Effect of manufacturing conditions on *in vitro* starch and protein digestibility of (cellular) lentil-based ingredients

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Abstract

(Cellular) pulse powders are being proposed as ingredients for different foods. However, the effect of manufacturing conditions on the properties of those powders remained unknown. Therefore, this study investigated the effect of specific manufacturing conditions (cooking time, application of cell isolation, and drying method) on the composition, microstructure, and *in vitro* starch and protein digestibility of lentil powders. Next to powders consisting of isolated cotyledon cells (ICC), this study proposes the production of precooked whole lentil powders (WL), without a cellular isolation step. In a model food system (heat-treated suspension), starch and protein digestion were significantly attenuated for both WL and ICC compared to raw-milled lentil flour. The applied cooking time determined macronutrient digestibility in the powders by *(i)* affecting the susceptibility of ICC to *in vitro* digestion, and *(ii)* determining the microstructural properties of WL. Freeze-dried ICC powder showed a stronger attenuation of amylolysis compared air-dried ICC. This study showed that WL powders have an important potential as innovative food ingredients higher in fiber but lower in starch compared to ICC.

Keywords

Pulses; lentil flour; cellular powder; whole precooked lentil powder; amylolysis; proteolysis; INFOGEST

List of abbreviations

PSD Particle size distribution

ICC Isolated cotyledon cells

WL Whole precooked lentils

SSF Simulated salivary fluid

SGF Simulated gastric fluid

SIF Simulated intestinal fluid

1 Introduction

Pulses are being put forward as an important part of healthy and environmentally sustainable diets (Willett, Rockström, & The Eat-Lancet Commission, 2019) as they are rich in complex carbohydrates (slowly digestible starch and dietary fiber), protein, vitamins, and minerals. Pulses are increasingly being proposed as plant-based protein sources, with an amino acid composition complementary to that of cereals (Boye et al., 2010). Regular intake has been associated with several health benefits, such as improved glycemic control and postprandial satiety (Jenkins et al., 2012; Mollard et al., 2012; Wong, Mollard, Zafar, Luhovyy, & Anderson, 2009).

Nutrients in pulses are bioencapsulated by a cell wall and intracellular protein matrix. During cooking, gradual pectin solubilization causes a shift in the mode of tissue failure upon mechanical disintegration from cell breakage to cell separation (Chigwedere et al., 2018; Njoroge et al., 2016). As a result, pulse cotyledon cells mostly remain intact when cooking is followed by mechanical disintegration (Pallares Pallares, Loosveldt, Karimi, Hendrickx, & Grauwet, 2019). This intrinsic barrier hinders enzyme diffusion towards the encapsulated substrate, decreasing the rate and extent of macronutrient digestion (Berg, Singh, Hardacre, & Boland, 2012; Dhital, Bhattarai, Gorham, & Gidley, 2016; Pallares Pallares, Alvarez Miranda, et al., 2018; Rovalino-Córdova, Fogliano, & Capuano, 2018).

While pulses are an essential part of the diet of many populations, they remain underutilized in most of the western world, e.g. due to sensory characteristics and lack of knowledge on how to

prepare them (Henn, Goddyn, Bøye Olsen, & Bredie, 2021). However, the incorporation of pulse-based ingredients into innovative foods can be a strategy allowing consumers to conveniently increase their pulse intake without drastically altering eating habits (Asif, Rooney, Ali, & Riaz, 2013). In this context, pulse flours are being used to enrich or replace traditional ingredients in mostly wheat-based product formulations (Monnet, Laleg, Michon, & Micard, 2019). Traditionally, these flours are produced through milling of raw pulses, mostly disrupting the inherent structural hierarchy and, depending on the obtained particle size, the barrier function for macronutrient digestion (Byars, Singh, Kenar, Felker, & Winkler-Moser, 2021;Edwards, Warren, Milligan, Butterworth, & Ellis, 2014).

Recently, researchers developed innovative powders from precooked pulses containing a high concentration of intact cells (PulseON®) and incorporated them into foods such as bread and biscuits (Bajka et al., 2021; Delamare et al., 2020; Edwards et al., 2020). The cellular integrity was maintained during food processing, as well as digestion, resulting in a (s)lower starch digestibility compared to a raw-milled pulse flour, both *in vivo* and *in vitro* (Bajka et al., 2021; Delamare et al., 2020).

As recently reviewed (Duijsens et al., 2021), the use of cellular powders opens up vast opportunities for the production of foods with attenuated macronutrient digestion patterns. However, since most studies only apply one specific manufacturing process to establish cellular pulse powders (Edwards et al., 2020; Pälchen, Bredie, et al., 2022), insights into the effect of manufacturing conditions (e.g. cooking time, procedure of cell upconcentration/isolation, drying method) on nutrient digestibility in these ingredients are currently missing (Pallares Pallares et al., 2021). Since pulses are an important protein source in most plant-based and sustainable diets, protein digestibility of these ingredients should be studied as well. However, research on protein

digestibility of pulse-based ingredients is missing, while research on protein digestion in whole pulses remains limited as well (Bhattarai, Dhital, Wu, Chen, & Gidley, 2017; Gwala, Pallares Pallares, Pälchen, Hendrickx, & Grauwet, 2020; Pälchen, Van Den Wouwer, et al., 2022; Rovalino-Córdova, Fogliano, & Capuano, 2019). Therefore, the current research investigated the effect of several manufacturing conditions (cooking time, drying method, and application of a cell isolation method) on both starch and protein digestibility in different lentil powders with (partial) cellular intactness, compared to a raw-milled flour. This is the first time the integrated effect of applying different manufacturing conditions on microstructural and related *in vitro* digestive properties of lentil ingredients was investigated.

2 Materials and Methods

2.1 Materials

Raw Dupuy-type green lentils (*L. culinaris*) grown and harvested in Canada in August 2019, were donated by Casibeans (Melsele, Belgium). The material (<10% moisture) was sorted and foreign material removed, after which seeds were stored below their glass transition temperature (-40°C), ensuring stability until usage (Kyomugasho, Kamau, Aravindakshan, & Hendrickx, 2021). Chemical reagents and enzymes were purchased from Sigma Aldrich (Belgium), except for the Total Starch Kit (Megazyme, Bray, Ireland), KCl, $MgCl_2(H_2O)_6$, NaOH, sodium potassium tartrate (Acros Organics, Geel, Belgium), $NaHCO_3$, NaCl, and KH_2PO_4 (VWR, Leuven, Belgium).

2.2 Lentil powder preparation

Lentil powders with different microstructural properties were produced from a single batch of whole lentils by altering and interchanging manufacturing steps (i.e. mechanical disintegration, thermal treatment, application of a concentration method, and the choice of drying method). After drying, the ingredients were stored in a desiccator until use.

To study the effect of cooking time, two cooking times (15 and 60 min) were selected for which the presoaked whole lentils showed softening to a palatable level. Moreover, preliminary research (*data not shown)* revealed that for both cooking times, the predominant mode of tissue failure was not cell breakage, indicating that significant pectin solubilization had occurred. However, the application of a shorter cooking time (15 min) was hypothesized to cause a significantly different particle size distribution upon mechanical disintegration, with a lower fraction of individual cells compared to the longer cooking time where cell separation was expected to be more complete (60 min). For comparison, cooking times of 8-43 min have been reported for soaked lentils (Jood, Bishnoi, & Sharma, 1998; Sharif et al., 2014; Singh, Erskine, Robertson, And, & Williams, 1988).

2.2.1 Raw-milled lentil powder

Raw-milled lentil powder was produced by milling (Cyclotec1093 sample mill, FOSS, Sweden) raw, whole lentils until passing through a 500 µm sieve mesh.

2.2.2 Isolated cotyledon cell powders

Isolated cotyledon cells powders (ICC) were produced following a procedure based on previous work by Dhital, Bhattarai, Gorham, & Gidley (2016), Edwards (2014), and Pallares Pallares et al. (2018). Raw whole lentils were soaked in an excess of demineralized water (1:10 w/v) for 16h at 25°C after which the soaking water was discarded. The lentils were cooked in demineralized water (1:10 w/v) for 15 or 60 minutes at 95°C. It was expected that a longer cooking time would lead to a higher yield of ICC, as wel as possibly different barrier properties of the individual cells to digestive enzymes (Gwala et al., 2020). Subsequently, the cooking water was discarded and the cooked lentils mixed with water (1:1 w/v) and homogenized (2 min, 3000 rpm) (IKA® T25 ultra-turrax, Janke and Kunkel, Germany). The lentil slurry was wet-sieved using a vibratory sieve system (amplitude 2.5 mm, 4 min) (AS200, Retsch, Germany). The fractions 40-125 µm contained

individual cotyledon cells, as confirmed microscopically (Olympus, Optical Co. Ltd, Tokyo, Japan) and by laser diffraction analysis (Beckman Coulter Inc., LS 13 320, FL, USA) (*data not shown).* The yield of cell isolation was calculated on a dry weight basis. The collected cells were lyophilized (Alpha 1-4 LSCplus, Martin Christ, Germany) to obtain freeze-dried isolated cotyledon cell powders with a cooking time of 15 min ($ICC15_f$), and 60 min ($ICC60_f$). Based on literature, it was hypothesized that the applied drying method would affect the digestive properties of cellular pulse powders (Yu et al., 2020). Therefore, additionally, part of the cells isolated from lentils with a cooking time of 15 minutes were dried by air convection ($ICC15_{air}$). For this, the cell slurry was thinly spread and dried to a stable moisture content under a fume hood (48h) (Aravindakshan et al., 2021).

2.2.3 Precooked whole lentils

Next to ICC powders, precooked whole lentil (WL) powders were prepared for cooking times of 15 and 60 min, aiming to obtain ingredients with different particle size distributions (PSD) and possibly distinct macronutrient digestibilities. Whole lentils were soaked, cooked, and homogenized as stated in **section 2.2.2.**. The obtained slurries were lyophilized as such (without cell isolation) after which the powder was sieved (mesh size 500 µm). The fraction >500 µm (mostly seed coat particles) was pounded in a blender mill (IKA Labortechnik-A10, Germany) for 30 s, re-sieved to generate a fine and homogenous powder, and merged with the <500 µm fraction.

2.3 Lentil powder characterization

2.3.1 Proximate analysis

The starch content of all powders was determined in duplicate using a total starch kit (AA/AMG, Megazyme Inc. Bray, Ireland). The nitrogen content was analyzed in duplicate by automated Dumas analysis (CE instrument, Thermofischer Scientific, Walthom, USA). Then, the crude

protein content was calculated using a conversion factor of 5.4 (De Almeida Costa, Da Silva Queiroz-Monici, Pissini Machado Reis, & De Oliveira, 2006). The moisture content was determined in triplicate as the difference in weight before and after vacuum drying (Nguyen et al., 2016). Briefly, 1 g of sample was dried in a vacuum oven (1445-2, 1400 W, Germany) at 70°C, in steps of 1 h at 0.8, 0.6 and 0.4 bar and 30 min at 0.2 bar. The ash content was determined in triplicate, as the loss of weight upon complete combustion in a muffle furnace (20h, 550 °C) (Park & Bell, 2004). The lipid content was determined gravimetrically according to Janssen et al. (2018), in triplicate. The fiber-rich residue, containing both fiber and sugars such as sucrose, glucose, and undigestible galacto-oligosaccharides,was calculated by subtracting the average starch, protein, moisture, ash, and lipid content from the total composition. For raw and cooked lentils, free sugar contents of 2 to 5 g/100 g have been reported, containing 50 to 74% undigestible galacto-oligosaccharides, respectively (Brummer, Kaviani, & Tosh, 2015; Kathirvel, Yamazaki, Zhu, & Luhovyy, 2019).

2.3.2 Microscopy

Representative micrographs of all lentil powders were taken upon resuspension in water, using an Olympus BX-51 light microscope (Olympus, Optical Co. Ltd, Tokyo, Japan) with cellSense Standard® software. Objectives of 10x and 40x magnification were used. Moreover, starch was visualized in digestion pellets using a 5% (w/v) Lugol's iodine solution.

2.3.3 Particle size distribution

The PSD of each powder was measured in duplicate using a particle size analyzer set up with a Universal Liquid Module (Beckman Coulter, LS 13 320, Miami, FL, United States), exactly as described by Noordraven, Bernaerts, Mommens, Hendrickx, & Loey (2021). The Fraunhofer optical model was used to calculate volumetric PSDs.

2.4 *In vitro* digestion of heat-treated lentil powder suspensions

Digestion of produced lentil powders was simulated *in vitro* following the static INFOGEST protocol (Brodkorb et al., 2019). Prior to digestion, the concentrations and activities of the used bile salts and enzymes were determined according the procedures proposed in the protocol (Brodkorb et al., 2019).

The lentil powders were digested in a model food system, i.e. heat-treated suspensions. One independent sample was prepared for each analyzed digestion time (5 for the gastric phase and 13 for the small intestinal phase). In each digestion tubes, 125 mg of lentil powder was suspended in 1.125 mL of demineralized water. 10% suspensions were selected as the viscosity of the system was not significantly affected at this concentration. After 1h of rehydration at 25°C, the suspensions were heat-treated (15 min, 95°C), mimicking a pasteurization process of a liquid food and aligning levels of starch gelatinization in raw-milled and thermally pretreated samples. Differential scanning calorimetry (DSC) analysis was carried out, exactly as stated previously by (Noordraven et al., 2021). While the data are not shown here, they indicated complete starch gelatinization upon heat-treatment of the suspensions. Moreover, microstructural properties of ICC and WL ingredients upon suspension preparation (secondary processing) were confirmed via laser diffraction and microscopy (*data not shown*).

2.4.1 Oral phase

The oral phase was initiated by adding 1 mL of simulated salivary fluid (SSF, pH 7), 0.0063 mL $CaCl_2$ (0.3 M), and 0.125 mL demineralized water to each of the digestion tubes. No salivary amylase was added, since the enzyme would immediately be inactivated upon initiating the gastric phase (pH 3) (Pälchen et al., 2021).

2.4.2 Gastric phase

2 mL of simulated gastric fluid (SGF) and 0.0013 mL $CaCl_2$ (0.3 M) were added to the oral mixture. The pH was adjusted to 3 using 2M HCl. Subsequently, a porcine pepsin solution and demineralized water were added to obtain a final pepsin activity of 2000 U/mL and a total volume of 5 mL. The tubes were incubated under end-over-end rotation (120 min, 37°C, 70 rpm). Independent samples were taken at 5 predetermined time points followed by enzyme inactivation (5 min, 98°C).

2.4.3 Small intestinal phase

The gastric chyme was mixed with 2.125 mL simulated intestinal fluid (SIF), 0.001 mL $CaCl_2$ (3 M) and 0.625 mL of a fresh bile solution (160 mM in SIF). The pH was adjusted to 7 (1 M NaOH). Subsequently, demineralized water and enzyme solution were added to the mixture to obtain a final digestion volume of 10 mL and enzymatic activities of 200 U/mL α-amylase, 100 U/mL trypsin, and 25 U/mL chymotrypsin, and 10 mM bile. These samples were incubated (180 min, 37°C). To study digestion kinetics, independent samples were taken at 13 predetermined time points and enzyme inactivation was carried out as stated above. Enzyme blanks (all enzymes and simulated fluids but no sample) were prepared for both considered digestive phases. After enzyme inactivation, all digestion tubes were centrifuged (5 min, 2000g, 25°C). The supernatant and pellet were separated and both snap frozen and stored at -40°C until analysis. Starch and protein digestion kinetics of both the gastric and small intestinal were then studied from each of the digests, since each tube represents an individual and independent repetition of the digestion experiment stopped at predetermined digestion times (**section 2.6**).

2.5 Quantitative evaluation of macronutrient digestion kinetics

2.5.1 Determination of digested starch

The dinitrosalicylic (DNS) method was used to determine the digested starch (%) (Englyst & Hudson, 1987; Miller, 1959). Since no salivary amylase was included, amylolysis was only evaluated for the small intestinal phase. For each digestion time, 2 mL diluted supernatant was mixed with 1 mL DNS reagent in duplicate, followed by 15 min incubation at 100°C. After cooling down, the mixture was diluted with 9 mL milliQ water and the absorbance was measured at 540 nm. The concentration of reducing sugars was calculated employing a maltose calibration curve (0.5-2.0 mg/mL). Maltose concentrations were then converted to starch equivalents using a conversion factor of 0.95 (Eq. 1).

$$Digested\ starch\ (\%) = \frac{maltose\ equivalents \times 0.95}{total\ starch\ content} \times 100 \qquad \text{Eq. 1.}$$

2.5.2 Determination of protein digestion products

Protein digestion was evaluated by quantifying α-amino groups released by proteolysis using the spectrophotometric o-phthaldialdehyde (OPA) assay (Nielsen, Petersen, & Dambmann, 2001; Zahir, Fogliano, & Capuano, 2018). The extent of proteolysis was always expressed relative to the total amount of α-amino groups present in the undigested sample (NH_{2total}). To determine this fraction, 5 mg of sample were hydrolyzed in duplicate in 1 mL 6 N HCl (24h, 110°C). The acid was removed by rotary evaporation (Hei-Vap Core, Heidolph, Germany), after which samples were diluted in 5 mL milliQ and filtrered (pore size 0.25 µm).

The extent of proteolysis was determined in the digestive supernatant for each considered digestion time in two distinct fractions were prepared from the digestive supernatant. Next to the digested samples ($NH2_{TCA}$ and NH_{2total}), blanks were analyzed containing all reagents but no sample

($NH_{2initial}$). Firstly, as widely reported, the *readily bioaccessible* fraction ($NH2_{TCA}$) was separated (Gwala et al., 2020; Zahir, Fogliano, & Capuano, 2020). To obtain this fraction, large peptides and proteins were precipitated from the digestive supernatant by adding 3.2% TCA followed by centrifugation (10 000g, 25°C, 30 min) (Microfuge 22R, Beckman Coulter, US). The resulting supernatant, containing small peptides and amino acids, is considered to be readily absorbable at the brush border without requiring any further hydrolysis (Webb, 1990). Secondly, the *hydrolyzed readily bioaccessible* fraction ($NH_{2TCA,hydro}$) was determined by hydrolyzing the TCA-soluble peptides into amino acid constituents (Pälchen et al., 2021). Briefly, the TCA-soluble supernatant was hydrolyzed in 6 N HCl (16h, 110°C), after which the acid was removed and samples redissolved and filtered. This fraction represents the readily bioaccessible protein as well, but in terms of the amino acids making up the small peptides in the supernatant. Therefore, this fraction can more easily be related to the total amount of α-amino groups present in the hydrolyzed, undigested sample (NH_{2total}).

For each analysis, a fresh batch of OPA reagent was prepared as described by Zahir et al. (2018). In duplicate, 0.4 mL of diluted sample ($NH_{2total,}$ NH_{2TCA}, and $NH_{2TCA,hydro}$) was thoroughly mixed with 3 mL of OPA reagent and incubated in the dark for 2 min, after which the absorbance was read at 340 nm. A L-serine standard curve (12.5-100 mg/mL) was used to express the detected α-amino groups in terms of L-serine equivalents. The extent of protein digestion could be expressed following equation 2 and 3.

$$bioaccessible\ protein = \frac{NH_{2TCA} - NH_{2initial}}{NH_{2total}} * 100 \quad \text{Eq. 2}$$

$$hydrolyzed\ bioaccessible\ protein = \frac{NH_{2TCA,hydro} - NH_{2initial}}{NH_{2total}} * 100 \quad \text{Eq. 3}$$

2.6 Statistical analysis and modelling of kinetic data

Proximate analysis data was statistically analyzed by one-way ANOVA. Pairwise comparison of the means was carried out using a Tukey's range test ($p < 0.05$), using JMP Pro 16 (SAS Institute, Cary, NC, United States). To compare calculated fiber-rich residue contents, 95% confidence intervals were used.

Macronutrient digestion kinetics were studied from each of the produced digests. In the applied kinetic approach, the digestive behavior of each sample is evaluated with independent measurements (digestion tubes) at different small intestinal digestion times (**section 2.4**). From a statistical point of view, each of these consecutive measurements can be seen as repetitions of characterizations of one system (Verkempinck et al., 2018). The information obtained from those different kinetic points was integrated by regression. Subsequently, modelled curves and estimated parameters (each with an insecurity and confidence interval) were used to assess differences in digestion kinetics between different powders. Additionally, particular kinetic digestion experiments were repeated confirming the repeatability of the procedure *(data not shown).*

Macronutrient digestion during the small intestinal phase was fitted using a first order fractional conversion model (Eq. 4) as applied by Edwards, Warren, Milligan, Butterworth, & Ellis (2014) and Gwala et al. (2019). In Eq. 4, C is the percentage of digested starch or protein at a certain digestion time. Both the final extent C_f (%) and rate constant k (min^{-1}) were estimated. C_0 was defined as the extent of hydrolysis at the end of the gastric phase/beginning of the small intestinal phase (t=0). Since no salivary amylase was added during the simulated oral phase, it was assumed no amylolysis took place during the gastric phase and the model could be simplified (C_0=0).

$$C(t) = C_f + (C_0 - C_f) * e^{-k*t} \quad \text{Eq.4}$$

Amylolysis was however not always suitably described by a first order reaction. Foods containing starch fractions with different susceptibilities to amylolysis can show distinct phases in which starch is hydrolyzed at different rates (W. Yu, Zhou, & Li, 2021). Edwards et al. (2014) proposed a modified (biphasic) model (Eq. 5) in which k_1, k_2, C_{f1} and C_{f2} are estimated as starch hydrolysis rate constants and end points of distinct consecutive reaction phases. t_{int} is estimated as the intersection between both phases (at which the extent of amylolysis is C_{int}), where the first (fast) reaction became negligible while the second phase became predominant. In this case, the estimated final extent of digestion is the sum of C_{f1} and C_{f2}.

$$C(t) = \begin{cases} C_{f1} * (1 - e^{-k*t}) & t \leq t_{int} \\ C_{int} + C_{f2} * \left(1 - e^{-k*(t-t_{int})}\right) & t > t_{int} \end{cases} \quad \text{Eq. 5}$$

C_f and k parameters were simultaneously estimated by nonlinear regression (SAS version 9.4, SAS Institute, Inc., Cary, NC, USA). Residual and parity plots *(data not shown)* and R^2_{adj} were drafted to assess the fit of the modelled curves. The initial rate of amylolysis was estimated by calculating the slope of the tangent to the modelled curve at time zero (Pallares Pallares, Rousseau, et al., 2018). 95% confidence intervals were employed to evaluate significant differences between estimated parameters.

3 Results and discussion

3.1 Characterization of lentil ingredients

PSDs of the produced lentil ingredients and representative micrographs, are shown in **Figure 1**. The raw-milled lentil flour showed a bimodal particle size distribution, with a first broad peak at 10-40 µm and a second peak around 400 µm. The peak at small particle sizes represents starch granules, protein bodies, and cell wall fragments released due to cell rupture. Indeed, (native)

starch granules have been reported to have dimensions around 19 to 35 µm (Do, Singh, Oey, & Singh, 2019; Ma et al., 2011). The peak at larger particle sizes likely represents tissue and seed coat fragments persisting upon milling. Similar PSDs have been reported for raw-milled pulse flours (Edwards et al., 2020).

As expected, ICC powders revealed a homogenous, unimodal PSD. Micrographs in **Figure 1** confirm these powders contained only individual cells, demonstrating elliptical to irregular shapes. The volume-weighted median particle size d_{50} was 111 µm for $ICC15_f$, 113 µm for $ICC60_f$, and 129 µm for $ICC15_{air}$, assuming a spherical diameter, in accordance with the previously reported average diameter of 101µm for lentil cotyledon cells (Do et al., 2019). This particle diameter of the lentil ingredient was slightly lower than previously reported average particle size (around 178 µm) for a cellular powder made of green lentils (Edwards et al., 2020), possibly due to differences in botanical origin and the manufacturing process (sieve mesh size and drying method).

WL powders showed a heterogenous distribution, with two distinct peaks. The first peak (around 110 µm) indicates the presence of individual cells and the tail towards smaller sizes implies that some cell breakage occurred releasing cytoplasmatic contents (i.e. starch granules, protein bodies). The volumetric fraction containing single cells (sizes between 40 and 300 µm) could be estimated to be around 60% and 69% for $WL15_f$ and $WL60_f$, respectively. The second peak (around 600 µm) represents cell clusters (intact tissue) as well as seed coat fragments, as confirmed by microscopic analysis. The (volumetric) fraction containing both seed coat material and cell clusters was around 34% and 24% for $WL15_f$ and $WL60_f$, respectively. Logically, a longer cooking time led to more cell separation and a shift in the PSD towards more individual cells but a lower amount of cell clusters. The remaining fraction (around 6-7%) can be attributed to smaller material, such as cell material solubilized upon cooking or released upon cell rupture (free starch and proteins) and. It

was hypothesized that more cell rupture occurred upon shorter cooking times, leading to more free starch in $WL15_f$. While this hypothesis seems confirmed by the micrographs in **Figure 1**, this was not clearly observed in the (volumetric) PSD.

The yield of ICC amounted to approx. 48 and 67 g/100 g on a dry matter basis for pre-soaked seed cooking times of 15 and 60 minutes, respectively. A similar yield (62.7%) was reported previously for an isolated cell powder from cooked green lentils (Edwards et al., 2020). Since ICC and WL were prepared from identically prepared lentil slurries, the ICC yield is also representative of the contribution of individual cells in WL powders (on a dry matter basis). Therefore, $WL60_f$ could be considered to contain more individual cells as compared to $WL15_f$.

The proximate analysis of the lentil powders with different process-induced microstructural properties was summarized in **Table 1**. While the raw-milled flour and $WL60_f$ were similar in overall composition (except for the ash content), $WL15_f$ showed a significantly higher starch, but lower ash and fiber-rich residue compared to raw-milled flour. During soaking and thermal processing included in the WL ingredient preparation, dry matter leached out, possibly facilitated by cooking (e.g., pectin solubilization and leaching of sugars) (Brummer et al., 2015; Chigwedere et al., 2019; Wang, Hatcher, Toews, & Gawalko, 2009). Since soaking and cooking liquids were discarded, shifts could occur in the relative dry matter composition. ICC powders showed an increased starch content compared to the raw-milled flour which can be attributed to *(i)* relatively higher leaching of other components as explained above, and *(ii)* upconcentration of cells (high in starch) during wet-sieving. Starch and protein contents of raw-milled and ICC powders are comparable to those reported by Edwards et al. (2020). The ash contents of raw-milled and WL powders were higher as compared to ICC, due to the presence of the seed coat, generally rich in minerals.

3.2 Starch digestion of heat-treated suspensions of different lentil powders

3.2.1 Effect of process-induced microstructure on starch hydrolysis

Figure 2A compares starch digestion kinetics of heat-treated suspensions of powders with the largest differences in microstructure, i.e. raw-milled flour, $ICC15_f$ and $WL15_f$. Modelled parameters for starch digestion are shown in **Table 2**. The data in **Figure 2A** indicate that amylolysis occurred fastest for the heat-treated raw-milled flour suspensions, while it was clearly attenuated for both $ICC15_f$ and $WL15_f$**.** Indeed, the initial rate of starch hydrolysis was significantly higher for raw-milled flour, compared to $WL15_f$ and $ICC15_f$.

Over the whole course of *in vitro* digestion, the $ICC15_f$ suspension showed the least complete amylolysis. Amylolysis was modelled using a fractional conversion model (Eq.4). The attenuation of starch hydrolysis can be attributed to its bioencapsulation by an intact cell wall and cytoplasmic (protein) matrix, as demonstrated earlier for whole pulses, isolated cells, and cellular powders (Dhital et al., 2016; Edwards et al., 2020; Rovalino-Córdova, Fogliano, & Capuano, 2018; Xiong et al., 2018). Moreover, amylolysis was possibly further slowed through interactions of amylase with cell wall material (Bhattarai et al., 2017; Dhital, Gidley, & Warren, 2015).

Contrarily, starch digestion in the (heat-treated) raw-milled flour suspension seemed to occur instantly and rapidly during the first 10 min of small intestinal digestion, followed by a second (slower) phase and was therefore modelled using the biphasic model (Eq. 5). The model assumes amylolysis occurred in two consecutive phases due the presence of distinct starch fractions with different susceptibilities to amylolysis (Edwards et al., 2014). For raw-milled flour, the PSD (**Figure 1**) indicated the presence of a fraction of free starch granules which could preferentially interact with amylases. Upon gradual exhaustion of that first fraction (after about 42 min (estimated t_{int})), amylases would have become available to migrate into larger tissue fragments

(which persisted upon milling) and hydrolyze the entrapped starch. The rate at which amylases could access the starch bio-encapsulated inside intact tissue fragments was probably determined by several factors, such as the porosity of the cell wall and its subsequent permeability to amylases (Pallares Pallares, Alvarez Miranda, et al., 2018) and the binding affinity of amylases to cell wall material (Bhattarai et al., 2017). Biphasic amylolysis has been reported earlier for raw-milled pulse flours (Edwards et al., 2014), with the rate increasing upon more intensive matrix disruption (Romano, Gallo, Ferranti, & Masi, 2021). Consistent with the results reported here, a lower rate and extent of starch hydrolysis (around 30%) were reported for lentil powder enriched in intact cells, compared to raw-milled lentil flour (95%) (Edwards et al., 2020).

The data for amylolysis in $WL15_f$ suspension showed a biphasic behavior as well, which could be explained by the presence of distinct starch fractions (**Figure 1**). It was hypothesized that some free starch (released from ruptured cells) was readily available for hydrolysis in a first phase of small intestinal digestion. In a second phase, starch entrapped in intact cotyledon material (individual cells, cell clusters, tissue fragments) could gradually be hydrolyzed. While the biphasic model presupposes two distinct and consecutive phases, it was clear from the measured datapoints that the transition did not occur instantly (at t_{int}). More accurately, we hypothesized that amylases could more and more penetrate into cells and cell clusters upon gradual exhaustion of the more easily digestible substrate. Therefore, comparison of the measured data to the estimated model revealed a slight underestimation of the degree of amylolysis in $WL15_f$ between approximately 30 and 60 minutes of small intestinal digestion.

Compared to heat-treated raw-milled flour suspensions, both the initial rate and rate constant of amylolysis were significantly lower for $WL15_f$, indicating significant attenuation of starch hydrolysis due to the presence of intact cotyledon material (**Table 2**). From the data and modelled

curves in **Figure 2,** it could be observed that the extent of amylolysis remained significantly lower for $WL15_f$ compared to the raw-milled flour during 120 min of small intestinal digestion (average *in vivo* small intestinal digestion time), after which the difference gradually levelled off. Differences in amylolysis extent between $WL15_f$ and $ICC15_f$ seemed small during the first 60 minutes of small intestinal digestion, however, the data revealed a different trend/mechanism of amylolysis which we attributed to different microstructural properties. The difference in digestive behavior between $WL15_f$ and $ICC15_f$ became more clear upon longer digestion times. The predicted estimated extent of starch digestion upon 90 minutes of *in vitro* small intestinal digestion, which was previously found to correlate to both *in vivo* final extent of amylolysis and GI value (Edwards, Cochetel, Setterfield, Perez-Moral, & Warren, 2019), was around 90% for the raw-milled flour suspension, compared to 68% for $WL15_f$, and 50% for $ICC15_f$. While the $ICC15_f$ suspension showed the least complete amylolysis upon *in vitro* digestion, the use of WL powders offers a strategy to limit processing steps and generation of waste streams to manufacture slowly digestible pulse ingredients. These precooked powders showed a clearly attenuated starch hydrolysis pattern due to the presence of bio-encapsulated starch (very similar to $ICC15_f$ in the first 60 minutes of small intestinal digestion). Moreover, though overall (final) starch digestibility may be a bit higher, the total amount of starch is lower in WL powders while maintaining a similar (or slightly higher) protein content and a higher fiber and mineral content compared to ICC powders (**Table 1**).

3.2.2 Effect of cooking time on starch hydrolysis

The *in vitro* starch digestibility of suspensions of powders derived from lentils with different cooking times can be found in **Figure 2 A and B.** The data was modelled using the (modified)

fractional conversion model (Eq. 4 and Eq. 5), with estimated kinetic parameters shown in **Table 2.**

The initial rate and rate constant of starch digestion was lowest for an isolated cell powder with a shorter compared to a longer cooking time ($ICC15_f$ *versus* $ICC60_f$). Similarly, the amylolytic susceptibility of isolated cells has been reported to increase with cooking time due to decreased barrier properties of the cell (i.e. increased cell wall permeability and/or denaturation of the (cytoplasmic) protein matrix) for common beans and Bambara groundnuts (Gwala et al., 2020; Rovalino-Córdova et al., 2019).

As elaborated in **section 3.2.1**, amylolysis of $WL15_f$ suspensions showed a biphasic behavior due to the presence of distinct starch fractions with different susceptibilities to hydrolysis. In contrast, a different course was observed for $WL60_f$. For the precooked WL powders, an increase in cooking time from 15 to 60 min caused a small but significant decrease in the initial starch hydrolysis rate (and thus the release of starch hydrolysis products). It was hypothesized that progressive middle lamella pectin solubilization upon prolonged cooking caused increased cell separation and less cell rupture upon manufacturing of $WL60_f$ powders. Indeed, the PSD of the powder revealed an increased volume fraction representing individual cells (69% for $WL60_f$ *versus* 60% for $WL15_f$). Previously, an increase in thermal treatment time was linked to increased cell separation upon mechanical disintegration affecting starch digestion kinetics for (mechanically disintegrated) common beans and Bambara groundnuts (Gwala et al., 2019; Pallares Pallares, Rousseau, et al., 2018). As a consequence of prolonged cooking and progressive pectin solubilization, both the microstructure (**Figure 1**) and the starch hydrolysis pattern (**Figure 2B**) of $WL60_f$ approached the properties of $ICC60_f$ (its most characteristic fraction).

To resume, all produced powders showed important attenuation of starch hydrolysis due to starch bioencapsulation, regardless of cooking time and performed isolation step. The applied cooking time affected starch digestibility in lentil-based ingredients with cellular intactness, plausibly through *(i)* changes in cell (wall and protein matrix) permeability to amylases ($ICC60_f$ *versus* $ICC15_f$), and *(ii)* shifts in the particle size distribution increasing the ICC yield/concentration of single cells in WL. It must be noted that even at a short cooking time ($WL15_f$), mechanical disintegration led to a rather significant amount of intact cellular material (cells and cell clusters) retarding starch digestion, next to only a limited extent of cell breakage (and free starch). After all, digestion kinetics represent the overall behavior, resulting from respective contributions of different microstructures present in the powder. In this case, it might be interesting to consider short cooking times to limit energy requirements and nutrient losses due to leaching for the production of lentil powders. For other (slow cooking) pulses (e.g. common beans), it might be useful to find the optimal cooking time at which cell breakage is minimal, while the yield of cells (and clusters) and their barrier properties in the precooked powder are maximized.

For both WL and ICC powders, the decreased amylolysis rates suggest that an attenuated starch digestion could be expected *in vivo* (Dupont, Le Feunteun, Marze, & Souchon, 2018). The link between *in vitro* and *in vivo* attenuated starch digestion has been made previously for bread containing cellular chickpea flour (Bajka et al., 2021), and should be made for other promising food ingredients such as these lentil powders and their applications.

3.2.3 Effect of drying method on starch hydrolysis

Small intestinal starch digestion of lentil ICC dried using different methods are shown in **Figure3**, with estimated kinetic parameters summarized in **Table 2.** While $ICC15_{air}$ and $ICC15_f$ were both produced from lentils with the same cooking time, the freeze-dried ingredient showed a

significantly lower initial rate of starch hydrolysis. Consequentially, after 30 minutes of small intestinal digestion, the extent of starch hydrolysis was remarkably higher for the air-dried ingredient, as confirmed qualitatively by the iodine-stained micrographs shown in **Figure 4**. Air-drying (and subsequent rehydration and thermal treatment) may have (partially) damaged the cell (wall or protein matrix) barrier, increasing permeability to amylases and causing an increased rate of amylolysis. Additionally, the fast initial starch hydrolysis for $ICC15_{air}$ (**Figure 3**) could possibly be linked to some cell damage and/or breakage during rehydration and/or thermal treatment of the air-dried ingredient, releasing free starch into the suspension. Correspondingly, from **Figure 4C,** damage to some cells as well as clustering of some fragments could be perceived qualitatively.

However, the (digestive) properties of the cellular powders highly depend on the conditions applied during drying, such as equilibrium moisture content and drying temperature (Yu, Fan, Wang, Xia, & Cai, 2020). Edwards et al. (2020) developed air-dried cellular powders (6% moisture) with more clearly attenuated starch hydrolysis patterns compared to the ingredient prepared here. However, the exact drying conditions are unclear, and a different *in vitro* digestion simulation was used, rendering comparison of the data difficult. In literature, the applied drying method has been reported to significantly affect starch digestibility of pulse powders (Edwards, Veerabahu, Mason, Butterworth, & Ellis, 2020; Z. Yu et al., 2020). Therefore, additional research is necessary to unravel the effect of the applied drying method on the microstructural and linked digestive properties of (cellular) pulse-based ingredients.

3.3 Protein digestion of heat-treated suspensions of different lentil powders

Protein hydrolysis upon *in vitro* digestion of heat-treated lentil powder suspensions is shown in **Figure 5** in terms of readily bioaccessible (NH_{2TCA}) and hydrolyzed bioaccessible protein ($NH_{2TCA,hydro}$), with parameters estimated following the fractional conversion model in **Table 3.**

3.3.1 Effect of process-induced microstructure on protein hydrolysis

At the end of the gastric phase, the amount of hydrolyzed readily bioaccessible protein ($NH_{2TCA,hydro}$) varied from 25-30% for powders with cellular intactness (ICC and WL) to approximately 41% for the raw-milled flour. The lower protein hydrolysis for suspensions of powders with cellular integrity compared to the raw-milled flour could be related to the (partial) bioencapsulation of protein. Similar extents of gastric formation of NH_{2TCA} and $NH_{2TCA,hydro}$ were reported earlier for other pulse types (Bhattarai et al., 2017; Gwala et al., 2020; Pälchen et al., 2021). In terms of NH_{2TCA}, the extent of proteolysis was slightly higher for $WL15_f$ compared to $ICC15_f$ for each considered gastric time point. A similar difference could be observed for $ICC60_f$ compared to $WL60_f$. However, the extent of gastric proteolysis in terms of $NH_{2TCA,hydro}$ at the end of the gastric phase is similar for those samples. The polymerization degree and thus size of the bioaccessible peptides formed during gastric digestion may be affected by the sample type. Further research is necessary to unravel proteolysis patterns of lentil ingredients with different microstructural properties, for example by applying SEC-HPLC and/or FTIR analysis (Barbana & Boye, 2013; Garcia-Valle, Bello-Pérez, Agama-Acevedo, & Alvarez-Ramirez, 2021; Wubshet et al., 2017).

From **Figure 5A**, it could be concluded that proteolysis was attenuated in the powders with cellular integrity (ICC_{15f} and WL_{15f}) compared to the raw-milled one, due to differences in the process-induced nutrient bioencapsulation (**Figure 1**). For the raw-milled flour, upon 120 minutes of small intestinal digestion, about 92% ($NH_{2TCA,hydro}$) of the protein, was converted into readily bioaccessible peptides. Interestingly, the ratio of the estimated final extent $NH_{2TCA,hydro}$ to NH_{2TCA} (around 30%) indicates that the bioaccessible protein was mostly made up of peptides with an

517 average polymerization degree of around 3. In comparison, around 65% of $WL15_f$ and 80% of
518 $ICC15_f$ protein was rendered bioaccessible during 120 min of small intestinal digestion. These
519 results suggest possible physiological differences for raw-milled *versus* cellular lentil powders
520 within realistic digestion times. However, as the digestion time increased even further, the
521 differences between the raw-milled flour and the flours with cellular integrity disappeared (no
522 significant difference between C_f values of raw-milled flour $ICC15_f$, and $WL15_f$ in terms of
523 $NH_{2TCA,hydro}$).

524 Generally, the proteolytic behavior observed for $ICC15_f$ and $WL15_f$ followed a similar trend, yet
525 the formation of bioaccessible protein seemed slightly faster for $ICC15_f$. Indeed, the digestive
526 behavior observed here could be seen as the summed behavior of the different microstructural
527 fractions present. While all $ICC15_f$ protein was bioencapsulated inside individual cotyledon cells,
528 $WL15_f$ also contained protein entrapped within cell clusters, which could form an additional
529 barrier slowing proteolysis (Byars et al., 2021; Zahir et al., 2020).

530 *3.3.2 Effect of cooking time on protein hydrolysis*

531 Small intestinal proteolysis kinetics of $ICC15_f$ and $ICC60_f$ followed a similar trend, showing little
532 effect of increasing cooking time. Similarly, ICC60f and WL60f showed very similar proteolysis
533 trends, due to their similar PSDs as described earlier (**section 3.1**). However, $WL15_f$ generally
534 showed a slightly lower extent of protein hydrolysis for each digestion time, compared to $WL60_f$.
535 In this case, it could be expected that protein digestibility was affected by the PSD of the samples,
536 with $WL15_f$ containing a relatively higher amount of cell clusters (possibly slowing proteolysis)
537 compared to $WL60_f$. In literature, contradicting trends regarding protein digestibility have been
538 reported with increasing cooking time for different whole pulses (Gwala et al., 2020; Torres,
539 Rutherfurd, Muñoz, Peters, & Montoya, 2016).

These results suggest that, while cooking time significantly affected amylolysis kinetics for ICC and WL powders, the effect on proteolysis appeared limited. However, the analyzed bioaccessible protein (NH_{2TCA} and $NH_{2TCA,hydro}$) only represents a specific fraction of solubilized (small) peptides and amino acids. There may be differences in substrate breakdown patterns occurring during proteolysis, which could not be detected here. Future studies should consider the analysis of additional fractions (such as solubilized protein (Gwala et al., 2020)) and/or by applying alternative methods as suggested previously. Effect of drying method on protein hydrolysis

The formation of bioaccessible protein followed a similar general trend for $ICC15_f$ and $ICC15_{air}$ (**Figure 5C**). While the air-drying seemed to increase susceptibility to amylolysis (**Figure 3**), this was not perceived for proteolysis. Possibly, an effect of the drying method on the cell wall porosity/cell intactness may have increased amylase permeation and subsequent amylolysis, while not affecting proteolysis. As shown earlier (Rovalino-Córdova et al., 2019), the ability of the smaller proteases (between 23.8 (porcine trypsin) and 35 kDa (porcine pepsin)) to permeate into cells might be less affected by processing as compared to amylases (51-54kDa for α-amylase from porcine pancreas). As mentioned earlier**,** follow-up experiments are necessary to elucidate the effect of the drying method on the digestibility of pulse-based ingredients.

4 Conclusion

This study showed that manufacturing conditions significantly affected structural, compositional, and *in vitro* digestion properties of lentil powders. Isolated cotyledon cell (ICC) and whole precooked lentil (WL) powders showed significantly slowed *in vitro* starch and protein digestibility compared to raw-milled flours, due to the presence of intact cellular material. Innovative WL powders can be prepared without a cell isolation step and are higher in ash and

fiber-rich residue, but lower in starch compared to ICC. Our findings illustrated that the cooking time applied during powder preparation (15 *versus* 60 min) determined digestive properties of *(i)* ICC powder potentially through their distinct susceptibility to digestive enzymes, and *(ii)* WL powders through their particle size distribution profiles. The applied drying method significantly affected amylolysis kinetics of ICC, though more research on the effect of drying conditions on digestive properties is required.

To conclude, WL powders are innovative ingredients with attenuated nutrient digestibility, even upon applying a short cooking step (15 min). This study highlighted that the choice of manufacturing conditions is crucial in modulating digestion kinetics. Future research should investigate the technofunctional properties of these food ingredients. Moreover, their application potential to produce healthy, pulse protein-rich, low glycemic foods which appeal to consumers, should be explored.

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Conflict of interest

None.

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