscopically visualised using *epi*-fluorescent light (excitation filter between 460 and 490 nm). An Olympus BX-51 microscope equipped with epifluorescence illumination (X-Cite® 120Q, X-Cite® Fluorescence Illumination, EXFO Europe, UK) was used. Representative micrographs of each sample were taken using the objective of $40\times$ magnification, with the aid of an Olympus XC-50 digital camera and the photo-analysing software (Cell^F). During the collection of fluorescence microscopy images, the strength of the light from the lamp providing the epifluorescence illumination and the live acquisition time to make images (ms) were kept constant.

2.4. *In vitro* starch digestion kinetics of isolated common bean cotyledon cells

2.4.1. Static *in vitro* digestion procedure. The protocol suggested by Minekus *et al.* (2014)³⁰ was used to evaluate the *in vitro* starch digestion kinetics of cotyledon cells isolated from thermally treated ($95 \text{ }^\circ\text{C}$) common beans using different processing times. An individual tube was used for each digestion time studied during the intestinal phase.

The *in vitro* digestion procedure consisted of three phases, namely oral, gastric, and small intestinal phases. Appropriate electrolyte solutions, mimicking digestion fluids, were added in each simulated phase. These solutions are denoted in following paragraphs as SSF (simulated salivary fluid), SGF (simulated gastric fluid), and SIF (simulated intestinal fluid). The composition of these solutions is described in detail by Minekus *et al.* (2014).³⁰ According to the authors, the electrolyte concentrations and other recommended conditions of the method are based on human *in vivo* data.

Initially, during oral phase simulation, 1.25 g of wet sample (*i.e.*, isolated cotyledon cells) was mixed with 1 ml of SSF ($\text{pH} = 7$), 0.125 ml of a 0.015 M CaCl_2 solution, and 0.125 ml of Milli-Q water. The oral bolus was placed in a horizontal stirring plate (Promax 1020, Heidolph, Germany) inside an incubator (IPP 500, Memmert, Germany) and mixed at $37 \text{ }^\circ\text{C}$ and 70 rpm for 2 min. At this stage, salivary α -amylase was not added because it was observed in a preliminary experiment that its addition did not affect the kinetics of starch digestion during the intestinal phase (data not shown).

The gastric phase was simulated by adding 1.6 ml of SGF ($\text{pH} = 3$) to the oral bolus, together with 0.025 ml of CaCl_2 (0.015 M), 0.4 ml of porcine pepsin solution prepared in SGF

(reaching 2000 U ml^{-1} in final digestion mixture), and volumes of 1 M HCl and Milli-Q water enough to, respectively, adjust the pH of the solution to a value of 3 and complete a final volume of 5 ml . Samples were incubated at $37 \text{ }^\circ\text{C}$ for 2 h, with horizontal mixing at 70 rpm .

For the simulation of the small intestinal phase, the gastric chime was mixed with 2.75 ml of SIF ($\text{pH} = 7$), 0.2 ml of 0.015 M CaCl_2 , 0.625 ml of bile solution (160 mM , prepared in Milli-Q water), and 1.25 ml of intestinal enzyme solution prepared in SIF (reaching $\approx 200 \text{ U ml}^{-1}$ (9 U mg^{-1} starch) of pancreatic α -amylase, 100 U ml^{-1} of trypsin, and 25 U ml^{-1} of chymotrypsin in a final digestion mixture), and volumes of 1 M NaOH and Milli-Q water enough to, respectively, adjust the pH of the solution to a value of 7 and complete a final volume of 10 ml . The tubes containing the samples, incubated at $37 \text{ }^\circ\text{C}$ with horizontal mixing at 70 rpm , were withdrawn as a function of digestion time and immediately heat shocked ($100 \text{ }^\circ\text{C}$, 5 min) to stop enzymatic activity. The supernatant fraction of the digests, in which starch digestion products were measured, was separated from the remaining solids by centrifugation (Sigma 4-16 KS, Sigma Laborzentrifugen GmbH, Germany) at $2000g$ for 5 min.

2.4.2. Assessment of *in vitro* starch digestion kinetics. The time-dependent behaviour of starch digestion was characterised qualitatively and quantitatively, as described in the following paragraphs.

Substrate disappearance (qualitative assessment). After being separated from the supernatants, the solids remaining after digestion were stained with a 5% (w/v) Lugol's iodine solution and observed under a light microscope as described earlier (section 2.2.2). The goal of this analysis was, on the one hand, to determine microstructural changes at cellular level during digestion and, on the other hand, to qualitatively assess where and to what extent were the starch granules of different samples being digested.

Product(s) formation (quantitative assessment). The quantification of starch digestion products was based on the measurement of reducing sugars present in the aqueous phase of the digests. For this purpose, the dinitrosalicylic acid (DNS) method was used.³¹ For each digestion time considered, 1 ml of (diluted) supernatant was mixed with 1 ml of colour reagent solution (containing 3,5-dinitrosalicylic acid, potassium sodium tartrate tetrahydrate, and 2 M NaOH) and incubated in an oil bath (ONE 10, Memmert, Germany) at $100 \text{ }^\circ\text{C}$ during 15 min. After cooling down, 9 ml of Milli-Q water was added before measuring the absorbance of the resulting coloured solution in a spectrophotometer (Shimadzu UV-1800, Shimadzu, Japan) at a wavelength of 540 nm . The amount of reducing sugars was measured in duplicate in each supernatant and expressed as maltose reducing sugar equivalents based on a maltose standard curve. Maltose equivalents were multiplied by a factor of 0.95 in order to be converted to starch equivalents, which were then used for calculation of the percentage of digested starch.

2.4.3. Kinetic modelling and statistical analysis. As described earlier, the determination of reducing sugars was

done in duplicate. However, for modelling purposes, the average value of each digestion time was considered. The kinetic data describing the experimental behaviour of *in vitro* starch digestion of isolated common bean cotyledon cells were mathematically fitted using the empirical, logistic model described by Palmers *et al.* (2015)³² (eqn (1)):

$$\% \text{starch} = \frac{\% \text{starch}_f}{1 + \exp \left[\frac{4 \times k_{\max}}{\% \text{starch}_f} (\lambda - t) + 2 \right]} \quad (1)$$

with % starch representing the amount of starch digested at digestion time t , % starch_f being a plateau concentration at long digestion times, k_{\max} denoting the maximum reaction rate constant at the inflection point, and λ symbolising the duration of the lag phase, for a given processing time. The three parameters of the model were simultaneously estimated using nonlinear regression (SAS version 9.4, SAS Institute Inc., US). The appropriateness of the model was evaluated by calculation of the R^2_{adjusted} and visual inspection of the parity and residual plots. The estimated model parameters of different samples (cotyledon cells isolated from common beans thermally treated using different processing times) were statistically compared by use of their 95% confidence intervals.

3. Results and discussion

3.1. Isolated cotyledon cells from thermally processed common beans: are they all the same?

The first aim of this study was the isolation and characterisation of cotyledon cells from common beans, previously subjected to thermal treatments (95 °C) of different intensities. Specifically, five processing times were applied to common beans before the isolation of cells: 30, 60, 90, 120, and 180 min. The isolated cotyledon cells were characterised by diverse quantitative and qualitative techniques, in order to establish their similarities and/or differences.

Initially, their microstructural properties were evaluated in order to confirm the preponderant presence of individual cells and to establish whether or not remarkable changes in their microscopic structure induced by different processing times could be observed. The results of such characterisation, including particle size distribution and the microstructure, are presented in Fig. 1.

As expected, all generated samples were highly homogeneous and constituted by intact free cells. During microscopic analysis, no striking differences in cellular size and/or shape were observed among the processing times considered. Additionally, it was clear from the laser diffraction analysis that all samples exhibited a single characteristic peak, comprising sizes between 50 and 200 μm . The median volume-weighted values (D_{50}) were highly similar for all cases, ranging between $91.19 \pm 0.02 \mu\text{m}$ and $102.97 \pm 0.74 \mu\text{m}$. These results are similar to others previously reported in literature.^{16,17,21}

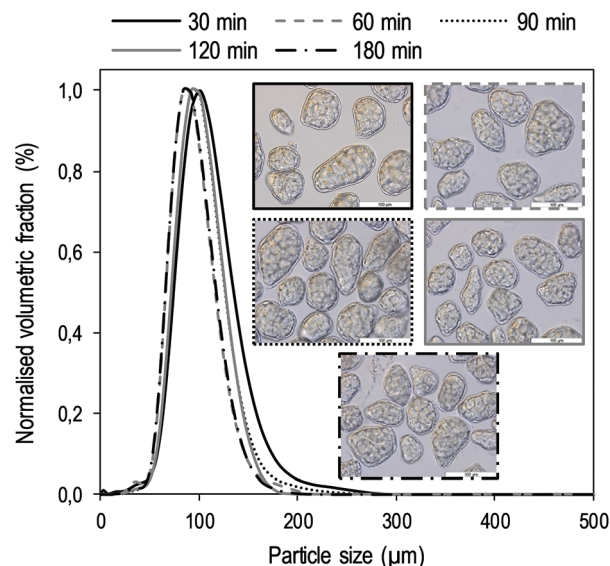


Fig. 1 Normalised volumetric particle size distribution (left) and representative microscopic images (right, scale bar: 100 μm) of cotyledon cells isolated from thermally treated common beans using different processing times (95 °C, t).

In terms of total starch and moisture content, not-significantly different values ($\alpha = 0.05$) were determined among samples when compared based on the processing time applied. The average total starch and moisture contents were, respectively, $0.62 \pm 0.01 \text{ g g}^{-1}$ dry sample and $0.70 \pm 0.01 \text{ g g}^{-1}$ wet sample.

Finally, the calorimetric analysis (Fig. S1†) of the isolated cotyledon cells revealed that complete starch gelatinisation had occurred irrespective of the processing time considered. In a similar way as reported in previous investigations,^{14,26,33} in this study it was found out that the residual gelatinisation enthalpy of all samples was negligible. On top of this, a peak ascribed to starch retrogradation was also identified. This peak had comparable enthalpy values for all samples. Therefore, if retrogradation had any effect on the results of the present study, such effect was likely to be the same for all the studied cases.

The presence of individual intact cells in the isolated samples is most likely a result of thermally-induced pectin solubilisation in the middle lamella leading to cell separation as preferred tissue failure mode upon mechanical disintegration.^{12,14,26–28} The solubilisation of pectin and other polymers, as a result of high temperature, could have also occurred at the level of the complex interacting network of pectin, cellulose and hemicellulose conforming the cell wall. Such solubilisation could have happened to different extents depending on the processing time applied, which in turn could have led to different degrees of cell wall permeability. Since these possible modifications were not distinguishable using light microscopy analysis, the hypothesis was qualitatively tested using *epi*-fluorescence microscopy. Aiming to evaluate the process-induced cell wall permeability of isolated

cells, pancreatic α -amylase (enzyme of interest for the substrate under investigation) was fluorescently labelled and put into contact with the generated samples. Characteristic micrographs reflecting the results obtained are presented in Fig. 2.

As clearly observed in the figure, marked differences were detected among common bean cotyledon cells in terms of enzyme diffusion into the cellular space. Upon short contact time with labelled pancreatic α -amylase, higher enzyme diffusion was observed in samples generated from long processing times as compared to those of shorter duration. In the case of samples produced after 30 min of thermal treatment, the labelled enzyme did not seem to immediately go through the cell wall and, instead, it appeared to accumulate on the outer border of the cells. As processing time increased, a reduction in outer accumulation occurred in parallel with an intensification of diffusion into the cellular space by pancreatic α -amylase. For those cells isolated after 180 min of thermal treatment, most of them seemed to be highly permeable to the enzyme.

In a similar way as expressed by Dhital *et al.* (2014),³⁴ Bhattarai *et al.* (2016),³⁵ and Yu *et al.* (2018),⁹ the micrographs in which the enzyme is inside the cellular space are likely to represent the catalytic and non-catalytic binding of the enzyme to starch granules, as well as its possible non-specific binding to some proteins around the granules. Regardless of the type(s) of interaction(s) being present, this qualitative approach demonstrates that thermal treatments of varying intensities can modify to different extents cell wall permeability of common bean cotyledon cells to pancreatic α -amylase.

The findings attained in the present study are not fully in agreement with the ones reported by Dhital *et al.* (2016)¹⁴ in their analysis of the location of FTIC labelled α -amylase during hydrolysis of chickpea intact cells isolated after a thermal treatment at 95 °C for 60 min. According to the authors, the enzyme was unable to penetrate the cellular space even after 4 h of hydrolysis. On the contrary, in this investigation it was observed some degree of enzymatic penetration in common bean cotyledon cells isolated using similar processing con-

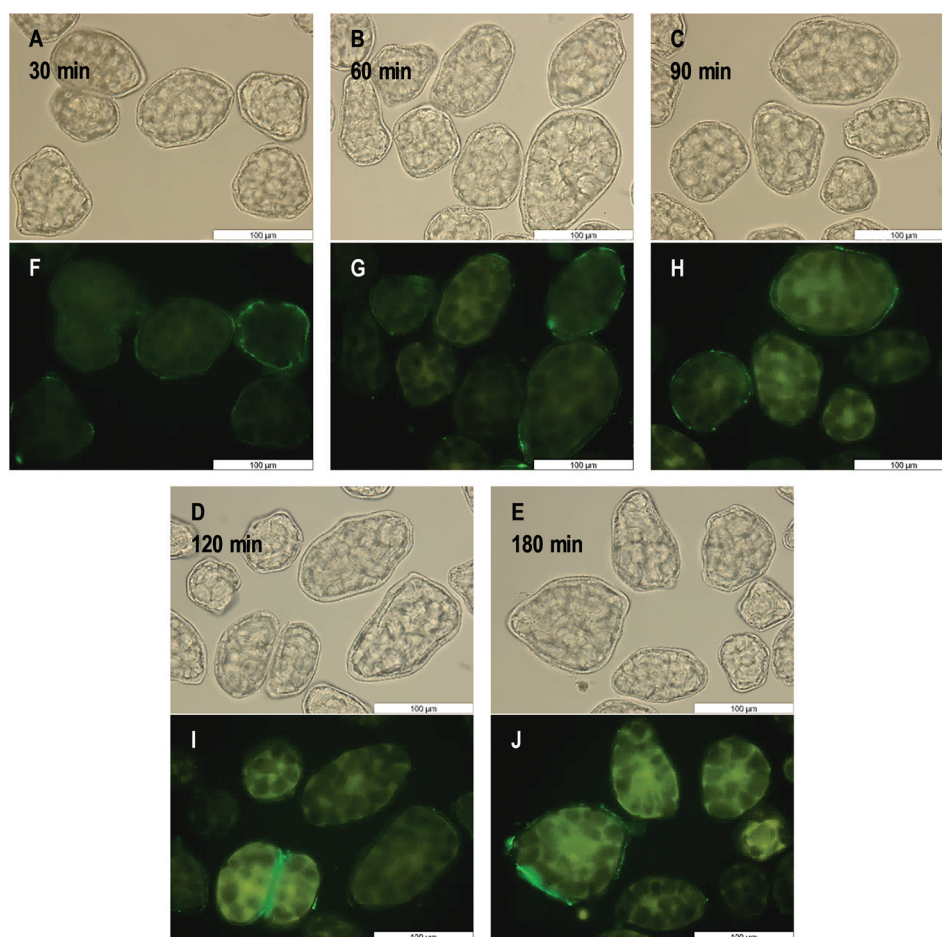


Fig. 2 Normal light (a–e) and epi-fluorescent (f–j) microscopic pictures of cotyledon cells isolated from thermally treated common beans using different processing times (95 °C, t) in contact with fluorescently labelled pancreatic α -amylase. Micrographs were taken on the same sample section using the two illumination sources. Scale bar: 100 μ m.

ditions (Fig. 2G), in combination with some accumulation in the cell wall. Possible causes for the observed divergence could be related to naturally-occurring differences in cell wall properties of common beans and chickpeas (due to varietal and harvest differences) and/or with the amount of enzyme used during the experiments. Regarding the latter, it could be determined that the amount of enzyme (mg) used in this study, relative to the amount of starch (mg) present in the system, was four times higher than the amount used by the cited authors.^{14,34} Since it is highly plausible that interactions between the enzyme and cell wall components were happening,¹³ a higher amount of enzyme added would have increased the likelihood of enzymes going inside the cellular space to (eventually) digest enclosed starch granules, and *vice versa*. In fact, the binding of fluorescently labelled α -amylase to the cell wall of common bean cotyledon cells was also identified in this study for samples isolated after thermal treatments of 30 min and 60 min (Fig. 3). The hexagonal-like pattern observed in the cell walls coincides with the results reported by Marconi *et al.* (2000)¹⁷ during the SEM (Scanning Electron

Microscopy) microstructure analysis of the cotyledon parenchyma of common beans after traditional cooking.

The results discussed in this section make evident that, despite being apparently equal from a microstructural point of view, intact cells isolated from common bean cotyledons exhibit process-induced differences in terms of cell wall permeability to pancreatic α -amylase. Based on these findings, different behaviours were expected for the samples under analysis upon *in vitro* simulated digestion.

3.2. *In vitro* starch digestion kinetics of common bean cotyledon cells: effect(s) of process-induced cell wall permeability?

Cotyledon cells isolated from thermally treated common beans using different processing intensities were subjected to *in vitro* simulated digestion in order to assess their starch digestion kinetics. A static *in vitro* digestion method of three phases was used for this purpose, during which the evolution (0–180 min) of starch breakdown was followed qualitatively and quantitatively in the small intestinal phase.

From a quantitative point of view, the amount of reducing sugars in the aqueous phase of digests was measured and utilised (together with the total starch content) for calculating the percentage of digested starch. The kinetic curves of *in vitro* starch digestion (% digested starch plotted against digestion time) for the microstructures considered in this study are shown in Fig. 4.

In general, an increasing trend in the percentage of digested starch followed by a plateau was observed in all samples. However, substantial differences among them were evident at early digestion times. Specifically, the experimentally determined data revealed the existence of a lag phase in some of the microstructures under evaluation. Such a delay was present in samples obtained from short processing times (*i.e.*, 30 min and 60 min), whereas for those generated after

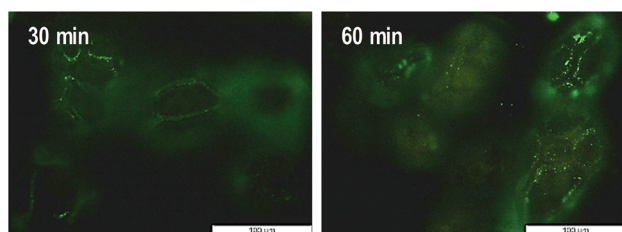


Fig. 3 epi-Fluorescent microscopic pictures of cotyledon cells isolated from common beans after 30 min (left) and 60 min (right) of thermal processing in contact with fluorescently labelled pancreatic α -amylase. Micrographs were taken on the same sample sections shown in Fig. 2, after changing the focus on the sample using the fine focus knob. Scale bar: 100 μ m.

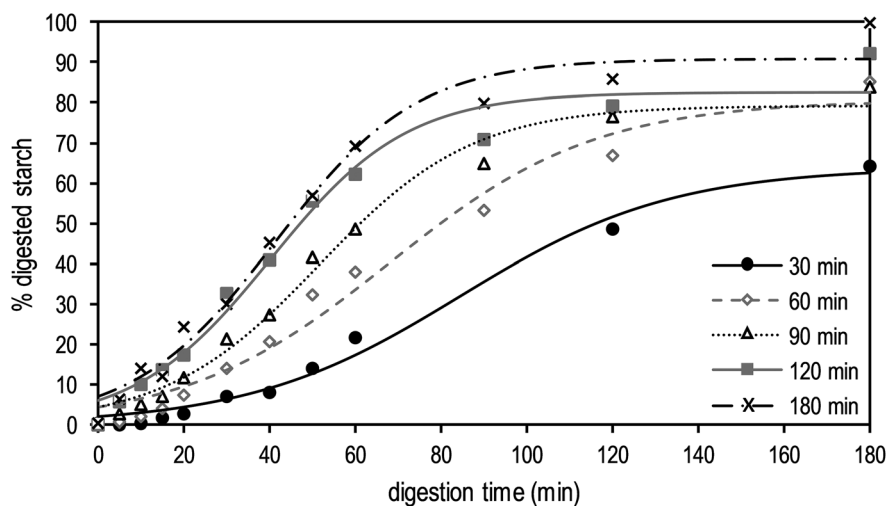


Fig. 4 *In vitro* starch digestion kinetic curves of cotyledon cells isolated from common beans after thermal treatments of varying processing times (95 °C, *t*). Experimental data points versus predicted values (using an empirical logistic model) are represented in the figure with symbols versus lines, respectively.

longer processing times its presence was not so evident. It has to be emphasised as well that despite following a similar trend in terms of increasing digested starch, the increase seemed to be happening faster for samples isolated from common beans treated for longer times. In other words, varying *in vitro* starch digestion rates could be already anticipated for the microstructures under analysis.

From the experimental data it was perceived that the *in vitro* starch digestion kinetics of the isolated common bean cotyledon cells deviated in its initial phase from a classical first-order kinetic behaviour. This observation was in agreement with a recent publication¹³ and led to the kinetic modelling of experimentally determined data using a range of empirical models. After a model discrimination procedure, an empirical logistic model was selected to fit the data. This model is characterised by three parameters that allow to quantitatively describe the consecutive digestion phases determined experimentally. Specifically, the model includes: (i) a parameter to characterise the lag phase, (ii) a parameter to describe the dynamic part of the curve, and (iii) a parameter to describe the final plateau value. Thus, an alternative model to describe the *in vitro* starch digestion kinetics of isolated legume cells is being proposed for the first time in the present study.

The calculated values of the three characteristic kinetic model parameters are shown in Table 1. In addition, a graphical representation of their 95% confidence intervals as a function of processing time is shown in Fig. 5.

From the results obtained, it was clear that the duration of the lag phase (λ) had a decreasing tendency followed by the reaching of a final constant value at long processing times. In contrast, the maximum reaction rate constant at the inflection point (k_{\max}) had a behaviour resembling a mirror-reversal across the horizontal axis of the trend presented by the lag phase. In other words, an increasing trend followed by a final constant value was observed in this case. For both kinetic parameters, significant differences were established between the shortest processing time (30 min) and the subsequent longer ones.

Regarding the maximum digestibility percentage attained (Starch_f), presence of a well-distinguished tendency was not clear and no major differences were observed among samples. In fact, we believe that the lower final digestibility

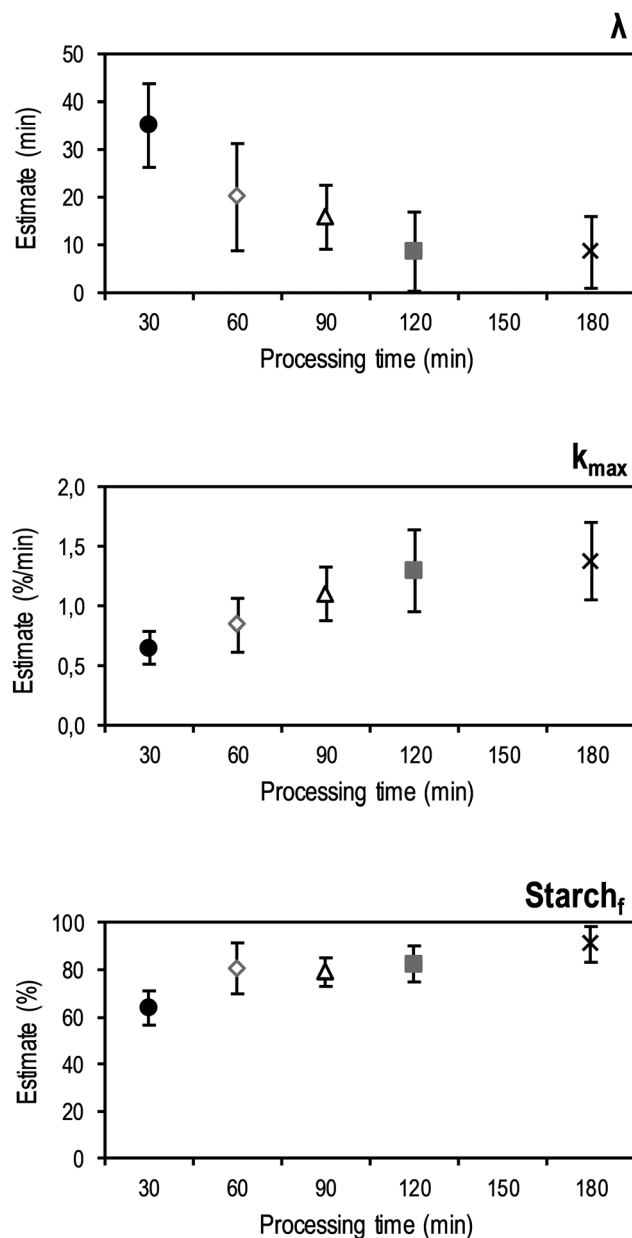


Fig. 5 95% confidence intervals of the kinetic parameters of the empirical logistic model used to describe the *in vitro* digestion kinetics of cotyledon cells isolated from thermally processed common beans using different processing times (95 °C, *t*).

Table 1 Kinetic parameters of the logistic model estimated for the *in vitro* starch digestion of common bean cotyledon cells obtained by thermal treatments (95 °C) with varying processing times. Values having different superscript letters are significantly different based on their 95% confidence intervals

Sample	λ (min)	k_{\max} (% min ⁻¹)	Starch_f (%)
30 min	35.01 ± 3.83 ^a	0.647 ± 0.059 ^c	63.91 ± 3.18 ^f
60 min	20.01 ± 4.96 ^{a,b}	0.839 ± 0.098 ^{c,d}	80.59 ± 4.83 ^{f,g}
90 min	15.65 ± 2.98 ^b	1.101 ± 0.100 ^d	79.08 ± 2.59 ^g
120 min	8.59 ± 3.64 ^b	1.299 ± 0.151 ^d	82.43 ± 3.28 ^g
180 min	8.41 ± 3.36 ^b	1.375 ± 0.143 ^d	90.76 ± 3.29 ^g

value predicted for the sample corresponding to 30 min of processing could be a modelling artefact due to the lack of sufficient experimental data to accurately calculate the plateau value.

The presence of natural (micro)structural barriers such as cell walls, preventing or delaying access of enzyme to starch, is thought to be the rate-limiting step of the starch digestion process.^{5,13} Specifically, it is believed that this structure controls enzyme diffusion into the cellular space, having a crucial effect on the formation of the enzyme-substrate complex. In this study we are demonstrating, for the first time, that the

barrier role of cell walls during digestion of enclosed starch granules in common beans changes as a result of different permeability degrees induced by distinct thermal processing intensities. This is clearly observable when analysing the trends followed by λ and k_{\max} , *i.e.*, the parameters describing (respectively) the lag phase and the rate constant of the logistic model used during the kinetic modelling.

In the case of the lag phase (min), this parameter is most probably giving information about how rapid pancreatic α -amylase encounters and exerts catalytic action on its substrate. In other words, it represents the period of time it takes for the enzyme to achieve diffusion through the cell wall and the protein matrix still present after gastric digestion, before adsorption on and hydrolysis of starch granules. As anticipated, common bean cotyledon cells with higher permeability exhibited lag phases of shorter duration, which led to an earlier detection of digestion products in the supernatant of their digests.

Regarding the reaction rate constant ($\% \text{ min}^{-1}$), it is believed to be related to the amount of enzyme being able to effectively bind and break down starch chains into reducing sugars per time unit. In a similar way as the lag phase, this parameter might be linked to the amount of pancreatic α -amylase reaching the starch granules after passing through the cell wall and the remaining protein matrix. Therefore, in those samples with lower cell wall permeability and/or higher amount of remaining protein after the gastric phase, lower rate constants are likely to be a result of less enzyme diffusing inside the cellular space (due to limited permeability) and/or less enzyme being able to reach the substrate (due to interaction with cell wall components and proteins).

The cell wall permeability of the samples under evaluation was assessed after thermal processing. Nevertheless, further changes occurring during the simulation of gastric conditions cannot be ruled out. For instance, cell wall permeability could have had an effect as well at the level of protein digestion by pepsin. In fact, the hydrodynamic radius of pepsin (around 3 nm)³⁶ is similar to the hydrodynamic radius of α -amylase (around 3–4 nm),^{37,38} which might imply that differences among samples at the level of protein digestion could be expected at the end of the gastric phase. Such differences could, in turn, have exerted varying effects at the level of starch digestion during the simulation of the small intestinal phase (in the ways postulated in previous paragraphs).

In summary, it could be hypothesised that process-induced cell wall permeability affects the *in vitro* starch digestion of common bean cotyledon cells either directly (cell wall permeability *per se* modulating diffusion of pancreatic α -amylase), indirectly (cell wall permeability affecting gastric digestion of the surrounding protein matrix to different extents), or by a combination of both effects.

As mentioned before, the *in vitro* starch digestion kinetics of individual cotyledon cells from thermally treated common beans was also qualitatively evaluated by microscopy. For these experiments, starch was stained in order to facilitate its identification. Representative micrographs of individual cells from

three processing times (30 min, 90 min, and 180 min), each analysed at different digestion times (0 min, 60 min, 120 min, and 180 min), are shown in Fig. 6.

The first distinctive observation from the figure is that samples maintained their cellular integrity during *in vitro* simulated digestion, irrespective of processing or digestion time. This is in agreement with recently published studies^{13,22} and strengthen the important role of process-induced cell wall permeability on modulating the *in vitro* starch digestion of common bean cotyledon cells. Secondly, the microscopic study as a function of digestion time for different processing times corroborated the existence of dissimilarities among samples generated from distinct processing intensities. At time zero of small intestinal digestion, as expected, no differences among samples were identified. However, two predominant changes were observed as digestion time progressed: on the one hand, cells became “emptier” and, on the other hand, some cells appeared to be less coloured than others. Emptying of cells was clear from the distance increase between the cell wall and cellular contents with the advance of small intestinal digestion, whereas decrease of colour intensity seemed to only occur on those cells in which some emptying had previously happened. Such discolouration is thought to be related to the amount of starch present, therefore giving qualitative information on the extent of *in vitro* starch digestion at the specific digestion time under analysis.

Both changes became visible at earlier digestion times for samples isolated from longer processing times (observation of

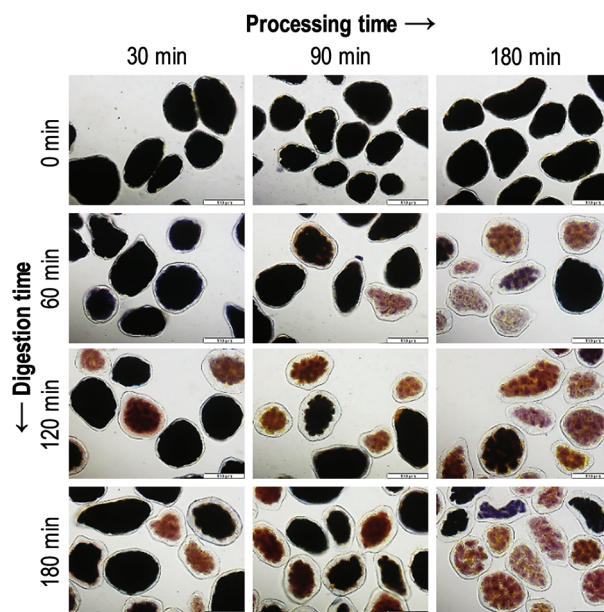


Fig. 6 Normal light microscopic pictures of cotyledon cells isolated from thermally treated (95 °C) common beans using different processing times (30 min, 90 min, and 180 min), taken at different digestion times of the small intestinal phase (0 min, 60 min, 120 min, and 180 min). Scale bar: 100 μm .

each row in Fig. 6), *i.e.*, faster emptying and discolouration of cells occurred in samples isolated from common beans thermally-treated during 180 min, followed by 90 min and 30 min, respectively. This observation is in agreement with the values calculated for the duration of the lag phase and the reaction rate constant during the kinetic modelling. Additionally, for each processing time (observation of each column in Fig. 6), more emptying and discolouration were present at longer digestion times, reflecting the time-dependent nature of *in vitro* starch digestion.

Overall, results from the qualitative analysis of *in vitro* starch digestion followed the same trend observed during the quantitative evaluation of this phenomenon. From this combined assessment, it is possible to ensure that starch granules were most probably digested inside the cells, which allows to suggest the following mechanism regarding the *in vitro* starch digestion of common bean cotyledon cells (regardless of process-induced cell wall permeability): (i) diffusion of pancreatic α -amylase through the cell wall and the protein matrix encapsulating starch granules; (ii) enzyme binding and hydrolysing starch granules following an “outside in” pattern; and (iii) digestion products leaving the cellular space due to concentration gradients.

4. Conclusions

In this study, we have demonstrated that the barrier role of cell walls during *in vitro* simulated digestion of starch in common bean cotyledon cells can be modified through variation of thermal processing intensity. Specifically, it has been proven that the application of thermal treatments of different durations results in distinct process-induced cell wall permeability degrees, which affect the *in vitro* starch digestion kinetics of intact cells. Although these events have been demonstrated for a specific variety of common beans, they could be expected to happen as well in other varieties and other types of pulses. The application of the techniques employed in the present study aiming to compare different common bean varieties or different pulses would be interesting.

We have proposed for the first time an alternative data analysis model to describe the time-dependent behaviour of *in vitro* starch digestion of legume cells. In addition, we have hypothesised that process-induced cell wall permeability affects the *in vitro* starch digestion of common bean cotyledon cells either by modulating the diffusion of pancreatic α -amylase inside the cells, by influencing the gastric digestion of the protein matrix surrounding starch granules to different extents, or by a combination of these two events. A mechanism of *in vitro* starch digestion in isolated common bean cotyledon cells has been proposed as well, allowing to improve understanding of the role of microstructure (as affected by targeted processing) on the digestive function of starchy foods. Finally, our investigation offers evidence of the potential of targeted thermal processing (using conditions normally applied in household situations) to modulate

starch digestion *in situ* through modification of structural properties.

Conflicts of interest

There are no conflicts of interest to declare.

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